Peer Review File

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Reviewer A

• Overall comments: This is a well-written paper about risk factors for delayed methotrexate clearance and infection. The flow of the paper, the explanation of the results, and the discussion are clear. With improvement, this paper can be a good addition to the extant literature.
• However, I have a few significant concerns about the analysis, such as how the thresholds of age >9 and 24hr mtx cutoff > 64 were determined. If there was exploration of the data to find these thresholds beyond what is reported in the paper, that needs to be disclosed and justified.
Response 1: A receiver operating characteristic (ROC) curve analysis was performed to select the optimal cut-off value for the various continuous characteristics.
Changes in the text: None.

• My other concern is about the nature of the presentation of the findings. None of these findings are new. There have been many, many such studies evaluating risk factors for HD MTX toxicity. Each of the primary findings is well attested in the literature but one would not know it from the "what is known and what is new" box nor the main body of the paper. Although the results are not novel, my concern is not lack of novelty. My concern is they are not well situated in the current body of knowledge, and this obscures their value. Their value is that they add to the literature as confirming (or perhaps cutting against) what has been described in larger patient cohorts around the world, yet very little of this work is discussed. My recommendation is that in the introduction and in the discussion for the sections regarding the risk factors for delayed excretion and for infection, the results should be better compared and contrasted to previous work. Where do they confirm what has been described? Where do they disconfirm? How is that relevant for the particular population being studied in this paper?
Response: Thank you for your advice. In addition to delayed excretion of methotrexate, other factors affecting infection include reduced blood pattern after chemotherapy and chemotherapy drugs with strong myelosuppressive effect. Few studies have analyzed the relationship between them separately, and this phenomenon is only listed in this paper, which has no relationship with the specific population studied in this paper.
Changes in the text: None.

Specific comments:
• Abstract
o Would be nice to know how they define delayed excretion in methods.
Response: In this study, MTX 44th hour blood-drug concentration C44h >1.0 μmol/L and C68h >0.3 μmol/L were used as the criteria for delayed excretion. (Page6 Line152-153)

• Introduction
o (Line 91) Where do the thresholds for delays excretion come from? The citation (3) is regarding a CBF AML study, which does not use methotrexate. The citation needs to be fixed. Moreover, this threshold needs to be contextualized. There are different thresholds for different doses and duration of MTX infusions. It is unclear to which setting your thresholds apply.

Moreover, in the United States for children with ALL who receive 5g/m2 of mtx, the threshold is less than or equal to 0.4 at 48 hours or 0.1 at 72 hours. It's ok that the thresholds are different but please clarify where these care from. Later in the discussion, it is mentioned the Chinese guidelines recommend a similar practice. It seems relevant for the reader to understand why there are differences in practice and the recommendation.

1. **Response:** Reference 3 has been corrected due to an incorrect DOI number provided in the original text. Our definition of delayed MTX excretion and rescue measures are referred to Chinese Children's Cancer Group study acute lymphoblastic leukemia study plan 2020. We defined the MTX concentration > 0.3μmol/L at the 72nd hour as delayed excretion because our instrument for detecting blood concentration could only measure 0.3μmol/L at the minimum.
   Changes in the text: Page 13 Line 408-409 Reference 3 （3. Hamed KM, Dighriri IM, Baomar AF, et al. Overview of Methotrexate Toxicity: A Comprehensive Literature Review. Cureus. 2022 Sep 23;14(9):e29518. ）

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o (Sentence starting line 91) There is abundant research about risk factors for delayed HDMTX excretion in children and adults. The authors should interact with this large body of work in their introduction because it does not correctly represent what is known about these risk factors.
Response: Studies in adults showed that high doses of MTX, high body surface area doses, and patient age > 60 years were risk factors for delayed MTX excretion.
Changes in the text: None.

o General comment: The introduction appears to be one large paragraph. Breaking up the discussion into smaller paragraphs would help the reader to understand the presented information.
Response: Thanks for your advice, the introduction has been divided into several paragraphs.

• Methods
o (Line 148) Clarification in introduction will help the reader understand where these threshold came from, as they are different than much of the literature.
Response: Specific rescue plans have been added.
Changes in the text: Page 5 Line 151-154（according to the Chinese Children's Cancer Group study mature B non-Hodgkin lymphoma study plan 2015 (CCCG-BNHL-2015) and the Chinese Children's Cancer Group study acute lymphoblastic leukemia study plan 2020 (CCCG-ALL-2020). ）; **Page 11 Line 348-353**（ CCCG-BNHL-2015 (MTX 5 g/m^2 d1 + etoposide 100 mg/m^2 d3–5 + vincristine 3 mg/m^2 d1 + isocyclophosphamide 1,200 mg/m^2 d1–5 + prednisone 60 mg/m^2 d1–7). Children with lymphomas on this regimen have a higher risk of infection because the chemotherapy intensity is higher than that in consolidation therapy for children with CCCG-ALL-2020(MTX 3–5 g/m^2 + mercaptopurine 25 mg/m^2/day).）

o What was considered the baseline value for creatinine when assessing toxicities? This should be clarified.
Response: The baseline value for creatinine is（9-88）umol/L.
Changes in the text: None.

• Results

o A statement or perhaps table with demographic information about demographic and clinical information in the cohort is needed. It can be inferred from Table 1, but the reader needs an easy way to understand who we are analyzing.

Response: We supplemented the results with patient age, gender, disease diagnosis and other information. (L193-196)

o A statement about how many patients were not analyzed due to never having a methotrexate level

Response: All patients had their MTX blood levels measured during the study period.

Changes in the text: None.

o The number of sessions of HDMTX is interesting. As the study period was only 6 months, the first dose of HD MTX may not be observed for some patients and steps may have been taken to avoid toxicity in subsequent doses such as reducing the dose of MTX, increasing the amount of fluids or the dose or duration of leucovorin administration, or the 24 hour dose titration practices mentioned in the discussion. The univariable analysis analyzes dose but not these other practices. How do we know these clinical practices are not confounding the reported associations (e.g. previous toxicity from the last cycle causing changes with

Response: Generally, if the effects of the toxicity of the previous chemotherapy on liver and kidney function, blood pattern, etc. still exist, chemotherapy will be delayed until the toxicity is cleared up to the standard of the previous chemotherapy.

Changes in the text: None.

o (Line stating 204) The terms "unifactorial/multifactorial" analysis are used to describe this portion of the analysis and "univariate/multivariate" in the abstract and methods. For the "multi" label, consider "multivariable" as there are recommendations for this label in the literature (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3518362/). Whatever you decide, please use consistent terminology.

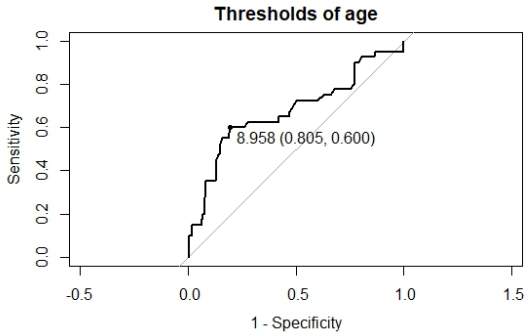
Response: Thank you for your advice. We have unified the use of the same term.

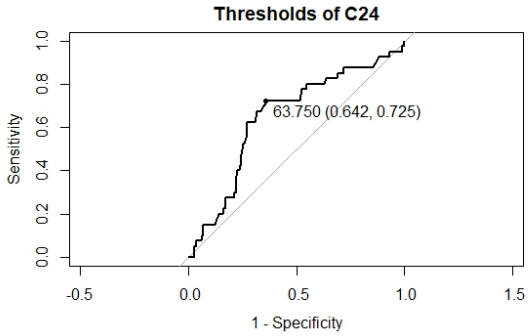
o How were the decisions to report age >= 9 years and mtx concentration >= 64 arrived at? Those appear to be arbitrary cutoff values. If these cutoffs were determined after exploration of the data with repeated tests across possible cutoff values, that should be disclosed, explained, and justified in the methods. Repeated testing to explore the space of possible cutoffs can inflate type I error and compromise the validity of the inferences, so a statistical explanation for how this was addressed is needed. As these are both continuous variables, an explanation why they were not analyzed as continuous variables should be included as well.

Response:

Thank you for raising this important concern. We determined the cutoff values using a receiver operating characteristic (ROC) curve analysis, selecting the optimal cut-off value for various continuous characteristics. This methodology has now been clearly described in the Methods section of the revised manuscript.

Your concern regarding the potential inflation of Type I error due to repeated testing across possible cutoff values is well-founded. However, our decision to use dichotomous variables instead of continuous ones was based on the non-uniform rise in hazard ratios, as evidenced by the lower p-values for dichotomous variables. For instance, when age was treated as a dichotomous variable with a cutoff at 9 years, the p-value was 1.58 x 10^-6, compared to 5.58 x 10^-4 when analyzed as a continuous variable. Similarly, for the 24-hour MTX concentration, a cutoff at 64 μmol/L as a dichotomous variable yielded a p-value of 1.42 x 10^-4, compared to 0.041 when analyzed continuously. The ROC curves clearly indicate significant inflection points for both age and 24-hour MTX concentration, supporting the use of dichotomous variables. Additionally, from a clinical perspective, dichotomizing these variables offers more meaningful guidance in defining high-risk patients for delayed MTX excretion.





Changes in the text: None.

o Table 2 & 5- please include which factors were used to adjust for these findings at least in a footnote in the table. Coefficient values are not necessary. "B value" is not a sufficient label. Are these log-odds? I suggest labeling it as "coefficient" value with clarification that these values came from a logistic model because it is unclear what "B value" means.

Response:

Thank you for your question regarding the factors included in the multivariate analysis as presented in Table 2 and Table 5. In our analysis, we only incorporated the factors listed in these tables, without adjustment for additional factors. This decision was made due to the presence of collinearity issues among certain variables. For example, when analyzing delayed excretion, there is a notable collinearity among height, weight, body surface area (BSA), and age.

Changes in the text: Page 16 Line 483-484 Table 2 and Page16 Line 489-490 Table 4

Table 2 Multivariate analysis of delayed methotrexate excretion

Factors	OR	95% CI for OR	P value
Age ≥9 years	4.725	2.053–10.873	<0.001
C_{MTX24h} ≥64 μmol/L	3.236	1.387–7.547	0.007

OR, odds ratio; CI, confidence interval; C_{MTX24h}, MTX 24th hour blood-drug concentration.

Table 4 Univariate analysis of methotrexate-induced infections in children

Variant	No infection group (n=106)	Infection group (n=93)	OR	95% CI for OR	P
MTX delayed excretion	14 (13.2)	26 (28.0)	2.55	1.24–5.25	0.011
MTX dose	3.63±1.82	4.18±1.65	1.2	1.02–1.41	0.029
MTX body surface area dose	3.86±0.989	4.54±0.753	2.3	1.65–3.21	<0.001
≥4 vs. <4	50 (47.2)	75 (80.6)	4.67	2.46–8.85	<0.001
C_{MTX24h}	59.7±35.5	74.8±38.4	1.01	1–1.02	0.006
≥64 vs. <64	35 (33.0)	49 (52.7)	2.26	1.27–4.01	0.005
C_{MTX44h}	0.426±0.882	0.955±1.68	1.52	1.08–2.14	0.015
Granulocyte deficiency	36 (34.0)	70 (75.3)	5.92	3.19–10.99	<0.001
white blood cell count	1.99±1.23	0.853±0.898	0.32	0.22–0.46	<0.001
Hemoglobin content	89.5±14.9	72.6±21.2	0.95	0.93–0.96	<0.001
Hemoglobin count <65 g/L	6 (5.7)	45 (48.4)	15.62	6.23–39.16	<0.001
Decreased platelet count	153±83.8	73.7±83	0.99	0.98–0.99	<0.001
Platelet count $<50\times10^9$/L	16 (15.1)	51 (54.8)	6.83	3.49–13.35	<0.001
Combination of drugs					
Prednisone	20 (18.9)	48 (51.6)	4.59	2.43–8.65	<0.001
Isocyclophosphamide	21 (19.8)	52 (55.9)	5.13	2.74–9.63	<0.001
Etoposide	18 (17.0)	48 (51.6)	5.21	2.72–9.99	<0.001
Vincristine	18 (17.0)	49 (52.7)	5.44	2.84–10.43	<0.001
Thiopurine	70 (66.0)	25 (26.9)	0.19	0.1–0.35	<0.001
Rituximab	7 (6.6)	25 (26.9)	5.2	2.13–12.7	<0.001
Pegaspargase	3 (2.8)	12 (12.9)	5.09	1.39–18.63	0.014

Data are presented as n (%) or mean ± standard deviation. OR, odds ratio; CI, confidence interval; MTX, methotrexate; C_{MTX24h}, MTX 24th hour blood-drug concentration

o Table 5 - factor "Decreased white blood cell count" with a negative log-odds makes it look like the presence of "decreased WBC" lowers the log odds of infection, which doesn't make sense. Please relabel the variable to clarify what this represents or invert the log-odds and OR to be consistent with the label (decreased WBC count was associated with increased odds of infection)

Response： I'm sorry, this is a misrepresentation, this should be white blood cell count and hemoglobin content.

Changes in the text: Page16-17 Line 489-490 Table 4

'white blood cell count' and "Hemoglobin content"

Table 4 Univariate analysis of methotrexate-induced infections in children

Variant	No infection group (n=106)	Infection group (n=93)	OR	95% CI for OR	P
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MTX dose	3.63±1.82	4.18±1.65	1.2	1.02–1.41	0.029

MTX body surface area dose	3.86±0.989	4.54±0.753	2.3	1.65–3.21	<0.001
≥4 vs. <4	50 (47.2)	75 (80.6)	4.67	2.46–8.85	<0.001
C_{MTX24h}	59.7±35.5	74.8±38.4	1.01	1–1.02	0.006
≥64 vs. <64	35 (33.0)	49 (52.7)	2.26	1.27–4.01	0.005
C_{MTX44h}	0.426±0.882	0.955±1.68	1.52	1.08–2.14	0.015
Granulocyte deficiency	36 (34.0)	70 (75.3)	5.92	3.19–10.99	<0.001
white blood cell count	1.99±1.23	0.853±0.898	0.32	0.22–0.46	<0.001
Hemoglobin content	89.5±14.9	72.6±21.2	0.95	0.93–0.96	<0.001
Hemoglobin count <65 g/L	6 (5.7)	45 (48.4)	15.62	6.23–39.16	<0.001
Decreased platelet count	153±83.8	73.7±83	0.99	0.98–0.99	<0.001
Platelet count $<50\times10^9$/L	16 (15.1)	51 (54.8)	6.83	3.49–13.35	<0.001
Combination of drugs					
Prednisone	20 (18.9)	48 (51.6)	4.59	2.43–8.65	<0.001
Isocyclophosphamide	21 (19.8)	52 (55.9)	5.13	2.74–9.63	<0.001
Etoposide	18 (17.0)	48 (51.6)	5.21	2.72–9.99	<0.001
Vincristine	18 (17.0)	49 (52.7)	5.44	2.84–10.43	<0.001
Thiopurine	70 (66.0)	25 (26.9)	0.19	0.1–0.35	<0.001
Rituximab	7 (6.6)	25 (26.9)	5.2	2.13–12.7	<0.001
Pegaspargase	3 (2.8)	12 (12.9)	5.09	1.39–18.63	0.014

Data are presented as n (%) or mean ± standard deviation. OR, odds ratio; CI, confidence interval; MTX, methotrexate; C_{MTX24h}, MTX 24th hour blood-drug concentration

o (Line 211) - Did 43% of children experience fever DURING the infusion or within the 3 week observation window afterward? Please clarify.

Response: 43% of children experience fever within the 3 week observation.

Changes in the text: Page 7 Line 222(within the 3 week observation window afterward)

• Discussion

o (Line 281) The meaning of the statement that certain risk factors were found to be "statistically different" is unclear, please clarify. Moreover, only age was "significant" on the multivariable analysis, so what other factors is this statement referring? If there are reasons to interpret findings from the univariable analysis (and there may be), those reasons should be provided.

Response: "statistically different" should be independent risk factors for delayed MTX excretion. The factors with statistical significance were included in univariate analysis, and multi-factor logistic regression analysis was performed by stepwise forward method. The results showed that age ≥9 years old and MTX concentration ≥64μmol/L at 24 hours were independent risk factors for delayed MTX excretion.

Changes in the text: None**.**

o General concern - There is a huge body of literature describing each of these

o A paragraph stating the limitations of the study is needed. The sentence in the conclusion should be separated, moved into the discussion, and expanded.

Response: Limitations of this study have been supplemented.

• Strobe checklist

o Key sections of the strobe checklist are missing and should be addressed in the paper. These are Items number 9 (extremely important), 11 (per comments about age and 24hr mtx cutoff above), 12c (no mention of missing data at all, if there were none this should be stated, 19 (extremely important)

Response: The missing important strobe checklist has been added.

Reviewer B

This report looks at the side effects seen in 4 groups of patients who seem to be receiving different protocols making analysis very much more difficult. Although they consider that the patients received high dose methotrexate they define such a dose as being at least 500 mg/m2. They continue to state that this dosage is frequently used to treat malignant neoplastic disorders such osteosarcoma …., I can assure them that this is most certainly not true (The citation given to support this contention was of a report of treatment of Osteosercoma with 12g/m2 MTX). The dose they used would today be regarded as intermediate dose MTX. In spite of the low doses used (2-5gm/m2), 20.1% showed what they referred to as delayed excretion. There seems to be some confusion about the use of this term. It is not clear if this is what they call acute kidney injury that they defined as a rise in creatinine level of more than 50% the baseline value. The manuscript does not state how often this occurred in their patients. When the urine ph drops, MTX can crystalise in the renal tubules and this will prevent MTX adequate excretion. This occurs when the creatinine to rise by 25%. It is not possible to keep the urine alkali without giving Bicarbonate (or Lactate) this must be given before during and after the MTX with adequate fluids. When antiemetics such as ondensatron are given (that cannot be given with bicarbonate) it is imperative to give the antiemetic in another line since renal shut down can occur even if the bicarbonate is discontinued by as little as half an hour. I am at a loss to comment on the findings in this report when they do not mention the fluids given and did not even give any bicarbonate during the treatment. All this is standard procedure with the treatment of pediatric malignancies for the last 20 years (see CohenIJ. high dose methotrexate is effective in Osteosarcome so what is the problem? J Pediatr Hematol Oncol 2009;31;892-4.)

Response：

Dear judges, the article said that MTX dose ≥500mg/m^2 is a high dose of MTX, and it is not wrong to be used in the treatment of osteosarcoma, because the dose of 12g/ m^2 you said is also ≥ 500mg/m^2, isn't it? In addition, the patients included in this paper do not have osteosarcoma patients, so you do not need to worry about this problem. In addition, you mentioned the use of carbonate alkalization of urine, we have made it very clear in the method, please refer to the original method section, thank you. With respect to creatinine values, none of the patients in this study exceeded the maximum creatinine value at baseline.

Changes in the text: None**.**

Reviewer C

It seems like an interesting article on a relevant topic in the treatment of hematologic oncology patients. I have some suggestions to add to your manuscript:
er
• I suggest describing the median number of methotrexate cycles each patient received and including this risk factor in the multivariate analysis.
Response: We did not compare the differences in this data due to the absence of more patient data.

• It is mentioned that MTX levels were continuously monitored every 24 hours until the blood-drug concentration was <0.3 μmol/L. A valuable addition to the article would be the follow-up at 48 and 72 hours regarding the levels of patients with delayed MTX excretion, as well as the clinical interventions performed in this patient group to prevent toxicity.
• Describe the modifications made in subsequent courses for the group of patients with delayed MTX excretion.
Response: Patients with delayed excretion were rescued strictly according to the CCCG-BNHL-2015 and CCCG-ALL-2020 protocols. See Line 149.

• It would be appropriate to report toxicity in grades to have a clearer understanding of the clinical impact on the patient.
Response: In our statistical analysis, adverse reactions are graded. For example, AST/ALT greater than three times the baseline value is considered an exception. There are four stages of platelet count reduction.
Changes in the text: None**.**

These suggestions address important aspects of the study, and incorporating them will likely enhance the depth and clarity of the manuscript. Good luck with the revision and improvement of your work!