Point-by-point Response to Reviewers:

Before providing a point-by-point response to the limitations raised by the reviewers of the article, we wish to express our deepest gratitude to them. Reviewing is a time consuming process ensuring high quality content. As a fact, all comments made have enabled us to enhance the overall quality of the manuscript, and the relevance of each underscores the time reviewers have dedicated to our article. Below are our attempts to respond to each comment hoping it will fully assess reviewers expectations.
Each point raised is transcribed in normal writing format and answered in italics.

Reviewer #1

1. P.3, L50- 51: Where is the reference for ceftriaxone's 'primarily nonlinear PK'? I've checked references 1 through 4 and none of them mention it.

We have added references concerning the nonlinear pharmacokinetics of ceftriaxone in its total form (see References 2 and 3 in the revised manuscript). We have also modified the sentence to specify that the pharmacokinetics is nonlinear when evaluating the total form of ceftriaxone, whereas the unbound form exhibits linear kinetics.

2. P.3, L56-58. Reference number 6 (Novy-E, 2023) does not state that increases in total CEF concentrations and/or a decrease in albumin levels may lead to an increase in free concentrations. I'm not clear on what you are trying to say here? Be especially careful as what I think you're saying is that because with saturable binding 'free-fraction' may increase; but that doesn't necessarily translate into free concentrations being elevated! You have to consider what is happening to volume of distribution (Vd) concurrently. Protein binding changes can also affect Vd. In any case, Novy-E is not the source for any of this discussion.

Answer: Unless we are mistaken, Nove-E (2023) was reference number 5, related to the complexity of the TDM process and the need for model-informed precision dosing to tailor individualized dosing regimens in ICU patients. Reference number 6 was:
Ulldemolins M, Roberts JA, Rello J, Paterson DL, Lipman J. The effects of hypoalbuminaemia on optimizing antibacterial dosing in critically ill patients. Clin Pharmacokinet 2011;50:99–110.
Regardless, you are absolutely right in mentioning that an increase in the free fraction is associated with increased volume of distribution (Vd) and clearance. This was highlighted by Ulldemolins et al., and we have modified the introduction section accordingly.

3. P.3, L59-62. You state that prior diverse mathematical models have been developed in the literature to model or identify CEFu from albumins and that they have all suffered

from a lack of external validation. But instead of validating those formulas with 'measured free' CEF concentrations you use existing formulas from the literature to re-predict them? How is that external validation? I don't think that validation of CEFu predictions is provided by your study design.

Answer: The initial aim of the manuscript was to study the between-model variability of unbound ceftriaxone (CEFu) prediction based on total ceftriaxone and albumin levels, highlighting the need for external validation since, for the same patient (same total ceftriaxone [CEFt] and albumin levels), models could lead to different conclusions (underexposure or target attainment). Based on your suggestion, external validation was performed on 62 samples for which albumin, total ceftriaxone, and unbound ceftriaxone levels were quantified. Dedicated sections regarding this part were added to the Results and Materials & Methods sections, and the overall manuscript was modified accordingly. Please note that our resubmission has been slightly delayed due to the time required to obtain approval from our local ethics committee (agreement number E2024-19 obtained on February 29, 2024).

4. P.3, L62. 'To establish optimal CEFt thresholds in plasma'. Please clarify. Why not add a statement in the introduction that most centers where TDM is available only measure total concentrations and not free pharmacologically active drug; that's why you choose this as a goal!

Answer: Thank you for this very relevant point, as it clarifies the aim of the manuscript concisely. We have added a statement in the introduction to highlight the problem that therapeutic drug monitoring (TDM) is usually performed on total drug concentrations rather than on the free, pharmacologically active drug.

5. P.4, L71. Please clarify. The last reference on P.3 was #6, however, after this point the references jump to # 24 - 26. What happened to the quoting of references #7 through to 23? Was this because those reference numbers jumped to Pages 10 to 12? I'm not familiar with this style.

Answer: Despite all our precautions, it seems a problem occurred with the quoting and formatting of references. This was a mistake on our part, probably due to the final proofreading by all authors. We would like to apologize for this, as it makes it difficult for readers (and reviewers) to properly assess the manuscript and references. Thanks to your suggestion, all references have been updated throughout the entire manuscript.

6. P.5, L101. One of your objectives was to ascertain total trough concentrations necessary to achieve free troughs over MIC of 100% (i.e. fT>MIC 100%). Now, you examined the performance of all 9 equations for fT>100% MIC and found that the total

concentrations ranged from 3.3 to 16.9 mg/L in normal albumin states, but 1.8 to 15.1 mg/L in hypoalbuminemic states. You repeated this for the PD target of fT>4xMIC 100% and found for all 9 equations that the total concentrations ranged from 13.1 to 56.2 mg/L in normal albumin states, but 6.6 to 50.1 mg/L in hypoalbuminemia states. As a reader, I'm saying 'are these really all that different'? My simply observation is that nothing is different. Can you comment?

Answer: Thank you for your useful comment. The aim of this section (and Table 2) was to provide an overview of between-model thresholds, but we did not elaborate much on the impact of hypoalbuminemia, which is also an important factor of variability depending on the model used. As a result of your valuable comment, we have modified both the Results and Discussion sections accordingly, and we have added the percentage decrease in the cut-off in Table 2. Results regarding the CEFt thresholds are now presented in two subsections: 1) Between-model variability, and 2) Impact of hypoalbuminemia for each model in a paired manner (normal vs. hypoalbuminemia for the same model, expressed as a percentage decrease in CEFt threshold).

7. P.5, L113- 115. Can you round everything to a single-decimal place as you have for all other concentrations reported above in the manuscript? It flips back and forth between 1 and 2 decimal places.

Answer: Thank you for pointing out this difference. We have now rounded all reported concentrations to a single decimal place.

8. P. 6, L116 - 117. This finding of greater variability in the CV around the thresholds for those with hypoalbuminemia (and thus predictions in that population) is not mentioned at all in your discussions. I think this is an important message.

Answer: Thank you for your valuable comment. We have added a sentence in the discussion section to highlight the greater variability associated with hypoalbuminemia.

9. Figure 1 - The ordinate scale for figures 1 A and 1C are just a little different. Can you adjust to make them the same?

Answer: Figure 1 has been updated according to the suggestions provided in the following comment.

10. Figure 1 - I find this very a busy figure examining the relationship between total and 'calculated' free concentrations. What is your message here? In order to better bring out comparative performance, please consider instead, plotting the ratio of free/total (as a percentage) across the range in total concentrations (on the x-axis). This will allow

one to clearly see how they are different or similar; and this should really bring out the nonlinear nature of protein binding for CEF. Additionally, you could then drop the lower total concentration ranges of figures B and D, of 0 - 60 mg/L. And repeat this for hypoalbuminemia (i.e. figure C).

Answer: We thank Reviewer #1 for this very interesting comment. Initially, we considered the best way to depict the between-model variability of the free fraction and chose to use unbound concentration on the Y-axis and total concentration on the X-axis. We agree that the figure is busy due to representing all nine models on the same plot. Following the reviewer's suggestion, we have modified Figure 1 to display the free fraction on the Y-axis and total ceftriaxone concentration on the X-axis, as requested.

11. Spelling errors: P.2, L32 'previously'; and P.10, Figure 1. 'literature'

Answer: We have thoroughly corrected typographical errors throughout the entire manuscript.

12. Table 1 - Under Model C, shouldn't this be 'bacterial meningitis', not meningitidis?

Answer: You are absolutely correct; the condition is bacterial meningitis and not exclusively related to Neisseria meningitidis. We apologize for this typographical error.

13. Table 3- Please clarify. a). What does the term 'Probabilistic' refer to here? b). what are 'Protides', is that protein?, and c). are Aminosides, aminoglycosides?

Answer:

a) "Probabilistic" has been replaced with "suspected" since it is the common term used to describe an infection that is presumed based on probability rather than confirmed through specific diagnostic tests.

b) "Protides" has been replaced with "protein" as suggested.

c) "Aminosides" has been replaced with "aminoglycosides" as suggested.

Reviewer #2

1. Line 32: previsouly, typo should be corrected

Answer: We have corrected the typographical errors and thoroughly reviewed the entire revised manuscript.

2. Lines 33 and throughout manuscript: The word "levels" should be replaced by the more scientific word "concentrations" where applicable.

Answer: We have replaced the word “levels” with “concentrations” throughout the entire manuscript wherever applicable.

3. Lines 38-9: "Optimal CEFt trough thresholds ranged from 1.8 mg/L to 16.9 mg/L (1xMIC) and from 6.6 mg/L to 56.2 mg/L (4xMIC)." It is not clear what this means, e.g. average or median, as an MIC is not 1.8 or 16.9 mg/L.

Answer: We have revised the sentence regarding CEFt trough concentrations to clarify that the CEFt trough concentration must reach levels sufficient to achieve an unbound concentration above 1 and 4 times the MIC, where the MIC is 1 mg/L, across the different studied models.

4. Lines "Age, daily dose, albuminemia and creatininemia were significant predictors of CEFt." Age as a demographic covariate should not be used and instead physiological covariates such as a measure of renal function and/or body weight and/or body composition should be tested; dose is obvious. It is unclear what is meant by CEFt, e.g. trough concentrations? Concentrations are not constant, unless dosing is by continuous infusion which was not the case here.

Answer: Thank you for pointing out the ambiguity regarding the term "CEFt." As you mentioned, "CEFt" could refer to both total Ceftriaxone concentration and trough Ceftriaxone concentration. To eliminate this confusion, we have changed “CEFt” to CEF_{tot}*"*

Additionally, we agree that age is a significant demographic covariate that can influence factors such as low cardiac output, renal dysfunction, hypoalbuminemia, and other comorbidities. We have included this discussion in the revised manuscript's discussion section.

5. Lines 41-2: "Importantly, 1g twice daily dosing achieved higher CEFt concentrations and improved PTA compared to 2g once daily dosing." It is not clear what is meant by CEFt concentrations, should it mean trough concentrations?

Answer: Thank you for your valuable feedback. We have updated the abbreviations by using "CEF_{tot}" to represent total ceftriaxone concentration, and we no longer abbreviate trough concentrations to avoid any confusion.

6. Lines 43-4: The conclusions don't seem to connect well with the methods and results of the abstract, as the authors did not perform an actual external evaluation.

Answer: The initial aim of the manuscript was to study the between-model variability of unbound ceftriaxone (CEFu) prediction based on total ceftriaxone and albumin levels, highlighting the need for external validation since, for the same patient (same total ceftriaxone [CEFt] and albumin levels), models could lead to different conclusions (underexposure or target attainment). Based on your suggestion, external validation was performed on 62 samples for which albumin, total ceftriaxone, and unbound ceftriaxone levels were quantified. Dedicated sections regarding this part were added to the Results and Materials & Methods sections, and the overall manuscript was modified accordingly. Please note that our resubmission has been delayed due to the time required to obtain approval from our local ethics committee (agreement number E2024-19 obtained on February 29, 2024).

7. Lines 138-42: "Additionally, clinical, demographic, and biological data from the retrospective cohort were analyzed as predictors of CEFt concentration at trough. Simple linear mixed effects regression identified age (p=0.033), intake dose (p<0.001), daily dose (p=0.001), albumin (p=0.002), and creatininemia (p=0.021) as significant predictors." This is not an appropriate analysis, a proper population PK analysis that models the concentration time profiles of ceftriaxone, with a covariate analysis needs to be performed.

Answer: Since ceftriaxone's pharmacodynamics are driven by the fraction of time (fT) that the free drug concentration remains above the MIC, we focused on trough concentrations of ceftriaxone as our primary endpoint. The aim of this analysis was to determine if our patient cohort exhibited the same predictors of ceftriaxone trough concentrations as reported in other studies and to examine how these predictors relate to population characteristics using linear mixed-effects regression.

You are absolutely correct that a population pharmacokinetic (popPK) analysis would allow a more thorough evaluation using nonlinear mixed-effects modeling. However, due to the retrospective nature of our data, the low number of patients with repeated ceftriaxone concentration measurements, and the heterogeneity of our patient population (encompassing both ICU and general ward patients), developing a popPK model for our cohort would likely introduce bias and yield inconclusive results. This limitation is now addressed in the discussion

section to emphasize the need for a comprehensive popPK analysis of both total and free ceftriaxone concentrations. Such an analysis would yield more robust insights, especially given the significant between-model variability highlighted in this study.

8. Lines 145-7: "ANOVA demonstrated that CEFt trough concentrations increased with dosing regimen. The mean CEFt concentrations were 52.6{plus minus}33.5 mg/L for 1g x1 /day, 62.9{plus minus}44.6 mg/L for 2g x1 /day, 84.2{plus minus}38.5 mg/L for 1g x2 /day, and 126.3{plus minus}69.1 mg/L for 2g x2 /day (Figure 3)." Again, this is not an appropriate analysis method. A population PK analysis with covariate analysis needs to be performed.

Answer: Since ceftriaxone's pharmacodynamics are driven by the fraction of time (fT) that the free drug concentration remains above the MIC, we focused on trough concentrations of ceftriaxone as our primary endpoint. The aim of this analysis was to determine if our patient cohort exhibited the same predictors of ceftriaxone trough concentrations as reported in other studies and to examine how these predictors relate to population characteristics using linear mixed-effects regression.

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