Author’s response to reviews

Title: Clinical and Biochemical Factors to Predict Biochemical Adrenal Insufficiency in Hospitalized Patients with Indeterminate Cortisol Levels: a Retrospective Study

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Responses to reviewers

We thank the reviewers for their careful reading of the manuscript and their constructive comments. We have taken all the comments to improve and clarify the manuscript. Please find below a detailed point-by-point response to all comments (reviewers’ comments in bold black, our replies in non-bold black).

Since the reordering and restructuring of the manuscript was substantial, we have written bullet points of our major changes to the manuscript. Also, line numbering was referred to the revised manuscript with track changes.

• According to reviewer#2, in order to exclude the patients with normal adrenal function, the inclusion criteria has been changed. Those with serum 0800 hr cortisol between 3-14.9 µg/dL were included instead of using 3-17.9 µg/dL. Therefore, the new sample size is 128 patients (10 patients excluded).

• New Table 1 (demographic data) was created accordingly.

• Statistical power for our final sample size was still more than 80%.

• According to reviewer#1, all continuous predictive factors were categorized by using median of each value (random cortisol, cholesterol, sodium).

• Therefore, the new predictive model which is easier to interpret has been created. Significant weight loss and systolic blood pressure as the predictors were removed from the model as they showed no statistically significant result. The AUC-ROC of the final model still demonstrated a high diagnostic accuracy (>80%).
• The main concept is still the same.

• The new Table 2, Figure 1 and 2 have been created accordingly.

Please see the full version with Tables and Figures of responses to reviewers in the attached supplementary file**

Claudine Angela Blum (Reviewer 1):

This is a small retrospective analysis on clinical and biochemical predictors of adrenal insufficiency in hospitalized patients. One very impressive result is that random cortisol in this cohort is a better predictor for adrenal insufficiency than 08h00 cortisol.

What I am missing here is a potential bias through a smaller number of patients having a 08h00 cortisol than the random cortisol sample. If this is the case, I would expect this to be a limitation. Thank you for raising this issue. In our cohort, all patients have performed both 0800h cortisol and 0-minute cortisol (random cortisol). We have further stated this issue in the method section (Page 5 Line 7-8). Therefore, this issue may not be a limitation.

Apart from random cortisol level, a higher cholesterol, if I understood correctly, lower creatinine, and lower serum sodium were biochemical predictors of adrenal insufficiency.

A large subset of patients had a history of exogenous steroids including herbal remedies. Therefore, I invite the authors to discuss whether elevated cholesterol and a lower creatinine could be a side effect of previous steroid exposure - lower creatinine through steroid-induced sarcopenia. I would rather suggest these two biochemical indicators are a surrogate marker for previous steroid exposure. Hyponatremia, however, is a well-known concomitant phenomenon of adrenal insufficiency.

Thank you for raising this interesting comment.

According to our previous results before revision, we found that the lower cholesterol level, the higher the risk of having AI (as the OR of serum cholesterol was <1). For serum creatinine, the higher serum creatinine level, the higher the risk of having AI (as the OR of serum creatinine was >1).

In this revised manuscript, the results are still in the same direction (Table 2). In those with CKD (high creatinine), the higher the risk of AI (based on OR>1) and the lower the cholesterol level (<150 mg/dL), the higher the risk of having AI.

Therefore, we suppose that the high prevalence of patients using exogenous steroid may not explain the association with low cholesterol and high serum creatinine.

We tried to discuss about the underpinning physiology for the association that we have found between serum cholesterol, creatinine, serum sodium and AI on the discussion section Page 10-11). Also, please see the response to the below comment.
It would be interesting to know whether these three markers were similarly distributed between patients with and without previous exogenous steroid exposure. However, I understand that due to the small number of patients with adrenal insufficiency, the subgroups might be too small for comparison.

As explained in the above comment, the results that we have found may not link to exogenous steroid use.

However, we have tried to analyse the distribution (by Student’s t-test) of serum creatinine, cholesterol and serum sodium categorized by history of exogenous steroid use. The data are as shown below.

<table>
<thead>
<tr>
<th>Exogenous steroid use (n=38)</th>
<th>No exogenous steroid use (n=90)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine 1.47±1.99 1.23±1.25</td>
<td>0.411</td>
<td></td>
</tr>
<tr>
<td>Serum cholesterol 161.31±46.49 144.81±48.31</td>
<td>0.060</td>
<td></td>
</tr>
<tr>
<td>Serum sodium 136.40±5.61 136.04±6.80</td>
<td>0.760</td>
<td></td>
</tr>
</tbody>
</table>

The baseline level of serum cholesterol, creatinine and sodium showed no significant difference between those who used exogenous steroid and those who did not use exogenous steroid.

In the limitations section, I encourage the authors to highlight that due to the retrospective nature of the study, no conclusion can be drawn on cause or effect of the results.

Thank you. We have further addressed this limitation in the discussion section (Page 14 Line 7-8)

As the authors aim to create a predictive model, I suggest giving cutoff values with probabilities. Crude numbers do not allow to calculate probability of adrenal insufficiency or individuals, as the crude values don't differ effectively between groups. Did you try cutoffs for developing a practical model?

Thank you for this very helpful comment.

Yes, we plan to conduct the predictive model in our future study. As suggested, we have re-analyzed the data and categorized all continuous data to the categorical data based on median values (cortisol <10 µg/dL, cholesterol <150 mg/dL, sodium <140 mEq/L and using CKD defined by eGFR instead of creatinine level).

After the data were categorized, some of the factors (weight loss and systolic blood pressure) were removed from the model as they showed no statistically significant result.

Thanks to your helpful comment, our new model still demonstrates high ROC level (0.83), easier to interpret and give a promising result for future development of scoring system. New data are shown in Table 2 and Fig 2.

Table 1: Lines with n (%) in the cell with indication of test shifted. Please adjust (separate lines for each item?)

All the Tables have been adjusted as suggested. Thank you.

In summary, this manuscript nicely describes clinical and biochemical predictors for adrenal insufficiency in a small cohort of hospitalized patients

Giuseppe Reimondo (Reviewer 2): The study addresses the interesting issue of the undiagnosed adrenal insufficiency in hospitalized patients. The Authors interestingly try to define an index of suspicion to perform ACTH stimulation test.
Some concerns derive from reading the study.

1. The Author consider as inclusion criteria a wide range of 8 h cortisol levels, without any distinction between the different categories defined by the guidelines (<3, 3-15, >15). Thus, patients with cortisol levels >15, that predict a normal adrenal function, are also included. My suggestion is to reconsider the results after having categorized the patients in the 3 groups.

Thank you for raising this issue.

As suggested, we have re-analyzed the data and excluded the patients with serum morning cortisol >15 from our cohort. Therefore, 10 patients had been excluded (new sample size=128). The statistical power of this new sample size is still the same. Only those with serum morning cortisol between 3-14.9 µg/dL were used in the analysis.

The predictive factors in the new model were cirrhosis, Cushingoid, CKD, random cortisol, cholesterol and serum sodium. Weight loss and systolic blood pressure were removed from the model as they showed no statistical significant. The new area under the curve of new ROC was 0.83 which showed high diagnostic accuracy.

2. How many days after admission was performed ACTH test? Are there any differences between patients evaluated in the morning or in the afternoon? Was the test repeated after discharge in some patients?

All patients had undergone ACTH stimulation tests within 2 days of admission. We apologized that the data regarding the time of the tests (morning or afternoon) and data on follow-up cannot be obtained. However, there was a study showed that ACTH stimulation tests can be performed anytime of the day with no effect on the results (Munro V.Clin Biochem.2018 Apr).

3. Which was the best predictive threshold of 8 h cortisol level for AI?

We did not incorporate 0800hr serum cortisol in our model as it showed lower diagnostic accuracy (AuROC) than using serum random cortisol. We instead used random cortisol together with other clinical and biochemical factors in our final model. Please see the comparing area under ROC of these 2 models below.

According to Reviewer#1 comment, we have categorized all continuous variables to categorical variable by using median values for each variable and re-analyzed the model. For serum random cortisol the best predictive threshold for AI is at 10 µg/dL.

4. The suggestion to use random cortisol levels require more caution since it is not demonstrated in a prospective head to head study and derive from a slight difference obtained by an area under the curve.
Thank you for raising this issue.

Previously, there were 2 studies (Struja et al. Endocr Pract. 2017 Aug;23(8) and Manosroi et al. PLoS One. 2019 Nov 18;14(11)) which conducted in outpatient department revealed that serum basal cortisol (random cortisol) showed high diagnostic accuracy.

Struja et al. demonstrated that serum basal cortisol had high diagnostic accuracy. Also, they have proposed the lower and upper cut-off for basal cortisol which gave fairly good accuracy. A retrospective study from Manosroi et al., showed that basal cortisol (random) gave higher diagnostic accuracy than 0800hr cortisol. However, these 2 studies were retrospective in nature and future prospective studies are needed to validate these results.

We have added these 2 publications and further discussed about this issue and limitation in the discussion section (Page 10 Line 12-15)

5. How many patients were in lipid lowering treatment? How this result can influence the correlation between low cholesterol levels and the risk of biochemical AI?

Thank you. This issue is very interesting. There were 16 patients (16/128=12.5%) in our cohort who were on lipid lowering medications.

Below tables are data of patients with or without lipid lowering medication therapy categorized by low cholesterol and adrenal insufficiency.

<table>
<thead>
<tr>
<th>Cholesterol &lt;150mg/dL</th>
<th>Cholesterol ≥150 mg/dL</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid lowering meds</td>
<td>9 7</td>
<td>0.892</td>
</tr>
<tr>
<td>No lipid lowering meds</td>
<td>65 47</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Adrenal insufficiency</th>
<th>No adrenal insufficiency</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid lowering meds</td>
<td>12 4</td>
<td>0.766</td>
</tr>
<tr>
<td>No lipid lowering meds</td>
<td>32 80</td>
<td></td>
</tr>
</tbody>
</table>

There is no significant difference between lipid lowering medication use and low cholesterol or adrenal insufficiency. Therefore, we presume that this lipid lowering medication may not interfere with the result.

We have further discussed this issue in the discussion section (Page 11 Line 1-3)