Author's response to reviews

Title: Profile of Blood Cells and Inflammatory Mediators in Periodic Fever, Aphthous Stomatitis, Pharyngitis and Adenitis (PFAPA) syndrome

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Author's response to reviews: see over
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Re: Revised manuscript No.: 3306945273932789  
Title: Profile of Blood Cells and Inflammatory Mediators in Periodic Fever, Aphthous Stomatitis, Pharyngitis and Adenitis (PFAPA) syndrome  
Corresponding Author: Dr. Kelly L. Brown

Dear Dr. Norton,

We thank you and the reviewers’ for feedback on our manuscript (3306945273932789). We take this opportunity to reply to the reviewers’ comments and submit a revised manuscript for publication in BMC Pediatrics. On the subsequent pages, we provide a response (plain text, no italics) to each of the reviewer’s points (in italics). Whenever possible we made changes in accordance with reviewers’ suggestions. Changes are highlighted in the revised manuscript. We believe that these changes have improved the article and it is suitable for publication in BMC Pediatrics.

Regards,

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**Reviewer:** Isabelle Kone-Paut  
**Reviewer's report:**  
This study is an attempt to identify modification in cells, serum acute phase reactants, cytokines and chemokines in PFAPA syndrome, that could help to understand the pathogenesis. Three small groups of children have been studied: controls, PFAPA afebrile and PFAPA febrile. The study is limited due to the low number of patients, the lack of information on patient’s selection and the absence of “febrile” control group: MKD patients for example.

**Patients and methods**

1. **How were these patients selected for the study?**

Patients that were enrolled in his study were selected according to a number of parameters such as age, ethnicity and clinical presentation. We provide additional details on pg. 5 of the manuscript (underlined sentences below) regarding the selection process.

‘Ten children with diagnosed PFAPA were selected to participate in this study based on age, ethnicity and clinical presentation. Participants 1) were less than 7 yr. of age, 2) fulfilled the standard clinical criteria for PFAPA syndrome [5], ..... 3) had febrile episodes lasting 3-5 days followed by 4) an asymptomatic interval between attacks (on average, 3-5 weeks) and 5) lacked additional features that would suggest a hereditary periodic fever syndrome such as skin rash, arthritis, severe abdominal pain, diarrhea, thoracic pain and splenomegaly, fever episodes longer than 7 days, a history of hearing loss or symptoms secondary to cold exposure [6, 17]. Moreover, patients with an ethnic origin in the Eastern Mediterranean basin, the Netherlands, Belgium or France were excluded to further minimize the risk of including patients with hereditary FMF or MKD. To our knowledge MKD has never been genetically diagnosed in a population of Swedish ethnic origin.’

2. **The authors say that PFAPA was diagnosed in accordance to clinical criteria of Thomas et al...and that they ruled out patients from Mediterranean countries Netherlands and Belgium were excluded to avoid FMF and MKD. For this reviewer the only way to rule out MKD is to measure the mevalonic acid in the urine during acute episodes. It is not guaranteed that Swedish patients may not have MKD.**
The reviewer makes a valid point; the only way to rule out MKD entirely is by genetic analyses or, to some degree, by analyzing mevalonic acid in urine during febrile episodes. The prevalence of MKD was actively searched in Sweden by analyzing mevalonic acid in urine during febrile episode in PFAPA cases, and by genetic analyses in suspected cases. As a consequence, not a single case of MKD was found. Further, the clinical presentation of our patients with “typical” PFAPA in the absence of symptoms indicative of a hereditary periodic fever makes it highly unlikely that any of our participants had MKD.

3. It is not clear if the patients have received steroids in both the febrile and afebrile group.

The patients did not receive steroids during febrile episodes or afebrile intervals during the study or prior to the study. Further, no prophylactic treatment for PFAPA was given to patients. In the event that patients received medication for a febrile episode, our study protocol dictated that those patients would be excluded from further study until a minimum of two febrile episode without medication were experienced. We have included the following statement on pg. 5 of the manuscript ‘The patients did not receive steroids or prophylactic treatment during febrile episodes or afebrile intervals either during the study or immediately preceding their participation in the study.’

Results
1. SAA: It is interesting to note that SAA levels are not completely normal in the control group in contrast to the CRP levels. The same observation is present in the afebrile PFAPA group even SAA is markedly elevated in the febrile PFAPA group. What happened with patient 3?

In our opinion, the slightly elevated level of SAA in the control group and afebrile PFAPA group demonstrates that SAA is a very sensitive inflammatory marker.

With respect to the control group, we cannot exclude the possibility that (i) the raised SAA level was caused by subclinical infection or inflammation despite the fact that our control group was comprised of children with no history of overt infections that were admitted to hospital for minor surgery, or (ii) that the surgical procedure caused an
increased SAA level; one control was sampled after the surgical procedure due to sampling difficulties while anesthesia was being initiated.

Elevated levels of SAA in the afebrile group are not entirely surprising as we and others (Stojanov et al Eur Cytokine Netw 2006, 17(2):90-97) found altered cytokine levels in PFAPA patients during the afebrile period, which is indicative of active disease during the afebrile phase. To this effect, we stated on pgs. 15-16 ‘While clinically asymptomatic, we, and others [22] demonstrate fluctuations in cytokines in the afebrile phase suggesting that the disease is active at the cellular level also between febrile flares.’ In addition, Lachmann et al demonstrated fluctuations in SAA levels in patients with FMF that were completely asymptomatic; the authors concluded that this was due to subclinical inflammatory activity (Rheumatology 2006 45(6):746-750).

With respect to the reviewer’s question about patient 3, blood samples from this individual were drawn <12 hours before the onset of fever, which could explain the low SAA level in this sample. The sampling time for this patient was stated, among other places, in the Subjects and Methods section (pg. 6, first paragraph), in Table 1 and in the footnote of Table 2.

As stated above, the elevated levels of SAA in the control and afebrile PFAPA group illustrate that SAA is a sensitive inflammatory marker. The results emphasize the importance of being able to strictly manage the control group in clinical studies. Moreover, they emphasize the need for investigations into the regulation and role of SAA in subclinical infection and inflammation. We have now commented on the elevated levels of SAA in some participants in the control and afebrile groups on pg. 8 and pg. 16 and cite the article by Lachmann et al.

2. Thrombocytosis in between febrile episodes is of interest (not observed in 2 patients). The problem is that if we compare the values in the same patients afebrile and febrile, for example patient 5 and 8, we do not find differences…..So is there a possible bias regarding patient selection?
Indeed, there was no increase in thrombocytes during the afebrile period in 2 of the 8 afebrile samples (patient 6 (not 5) and patient 8). As stated on pg. 14 of the manuscript, ‘thrombocytosis may be a delayed, acute phase reaction to the previous febrile episode’. Alternatively, thrombocytosis could be an indicator of subclinical inflammation during the symptom-free interval. Daily blood samples would be required to conclusively determine if we simply missed the window in which thrombocytes were elevated in these two patients or if there was a bias regarding patient selection.

3. The control group seems to have higher eosinophils count than PFAPA patients. There is a decrease in eosinophils count in between attacks, however the variation is within a range of 40 to 190/mm3 which is in the normal values? Is there a role in steroids administration to PFAPA febrile and afebrile patients? Indeed there is an hypothesis for a link between this syndrome and atopia and it is well known than eosinophils from atopic patients bear high levels of receptor to steroids.

The reviewer has interpreted the data correctly. While absolute eosinophil counts (AEC) were lower in the afebrile patients they were considered normal for children this age and were not significantly different than AEC in the control group. Diminished AEC in febrile samples however were below the lower limit of normal AEC and were statistically distinct from the control group. While exogenous administration of steroids is not responsible for the diminished AEC in febrile patients (our patients did not receive steroids), it is known that endogenous glucocorticoids inhibit eosinophil release from the bone marrow and enhance their removal from circulation. Therefore, it is possible that endogenous glucocorticoids released during the inflammatory process are responsible for lower AEC in PFAPA patients.

4. As the biological pattern of PFAPA is very proinflammatory, it is not clear why IL-1b is not elevated. One reason could be that IL-1b is generally undetectable in the serum even in clear IL1b linked disorders. Probably in vitro experiments with patient’s stimulated monocytes could show increased release of this cytokine. The concomitant elevation of the IL1RA and IL-6 in febrile patients could support this hypothesis.

We wholeheartedly agree with the reviewer. We predict that the pro-inflammatory
cytokines IL1β and TNFα are elevated early after the onset of fever, but as pointed out by the reviewer, it is difficult to detect these cytokines in the serum. To this effect, we stated in the manuscript (pg. 10), ‘Given that the expression of IL-6 is often induced by TNFα or IL1β [21] and these cytokines were found in PFAPA sera 4 hours after the onset of fever [22], we anticipate that TNFα and IL1β peak early in the fever period then quickly approach homeostatic levels. A rapid oscillation of TNFα and IL1β in vivo occurs in response to infection [23, 24].’ In the discussion on pg. 15, we go on to say ‘The classic pro-inflammatory cytokines TNFα and IL1β are typically associated with an inflammatory response and play cardinal pathogenic roles in monogenic, hereditary periodic fevers [30]. In contrast, we did not find elevated levels of TNFα or IL1β in FP sera ~15 hours after the onset of fever. It is however plausible that these cytokines appeared earlier after the onset of fever…….We however predict that cytokines TNFα, IL1β and IFNγ rise and fall rapidly in the early hours of fever….’

As suggested by the reviewer, we stimulated PBMC ex vivo with 10 ng/ml of LPS and indeed saw an increase in TNFα and IL1β produced by PBMC from febrile patients compared to control and afebrile PBMC. On pg. 15 we state ‘In response to ex vivo stimulation with LPS, PBMC from febrile patients produced more TNFα and IL1β compare to control and afebrile PBMC over a 24 hr period (data not shown).’

Discussion

1. Methodological limitations cited previously should be included in this section

We have added sentences to pgs. 14-16 of the manuscript (Discussion) that cite the limitations of our study and make suggestions for the design of future investigations.

‘We recognize that by selecting a well-defined group of patients, our cohort is relatively small thus we encourage independent confirmation of the results presented herein.’
‘Whether absolute cell counts that fall in the normal range but are significantly different between healthy and PFAPA children carry any biological significance remains to be proven.’

‘Future investigations require additional controls from children with other periodic fevers and acute infections.’

‘Due to technical and ethical restrictions associated with sampling at multiple time points after the onset of fever, it may be advisable to investigate cytokine profiles of disease [32] during the afebrile interval.’

2. As the classification of auto-inflammatory disorder is a challenging task, the authors should state if after this study they can give their opinion as classifying PFAPA in this category of disorders.

3. Moreover are the findings in accordance with an “immuno-allergic” mechanism?

The reviewer raises points in questions 2 and 3 that typify problems with diagnosing and describing PFAPA syndrome in the absence of biological markers of disease or an understanding of disease etiology. We are of the opinion that PFAPA syndrome is an auto-inflammatory disorder and, while lymphocytes may be involved, we have no evidence at present to suggest that the response is antigen-specific and in accordance with an immunoallergic response. Since our study was not intended to discern whether PFAPA has autoinflammatory or immunoallergic properties, we feel that this type of philosophical discussion, while interesting, would not add any value to the discussion.
Reviewer: Marco Gattorno

Reviewer's report:
The paper by Brown et al analyze the a number of laboratory parameters and cytokine serum levels in PFAPA patients either during fever episodes and in the inter-critical periods in comparison to age-matched healthy controls. The paper is generally well written and results are clearly presented. However a number of points should be raised:

Results
1) The first paragraph is not completely clear and could be deleted or incorporated to the second one. In fact, the laboratory parameters reported in this paragraph cannot be considered as markers of a “typical PFAPA”. This definition is mainly done on the clinical ground (see patients and methods).

We agree with the reviewer that ‘typical PFAPA’ is made primarily on the basis of clinical, not laboratory, findings. The first two paragraphs of the results section (pg. 7 and 8) have been placed under new headings to separate the clinical grounds that define ‘typical PFAPA’ and the laboratory findings (acute phase proteins) in our cohort of patients and controls. We also added the following text (underlined) to pgs. 7-8:
‘Children diagnosed with PFAPA syndrome according to the standard clinical criteria [5] were included in this study. They were assessed by a pediatrician experienced in the field and were described as having “typical PFAPA”. This designation was based primarily on clinical grounds; patients had recurring fever for, on average, 4 days with a fixed interval between febrile episodes and symptoms consistent with the acronym for PFAPA. Moreover patients lacked symptoms of, and ethnic predisposition to, hereditary fevers (see Subjects and Methods for details).’

2) Data shown on additional Figures 2 and 3 are much more convincing than Table 3 and give a clearer idea or the statistical differences among the three groups. I would leave Table 3 as a supplemental file.

In accordance with the reviewer’s recommendation, Additional Figure 2 and Additional Figure 3 now appear as Figure 1 and Figure 2 in the manuscript and Table 3 has been moved to a supplemental file, named Additional Table 2.

3) It is rather surprising to find low levels of WBCs in two febrile PFAPA patients.
How the Authors can be sure that the febrile episodes was not related to a viral infection? At least Authors should comment on it.

This is an interesting observation by the reviewer. Patients P05 and P06 had low levels of WBC yet high levels of CRP and SAA in blood samples that were drawn during a febrile episode. One limitation of our study is that the patients were not routinely re-assessed on the day of sampling if the febrile episode was consistent with past episodes. These two patients (P05 and P06) have a history and clinical features of “typical” PFAPA and, according to the parents, the febrile episode (that corresponded with a low WBC count) was not different than previous ones. From a clinical perspective, the high concentration of CRP and SAA are rarely seen in the early stage (< 24 hr) of a viral infection. In this context we are convinced that the febrile episodes experienced by patient P05 and P06 were caused by PFAPA. As the clinical picture of PFAPA can often be clearer later in a PFAPA episode, we suggest that sampling preceded an overt rise in WBC in these particular patients (P05 and P06). We have commented on these patients on pg. 9 as follows ‘Two notable exceptions were febrile patients P05 and P06 that did not have elevated WBC at the time of sampling even though the febrile episode was consistent with previous febrile attacks and CRP and SAA levels were elevated.’

4) Data on cytokine serum levels are quite interesting. However, even is statistically significant, it is rather difficult to draw a conclusion based on 3 points for each group only. Indeed, is not clear to me why only three patients for group were analyzed for each cytokine, whereas IP-10 serum levels are available from an higher number of patients and controls. How did the Authors selected the patients for the cytokine assay? Maybe it would be better to use a multiple cytokine kit as a screening method to identify the more relevant features. After this screening phase a dedicated kit should be used on a larger number of patients to verify the hypothesis arising from the first screening procedure (as correctly done for IP-10)

Our intention was exactly as the reviewer suggests, that is, to screen a small number of samples with the multiplex cytokine kit then verify the results on a larger number of samples using an independent (and less expensive) method. Samples collected subsequent to the multiplex analysis were included in the secondary analysis of IP-10 (Figure 4C, lower right panel), which is why more samples were available for this
analysis than for multiplex ELISA.

To clarify our choice of samples for multiplex analysis, we added text to pg. 7 (underlined below) as follows: Inflammatory mediators were analyzed in a subset of sera samples and supernatants …. To minimize variability in cytokine levels to the time of sampling, three febrile samples acquired at a similar time after the onset of fever (~15 hr.) were selected for multiplex analysis. An IP10 cytokine bead array assay (BD Biosciences) was used according to manufacturer’s protocols to confirm multiplex results on a larger sample set.

The rationale for selecting samples was reiterated on pg. 10 where it is stated that to reduce variability in results due to sampling time, ‘A multiplex bead ELISA was used to determine the concentration of classic pro-inflammatory cytokines TNFα, IL1β and IL6 in FP sera that was drawn at approximately the same time after the onset of fever (~15 hours, n=3)’

5) The experiment on monocytes is rather interesting. However it is really a pity that the Authors did not chose to add also a stimulation to the assay (LPS? PMA + inomycin?). This would add further interesting insights on the function of circulating PBMC from PFAPA patients.

We performed the experiment that the reviewer suggested; PBMC from controls and patients were stimulated 24 hr with 2 ng/ml PMA + 500 ng/ml ionomycin and with 10 ng/ml LPS then the tissue culture supernatant was analyzed with a multiplex cytokine kit. Of the 25 cytokines in the multiplex ELISA, 22 were induced by at least one of these two stimuli, thus PBMC from controls and PFAPA patients were viable and responsive to stimuli. We have added the following sentence to pg. 12: ‘Viability and responsiveness of PBMC was confirmed by an increase in cytokine production in response to LPS or PMA/ionomycin (data not shown).’ The effect of stimuli, in particular TLR agonists, on PBMC from PFAPA patients is the focus of future investigations by our group.