Author’s response to reviews

Title: MACROPHAGE ACTIVATION-LIKE SYNDROME: AN IMMUNOLOGICAL ENTITY ASSOCIATED WITH RAPID PROGRESSION TO DEATH IN SEPSIS

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Reviewer #1

- General comments In the present study 3,417 sepsis patients were identified from a Greece cohort by screening for infection and at least two SIRS criteria (study cohort). A second cohort of 109 patients from a previously published Swedish study with severe sepsis/septic shock was supposed to serve as validation cohort. In a second step the 3,417 and 109 patients were screened for meeting the Sepsis-3 definition and features of macrophage activation-like syndrome (MALS). MALS was diagnosed when the Sepsis-3 criteria were fulfilled and either a "HS score 2014" of more than 151 points (score for diagnosis of reactive hemophagocytic syndrome by Fardet et al.) and/or disseminated intravascular coagulation (DIC) along with hepatobiliary dysfunction (HBD) were present. HBD was defined by the presence of at least two of the following criteria: serum bilirubin > 2.5mg/dl, aspartate aminotransferase at least two times higher than the upper normal limit, and international normalized ratio (INR) higher than 1.5. DIC was defined as the presence of both: an absolute platelet count lower than 100,000/mm³ and activated partial thromboplastin time higher than 45 sec (p.9, l.9-12). The aim of the study was to investigate the frequency of MALS in septic patients as well as the development of a biomarker (ferritin) for MALS diagnosis and prognosis. Finally, 115 patients (3.4%) were diagnosed with MALS in the study cohort and 68 patients (4.0%) in the validation cohort. The mortality within 10 days was 48.9% and 45.2%, respectively. In the context of a ROC analysis in the study cohort, Figure 3B indicates a specificity of 97.4%, a sensitivity of 29.7%, NPV of 97.8% and PPV of 26.1% for a ferritin level above 4,420 ng/ml. The authors hereby conclude that ferritin may serve as a reliable biomarker for early detection of MALS (p.11, l.51-56). The OR for death after 28 days when ferritin exceeds 4,420 ng/ml was 4.07 in the study cohort and 3.75 in the validation cohort. Furthermore, the authors claim a correlation between elevated ferritin and a pro-inflammatory state indicated by IL-18, sCD163 levels and IL-10/TNFα ratio. Additionally, in 36 patients repeated ferritin measurements after 48 h were performed. Survivors after 10 days showed a significant decrease of serum ferritin in contrast to non-survivors (Figure 6A). According to the authors a second ROC analysis promised a reliable prediction of death after 10 days with a sensitivity of more than 90% when serum ferritin decreased greater than 20%. Finally, the OR for early death was shown to be significantly lower in patients with a decrease of serum ferritin more than 20% within the first 48 h (Figure 6C) Although this investigation tries to provide a solution for a clinically relevant issue (delayed identification of MALS in septic patients resulting in a delayed therapy initiation with a new and promising therapeutic approach with anakinra) there are several important concerns which need to be addressed in
connection with the presented manuscript: There are relevant weaknesses with regard to statistical analyses of the study as well as ensuing data interpretation: Based on the results displayed in figure 3B, the statement that serum ferritin (cutoff 4 420ng/ml) may serve as a reliable biomarker for early identification of patients suffering from MALs is misleading. A high specificity >97% permits a good performance in order to rule out MALs, whereas the performance of ferritin for the identification of MALS positive patients is low (with a sensitivity of <30%). Or in other words, the likelihood for false negative test results is high. Thus, the statement "our study shows that ferritin serves as a reliable biomarker……"(p. 11, l. 51-56) is misleading and needs to be revised. In addition, in the same sentence the author falsely stated a sensitivity of higher than 97% (p.11, l.53-56) instead of a specificity higher than 97% (again misleading).

Reply: Both statements were corrected, as suggested.

• Furthermore, if the displayed numbers in figure 3B (n patients) are correct, the results for sensitivity and PPV as well as specificity and NPV are mixed up. The sensitivity should be 26.1%, PPV 29.7%, specificity 97.8% and NPV 97.4%. But still, this does not alter the statement above.

Reply: Figure 3B was fully revised to address all discrepancies.

• In figure 5 the authors show a potential relation between ferritin and proinflammatory cytokines and postulate a correlation by comparing mean cytokine levels of patients with ferritin >4 420ng/ml and ≤4 420ng/ml. Although a correlation seems to be reasonable, I would suggest to perform a standard correlation analysis (Pearson, Spearman).

Reply: Spearman correlation tests were done, as suggested. This is described in the Methods on pg10, ln6-7 of the revised manuscript. Results are given on pg11, ln21-25 of the revised manuscript.

• But the displayed data is still of clinical relevance. A negative test result (ferritin ≤ 4,420ng/ml) seems to exclude a MALs in nearly every case

Reply: We do thank the reviewer for his comment. The manuscript now reads on pg15, ln3-9: "In light of the published evidence for sepsis-induced immunosuppression [3], someone may wonder to which state of immune activation a patient with high ferritin and low expression of HLA-DR on circulating monocytes lays. Our analysis outscored that ferritin above 4,420 ng/ml has very high NPV to exclude MALs. To this end, even when a patient presents with traits of
sepsis-induced immunosuppression, high concentrations of ferritin above 4,420 ng/ml should be considered diagnostic of MALS.”

- After critical evaluation of the manuscript, one might wonder about the diagnostic criteria for DIC. The diagnostic criteria used within the presented investigation (p. 9, l. 9-12) - only based on aPTT and platelet count - appears to be rather insufficient and might overestimate the incidence of DIC. A well-established scoring system with high sensitivity and specificity should be used instead (DIC Score of International Society of Thrombosis and Hemostasis (ISTH) or Japanese Association for Acute Medicine (JAAM)).

Reply: We agree with this reviewer that the generation of the MALS score should rely on robust criteria. To this end, we have rescored all our patients using the DIC score of the ISTH and we have repeated all calculations. As a consequence, Figures 2, 3 and 6 were fully revised. However, since cut-off criteria relied on the best specificity, the ferritin cut-off of 4,420 ng/ml still applied. Finally, through the changes of the number of patients with MALS, the change of ferritin on day 3 that was associated with unfavorable outcome after 10 days was found to be 15%. These are addressed on pg9, ln10-16, on pg10, ln22 to pg11, ln1 and on pg11, ln25 to pg12, ln2 of the revised manuscript. Furthermore, a new reference, namely reference 16, was added to cite the DIC ISTH score. Consecutive enumeration of the remaining references was changed accordingly.

- In contrast to non-survivors on day 10, a significant decrease in serum ferritin is described for survivors in the same period. Additionally, the OR for early death for patients with a decrease of serum ferritin over 20% within 48h is significantly lower. In this context, the interpretation of the second ROC analysis on p. 11, l. 24-29 is confusing. How can a decrease in ferritin greater than 20% predict early death after 10 days with a sensitivity >90%? Do we talk about survival?

Reply: We do apologize for the bad wording leading to misinterpretation of the ROC curve. This has been changed in the legend of Figure 6, on pg12, ln3-9 and on pg12, ln19-21 of the revised manuscript.

- The author showed, that the ferritin levels differed significantly between (MALS) survivors and non-survivors on day one and three (Figure 6A). In this context, an initial analysis of the ferritin levels of sepsis patients with and without MALS would be of great interest.

Reply: Figure 6 was revised to include panel B that refers to respective changes of ferritin among patients without MALS. The figure legend and the text on pg12, ln3 were revised accordingly.
• The total numbers (n-patients) on the right side in figure 3B seem to be wrong (287 instead of 101, 3130 instead of 3316).

Reply: Figure 3B was fully revised, as suggested.

• Why have IL-6 and IFNγ not been considered for further analysis of the pro-inflammatory state? Especially IFNγ levels might be of relevance. There is evidence that IFNγ plays a critical role in MAS pathogenesis and blockade of IFNγ is discussed as novel MAS therapy. Thus, levels of IFNγ could also be of great interest in MALS.

Reply: IL-6 and IFNγ were also measured, as suggested. This is reported in the methods on pg7, ln24 to pg8, ln1 and on Figure 1 of the revised manuscript. Results are provided in panels A and D of the revised Figure 5 and discussed on pg14, ln8-9 of the revised manuscript.

Reviewer #2:

• The topic of the study is very interesting, however, I have the following comments on its content and implementation: Background …poor definition of anakinra: anakinra is a recombinant, nonglycosylated form of the human interleukin-1 receptor antagonist (IL-1Ra).

Reply: The manuscript now reads on pg5, ln13-14: “Anakinra is a recombinant, nonglycosylated form of the human interleukin(IL)-1 receptor antagonist.”

• Patients and Methods: Study design: Did the study involve patients with sepsis or infection?

Reply: We thank the reviewer for this comment. Our study design involved patients defined with sepsis by the old 1991 definition. They were re-classified into infection and sepsis using the new Sepsis-3 definitions. These points are fully clarified on pg6, ln23-25 and on pg9, ln16-18 of the revised manuscript. The correct reference of the 1992 sepsis definitions has been used in the list of references.

• P. 8 - study endpoints……Why did the authors divide the patients into two cohorts and subsequently use the Swedish validation cohort?

Reply: The manuscript now reads on pg8, ln13-14: “To demonstrate robustness of findings, it was considered that a cohort coming from a different geographical region should be used.”
• Definitions of MAS and MALS are confusing. What exactly is the difference between MALS definition here and MAS definition commonly used in the literature? And why were two definitions used to fulfill the criteria of MALS? (lit 5 a 15)

Reply: The manuscript now reads on pg6, ln6-10: “Presence of bone marrow hemophagocytosis is one of the criteria taken into consideration for the diagnosis of MAS [6, 8, 9]. Since this criterion was investigated neither by Shakoory et al [5] nor by us in the current study due to the difficulty of performance in every critically ill patient, we prefer to call this entity as macrophage activation-like syndrome (MALS).”

• How is "immunodeficiency" defined in the authors´ HS score? In the original study referred to, it indicates clinical characteristics of the patients.

Reply: This is fully defined on pg8, ln22-24 of the revised manuscript.

• It is incorrect what the authors did in the case of MALS definition….i.e. compliance with the criteria for HS (p.8). HS is based on a certain number of parameters, each of them having a specific point score. A certain number of points has 90% sensitivity for the compliance with the criteria of the syndrome. The authors, unable to measure one parameter, deducted the number of points assigned to this character and considered the total "diminished" in this way to be of equal value as the original. However, the evaluation of 9 parameters is not the same as the evaluation of 8 characters…In addition, I do not understand why 18 points were deducted, when bone marrow aspiration is associated with 35 points???…..

Reply: This deduction by 18 points is explained on pg9, ln4-7 of the revised manuscript reading the following: “Since bone marrow aspiration providing 35 points (i.e. 10.4%) the maximal points was not done routinely in our patients, a deducted cut-off by 10.4% i.e. 151 points was applied in analogy to be diagnostic of MALS.”

• The ratio of IL10/TNF - is not used in clinical practice. The measurement of HLA-DR expression on monocytes would be far more beneficial.

Reply: We do agree with the reviewer. The manuscript now reads on pg14, ln11-19: “Usually a low ratio is compatible with hyper-inflammation. Our analysis showed that this ratio decreased when ferritin exceeded 4,420 ng/ml whatever was further supportive of high ferritin as a biomarker of hyper-inflammation. It should, however, be outscored that in everyday clinical
practice, the expression of the major histocompatibility complex II HLA-DR molecule on CD14-circulating monocytes by flow cytometry using fresh blood is a more easy way than the IL-10/TNFα ratio to assess the drive of anti-inflammation. Expression less than 30% is usually indicative of sepsis-induced immunosuppression [24].” A new reference, namely reference 24, has been added in the list of references. Consecutive enumeration of the remaining references has been changed accordingly.

- The following points should be discussed in more detail: In the studies published by Hotchkiss et al, dominant immunosuppression characteristic for patients with sepsis has been demonstrated. What is the authors´ opinion of the presence of MAS in the context of this immunosuppression and why is MAS present in such a small percentage of patients with sepsis? What explanation do the authors have for the dependence of this mortality on the decrease in ferritin on day 10?

Reply: The manuscript now reads on pg15, ln3-11: “In light of the published evidence for sepsis-induced immunosuppression [3], someone may wonder to which state of immune activation a patient with high ferritin and low expression of HLA-DR on circulating monocytes lays. Our analysis outscored that ferritin above 4,420 ng/ml has very high NPV to exclude MALS. To this end, even when a patient presents with traits of sepsis-induced immunosuppression, high concentrations of ferritin above 4,420 ng/ml should be considered diagnostic of MALS. This is further outscored by the association between increase of ferritin and early 10-day mortality that is an intrinsic characteristic of MALS.”

- Overall assessment: As has already been mentioned, the subject of the study is interesting but the general conception of the manuscript is confusing. It contains serious inaccuracies both in the form and content, and therefore requires substantial revision.

Reply: Every effort was done to fully revise the manuscript along the lines of suggestions of both reviewers.