Association between high mobility group box-1 circulation level and Graves' ophthalmopathy [version 1; peer review: 1 approved with reservations]

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Abstract

Background: Graves' disease is a prevalent autoimmune disorder that causes hyperthyroidism. Despite being widely recognized, the risk factors for its associated condition, ophthalmopathy, are not well understood. High Mobility Group Box 1 (HMGB1), a damage-associated molecular pattern biomarker, has been linked to autoimmune diseases and may play a role in Graves' ophthalmopathy. The aim of this study is to assess the correlation between the levels of circulating HMGB1 and the occurrence of Graves' ophthalmopathy (GO).

Methods: This cross-sectional study evaluated 44 recently diagnosed Graves' disease patients at Sardjito Hospital. The presence of Graves' ophthalmopathy (GO) was determined using criteria set by Bartley and Gormans. The levels of HMGB1 were measured in the blood of both groups (22 GO patients and 22 controls without GO) using ELISA. Statistical analysis, including binomial logistic regression and Mann-Whitney test, was conducted to analyze the data and adjust for confounding factors with multinomial logistic regression.

Results: The baseline characteristics of 22 GO patients and 22 non-GO patients were similar, including age (30.91±6.06 vs. 30.68±6.63 years, p>0.05), gender distribution (77.3% vs. 81.8% female, 22.7% vs. 18.2% male, p>0.05), and duration of diagnosis (5.13±2.21 vs. 4.82±1.89 months, p>0.05). However, a significant difference (p<0.001) was found in the levels of circulating HMGB1, with GO patients having a median value of 15.49 pg/mL (5.12-47.59 pg/mL) compared to 2.33 pg/mL (0.82-15.66 pg/mL) in the control group. The risk of developing...
ophthalmopathy increased 12 times when Graves disease patients had HMGB1 levels above 8.86 pg/mL.

**Conclusion:** The study found a significant association between elevated levels of HMGB1 (> 8.86 pg/mL) and an increased risk (12 times) of Graves' ophthalmopathy in newly diagnosed Graves' disease patients. The results suggest that HMGB1 may be a potential biomarker for predicting the development of ophthalmopathy in Graves' disease patients.

**Keywords**
Graves' disease, ophthalmopathy, High Mobility Group Box-1
**Introduction**

Graves’ disease is the most common form of hyperthyroidism, occurring in 1% to 1.5% of the general population. The underlying causes and mechanisms of Graves’ disease remain elusive. Autoimmune thyroid disorders like Graves’ disease involve complex immune processes. In Graves’ disease, the autoimmune response affects the orbital fibroblast tissue and triggers inflammation, leading to orbital fibroblast remodeling. Graves’ ophthalmopathy is a hallmark of Graves’ disease and an additional thyroid manifestation that is of great importance.

Graves’ ophthalmopathy occurs in 23% of cases before diagnosis, 39% after diagnosis, and 37% concurrent with diagnosis. The exact pathophysiology of Graves’ ophthalmopathy is not well understood, with numerous factors influencing its progression and contributing elements. The involvement of damage-associated molecular patterns (DAMPs), endogenous compounds released during cellular stress and injury, as well as non-apoptotic cell death, may contribute to the inflammation in Graves’ ophthalmopathy.

The connection between DAMPs and autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, psoriatic arthritis, and others, has been established. However, the role of DAMPs in the pathogenesis of autoimmune thyroid disease and Graves’ ophthalmopathy is not well understood. The immunological pathways involved in the progression of Graves’ disease and the onset of Graves’ ophthalmopathy, as well as the high incidence of Graves’ ophthalmopathy in patients who experience remission, suggest that DAMPs may play a crucial role in the development of severe Graves’ ophthalmopathy.

Studies in individuals with autoimmune thyroid disease showed elevated levels of HMGB1, which correlated with thyroid antibody levels in the body. Lacheta et al. investigated the impact of DAMPs on inflammation in Graves’ ophthalmopathy by conducting an orbital tissue biopsy to assess HMGB1 expression in orbital fatty tissue. They found an increase in HMGB1 expression that corresponded to the severity of Graves’ ophthalmopathy.

**Objectives**

This study aims to compare circulating levels of HMGB1 in two groups of Graves’ disease patients: those with ophthalmopathy and those without. The goal is to examine the relationship between HMGB1 levels and the presence of Graves’ ophthalmopathy.

**Methods**

This cross-sectional study compared two groups to assess the difference in blood circulation levels and the effect of HMGB1 in Graves’ ophthalmopathy. Graves’ Disease patients with ophthalmopathy comprised the case group, while Graves’ Disease patients without ophthalmopathy comprised the control group.

Participants were identified through medical records and assessed for eligibility. Eligible participants were scheduled for a screening visit where they provided informed consent and underwent a medical history, physical examination, and Thyrotropin Receptor Antibody (TRAb) level measurement. To be eligible, participants had to have been diagnosed with Graves’ disease within the past six months, be between the ages of 18 and 60, and have a Thyrotropin Receptor Antibody (TRAb) level greater than 1.75 IU/L. These criteria suggest that the study was focused on individuals who were in the early stages of Graves’ disease and had elevated levels of TRAb, which is a biomarker associated with the disease. Those who met eligibility criteria were enrolled and underwent baseline assessments including laboratory tests and questionnaires. Exclusion criteria included chronic comorbidities such as diabetes, heart failure, stroke, kidney failure, cancer, thyroid ablation/surgery, other eye conditions, and active autoimmune diseases to ensure participant safety and study validity.

In 1995, the Bartley and Gorman criteria were developed to diagnose ophthalmopathy. The criteria are composed of three main components: eyelid retraction, thyroid dysfunction, and specific eye-related symptoms. Eyelid retraction is characterized by the abnormal elevation of the upper eyelid. The second criterion, thyroid dysfunction, refers to the improper functioning of the thyroid gland, which can cause a variety of symptoms, such as weight gain or loss, fatigue, and temperature sensitivity. The third criterion involves the presence of eye-related symptoms, such as optic nerve dysfunction, exophthalmos, or extra-ocular muscle involvement. Optic nerve dysfunction can cause visual disturbances, while exophthalmos is a condition in which the eyes protrude from the sockets, resulting in a “bulging” appearance. Lastly, extra-ocular muscle involvement can lead to double vision or difficulty focusing due to weakened or dysfunctional eye muscles.

Circulating HMGB1 levels were measured using Enzyme-Linked Immunosorbent Assay (ELISA) method. A plasma blood sample was collected from the patients and analyzed using the HMGB1 express ELISA Kit from TECAN IBL International in Hamburg, Germany.
This study took place from September 2021 to February 2022 at RSUP Dr. Sardjito, in the Internal Medicine Clinic of Endocrinology Division, the Eye Clinic of the Oculoplasty Reconstruction Division, and the Oncology.

This study was previously approved by the subjects with written informed consent. The ethical committee of the Faculty of Medicine, Public Health, and Nursing at Universitas Gadjah Mada in Yogyakarta, Indonesia reviewed and approved this study under protocol number KE/FK/0980/EC/2021. The research adheres to the 2013 version of the Declaration of Helsinki, which outlines ethical principles for medical research involving human subjects.

Statistical analysis
The statistical methods used in this study include univariate and multivariate linear regression analyses to determine the standardized beta coefficients of independent variables and their association with Graves’ disease ophthalmopathy status, as well as independent sample t-tests, Mann-Whitney tests, and Fisher’s exact tests to compare characteristics between subjects with and without ophthalmopathy.

The statistical analysis for this study was conducted using R Studio, with support from the tidyverse and gtsummary packages. The tidyverse package was utilized for data manipulation and cleaning, while gtsummary was used to generate easily readable summary statistics and tables. It is possible that additional R packages were used for further data analysis.

Results
Characteristics of the research participants
The study included 22 patients with Graves’ disease and ophthalmopathy and 22 patients with Graves’ disease without ophthalmopathy who met the inclusion criteria. The clinical characteristics of the Graves’ disease patients without ophthalmopathy are outlined in Table 1.

Basic characteristics, including demographics, comorbidities, family history, and disease duration, were not significantly different between Graves’ disease patients with and without ophthalmopathy.

Differences in circulation level of HMGB1 between groups of Graves’ disease with ophthalmopathy compared no ophthalmopathy
HMGB1 levels were significantly higher in Graves’ patients with ophthalmopathy compared to those without, with a median value of 15.49 pg/mL in the ophthalmopathy group and 2.33 pg/mL in the non-ophthalmopathy group (p<0.001). The lowest and highest values were 5.12 pg/mL to 47.59 pg/mL in the ophthalmopathy group and 0.82 pg/mL to 15.66 pg/mL in the non-ophthalmopathy group.

According to the findings of this study, the levels of HMGB1 in the blood circulation were found to be significantly different between Graves’ disease patients with ophthalmopathy and those without ophthalmopathy (Figure 1).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall, N = 44</th>
<th>Ophthalmopathy Status</th>
<th>p-value²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yes ¹</td>
<td>No ¹</td>
</tr>
<tr>
<td>Age (years)</td>
<td>30.8 (6.3)</td>
<td>30.9 (6.1)</td>
<td>30.7 (6.6)</td>
</tr>
<tr>
<td>Gender (% male)</td>
<td>9/44 (20%)</td>
<td>5/22 (23%)</td>
<td>4/22 (18%)</td>
</tr>
<tr>
<td>Smoking (% yes)</td>
<td>6/44 (14%)</td>
<td>3/22 (14%)</td>
<td>3/22 (14%)</td>
</tr>
<tr>
<td>Free T-4 (ng/dL)</td>
<td>3.2 (1.1)</td>
<td>3.3 (0.8)</td>
<td>3.0 (1.2)</td>
</tr>
<tr>
<td>TRAb (IU/L)</td>
<td>7.6 (3.2)</td>
<td>9.0 (3.2)</td>
<td>6.1 (2.6)</td>
</tr>
<tr>
<td>Diabetes Mellitus (% yes)</td>
<td>4/44 (9.1%)</td>
<td>2/22 (9.1%)</td>
<td>2/22 (9.1%)</td>
</tr>
<tr>
<td>Hypertension (% yes)</td>
<td>4/44 (9.1%)</td>
<td>3/22 (14%)</td>
<td>1/22 (4.5%)</td>
</tr>
<tr>
<td>Family History (% yes)</td>
<td>7/44 (16%)</td>
<td>4/22 (18%)</td>
<td>3/22 (14%)</td>
</tr>
<tr>
<td>Dermopathy (% yes)</td>
<td>4/44 (9.1%)</td>
<td>2/22 (9.1%)</td>
<td>2/22 (9.1%)</td>
</tr>
<tr>
<td>Duration since diagnosis (months)</td>
<td>5.0 (2.0)</td>
<td>5.1 (2.2)</td>
<td>4.8 (1.9)</td>
</tr>
<tr>
<td>HMGB Box 1 (pg/mL)</td>
<td>9.1 [2.5-15.7]</td>
<td>15.5 [9.7-18.8]</td>
<td>2.3 [1.1-7.7]</td>
</tr>
</tbody>
</table>

¹n/N (%); Mean (SD); Median [Q1-Q3]
²Independent Sample t-test; Mann-Whitney-test; Fisher’s exact test
Before the regression analysis was performed, it was important to identify an appropriate cut-off value for the independent variable (HMGB1 level) which would be evaluated. This value was determined through analysis of the Receiver Operating Characteristic (ROC) curve, which aimed to find a cut-off value that had a high degree of both sensitivity and specificity. The results of the ROC analysis revealed a cut-off value of 8.86 pg/mL, with a sensitivity of 81% and a specificity of 73%.

Binary logistic regression analysis was then conducted to determine the impact of HMGB1 levels in circulation on the incidence of Graves’ ophthalmopathy. The results of this analysis, showed that patients with Graves’ disease and HMGB1 levels greater than 8.86 pg/mL had a 12 times higher risk of developing ophthalmopathy compared to those with HMGB1 levels less than 8.86 pg/mL (Table 2).

A stepwise analysis was conducted to determine which variables had the greatest role as predictors of the onset of Grave’s disease ophthalmopathy. The results showed that high levels of TRab and HMGB1 were the variables with the greatest impact on the status of ophthalmopathy in Grave’s patients. Multivariate analysis showed that even when controlling for TRab, there was still a significant statistical relationship between Grave’s ophthalmopathy and HMGB1. Table 2 showed that a high level of Hmgbox1 can increase the risk of developing Grave’s ophthalmopathy by 7.06 times with a p-value of 0.012 (95% CI 1.61-36.1)

Discussion
In the study, a significant difference was observed in the levels of HMGB1 between patients with Graves’ disease and ophthalmopathy and those with Graves’ disease without ophthalmopathy. The median levels of HMGB1 in the group with ophthalmopathy were considerably higher compared to the group without ophthalmopathy, with a median value of 15.49 pg/mL and 2.33 pg/mL, respectively. The results of the ROC curve analysis suggested that a cut-off value of 8.86 pg/mL for HMGB1 levels had a high degree of sensitivity and specificity in predicting the presence of ophthalmopathy in patients with Graves’ disease. Binary logistic regression analysis revealed that patients with Graves’ disease who had elevated levels of HMGB1 (above 8.86 pg/mL) had a risk of developing ophthalmopathy that was 12 times higher compared to patients with lower levels of HMGB1. These findings align with the previous study conducted by Han et al. that concluded that patients with symptomatic Graves had higher levels of HMGB1 compared to those with stable Graves or healthy individuals.
The study results showed a significant difference in the levels of HMGB1 in the circulation of Graves’ disease patients with and without ophthalmopathy. HMGB1, a known effector molecule involved in inflammation, is believed to play a role in the development of Graves’ disease and its associated complications, including ophthalmopathy. Its high levels have been linked with other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, where increased HMGB1 levels and increased numbers of HMGB1-producing cells are found in inflammatory areas. Through its interaction with RAGE and TLR receptors, HMGB1 contributes to the pathogenic inflammatory processes. The study findings suggest that HMGB1 may be a useful biomarker to reflect the level of inflammation in Graves’ disease patients, especially those with active disease, as increased HMGB1 secretion was found in response to high levels of pro-inflammatory cytokines such as IL-1 or TNF.13

The presence of elevated levels of HMGB1 in patients with Graves’ disease and ophthalmopathy suggests a potential role of HMGB1 in the development of ophthalmopathy. HMGB1 is known as a Damage-Associated Molecular Pattern (DAMP) molecule and is released by damaged or necrotic cells, or cells that die without undergoing apoptosis. This provides a theoretical explanation for the high levels of HMGB1 found in patients with Graves’ disease and ophthalmopathy.14

Damage-associated molecular patterns (DAMPs) play a dual role in the body as both intracellular regulators and extracellular signals of cell damage. They are typically not recognized by the immune system as long as they remain inside the cell, but are detected when they are released into the extracellular environment and trigger an immune response.14

The role of damage-associated molecular patterns (DAMPs) in the initiation of inflammation is essential for the survival of an organism. When cells are damaged or dying, DAMPs are released, acting as alarm signals to surrounding cells and activating the immune system. This process is known as the “danger signal hypothesis”.15 The immune system’s response to DAMPs includes the activation of both the innate and adaptive immunity, where innate immunity acts as the first line of defense and adaptive immunity provides a more specific and prolonged response. DAMPs activate immune cells, such as macrophages and neutrophils, which phagocytose cellular debris and release cytokines and chemokines, leading to an increase in blood flow and immune cell recruitment to the site of injury. This process helps to remove damaged tissue and promote tissue repair and healing.13 Additionally, the presence of DAMPs can also aid in the formation of immunological memory, helping the immune system to respond more effectively in the future to similar threats.15 Overall, DAMPs play a crucial role in the body’s defense mechanisms against cell damage and contribute to the regulation of tissue repair and healing.

The findings of Han et al. showed a strong correlation between the high expression of HMGB1 and its receptors with the inflammatory processes in Graves’ disease with ophthalmopathy. Their research also indicated that the high expression of receptors in orbital fibroblast tissues can lead to excessive inflammation, which contributes to the growth and

### Table 2. Univariate and multivariate linear regression analysis: standardized Beta coefficients of independent variables and Grave disease ophthalmopath status (n=44).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>OR 1</td>
</tr>
<tr>
<td>TRAb (IU/L)</td>
<td>44</td>
<td>0.69</td>
</tr>
<tr>
<td>HMG Box 1 (&gt;88.6 pg/ml)</td>
<td>44</td>
<td>12</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>44</td>
<td>0.99</td>
</tr>
<tr>
<td>Gender (Male)</td>
<td>44</td>
<td>1.32</td>
</tr>
<tr>
<td>Smoking (Yes)</td>
<td>44</td>
<td>4.4</td>
</tr>
<tr>
<td>Free T-4 (ng/dL)</td>
<td>44</td>
<td>0.73</td>
</tr>
<tr>
<td>Diabetes Mellitus (Yes)</td>
<td>44</td>
<td>1.32</td>
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<tr>
<td>Hypertension (Yes)</td>
<td>44</td>
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<tr>
<td>Family History (Yes)</td>
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<td>1.41</td>
</tr>
<tr>
<td>Dermopathy (Yes)</td>
<td>44</td>
<td>1.32</td>
</tr>
<tr>
<td>Duration since diagnosis (months)</td>
<td>44</td>
<td>0.92</td>
</tr>
</tbody>
</table>

1 OR = Odds Ratio, CI = Confidence Interval.

The role of damage-associated molecular patterns (DAMPs) in the initiation of inflammation is essential for the survival of an organism. When cells are damaged or dying, DAMPs are released, acting as alarm signals to surrounding cells and activating the immune system. This process is known as the “danger signal hypothesis”.15 The immune system’s response to DAMPs includes the activation of both the innate and adaptive immunity, where innate immunity acts as the first line of defense and adaptive immunity provides a more specific and prolonged response. DAMPs activate immune cells, such as macrophages and neutrophils, which phagocytose cellular debris and release cytokines and chemokines, leading to an increase in blood flow and immune cell recruitment to the site of injury. This process helps to remove damaged tissue and promote tissue repair and healing.13 Additionally, the presence of DAMPs can also aid in the formation of immunological memory, helping the immune system to respond more effectively in the future to similar threats.15 Overall, DAMPs play a crucial role in the body’s defense mechanisms against cell damage and contribute to the regulation of tissue repair and healing.

The findings of Han et al. showed a strong correlation between the high expression of HMGB1 and its receptors with the inflammatory processes in Graves’ disease with ophthalmopathy. Their research also indicated that the high expression of receptors in orbital fibroblast tissues can lead to excessive inflammation, which contributes to the growth and
development of ophthalmopathy symptoms such as angiogenesis and changes in the connective and adipose tissue. Based on these findings, it is proposed that the HMGB1 signalling pathway can be targeted and inhibited through its receptors as a potential treatment option for reducing the symptoms of ophthalmopathy in patients with Graves’ disease.\textsuperscript{13}

The study by Peng et al. demonstrated that the levels of HMGB1 and RAGE were elevated in monocytes collected from patients with autoimmune thyroid disease, including Graves’ disease, compared to monocytes obtained from healthy individuals. This indicates a crucial role of RAGE and HMGB1 in the development and progression of Graves’ disease.\textsuperscript{16}

**Association between HMGB1 levels in circulation and the occurrence of Graves ophthalmopathy**

The results of this study indicated that patients with Graves’ disease who had circulating HMGB1 levels greater than 8.86 pg/mL had a 12 times higher risk of developing ophthalmopathy compared to those with levels less than 8.86 pg/mL. This highlights the significant role of HMGB1 in the development of ophthalmopathy in Graves’ disease patients.

High levels of HMGB1 in the bloodstream can indicate a heightened level of DAMPs production, which acts as a marker for cell damage caused by overactive inflammation. The exact mechanism behind HMGB1 secretion is not fully understood. One possible explanation involves an inflammatory environment that triggers an increase in HMGB1 acetylation within cells, leading to its cytoplasmic translocation and release in response to a second stimulus. Research by Lu et al. suggests that inflammasomes, particularly NLRP3, play a role in the release of HMGB1 from cells.\textsuperscript{17}

HMGB1 functions as a DAMP molecule and signals damage to cells in the surrounding environment. It triggers inflammation and activates both innate and adaptive immunity through its interaction with various receptors. RAGE (receptor for advanced glycation end products) is one of the first identified receptors for HMGB1 and is a multifunctional protein of the immunoglobulin superfamily. In normal conditions, RAGE expression is limited in most tissues, but it increases significantly in pathological situations like inflammation.\textsuperscript{15}

Fibroblasts located in the connective tissue of the orbit are called orbital fibroblasts and have been identified as target cells on Graves’ ophthalmopathy. Orbital fibroblasts play an important role in lymphocyte infiltration and differentiation of B cells. IL-1β also regulates the expression of cyclooxygenase-2 by increasing its gene promoter activity and mRNA stability in fibroblast and encourages the synthesis of prostaglandin-E2 in orbital fibroblasts. CD40L cells promote the synthesis and secretion of hyaluronic acid, IL-6, IL-8, and CCL2 in fibroblast. IL-6 can facilitate immunoglobulin synthesis, plasma cell development, IL-4 production and differentiation of subsets of T cells into Th2 cells. CCL2 and IL-8 are powerful monocyte chemotactic factors that increase the infiltration of monocytes into orbital connective tissue in Graves’ disease with ophthalmopathy patients.\textsuperscript{19}

Orbital fibroblasts, fibroblasts found in the connective tissue of the orbit, play a crucial role in the development of Graves’ ophthalmopathy. These cells are involved in B cell differentiation, lymphocyte infiltration, and cytokine production. They are known to be responsive to signals from cytokines, such as IL-1, CD40L, and IL-6 (17). IL-1, an inflammatory cytokine, affects the expression of the enzyme cyclooxygenase-2 (COX-2) in fibroblasts, which boosts its gene promoter activity and mRNA stability. It also stimulates the synthesis of prostaglandin-E2 in orbital fibroblasts. CD40L, another cytokine, stimulates fibroblasts to produce and secrete hyaluronic acid, IL-6, IL-8, and CCL2. IL-6, in particular, has the ability to promote immunoglobulin synthesis, plasma cell development, IL-4 production, and Th2 cell differentiation from certain subsets of T cells. Meanwhile, CCL2 and IL-8 are powerful monocyte chemotactic agents that increase monocyte infiltration into the orbital connective tissue of Graves’ disease patients with ophthalmopathy.\textsuperscript{19}

These findings highlight the significance of orbital fibroblasts in the pathogenesis of Graves’ ophthalmopathy and emphasize the importance of targeting these cells to control the progression of the disease.

While the study’s strict inclusion and exclusion criteria and standardized diagnostic criteria enhance the internal and diagnostic validity, the study’s limitations including a small sample size, cross-sectional design, single-point measurement of HMGB1 levels, lack of control for potential confounding variables, and single-center location may constrain the generalizability and causal interpretation of the findings.

**Conclusion**

The results of the study indicate a strong correlation between elevated levels of circulating HMGB1 and the occurrence of Graves’ ophthalmopathy. The findings offer new perspectives on the management and treatment of ocular disease in Graves’ patients by administering anti-HMGB1. By reducing HMGB1 levels, the study suggests that it may be possible to prevent the development of ocular disease and control its clinical activity in patients diagnosed early on. These results provide important implications for the clinical management of Graves’ disease patients with ocular manifestations.
Data availability

Underlying data

Figshare: hmgb1_robi.xlsx, https://doi.org/10.6084/m9.figshare.22126892.19

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

References

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Current Peer Review Status: ?

Version 1

Reviewer Report 18 July 2023

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The manuscript “Association between high mobility group box-1 circulation level and Graves’ ophthalmopathy” investigated the correlation between circulating HMGB-1 with ophthalmopathy associated with Graves’ disease with additional inclusion of basic parameters in their analysis. While the findings in this study are interesting, there are several points that need to be addressed.

Major points:
1. The authors might be better served to expand their analysis to include the clinical parameters of the patients in their analysis. For example, they have already mentioned in the discussion themselves, other factors could be involved in GO pathogenesis, such as inflammatory cytokines that could be produced by the immune cells. As such, it would be wise for the authors to also take this into consideration and include immune cells-related clinical parameters (e.g. WBC counts or leukocyte differential counts) in their model.

2. Of course, inclusion of other basic clinical and laboratory findings would also strengthen their results.

3. Saying in the conclusions (both abstract and in the main text) that their results may be able to “predict” GO or “reducing HMGB-1 can prevent” GO might be jumping the gun a little, as their current study design is not able to do much more than simple association. The authors might want to be a little careful in their choice of words.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate? Yes

Are all the source data underlying the results available to ensure full reproducibility? Yes

Are the conclusions drawn adequately supported by the results? Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Cardiology, Respiratory Medicine, Lung Development

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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