Peer Review File

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Reviewer Comments

Reviewer A

Comment 1: The authors should indicate how they determined the normal range; did they use the values indicated by the manufacturer or did they perform an analysis on healthy controls?

Reply 1: At page 3 line 85 we report: The concentration of FLC and urine κ/λ ratio were considered abnormal when results were outside of the reference interval provided by the manufacturers (κ/λ ratio 1.4-6.2) and, as suggested by reviewer, we added at page 5 line 131: "… the reference value provided by the manufacturers…"

Comment 2: It should be of interest to indicate, for discrepant results, which diagnosis was finally considered. because, in contrast to the conclusions of the authors, the reviewer is not convinced by the conclusion, On this small series, there are a lot of discordant results, and it could be interesting to analyze which result was the most accurate for the patient.

Reply 2: We agree with this argument of Reviewer but unfortunately, we don't have a final diagnosis for all subjects. Our intention was to highlight/confirm the presence of a monoclonal component and this was done using the other available laboratory data (IFI, serum FLC, electrophoresis.) as for patient number 6, 14 and 30. For patient 32, as we reorted in the text, a final diagnosis was available

Comment 3: There are several typos (abstract, line 34, "and" is repeated twice, in English the decimal part of numbers is separated by a period and not a comma (abstract line 25 and in the tables).

Reply 3: Done

Reviewer B

Comment 1: The authors evaluated the concordance between BJP and urine FLC (Siemens Attelica). In a rather small cohort discrepancies were observed between the two methods and authors conclude that there is no added value for urine FLC besides BJP. BJP is labor-intensive and a good immunological assay would be very welcome to reduce costs and time. The article is concise but missing some important discussions:
1. Could discrepancies be solved by wider reference ranges for urine FLC?

Reply 1: This is a nice point, thanks. We used the reference interval provided by the manufacturer (Siemens). Siemens does not distinguish a different range based on kidney function unlike other providers (the Binding Site, Birmingham, UK). It would certainly be interesting to evaluate a different range, but it would be necessary to design a study for this purpose. A sentence was added in the test: The concentration of FLC and urine κ/λ ratio were considered abnormal when results were outside of the reference interval provided by the manufacturers (κ/λ ratio 1.4-6.2) which does not distinguish a

different range based on kidney function, unlike other providers.

Comment 2: Could BJP be replaced by urine FLC if free kappa or lambda concentrations were higher?
Reply 2: This is another nice point. A higher concentration of monoclonal FLC is likely to result in easier BJ detection but current IMWG guidelines recommend BJ protein research by urinary immunofixation. Automated measurement of urinary FLC is currently not yet recommended.

Comment 3: Urine FLC is a quantitative result while BJP is only qualitative (POS or NEG). Are the quantitative results not important for the following treatment?
Reply 3: For BJP testing by IFI, the early morning urine sample is indicated. If BJP is detected then its quantification becomes important and for quantification, the recommended sample is a 24-hour urine collection (Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma
Working Group Updated criteria for the diagnosis of multiple
myeloma. Lancet Oncol. 2014;15:e538-e548.). The aim of our study was to evaluate whether simultaneous measurement of urine FLC with automated assay and BJP detection by urine-IFI, i.e. the first step in the diagnostic process, can provide additional clinical information in the management of patients with suspected MG.

Comment 4: Could the difference between involved and uninvolved FLC be of diagnostic importance (follow-up)? Would it be possible to reflect on these questions or is there a restriction in word count?
Reply 4: The difference between involved and uninvolved serum FLC is an International Myeloma Working Group diagnostic criteria for multiple myeloma. (Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma
Working Group Updated criteria for the diagnosis of multiple
myeloma. Lancet Oncol. 2014;15:e538-e548.). What is important to emphasize is that the guidelines report the importance of **serum** FLC measurement and not urinary FLC. The difference between involved and uninvolved serum FLC (not urine FLC) is a validated criterion for response assessment in AL amyloidosis

Comment 5: Line 75: male 30/40? What does this mean? Should it be 30/39? Maybe 30 males and 9 females?
Reply 5: We apologize for the mistake, we have corrected in the text (see line 75)

Comment 6: Line 116: I counted 11 samples and 8, respectively.
Reply 6: The presence of BJP was detected in 9 urine specimens and of these 9 specimens, seven had also an altered κ/λ ratio. The urine κ/λ ratio measured with the automated nephelometric assay was abnormal in 10 urine samples and of these specimens, only seven also had detectable BJP.

Comment 7: Line 122: K should be the Greek symbol kappa.

Reply 7: Done as requested.

Comment 8: Line 129: FLC k is 89 mg/L and FLC 'lambda' 80.4. --> Use instead of the k symbol for kappa and put mg/L after 80.4.
Reply 8: Done as requested.

Comment 9: Line 130-131: How can 1 exceed the reference range 0.31-1.56?
Reply 9: We apologize for the mistake, the sentence was deleted.

Comment 10: Line 138 and in Table 3: use the kappa symbol instead of k
Reply 10: Done as requested.

Comment 11: Table 3: Please comment on ptnt 32. Kappa in BJ en lambda in urine-ratio? Were samples mixed up?
Reply 11: In the text, we reported (line 135): Patient 32 showed normal serum electrophoresis, serum IFE did not reveal the presence of MC and absence of BJP but an abnormal serum κ/λ of 0.29 was observed (reference interval provided by the manufacturers for serum κ/λ ratio: 0.31-1.56)…

Reviewer C
Comment 1: The work is poorly designed because it does not use 24-hour urine, which is the necessary sample.
Reply 1: For BJP testing by IFI, the early morning urine sample is indicated. If BJP is detected then its quantification becomes important and for quantification the recommended sample is a 24-hour urine collection (Rajkumar SV, Dimopoulos MA, Palumbo A, et al. International Myeloma
Working Group Updated criteria for the diagnosis of multiple
myeloma. Lancet Oncol. 2014;15:e538-e548.). Aim of our study was to evaluate whether simultaneous measurement of urine FLC with automated assay and BJP detection by urine-IFI, i.e. the first step in the diagnostic process, can provide additional clinical information in the management of patients with suspected MG.

Comment 2: The number of cases is small.
Reply 2: We agree with the reviewer as we reported in the text: "The main limit of our preliminary study is the small number of the patients included and obviously our conclusion must corroborate by further studies".

Comment 3: There are no references to works that have used the K/L ratio in urine.
It does not provide bibliographic references on obtaining the K/L ratio in urine.
Reply 3: We used the reference interval provided by the manufacturers. In the Methods paragraph, we report: "The concentration of FLC and urine κ/λ ratio were considered abnormal when results were outside of the reference interval provided by the manufacturers (κ/λ ratio 1.4-6.2)"

Comment 4: The introduction is brief and does not provide information related to the objective of the study.
Reply 4: This is an interesting observation. For better explain the aim of this preliminary study we added "… provide additional clinical information in the management of patients with suspected MG, and whether it is possible to think of replacing the search for BJP in IFE in the near future with a fully automated method such as the nephelometric measurement of urine FLC".

Comment 5: The objective of the study is not met. The discussion is poor and refers more to FLC in serum than to FLC in urine.
Reply 5: We basically agree with the reviewer, but this paper represents a first preliminary evaluation as underlined by the title of the study. We also hope that our preliminary study will be a stimulus for other authors to evaluate the real contribution of urine FLCs

Comment 6: Many errors are made in the writing. Some are these:
- Clinical Biochemical Laboratory or Clinical Biochemistry Laboratory.
- Short title: urineflc or urine FLC.
Reply 6: We apologize for the mistake; we have corrected the short title.

Comment 7: Abbreviation list: Immunofixation must be IFE and not IFI. IFI is indirect immunofluorescence.
Reply 7: Done as requested. See Text

Comment 8: Line 124-5
Polyclonal hypergammaglobulinemia (0.97 g/dl, normal range (NR): 0.8-1.40): this is not hypergammaglobulinemia.
Reply 8: We apologize for the mistake. It was an expression error (1.97g/dl)

Comment 9: Line 130-1
The serum κ/λ ratio is 1 which exceeds the reference value of 0.31- 1.56. Are you sure, it exceeds?
Reply 9: We apologize for the mistake, the sentence was deleted.

Comment 10: Line 139-140
an abnormal serum κ/λ ratio (0.83): Are you sure that this value is abnormal?
Reply 10: We apologize for the mistake, the sentence deleted.

Reviewer D
Comment 1: This is a study analyzing the concordance of urine immunofixation and urine-free light chains using the N-Latex Siemens assay. While the literature appears sparse for this type of study, there are several important limitations. First, there is no gold standard routinely available for discrepancy analysis other than clinical assessment and review of serum electrophoresis and immunofixation. Also, only qualitative Bence

Jones analysis was conducted (owing to spot urine samples), further limiting the discrepancy analysis. However, the primary limitation of this study is the sample size including only 9 patients with evidence of Bence Jones protein by immunofixation. Even with such a small sample size, there were actually 30% of cases with an abnormal urine FLC that did not show BJP. That's a large number, but the uncertainty on that number is very high considering the low sample size. I realize that clinical assessment favored no additional clinical value, but without a larger sample size, this is a tenuous conclusion, as written also by the authors.

The authors write: "that undergoing routine clinical analysis for urine FLC and BJP and other analytes performed on the Clinical Biochemical Laboratory at the Hospital of Bolzano" This reads as if it is current clinical practice to run urine FLC analysis at this institution. Is this the case? If so, why are so few patients summarized in this study?
Reply 1: This is an interesting observation. The study included only patients who undergoing simultaneously BJP and urine FLC during the 11-month period between January and November 202. For this reason, the number of patients is few.

Comment 2: The authors write: "but the measure of urine FLC adds nothing from a diagnostic point of view, as the CM was already highlighted by electrophoresis" However, it's possible now that there will be increased vigilance about looking for Bence Jones protein. I suppose it remains to be seen whether there is possible renal damage from FLC when it is only detectable by urine FLC ratio. However, this abnormal ratio is very mildly abnormal so I do not disagree that it's probably not very relevant.
Reply 2: This is an interesting observation, and we completely agree with the reviewer.

Comment 3: The authors write: "Seven samples tested positive for the presence of BJP showed an altered κ/λ ratio, while two BJP positive samples showed a normal κ/λ ratio (one sample with a FLC κ and one with FLC λ)." But then in Table III, they write: "Patients with both positive results at the BJP detection and abnormal urine κ/ λ ratio (7 patients), negative BJP and abnormal urine κ/λ ratio (3 patients) and positive BJP detection and normal urine κ/λ ratio (1 patient)." Were there two or only one BJP sample that were normal by urine FLC testing?
Reply 3: We are thankful to the referee for this observation. We apologize for the mistake; The data reported in Table III are correct. We have corrected the text-

Comment 4: The authors write: "evident a pattern of Polyclonal hypergammaglobulinemia (0.97 g/dl, normal range (NR): 0.8-1.40)" It appears 0.97 is normal, and not hypergammaglobulinemia, which would be > 1.4. Please clarify.
Reply 4: We apologize for the mistake; it was an expression error (1.97g/dl)

Comment 5: Patient 6 has an abnormally low ratio (according to the reference interval in the study), but both hyper-gamma and renal failure tend to increase the FLC ratio, not decrease it, at least in serum and using the Binding Site assay. However, the results

interpret the low ratio as due to these factors (also, the interpretation should be in the discussion). Please clarify or provide some literature.
Reply 5: This is an interesting observation. The rate of false positive kappa/lambda ratio in serum is greater than 50% in patients with polyclonal hypergammaglobulinemia, at least in serum, and using the Binding Site assay, as underlined by the reviewer. Less well-known is the behavior of urine FLCs in cases of hypergammaglobulinemia and renal failure. Text revised accordingly: "In one recent study, Xu et al, reported that urine FLC showed an increase proportional to the decrease in renal function by the N-Latex FLC method, but with a weakly correlation and a relative stable urine κ/λ ratio within the reference interval provided by the manufacturers, while the κ/λ ratio obtained with another method was strongly affected by renal failure" Xu L, Zhao B, Sun Y, Wang S, Chen X, Mao Y. Using two detection methods to observe the changes and significance of free light chain in serum and urine in patients with renal insufficiency. BioMed Research International 2022;5536199

Comment 6: The authors write: "The serum κ/λ ratio is 1 that exceeds the reference value of 0.31-1.56." how does 1 exceed this reference interval?
Reply 6: We apologize for the mistake, the sentence was deleted.

Comment 7: It also raises the question of whether urine FLC ratio is more or less sensitive than serum FLC ratio, but I don't think that was directly addressed at any point in this manuscript.
Reply 7: This is an interesting observation. Further studies on this topic would be needed

Comment 8: Regarding comments about limitations of serum FLC testing (e.g. Singh et al.), they need to be reassessed with so many studies showing that the IMWG reference interval of 0.26-1.65 is no longer relevant. See
https://pubmed.ncbi.nlm.nih.gov/33662349/,
https://pubmed.ncbi.nlm.nih.gov/29885308/,
https://pubmed.ncbi.nlm.nih.gov/37394225/,
https://pubmed.ncbi.nlm.nih.gov/37221866/
Reply 8: We are thankful to the referee for this observation. The text was revised accordingly. We added the references suggested.

Comment 9: Instead of IQR on the median, I suggest reporting the 25th and 75th percentile. Table II should also include the statistics for the FLC ratio. Clarify whether Table II is for all patients or just those in Table III. Further, what are readers expected to get from Table II (I suggest adding this to the discussion)?
Reply 9: Table II is for all patients. Table II revised accordingly. (we added the 25th and 75th percentile, and the statistic for κ/λ ratio) as requested

Comment 10: There is only one reference to the literature in this manuscript regarding urine FLC testing (Bradwell, 2001). The authors should either reference additional

publications or comment on the absence after conducting a structured search.
Reply 10: This is a very nice point. data on urinary FLC (uFLC) assays are limited. We added an additional recent study regarding urine FLC: Cho J et all. Comparison of serum and urine free light chain analysis in clinical diagnosis. (Letter) Blood Res 2022;57:278-296 (see: Discussion paragraph and references)

Comment 11: Acronyms should be described, or not used, in the abstract.
Reply 11: Abstract revised as requested