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Reviewer A

The review covers all important aspects of fetal blood group genotyping, including methodologies, pre-analytical issues and quality assurance. I have only a list of minor suggestions for improving the manuscript, which are mainly alternative wordings or structure-related.

Comment 1. Page 1, Abstract, last part: Here you mention blood groups, but you are listing blood group systems. Please consider changing the sentence to: "…fetal genotyping, and assays for predicting blood groups within the blood group systems ABO, Rh…"

Reply 1. I have corrected the text as advised, (see Page 1, line 25)

Changes in the text: added: "within the blood group systems"

Comment 2. Page 2, Introduction, first line: Please consider using the abbreviation RBC for red blood cell (here and throughout the manuscript). Here you can introduce the abbreviation as "red blood cell (RBC)".

Reply 2. I have abbreviated "red blood cell" as advised, (see Page 2, lines 33, 34, 35 and 41)

Changes in the text: ~~red blood cell~~ RBC

Comment 3. Page 2, Introduction, last section: It reads: "…that use fetal cell-free DNA as template." Please consider using an alternative phrase, for example "…that analyze fetal cell-free DNA."

Reply 3. I have corrected the text as advised, (see Page 2, lines 46-47)

Changes in the text: genotyping assays that analyze fetal cell-free DNA

Changes in the text: Thus, particularly in the case of a negative fetus, the sample should be shown to really contain fetal DNA or otherwise ensure the result.

Comment 4. Page 2, Blood group genetics, fourth line: Please consider rephrasing the wordings here to: "…polymorphisms affecting gene expression, rather than a gene deletion."

Reply 4. I have modified the text as advised, (see Page 2, line 54)

Changes in the text: RhD-negativity is often caused by polymorphisms affecting gene expression, rather than a gene deletion.

Comment 5. Page 3, third line: Here you say that in case of a negative fetus, the sample must be shown to really contain fetal DNA. I think this is very strong words (the must) leaving no room for not having a fetal control. Please consider if you can find a milder phrasing, fx highly recommended or likewise.

Reply 5. I have modified the text as advised, (see Page 3, line 70-71)

Changes in the text: Thus, particularly in the case of a negative fetus, the sample

should be shown to really contain fetal DNA or otherwise ensure the result.

Comment 6. Page 3, Cell-free DNA, middle: It is stated that "A threshold for fetal fraction is usually 4%...". Is this not something coming from NIPT for aneuploidies?

Reply 6. I agree, the threshold of 4% is mainly applied in aneuploidy testing. I removed the sentence as it is not relevant (see Page 4, line 85-86).

Changes in the text:

Comment 7. Page 4, middle. Here, the word 'scrutinized' seem a bit off in my ears. I would suggest using 'looked at'.

Reply 7. I have corrected the text as advised, (see Page 4, line 109)

Changes in the text: In their excellent review, Runkel et al looked at fetal *RHD* screening studies (20).

Comment 8. Page 4, bottom, fourth last line: It says: "…due to fetal DNA fraction below the detection limit…". Should it not be "…due to fetal DNA fraction falling below the detection limit…" ?

Reply 8. I have corrected the text as advised, (see Page 5, line 121)

Changes in the text: False-negative results are often due to the fetal DNA fraction falling below the detection limit in maternal plasma.

Comment 9. Page 6, Methods, first line: Please consider abbreviating real-time PCR as qPCR in stead of rtPCR.

Reply 9. I have changed the abbreviation to qPCR (see page 6, line 160 and thoroughly the text)

Changes in the text: ~~rtPCR~~ qPCR

Comment 10. Page 6, Methods, first line: Please consider, if it would be a good idea to insert a reference to Table 1 already here, as the first line of the section. Fx: "Several technological platforms are used for noninvasive fetal blood group genotyping (Table 1)."

Reply 10. I have added a reference to Table 1 (see page 6, line 157).

Changes in the text: Several technological platforms are used for noninvasive fetal blood group genotyping. They are listed in Table 1.

Comment 11. Page 6, middle. Please put RHCE in italics.

Reply 11. I have changed "RHCE" into italics (see page 7, line 176)

Changes in the text: *RHCE*

Comment 12. General remark: Often times, your references are placed early in a sentence. This may be a matter of style and likings, but I would suggest moving your reference to behind the statement. For example page 6 (bottom), you write "Orzinska et al (32) published…" The reference could easily be at the end of the sentence. Other

examples are page 6 same section, page 6 middle (newly published (29)), page 7 (bottom) ref 37, and page 4 bottom and middle.

Reply 12. I have moved references to the end of sentence (thoroughly the text)

Changes in the text: for example: Orzińska et al ~~(32)~~ published diagnostic genotyping of D, C, c, E, and K blood groups for fetuses of immunized women (35).

Comment 13. Page 9, middle. Please check the sentences about the fetal fraction and see if all wordings are ok.

Reply 13. I have modified the text, I hope it is clearer now (see page 10, line 176)

Changes in the text: Because fetal fraction is crucial for the accuracy of the sequencing assay, it should be evaluated (54). As it is an inherent feature of sequencing (53) estimation of fetal fraction is relatively easy to implement. The recommended minimum fetal fraction is 4% (53).

Comment 14. Page 12, top. Reference 37 should be published soon, so please have a look out for this, and then rephrase the sentence accordingly (not using 'will publish').

Reply 14. I have corrected the text as advised, (see Page 13, line 330)

Changes in the text: the cfDNA Subgroup published recommendations for assay validation

Reviewer B

General Comments:

Thank you for the opportunity to review this manuscript. This narrative review is informative, with a good structure and well-referenced. There was no completed checklist received for review as per the Author submission guidelines and the abstract is unstructured, which will be issues for the Editorial Office to adjudicate. Corrections, suggested changes and questions can be found under 'Specific Feedback'.

Specific Feedback:

Abstract

Comment 15. Line 19 ‘Non-invasive prenatal testing is commonly used in feto-maternal blood group allele discrepancy’ Replace ‘in’ with ‘to identify’.

Reply 15. I have corrected the text as advised, (see Page 1, line 15)

Changes in the text: Non-invasive prenatal testing is commonly used to identify feto-maternal blood group allele discrepancy.

Comment 16. Line 20 ‘DNA in maternal sample has two important uses’ Insert the word ‘blood’ before maternal to distinguish from other types of biological samples eg amniocytes

Reply 16. I have corrected the text as advised, (see Page 1, line 16)

Changes in the text: Fetal cell-free DNA in maternal blood sample has two important uses:

Introduction

Comment 17. Line 35 Either change ‘KEL’ to Kell or change ‘Rh’ to RH for consistency between nomenclature ie system name or system symbol – refer Table of blood group systems v. 9.0 03-FEB-2021.pdf (isbtweb.org).

Suggest that in first paragraph, briefly name antibodies that (more rarely) can cause mild/moderate/severe (since some of them are mentioned later)

Reply 17. I have modified the text as advised, (see Page 2, lines 36-38)

Changes in the text: Anti-D (Rh), anti-c (Rh), anti-K (Kell) create the most significant risk for a severe HDFN. Also, antibodies against other Rh antigens or Duffy and Kidd antigens may cause HDFN albeit less often and typically with milder symptoms. Anti-A and anti-B (ABO) antibodies may be a risk for HDFN after birth (1).

Blood group antigens A and B (ABO) are produced by a glycosyltransferase (coded by the ABO gene); the blood group O phenotype results from the unfunctional glycosyltransferase. The difference between *ABO*A1.01* (*A1*) and *ABO*B1.01* (*B1*) alleles is based on a few nucleotide exchanges in exon 7 of the ABO gene and the most common inactive *ABO*O.01* (*O1*) allele is caused by a frameshift deletion in exon 6. In addition, many other inactivating mutations occur in the *ABO* gene, therefore, designing robust genotyping methods for *ABO* blood group alleles is challenging (3).

Comment 18. Line 52 '*Also, the homologous RHCE gene poses a challenge for Rh genotyping*.' Reword to explain a little further for the reader eg 'Also the RHD and RHCE genes of the Rh system are highly homologous and pose a challenge for Rh genotyping'

Reply 18. I have modified the text as advised, (see Page 2, line 56-57)

Changes in the text: Also, the *RHD* and *RHCE* genes of the Rh system are highly homologous and pose a challenge for Rh genotyping.

Comment 19. Line 54 '*The majority of blood groups, such as KEL, result from single nucleotide polymorphisms, the smallest possible…*'

Change 'blood groups' to 'blood group antigens' and change 'KEL' to 'K' to give a single example of a SNP-based Ag using ISBT nomenclature

Suggestion: The review covers ABO so a brief mention of ABO genetics is warranted

Reply 19. I have corrected the text as advised (see Page 3, line 64) and added a brief mention of ABO genetics (see Page 3, line 57-63)

Changes in the text:

The majority of blood group antigens, such as K, result from single nucleotide polymorphisms, the smallest possible variation between genetic alleles.

Comment 20. Line 57 '*essential to be able to distinguish between true negative and false negative results*'

Within an assay, it isn't possible to distinguish between between false negative and

false positive results but I understand what the author is trying to convey. Would it be better to say that tests need to be designed and validated to ensure they are robust enough to avoid false negatives (in particular) and false positives?

Reply 20. I have modified the text as advised, (see Page 3, line 67-68)

Changes in the text: A negative result is based on not detecting a positive signal, so it is essential to design and validate tests to ensure they are robust enough to avoid false negatives and false positives.

Comment 21. Line 59 '*particularly in the case of a negative fetus, the sample must be shown to really contain fetal DNA*'

Reconsider use of the word 'must' as many sites use other strategies such as recollection, stabilised tubes or whatever their validation work has demonstrated

Reply 21. I have modified the text as advised, (see Page 3, line 70-71)

Change in the text: Thus, particularly in the case of a negative fetus, the sample should be shown to really contain fetal DNA or otherwise ensure the result.

Cell-free DNA

Comment 22. Line 63 '*of circulating cfDNA in 1997 (5) has significantly changed fetal*'

Insert 'fetal' before cfDNA

Reply 22. I have corrected the text as advised, (see Page 3, line 75)

Changes in the text: The discovery of circulating fetal cfDNA in 1997

Comment 23. Line 64 *'traditional amniocentesis carries a risk of miscarriage and may enhance maternal immunization*'

Include a current reference and add CVS as an invasive risk procedure

Reply 23. I have added chorionic villus sampling and included a reference. I removed a risk for immunization, because the risk is very low (Kristensen et al, 2019).

Changes in the text: As invasive methods, traditional amniocentesis and chorionic villus sampling carry a slight risk of miscarriage (7).

Suggestion: When talking about technical factors including 'shipping time that effect fetal fraction, it would be worthwhile briefly explaining the link between shipping time and maternal cell lysis. This would be helpful for later in the paper when methods to manage FF are mentioned

Reply: I carried the suggestion out in the other paragraph, see Page 5, lines 123-125.

Changes in the text: To avoid risks, several practices (7,18,19) have been implemented, starting from pre-analytics: collecting blood samples in special cfDNA stabilizing tubes and processing them within a specific time limit (20,21). These measures help prevent lysis of maternal cells, which would otherwise induce an increase in total cell free DNA and thereby reducing the fetal fraction (7).

Comment 24. Line 65 '*to detect of fetal cfDNA in a maternal sample has expanded the applications of non-invasive prenatal testing*.

Remove 'of'

Reply 24. I have removed the word "of" (see Page 3, line 77)

Changes in the text: The possibility to detect fetal cfDNA in a maternal sample has expanded the applications of non-invasive prenatal testing.

Comment 25. Line 72 '*A threshold for fetal fraction is usually 4%, and for samples below this limit, a risk for test failure is high*'

This statement needs a reference and some clarifiers. This threshold is mostly applied in commercial fetal aneuploidy screening tests

Reply 25. I agree, the threshold of 4% is mainly applied in aneuploidy testing. I removed the sentence as it is not relevant (see Page 4, line 85).

Comment 26. Line 74 '*As a result, amplicons are short, which must be taken account in assay design*' Add 'into' after the word 'taken'

Reply 26. I have corrected the text as advised, (see Page 4, line 89)

Changes in the text: As a result, amplicons are short, which must be taken into account in assay design.

Screening fetal RHD for targeting anti-D prophylaxis

Comment 27. Line 79 '*28-32 to prevent immunization during the last trimester of pregnancy*' Reference a guideline or publication here

Reply 27. I have added a reference: Sperling et al. Prevention of RhD Alloimmunization: A Comparison of Four National Guidelines. Am J Perinatol. 2018 Jan;35(2):110-119. (see Page 4, line 93)

Changes in the text: Routine antenatal anti-D prophylaxis (RAADP) is recommended for all RhD-negative women at gestation weeks 28-34 to prevent immunization during the last trimester of pregnancy (13).

Comment 28. Line 80 '*those RhD-negative women who benefits from it i.e.*'

remove the 's' from benefits

Reply 28. I have corrected the text as advised, (see Page 4, line 94)

Changes in the text: those RhD-negative women who benefit from it

Comment 29. Line 92 '*Cost-effectiveness of targeted RAADP programs has been widely discussed in a review by Saramago et al (16).*'

Is it possible to say a little more on this?

Reply 29. I have added the main conclusion regarding cost-effectiveness in the review (see Page 4, lines 106-108).

Changes in the text: The targeted RAADP may be cost-effective compared to the untargeted prophylaxis, depending mainly on the unit cost of the screening test and on whether the result of the test also guide post-partum administration of anti-D prophylaxis (19).

Genotyping blood groups of fetuses of immunized women

Comment 30. Line 108 '*small in the early gestation but already at weeks 9-12, the*'

Remove 'the'

Reply 30. I have corrected the text as advised, (see Page 5, line 126)

Changes in the text: but already at weeks 9-12, results have been shown

DNA Extraction

Comment 31. This reviewer suggests moving this section to 'Methods' section since DNA Extraction methods are discussed. Alternatively changing the 'Methods' heading to 'Genotyping Methods'

Reply 31. I have moved DNA extraction section to Methods section (see Page 5, line 130)

Changes in the text:

Comment 32. Line 124 '*It is also prone to aberrations. Also, Ordonez et (25)*'

What is meant by aberrations? Also please add 'al' after 'et' for Ordonez reference

Reply 32. "Prone to aberrations" was too strong expression. I have removed the sentence. A manual method is reliant on the competence of the operator and an

Comment 33. Line 126 '*the automated COBAS AmpliPrep (Roche) method, although the cfDNA yield was better with the manual method.*'

Though there were lower cfDNA yields using automated methods I would think the main point to make is the efficient detection of fetal DNA sequences in both the methods

Reply 33. I have corrected the text as advised, (see Page 5, line 126)

automated system is steadier; these are the main points. (see Page 6, line 143-146)

Changes in the text: Also, Ordonez et al chose a steady automated system for routine use when they compared the manual QIAamp DSP Virus Kit and the automated COBAS AmpliPrep (Roche) method (28). Both methods were efficient and suitable for cfDNA extraction.

Changes in the text: Also, Ordonez et al chose a steady automated system for routine use when they compared the manual QIAamp DSP Virus Kit and the automated COBAS AmpliPrep (Roche) method. Both methods were efficient and suitable for fetal cfDNA extraction.

Comment 34. Line 130 '*(Perkin Elmer) with LABTurbo and IDEAL found to be slightly more efficient*' What is meant by slightly more efficient – better cfDNA yields or better fetal DNA yields?

Reply 34. More efficient in fetal cfDNA yields, I have corrected the text as advised, (see Page 6, line 149)

Changes in the text: with LABTurbo and IDEAL found to be slightly more efficient in fetal cfDNA extraction than the other two methods

Methods

Comment 35. Line 149 'Besides RHD, genotyping of the RHCE gene alleles, and also KEL, is required in the care of immunized pregnancies.'

Italicise 'RHCE' and 'KEL'

Substitute 'performed' or 'often requested/often required' for 'required' (not always required)

Reply 35. I have corrected the text as advised, (see Page 7, line 171)

Changes in the text: Besides *RHD*, genotyping of the *RHCE* gene alleles, and also *KEL*, is often requested in the care of immunized pregnancies.

Comment 36. Line 160 '*result (KEL) was detected among*'

Changes in the text: The situation regarding requirement of CE certification will change very soon, because a five-year transition period of the Regulation (EU) 2017/746 on in vitro diagnostic medical devices (IVDR) will end 27th of May 2022. This new regulation restrains use of laboratory developed tests for the determination of the blood groups belonging to the ABO, Rh- (C, c, D, E, e), Kell, Kidd and Duffy -systems as they are classified to the highest risk category D. All the laboratories in the EU countries should utilize CE certified kits for fetal blood group allele genotyping in the above-mentioned blood group systems. New kits may be CE IVD certified as demand will grow. Interpretation of the Regulation 2017/746 is still unclear with regard to whether the requirement of CE certification also concerns fetal *RHD* screening tests. Wide group of experts agree, that fetal *RHD* screening for the purpose of targeting prophylaxis should be under the lower risk category C and the use of laboratory developed tests should be allowed in screening (40).

change KEL to K

Reply 36. I have corrected the text as advised, (see Page 7, line 181)

Changes in the text: Only one false-positive result (K) was detected.

CE Certified RHD typing Kits

Comment 37. Lines 178-184

This reviewer finds this paragraph difficult to follow/understand. Perhaps it could start with the current regulation constraints, followed by the expert recommendations about class C, followed by the comment about the implications for future certification, demand etc?

Reply 37. I tried to clarify the paragraph (see page 8, lines 201-209)

Mass Spectrometry

Comment 38. Line 189 '*Li et al (39) set up an assay to detect KEL1 allele with an accuracy of 94%*.' Add current, correct allele nomenclature in brackets (KEL*01.01) or reference the K antigen for reader understanding

Reply 38. I have corrected the text as advised, (see Page 8, line 214)

Changes in the text: Li et al set up an assay to detect KEL1 allele (corresponding K) with an accuracy of 94% (42).

Droplet digital PCR

Comment 39. Line 196 '*Droplet digital PCR (ddPCR) is based on the partitioning of a sample into thousands or even millio*n'

Change the word 'sample' to 'PCR reaction'

Reply 39. I have corrected the text as advised, (see Page 9, line 222)

Changes in the text: Droplet digital PCR (ddPCR) is based on the partitioning of a PCR reaction into thousands or even millions of uniform droplets.

Comment 40. Line 197 '*Each droplet is subjected to a separate real-time quantitative PCR reaction*' ddPCR droplets/reactions are measured at end-point, ie not in real-time. There may be other digital PCR systems that measure in real-time.

Reply 40. I have corrected the text as advised, (see Page 9, line 223)

Changes in the text: Each droplet is subjected to a separate quantitative PCR reaction

Comment 41. Line 198 '*While the aim is to dilute samples so that each droplet contains a single template molecule on average, some of*'

The aim is not to have a single template in each droplet, each droplet should ideally contain zero or one (or, at most, a few) template molecules. See Introduction to Digital PCR | LSR | Bio-Rad

Reply 41. I have corrected the text as advised, (see Page 9, line 224)

Changes in the text: The aim is to dilute samples so that each droplet contains zero, a single or maximum a few copies of the template molecule.

Comment 42. Line 202 '*By diluting highly abundant maternal DNA, sample partitioning allows the detection of just a few copies of paternal allele in fetal*'

Reword – cfDNA, containing a high abundance of maternal DNA, is diluted….. etc etc

Reply 42. I have corrected the text as advised, (see Page 9, line 229)

Changes in the text: A cfDNA sample, containing a high abundance of maternal DNA, is diluted allowing the detection of just a few copies of paternal allele in fetal DNA.

Sequencing

Comment 43. Line 220 '*The strength of next-generation sequencing/massively parallel sequencing lies in its ability to enable unbiased amplification of alleles*'

Sequencing without a suitable design and feature can fall victim to PCR bias during enrichment steps depending on the library preparation method used. You may wish to qualify the statement a bit. If molecular barcodes are used before enrichment the biases can be managed in data analysis.

Would the Author consider including that a major strength of MPS is the large number of patients and targets that can be multiplexed in a single test

Reply 43. I have modifed the text as advised, (see Page 9, line 246-250)

Changes in the text: Unbiased PCR amplification is achieved by using a primer pair without allele specificity amplifying both anthetical alleles in same time (52). The strength of next-generation sequencing/massively parallel sequencing lies in possibility to multiplex the large number of samples and targets in a single test.

Comment 44. Line 226 '*minimum fetal fraction is be 4%*'

Remove the word 'be'

Reply 44. I have corrected the text as advised, (see Page 10, line 255)

Changes in the text: The recommended minimum fetal fraction is 4%.

Comment 45. Line 228 '*method is promising and under continuous to develop*'

Change to 'under continuous development'

Reply 45. I have corrected the text as advised, (see Page 10, line 257)

Changes in the text: However, the method is promising and under continuous development.

Controls Used in Assays

Comment 46. Line 280 '*CCR5 and albumin, are listed in Table 1*'

Add a full stop after 'Table 1'

Reply 46. I have corrected the text as advised, (see Page 12, line 312)

Changes in the text: are listed in Table 1.

Quality Assurance

Comment 47. Line 298 '*collaboration with international colleagues, the cfDNA Subgroup will publish (37) recommendations for assay validation in order to secure clinical applicability of fetal genotyping*.

Move reference 37 to the end of the sentence as it looks like there are 37 recommendations.

Reply 47. I have corrected the text as advised, (see Page 13, line 331)

Changes in the text: In collaboration with international colleagues, the cfDNA Subgroup published recommendations for assay validation in order to secure clinical applicability of fetal genotyping (40).

Conclusion

Comment 48. Line 306 '*pregnancies has substituted invasive methods*'

Use the word 'replaced' instead of 'substituted

Reply 48. I have corrected the text as advised, (see Page 13, line 338)

Changes in the text: Fetal blood group genotyping in immunized pregnancies has replaced invasive methods

References

Comment 49. Reference 37 (Clausen et al) is now published so can be updated

Reply 49. I have corrected the text as advised, (see Page 13, line 330)

Changes in the text: In collaboration with international colleagues, the cfDNA Subgroup published recommendations for assay validation in order to secure clinical

applicability of fetal genotyping (40).

Reviewer C

I found this review manuscript very informative for a wide variety of readers including those who involved in diagnostic testing and those who are developing new methods of non-invasive fetal blood group genotyping. Although the manuscript is well written overall, I suggest the author further polishes the sentences listed below:

Comment 50. Page 5, line 16:
I suggest that the author describes what types of aberrations Londero et al (24) observed.
Reply 50. "Prone to aberrations" was too strong expression. I have removed the sentence. A manual method is reliant on the competence of the operator and an automated system is steadier; these are the main points.
Changes in the text:

Comment 51. Page 9, lines 10 to 13:
"As it is an inherent feature of sequencing (49) it is relatively easy to implement." It is unclear to me what the first and the second "it" refer to, respectively. "is be 4%" should be "is 4%"? "under continuous to develop" should be "under continuous development"?
Reply 51. I have corrected the text as advised, (see Page 10, line 254-257)
Changes in the text: As it is an inherent feature of sequencing (53) estimation of fetal fraction is relatively easy to implement. The recommended minimum fetal fraction is 4% (53). Sequencing as a technique is still rather laborious and, expensive and requires experience, and consequently the numbers of sequenced cases still remain small. However, the method is promising and under continuous development.

Comment 52. Page 10, line 10
"Marker allele panels consist of a set of genetic markers that potentially differ between

two individuals, in this context the mother and the father and consequently the fetus." This sentence should be rephrased so that readers can easily understand expected genotype patterns in a trio when a genetic marker is informative for confirming the presence of fetal DNA.

Reply 52. I have modified the text as advised, (see Page 11, line 283-284)

Changes in the text: Marker allele panels consist of a set of genetic markers that potentially differ between two individuals, in this context the mother and the father and consequently the fetus. The fetus may have inherited a few differing markers from the father and thus detection of these paternal markers indicates the presence of fetal DNA.