

Answers to Reviewer 2

Thank you very much for acknowledging of our research data. In accordance with your suggestions, we have revised the paper and supplemented the limitations section which we are convinced contributed to the improvement of the quality of our manuscript. Since the comments and suggestions for improvement largely overlap, we will answer them together. Please find our point to point responses below.

Constructive points on how to improve this study:

1. (and Question 12) The sample size is very small ($n=7$), making it difficult to draw definitive conclusions. A larger sample would allow for more robust statistical analysis.

We thank the reviewer for this comment. As aHUS is a rare (orphan) disease with a very small annual number of cases, in Hungary, we can expect the occurrence of 1-2 cases per year, the number of cases that are actually diagnosed is around 0-1.

We treat all pediatric patients with aHUS in our center. Due to the small number of cases, only descriptive statistics are possible in this study.

Accordingly, we have supplemented the "limitations" chapter.

2. (and Question 13) There is no control group for comparison to the treatment group. Including a control or comparison group could strengthen the evidence for the efficacy of the treatment protocol.

We fully agree with the reviewer on this issue. However, referring to the previous question and answer, aHUS is an orphan disease, so it would be very difficult to compare patients with an untreated control group. After the introduction of C5 blockade, eculizumab therapy is the first-line treatment for aHUS, if available. (Pediatr Nephrol. 2023; 38(1):61-75.). Thus, it wouldn't be ethical to treat patients only with immunosuppression and PE. For comparison, we also refer to international data before the introduction of C5 blockade, concluding, that prompt plasma exchanges and immunosuppressive treatment improves the outcomes of anti-factor H autoantibody-associated hemolytic uremic syndrome in children. (Kidney Int. 2014; 85(5):1151-60. <https://doi.org/10.1038/ki.2013.373>., see reference 11)

3. (and Question 14) The study design is retrospective and observational. A prospective, randomized controlled trial would provide higher quality evidence.

We also fully agree with this comment. However given the nature and rarity of the disease in this study, we had the opportunity to analyze the data of all patients with antibody-mediated aHUS and TTP in Hungary. In the case of rare diseases, different levels of evidence - so retrospective analyzes and case series can form the basis of expert opinion. Our results could provide further data and impulse to plan a future international study in the era of C5 blockade to analyze the effect of additional immunosuppressive treatment.

We have supplemented the limitations paragraph with the shortcomings of the study design.

4. (and Question 15) Details of the treatment protocol and dosing are sparse. Providing more details would improve reproducibility.

Thank you for your comment, the description of the therapy has been supplemented as requested in the Treatment protocol paragraph.

5. (and Question 16) Longer follow-up of patients would help better determine long-term outcomes like kidney function.

We thank the reviewer for this comment. Of course, lifelong follow-up is the ultimate goal, however, in pediatrics and in this rare disease, a 10-year follow-up is also rare in the literature. There are 15 years of follow-up data available on HUS caused by EHEC (single acute kidney injury due to verotoxin), where serious late consequences were also described. [Pediatric Nephrol. 2023 Jul;38(7):2131-2136.]

The fact that in our study we see the development of CKD and hypertension after 10 years draws attention to the importance of regular follow-up, which is also the main message of the paper: the long-term effects of the initial damage must be taken into account even in remission without the return of the underlying disease. That statement is already included in the discussion

6. (and Question 17) The role and necessity of each component of the immunosuppressive regimen is unclear. Further study on optimal treatment combinations could help refine the protocol.

We fully agree with the reviewer's comment, which also reflects current recommendations for research to be conducted.

Currently complement blocking therapy, eculizumab appears to be effective in anti-CFH antibody-HUS. However, eculizumab is not expected to inhibit anti-CFH antibody production and a spontaneous decline in anti-CFH antibodies is rare. Since our observational study, mycophenolate mofetil was used as well as a maintenance therapy for patients with antibody associated aHUS (ref 11). According to the international guideline, additional studies are required to establish the respective place of eculizumab, plasma exchange, cyclophosphamide pulses, rituximab and MMF for optimal treatment of anti-CFH antibody-associated HUS. (Pediatr Nephrol 2016; 31:15–39). Our case series can provide additional follow-up data in this regard.

7. (and Question 18) Objective criteria for remission should be defined, rather than stating "clinical remission."

Thank for this suggestion. Patients were classified as achieving complete thrombotic microangiopathy response if they reached haematologic normalization and a $\geq 25\%$ decrease in serum creatinine persisting for ≥ 4 weeks. A haematologic normalization was defined as platelet count normalization ($\geq 150 \times 10^9/L$) and LDH normalization (< 1.5 times the age-matched upper limit of normal. Each normalization should be maintained for ≥ 4 weeks. (Nephrol Dial Transplant. 2023; 38:414-424. PMID 35438790)

The definition of complete thrombotic microangiopathy response has been added to manuscript Methods. A new reference was added to manuscript as well.

8. (and Question 19) Statistical analysis is limited. More rigorous statistical approaches could identify significant relationships in the data.

Thank you for this suggestion. As we pointed out, aHUS and TTP are orphan diseases with a very low incidence. Due to the rarity of antibody associated aHUS and TTP, only case series are available from other pediatric nephrology centres (Kidney Int. 2014 May;85(5):1151-60.; Pediatr Nephrol. 2021;36(4):917., Pediatr Nephrol 2016; 31:15–39)

During the follow-up period, all patients with antibody-mediated TMA were involved in our center in Hungary. Based on the results of the case series, only an international multicenter study could answer other relationships. Our results could help plan another randomized study. (and Question 20)

A note referring to this is included in the limitations.

9. The study population is quite heterogeneous, including aHUS and TTP patients. Focusing on one disease may allow stronger conclusions; or may provide subgroup analysis

We agree with the Reviewer's comment, however, since both diseases are orphan, a larger number of cases is not expected within one center, and the number of cases does not allow subgroup analysis either.

The common point of the two patient groups is, that both have have antibody-mediated TMA and similar immunosuppression was applied.

10. (and Question 21) Mechanistic studies on how the treatment works would be informative. Assessing antibody levels over time may provide insights.

Thanks for this comment, to which we have given a theoretical answer in the discussion, indeed the small number of cases does not allow for further conclusions.

The combination of individual immunosuppressive agents is based on the following:

Steroid pulses were used as first-line immunosuppression combined with PE. Cyclophosphamide was used to achieve long-term effects, while RTX was used as specific anti-B cell treatment in a non-specific attempt to block the reappearance of the FH antibodies.

The rationale for combining RTX with cyclophosphamide is based on the differential effect of these indications across the B-cell lineage. RTX is a B-cell depleting monoclonal antibody which binds to cluster of differentiation (CD) 20 expressed on the surface of B-cells while it has little direct effect on the antibody-producing plasmablasts and plasma cells that do not express CD20. [Ref 26,27]

The addition of cyclophosphamide can target autoantibody-producing plasmablasts and short-lived plasma cells, targeting cells not affected by anti-CD20 antibodies. [Ref 28] In our study with regard to the combined treatment the number of cyclophosphamide shots (and its cumulative dose) did not reach the dose used in immune-mediated glomerular diseases (systemic lupus erythematosus vasculitis, rapidly progressive glomerulonephritis). [Ref 29]

As for the antibody titers, as shown in the tables that they were within the normal range at the end of the observation period.

Complement profile and the presence of antibodies was reassessed at all patient visits. Without maintenance therapy, the complement profile was balanced in Patient 2-5, and there was no evidence of complement consumption or complement activation. Factor H titers and C3 levels were within normal range.

Accordingly, we supplemented the limitations with a relevant short statement