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Reviewer A

Comment 1: The authors review comparisons of manual semen analysis with computer assisted semen analysis (CASA). This an important review, that could help researchers identify challenges with current CASA, and come up with possible solutions.
Reply 1: Authors thank Reviewer A for his/her positive feedback.

Comment 2: It will be useful to include costs per sample for semen analysis for CASA systems and manual semen analysis, as it seems that cost/sample could be an important factor for the use and development of CASA by andrologists.
Reply 2: Including the cost is quite challenging. For CASA analysis, the cost varies across the different companies and depends on the instrument, whether it is equipped with additional accessories and software programs. For manual semen analysis also the cost is highly variable between the countries and this is well-known to the authors based on their personal experience interacting with Andrologists and physicians who come from USA, Italy, South Africa and UK. For the above-mentioned reasons, we prefer not to include any information about the price, and hence we have removed any reference to the cost from the manuscript (page 17, lines 319-321).

Reviewer B

Comment 1: I have read with interest the article by Finelli et al. : The validity and reliability of computer assisted semen analyzers in performing semen analysis : a systematic review.
It is indeed a systematic review using the PRISMA 2009 check list, not a meta-analysis. It deals about routine sperm analysis that is now possible using computer-aided sperm analysis (CASA) systems which are supposed to give more reliable results than the manual analysis (gold standard). The aim if this review is to summarize the data already published comparing manual and CASA systems for sperm concentration, sperm motility and sperm morphology. After a drastic selection, 14 articles were analysed here. Briefly it is suggested that CASA systems are valid for sperm concentration and sperm motility although they are less reliable for sperm morphology. This conclusion is agreement with what is known about the CASA devices.
This work is of interest because of the rapid growth of such devices, then a review is welcomed to evaluate their place in the andrology laboratory. However, before acceptance for publication several important points need to be addressed, in particular in the results and discussion sections. Moreover, there are numerous errors in the references and the articles cited, that are the main purpose of this article.
The aim the study is well introduced.
Reply 1: Authors thank Reviewer B for his/her positive feedback and useful suggestions.

Comment 2: Results: this section need extensive revision. It is a systematic review which

considers 14 articles but only 13 are cited in this section: the ref # 26 is missing in all the parameters evaluated.

Reply 2: Authors apologize for the mistake. Results from Schubert et al. (reference 15) have been reported in the results section.

Changes in the text 2:

"On the contrary, Schubert et al. reported no significant difference in the results (n = 150) when SCA system was used for analyzing either samples concentrated less than 1 million/mL or more than 80 million/mL (15). Also, results provided by SCA system showed higher repeatability in both oligo- and normozoospermic samples (15)" (page 8, lines 152-155).

"An agreement between both manual and SCA analysis was reported by Schubert, analyzing 30 semen samples (15). Particularly, authors reported a better repeatability when SCA was used for analyzing either astheno- or normozoospermic samples (15)" (page 11, lines 202-205).

"Moreover, results for sperm morphology analyzed by SCA were reported by Schubert et al. to be in agreement with those obtained by manual analysis (15). Finally, SCA analysis was associated with a lower variation in the results for either severely teratozoospermic or normozoospermic samples compared to manual semen analysis (15)" (pages 11-12, lines 221-224).

Comment 3:

Moreover there are numerous errors in the references cited:

- the publication by Baig et al., 2019, cited in the table 1 and 2, appears to be included in the 14 selected articles but the complete reference is missing (not cited in reference section!).
- the references 18 and 25 are the same, please delete one and modify the text line 170, 213, 226.
- the reference #23 cited in the progressive motility paragraph is not about motility at all. Please delete it in line 193 and line 198. it seems to be ref #22 instead. Please verify.
- The reference Singh et al. is noted #24 line 204 but it is actually ref #23.
- the ref #22 line 287 seems to be wrong and seems to be #21. Please verify.

All these errors made the text difficult to read and raises concerns about the validity of the analysis. Please check all reference numbers. However, the data and the conclusions made are in agreement about what is known.

Reply 3: Authors apologize for such mistakes. All the above references have been corrected, and the reference numbers verified.

Comment 4: Please, revise and add the reference 26 in the results section about sperm concentration, motility and morphology (as these parameters are studied considering what it is mentioned the table 1).

Reply 4: This has been done; please, refer to Reply 2.

Comment 5: - Sperm concentration and total sperm count: it is not obvious to separate these 2 paragraphs. As you know, total sperm count is obtained by volume X sperm concentration. As the volume is not assessed by the CASA system, only sperm concentration has to be evaluated here. Please gather these 2 paragraphs under "sperm concentration" title and delete or explain

why total sperm count add relevant data here.

Reply 5: Authors agree with the reviewer that total sperm count is a derivative parameter, not directly assessed by CASA system. Following his/her suggestion, we have created just one single section entitled “Sperm Concentration and Total Sperm Count” (pages 8-10, lines 143-180). We decided to include “Total sperm count” in the title, as the study published by Dearing et al. (2014) (reference 23) does not report data for sperm concentration, but compares manual and CASA analysis results based on total sperm count.

Comment 6: One of the main problem about CASA devices compared to manual analysis is that they are usually not very good for low and very low sperm concentration and for (very) high concentrations (but usually not a real matter of concern for the clinicians, more for the QC). Usually there is a lower limit under which manual analysis is mandatory. As your article is focused on the use in routine, this point needs to be assessed. Indeed the results presented show differences for low and high concentration (line 146, 155, 156, 170). Therefore there is likely a range of values where correlation is very good and lower for very low and very high concentration. For example, very low values such as cryptozoospermia and azoospermia cannot be assessed using CASA. Please add a paragraph about the lowest and highest valid limit (if some are described) or at least comment on that in the discussion section. It is useful for your publication.

Reply: 6

We agree with the reviewer. Any results below 15 million/mL (cryptozoospermic or oligozoospermic samples) or concentration greater than 60 million/mL may show significant differences. A total of 5 out of 14 studies reported a sperm concentration range where correlation was the highest, or a cut-off value above which results from manual and CASA system analyses mainly diverged. This data has been included in the discussion.

Changes in the text 6: “Hence, it would be of utmost clinical interest to identify the range of sperm concentration values which can be accurately detected by using a CASA system. Lammers et al. reported both SQA-V Gold and CEROS system to significantly overestimate the sperm concentration when lower than 15 million/mL (11). This is in agreement with the study published by Dearing et al., which reported a similar overestimation at the same value of sperm concentration (<15 million/mL) (23). Although Tomlinson et al. reported a general agreement between results from manual and CASA analyses, this was less pronounced when sperm concentration was higher than 60 million/mL (17). Whereas, Vested et al. and Schubert et al. reported increasing differences in the results when sperm concentration analyzed was higher than 100 million/mL (13,15). Therefore, the use of a CASA system for routine semen analysis is not accurate in samples which show very low (<15 million/mL) or high sperm concentration (>100 million sperm/mL)” (pages 14-15, lines 265-277).

Comment 7: Line 152: it is difficult to compare sperm concentration assessed by real motile sperm and Accu beads. As the latter are well characterized and immotile it is obvious to have a very high correlation with them. Please comment on that in the discussion section, as the ref#25 appears to solely rely on Accu beads. Using Accu beads for QC is a different point, that is not your topic here. Please explain.

Reply 7: All CASA machines have to be calibrated to validate their accuracy for sperm concentrations. The use of latex beads or Accubeads with two different concentrations (low 18±2.5 million/mL and high 35±5 million/mL) are recommended by the manufacturers of these machines as well as the accrediting agencies. The above mentioned point has been highlighted in the discussion.

Changes in the text 7: "In 2 studies, authors reported a high correlation by using Accu-beads (16,17). These are latex beads, commonly used for laboratory quality control or for training of junior technicians. As they are immotile, suspended in aqueous solution and well-characterized, it is not surprising that a high rate of correlation was observed between manual and CASA results. However, when semen samples with motile sperm are examined, the analyzers are not free of errors" (page 13, lines 237-242).

Comment 8: - Motility : please check the references and in particular # 23 which is not about motility. - Morphology: please check the references and in particular # 24 which is not Singh et al.

Reply 8: The numbering of the references has been verified and corrected.

Comment 9: More important, there are different sperm morphology classifications, do the CASA devices reviewed give a value using the same classification? For example, the ref#26 (that is not evaluated) mentions the David's classification which is likely not used in the other articles (to be verified). Please add this point in this section. It is important to know that different classification are used and can be adapted and read.

Reply 9: This is an important point to discuss, thank you for the suggestion. Most CASA devices use morphology algorithms based on WHO 4th and 5th editions. The David classification is not common and is used only in some European countries.

Changes in the text 9: "Besides the paucity of studies analyzing the performance of CASA systems for sperm morphology evaluation (n=5), a different classification system was used by Schubert et al.(15), who analyzed sperm morphology based on David's modified criteria (30), and not the strict criteria recommended by the WHO and used in the other studies (4). A poor correlation was reported between these two classification systems (31). We can assume that any device can "learn" how to classify sperm morphology based on different criteria. However, this represents an additional challenge to compare studies using different classification systems, and increases the uncertainty about the real capacity of a CASA system to classify sperm morphology correctly" (page 16, lines 301-310).

Discussion:

Comment 10: Line223 : discuss the range of the values where the correlation is the best.

Reply 10: This has been addressed in a previous comment. Please refer to 'Reply 6' above.

Comment 11: Line 265: dilution is an important point as it is mandatory for sperm concentration in manual analysis and not performed (always?) in CASA devices. So it is a clear bias with manual analysis (with increasing dilution there an increase in errors). Please discuss this point and in particular for the sperm concentration parameter at the beginning of the discussion section.

Reply 11: Samples are diluted if the sperm concentration is high, generally greater than 60 million/mL. The dilution factor is included in the final calculations both manually and by the CASA systems (Hamilton and SCA). The importance of the proper sample dilution in semen analysis has been highlighted accordingly in the discussion.

Changes in the text 11: "Also, samples with concentrations greater than 60 million/ mL must be diluted for accuracy. Once the sample is diluted, the same aliquot is loaded on the counting chamber and analyzed manually as well as by CASA system. The dilution factor is included in the final calculations for both techniques. There is no bias as the same sample that is diluted is counted both manually and by CASA device. The incorrect dilution of the sample may introduce a technical bias, due to the use of uncalibrated pipettes, along with incorrect pipetting performed by the technician, or the viscous nature of some samples, significantly affecting the results. However this bias is identical for both manual and CASA device as it is the same sample that is analyzed (27)" (page 14, lines 250-259).

Comment 12: Line 277: "manual analysis is not universally considered as the gold standard." Please explain why.

Reply 12: In this section, we want to highlight the fact that the performance of the CASA systems is compared to a control (manual semen analysis) which is not 100% accurate itself. Hence, the sentence has been revised as below.

Changes in the text 12: "Despite all the above-mentioned considerations, this systematic review of the literature assumes that the manual semen analysis is the gold standard. The manual semen analysis is considered the gold standard when performed by highly trained competent technologists working in accredited lab and participating in a proficiency testing program monitored by external agencies. However, large variability has been reported between different operators or laboratories analyzing the semen parameters due to the lack of an external quality control program, not following the standardized WHO procedures, or lack of appropriately trained technologies (33-35). Therefore, the control used to compare CASA systems performance may be itself inaccurate and show great deal of variability, and this represents a limitation for our analysis" (page 17-18, lines 332-345). The following references have been included supporting this statement:

33.Filimberti E, Degl'Innocenti S, Borsotti M, et al. High variability in results of semen analysis in andrology laboratories in Tuscany (Italy): the experience of an external quality control (EQC) programme. Andrology. 2013 May;1(3):401–7.
34. Punjabi U, Wyns C, Mahmoud A, et al. Fifteen years of Belgian experience with external quality assessment of semen analysis. Andrology. 2016;4(6):1084–93.
35. Brazil C, Swan SH, Tollner CR, et al. Quality control of laboratory methods for semen evaluation in a multicenter research study. J Androl. 2004;25(4):645–56.

Comment 13: Line 282-288: why do you highlight this specific device at the end of this section/article? Moreover, concerns remain about it as it is mentioned lines 184-187. In addition, it seems that 2 authors of the present paper were also authors of this publication. Please delete or comment to avoid any conflict of interest.

Reply 13: The LensHooke device is highlighted towards the end because it represents the most

recently developed device using artificial intelligence for the analysis of semen parameters. In order to avoid any conflict of interest, the section has been revised as follows, to underline the need for further improvement in the algorithm used in this device in place of manual semen analysis.

Change in the text 13: "In the Andrology laboratory, LensHooke X1 PRO represents the most recently developed device which integrates AI algorithms and autofocus optical technology for the analysis of semen parameters. In comparison with the existing CASA systems, this device is compact, portable, easy-to-use, and results correlate highly with those obtained by the manual semen analysis. Although pilot study showed some differences in manual and CASA sperm motility (22), in a recent study the Lenshooke X1PRO has reportedly shown the ability to detect a very large range of sperm concentration (between 0.1 – 300 million/mL). Moreover, it demonstrated higher sensitivity and specificity (>90%) in identifying oligo- and asthenozoospermic samples when compared to IVOS II system (36). Hence, further development of AI-based devices represents step in the right direction for the automation of semen analysis (page 18, 348-361).

Comment 14:

Minor revision:

Harmonize: CASA stands for Computer-aided Sperm Analysis : line 37, line 69

Reply 14: This has been modified accordingly.

Comment 15:

Discussion : Line 214 : references with an s

Reply 15: We have not found the word "reference" at the line indicated by the reviewer.

Comment 16:

Line 226 : Yes, I agree. Good point to address.

Line 233-236: it is true but the limits are wide and it is not a real problem in clinical practice. Please mitigate this sentence.

Reply 16: The sentence has been modified.

Changes in the text 16: "This may influence the clinical management of these patients, as the evaluation of semen quality is important in clinical diagnostics and management, including the type of ART technique to be used (IVF/ICSI or IUI)". (page 14, lines 262-265)

Comment 17:

Line 239 : good point.

Line 280, 283, 287: AI is integrated in all CASA devices not only in X1PRO as it is suggested here. Please modify.

Reply 17: The section has been modified accordingly.

Changes in the text 17: "As the technology is evolving continuously, artificial intelligence (AI)-based devices promise to improve the efficiency of the analysis and the reliability of the results. In the Andrology laboratory, the LensHooke X1 PRO represents the most recently developed

device which integrates AI algorithm and autofocus optical technology for the analysis of semen parameters" (page 18, lines 346-351).

Comment 18:
Figure 2 : Articles excluded in the 3rd box on the right is 158, not 157. Then 172-158=14 articles selected.
Comment 18: Thank you, the figure has been modified accordingly.

Comment 19:
References :
The Baig et al, 2019 reference is not included. Please add it.
Ref 18 and 25 are the same, please delete one and modify the reference in the article.
Comment 19: The study published by Baig et al., 2019 (reference 20) has been cited at page 9, line 165. The duplicated reference has been corrected.

Reviewer C

Comment 1: The review "The validity and Reliability of Computer-Assisted Semen Analyzers in Performing Semen Analysis: A Systematic Review" submitted for publication to Translational Andrology and Urology summarizes the results of original articles that deal with the comparison of manual semen analysis and computer-assisted systems for semen analysis published in the past 10 years. The review is written according to the PRISMA guidelines and meets the author guidelines.
After screening certain databases, the authors included 14 out of 2046 search results.
The review is well written and understandable and probably of great interest to the community.
I only have some minor remarks.
Reply 1: Authors thank Reviewer C for his/her positive feedback.

Comment 2: How about the operating temperatures of the CASA machines? Are there differences between the systems? Please also include a sentence or paragraph on that issue.
Reply 2: The operating temperature is 37°C for Hamilton Thorne devices. Also, the temperature can be adjusted accordingly in most of other devices. This information is not available for all the systems cited in our review. However, we have highlighted in the discussion the importance of avoiding changes in temperature during the analysis.
Changes in the text 2: "On the other side, some CASA systems (i.e. IVOS II) are equipped with heated specimen stage, which allows precise control of temperature during the analysis, maintaining it at 37±0.5°C, whereas manual semen analysis is usually conducted under a phase-contrast microscope at ambient temperature. This represents an unquestionable advantage, as changes in temperature may significantly affect the analysis of sperm parameters, particularly motility assessment" (page 17, lines 321-327).

Comments 3:

lane 98: The abbreviation should be put in parentheses.
lane 103: Maybe better: "An approval by the Institutional Review Board was, therefore, not required."
lane 114: better: …using this search strategy…
lane 148 a.o.: "et al." should be written in italics throughout the manuscript
lane 145 a.o.: Please decide whether you use p value or P value, ml or mL and make it consistent throughout the manuscript.
lane 149: …by using the SCA system…
lane 156 ff: Please change x to ×
lane 158: …CASA-based…
lane 162: Better write the word three not the number 3.
lane 226: Delete the space between the comma and 13.
Please make it consistent throughout the manuscript. Either put spaces before = and < or not.
lane 284: AI has already been abbreviated in lane 280.
References: According to the author guidelines, it should be for example: … J Thorac Dis 2012;4:247-58. Furthermore, the titles should be written in lower case letters. Please check ref. 5 and 8.
Reply 3: All the suggested changes have been included in the manuscript.

Reviewer D

The systematic review on CASA systems is holistically and concisely written. There are only a few changes that have to make so minor revision is required.
Reply: Authors thank Reviewer D for his/her positive feedback.

Comment 1: Baig et al., 2019 is not listed in the reference list;
Reply 1: The reference Baig et al., 2019 (reference 20) has been cited at page 9, line 165.

Comment 2: Has the LensHooke data been integrated into to text?
Reply 2: The study published by Agarwal et al., 2019 (reference 22) using LensHooke device is reported and cited on pages 10 (line 195), 11 (lines 201, 207), and 18 (line 355).

Comment 3: It will be more readable if the average time is given/available for preparation on each CASA system for each SA.
Reply 3: Unfortunately we do not have this information for all the systems used in the articles included in our review.

Comment 4: More readable if a table to compare the pros and cons between manual and CASA system in general.
Reply 4: Thank you for this suggestion; we have included Table 3 to summarize the pros and cons in using CASA systems.

Reviewer E

The manuscript is suitable to be published in the journal

Reply: Authors thank Reviewer E for his/her positive feedback.