CORRESPONDENCE

Response to "The importance of determining the limit of detection of non-invasive prenatal testing methods"

We thank Dr Lüthgens and collaborators for their interest in our article.[1] We welcome the opportunity to provide clarification and further stimulate the discussion on this important topic.

The authors raised concerns regarding the accuracy of the method used in our study to determine the fetal fraction (FF). The reason for their criticism is related to the higher proportion of samples with an FF <4% reported in our article, compared with that observed in other studies.

Different approaches are currently available for estimating the FF, either from the isolated DNA[2–4] or the sequencing data.[5–8] Among these methods, no two are alike. Thus, it is not surprising that these approaches show a relatively high variation in FF values, even at similar mean gestational age or when comparing similar populations. Recently, Gil *et al.*,[9] in a meta-analysis of published studies, reported that the incidence of test failures due to low FF (<4%) ranged from 0.5 to 6.1%. For instance, Pergament *et al.*[10] described a 6.1% rate of the samples with FF <4% in a population of patients with a mean gestational age of 17.0 weeks ± 4.1 days. This value is not so different from that reported in our study (8.5%), involving a significantly lower mean gestational age (12.8 weeks ± 2.3 days).

Lüthgens and collaborators found in their clinical practice only 0.6% of samples with FF <4%, a proportion that is also much lower than expected if compared with the 2 to 5% observed in the studies cited by the authors. This finding may be explained by the specific method used for FF determination or the characteristics of the population under evaluation (e.g. higher mean gestational age or lower mean maternal weight, as compared with our study). Alternatively, it could be assumed that FF is overestimated with their method, although no definitive conclusions can be drawn from the authors' data set because such details have not been provided.

The aforementioned methods for FF determination have not been compared with the same set of samples nor validated using standard reference DNAs; therefore, it is not currently possible to define which approach works best.

In addition, each non-invasive prenatal testing (NIPT) approach uses a different bioinformatics method to compute aneuploidy risk, their own protocol for FF estimation, and different sequencing depths (i.e. the number of sequence tags counted for each chromosome tested). The combination of such parameters characterizes the limit of detection (LOD; i.e. the lowest FF with a detectable aneuploidy), which is different for each specific NIPT approach. Therefore, several NIPT protocols may need a minimum FF level of 4%. Other methods may be able to reliably detect aneuploidies at a lower FF, because they involve a higher sequencing depth or a more sensitive bioinformatics analysis.[11]

Lüthgens and collaborators believe that setting a lower threshold for FF below 4% will necessarily affect the sensitivity of the cell-free fetal DNA (cfDNA) testing. However, this assumption does not take into consideration the fact that the reliability of cfDNA testing results depends not only on the FF level but also on the sequencing depth employed in testing,[12] both impacting the sensitivity or specificity of the cfDNA assay. If there is a low FF in the sample and a sufficiently high sequencing depth, then the assay can still provide accurate counting of the available chromosome fragments. The higher the number of sequence tags counted, the better the ability to distinguish euploid from aneuploid fetuses, thereby the better the test performance.

Recently, Benn and Cuckle[13] demonstrated that a low FF can partly be compensated by a higher sequencing depth, and the number of generated reads may thus overcome the statistical noise. In fact, when either depth or fetal fraction is high, expected cfDNA-based aneuploidy screening detection rates are high. However, when FF is low, deeper sequencing (at least ten millions of tags) is required to obtain high detection rates.

The NIPT protocol used in our study relied on a high sequencing depth (an average of 16 ± 1.6 millions of unique tags). Such a level of resolution was sufficient to reliably detect aneuploidies in samples with low FF. This was demonstrated by the results of the LOD experiments and confirmed by the chromosomally abnormal pregnancies involving a $2\% < FF < 4\%$, which were consistently identified, with no false negative results. It is unlikely that the two false positive cases that occurred in our study could be related with the low FF, as assumed by the authors. In fact, a low FF may potentially decrease the detection rate (or

sensitivity), and it is unlikely to have much impact on the false positive.

Lüthgens and collaborators also raised concerns on the size of samples included in the $2\% < FF < 4\%$ group. We recognize that such number is not high; however, the aim of our study was not to compare the cfDNA testing performance in samples with $2\% < FF < 4\%$ versus $FF \geq 4\%$. In fact, we aimed to demonstrate that each specific NIPT approach should use an FF cut-off related with its actual LOD and not a fixed value that is too low or too high. Nevertheless, updated data with follow-up from our study, involving a cohort of 16 856 samples, may be helpful in addressing the authors' criticisms. Out of 8540 pregnancies with a male fetus, 25/570 samples with $2\% < FF < 4\%$ and 89/7970 with $FF \geq 4\%$ were chromosomally abnormal. The sensitivity was 100% for both the $2\% < FF < 4\%$ group (95% CI 86.35–100%) and $FF \geq 4\%$ (95% CI 95.94–100%). Two false positive cases resulted in the $2\% < FF < 4\%$ group and one in the $FF \geq 4\%$ group, with a specificity of 98.63% (95% CI 98.7–99.96%) and 99.99% (95% CI 99.93–100%), respectively. Therefore, our updated data still indicate that lowering the FF threshold below 4% did not decrease the accuracy of cfDNA screening.

Finally, Lüthgens and collaborators raised concerns on the incidence of pregnancies with trisomy (T) 21 in the $2\% < FF < 4\%$ group compared with the euploid pregnancies in the same cohort of samples. In our data set of pregnancies with $2\% < FF < 4\%$, those with euploid results had an average FF of $2.8\% \pm 0.04$, while those with T21 had an average of $3.1\% \pm 0.03$. In the group with $FF \geq 4\%$, euploid pregnancies had an average FF of $9.7\% \pm 0.04$, while those with T21 had $10.9\% \pm 0.04$. Therefore, the mean FF for fetuses with T21 was higher than for euploid male fetuses. In addition, considering the entire cohort of patients, the FF was higher in pregnancies with T21 ($9.9\% \pm 0.04$) compared with T18 ($6.6\% \pm 0.04$) and T13 ($5\% \pm 0.034$), thus confirming the data reported in the literature.

In conclusion, we strongly feel that the minimum FF level necessary for accurate aneuploidy assessment should be related to the actual LOD of each specific NIPT approach used and not necessarily fixed at 4% for all cfDNA testing methodologies. The determination of the LOD represents a basic quality metric advisable for any NIPT method, which is required to ensure that the appropriate FF cut-off value can be used.

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