Supplementary Online Content

Malan V, Bussières L, Winer N, et al. Effect of Cell-Free DNA Screening vs Direct Invasive Diagnosis on Miscarriage Rates in Women With Pregnancies at High Risk of Trisomy 21: A Randomized Clinical Trial. *JAMA.* doi:10.1001/jama.2018.9396

eAppendix. Supplementary Methods

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This supplementary material has been provided by the authors to give readers additional information about their work.

eAppendix. Supplementary Methods

Sample collection

Peripheral venous blood samples for cfDNA tests were collected in Streck Cell-free DNA BCT 20ml-bottles (Streck, Omaha, NE, USA), and labelled with a bar code. Samples were sent to the Cytogenetic laboratory of Necker-Enfants Malades hospital within two days of being taken. Plasma was separated from whole blood initially by centrifugation at 1,600g for 10 minutes at +4°C and then transferred to micro-centrifuge tubes. A second centrifugation was performed at 16,000g for 10 minutes at +4°C. Plasma was transferred to fresh tubes to be stored at -80°C until further processing. In most of cases, DNA from plasma was extracted immediately or within 24 hours. All samples were visually inspected for haemolysis and haemolysed specimens were discarded.

DNA extraction

For each sample, genomic DNA was extracted from 5 ml of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The excess plasma was also frozen at -80°C. DNA was eluted into a final volume of 60µl Tris-EDTA buffer and stored at -20°C until further processing. DNA quantification was performed with the Qubit™ fluorometer (DNA high sensitivity assay) (Thermo Fischer Scientific, Waltham, USA) and samples with a high level of DNA (1.5 ng/µl) were rejected as contamination with maternal DNA was suspected.

Library preparation

Library preparation was achieved using TruSeq® Nano DNA LT Sample Preparation Kit (24 Samples) (Illumina, San Diego, CA, USA). 48µl of extracted DNA was used for the Illumina library preparation. End- repair, A-tailing and adaptors ligation with specific tag were performed. Purification was carried out prior to enrichment of the ligation product by PCR. Finally, elution in 100µl was completed after a new purification. The samples were stored for up to 12 hours at 4°C before library quantification and pooling. Libraries were quantified using the Qubit™ fluorimeter and their profiles were assessed using the Agilent 2100 Bioanalyzer system (Agilent Technologies, Palo-Alto, CA, USA). Each sample was normalized at 2nM.

Sequencing and Bioinformatics

Pooling was performed at a 10pM concentration and included 11 patients with one case of trisomy 21, as a control sample. Sequencing was performed with a HiSeq 1500 (Illumina, San Diego, CA, USA) using a rapid run for single reads of 50 base-lengths. After demultiplexing, sequenced files were aligned using bwa mem v0.7.6a on the UCSC human genome reference hg19[1]. PCR duplicates were removed using the rmdup command of the Samtools package v0.1.19 and then filtered using the R Bioconductor Rsamtools package v1.18.3 to allow only reads with a perfect match and a mapping quality score (mapq) of 60[2,3]. Only samples with at least 10 million filtered reads were considered compliant with our quality standard. This corresponded to an average coverage of 0.16 reads/base and more than 120000 reads mapped on the chromosome 21. Read counts per chromosome and z-score computations were then performed with the R RAPIDR package v0.1.1[4]. Read counts, for each evaluated sample, were computed using the 'GC bin correction' method and the default chromosomal 20kb bin size of RAPIDR. To compute the z-scores, the ratio of the weighted read counts on chromosome 21 and the sum of all autosomes was standardised by the mean and standard deviation of the same quantities derived from a reference set of 120 euploïd pregnancies (60 male and 60 female foetuses).

Interpretation and rendering of the cfDNA results

The results were given as positive or negative according to the z-score value and were available within 7-21 working days following blood sampling. A result was considered positive when the z-score was above +1.645. A z-score above this value indicated that the fraction of chromosome reads was higher than the 95th percentile of the set of the reference samples for a one-tailed distribution. Using a z-score of 1.645 anticipated sensitivity and specificity over 99% and 95% respectively with a false positive rate of 5%. This threshold was set-up at this value to reduce the number of invasive procedures, while limiting the risk of false negatives. All results were transmitted from the central laboratory to the referring clinical coordinator by facsimile and email. Positive results were also communicated by telephone by the cytogeneticist upon diagnosis. Women were informed that a positive test result would increase their risk for Down syndrome 50-100-fold and that an invasive prenatal diagnosis would be recommended in that situation. The decision to terminate the

pregnancy in the event of an affected foetus was only based on an actual cytogenetic result obtained by either karyotype or FISH analysis.

In cases with of a negative cfDNA result, women were reassured and advised that their risk for Down syndrome was reduced by at least 50 folds. Nevertheless, invasive prenatal diagnosis remained a possibility for women who were still anxious following cfDNA testing. Invasive testing was also proposed in all positive cfDNA cases, in addition to those with an abnormal follow-up ultrasound examination. Lastly, a choice was given to patients with an inconclusive cfDNA test whether to undergo either repeat-cfDNA test or proceed directly to invasive testing.

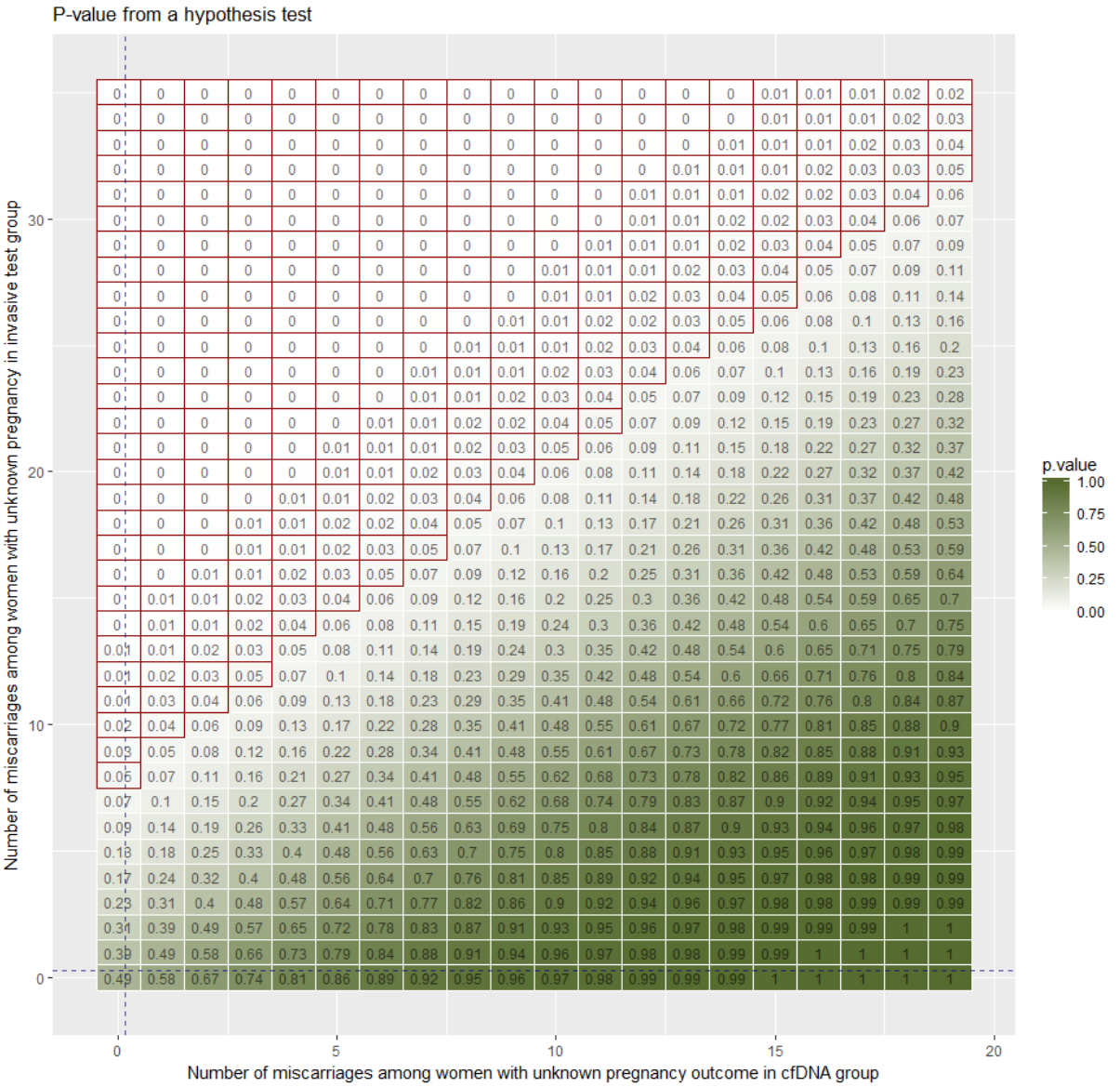
1. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *ArXiv13033997 Q-Bio*. March 2013. http://arxiv.org/abs/1303.3997.
2. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinforma Oxf Engl*. 2009;25(16):2078-2079. doi:10.1093/bioinformatics/btp352
3. Huber W, Carey VJ, Gentleman R, et al. Orchestrating high-throughput genomic analysis with Bioconductor. *Nat Methods*. 2015;12(2):115-121. doi:10.1038/nmeth.3252
4. Lo KK, Boustred C, Chitty LS, Plagnol V. RAPIDR: an analysis package for non-invasive prenatal testing of aneuploidy. *Bioinforma Oxf Engl*. 2014;30(20):2965-2967. doi:10.1093/bioinformatics/btu419

eTable. Detail of the Chromosomal Anomalies (Other Than Trisomy 21) Detected by Karyotyping in the 751 Women According to the ISCN (International System for Human Cytogenomic Nomenclature) 2016

Case	*Chromosomal anomaly*	*Karyotype (ISCN 2016)*
1	Apparently balanced reciprocal translocation between the chromosomes 5 and 17 inherited from the father	46,XX,t(5;17)(q32;p13).ish t(5;17)(wcp5+,wcp17+;wcp17+,wcp5+)pat
2	Balanced Robertsonian translocation inherited from the father	45,XY,der(13;14)(q10;q10)pat
3	Type II confined placental mosaicism (trisomy12)	Chorionic villi culture: mos 47,XY,+12[12]/46,XY[5]
4	Apparently balanced reciprocal translocation between the chromosomes 2 and 13 which occurred *de novo*	46,XX,t(2;13)(p12;p12).ish(2pter,wcp2+,2qter+;2pter+,wcp2+;wcp13+,13qter+)dn
5	Mosaic trisomy 13	mos 47,XY,+13[4]/46,XY[17]
6*	3q11.2q13.11 mosaic deletion: one clone with an interstitial deletion of the long arm of a chromosome 3 and a marker derived from a chromosome 3 and one clone with normal chromosomes 3 and the same marker	47,XX,del(3)(q11.2q13.11),+r(3)(q11.2q13.11)[15]/46,XX,del(3)(q11.2q13.11)[9].ish arr [GRCh37] 3q11.2q13.11(93537290_103985553)X1~2 dn
7	Two distinct clones: one with a small ring X (containing *XIST* locus) and one with the small ring X and a large ring X (which not contains *XIST* locus)	mos 46,X,+r1[5]/47,X,+r1,+r2[5].ish r1(X)(wcpX+,XIST+),r2(X)(wcpX+,XIST-)
8	Mosaic trisomy 13 resulting from a homologous Robertsonian translocation	mos 46,XY,+13,der(13;13)(q10;q10) [2]/46,XY[44]
9	Monosomy X	45,X
10	Mosaic 45,X and 46,XY	mos 45,X[8]/46,XY[22]
11	Klinefelter syndrome	47,XXY

*Chromosomal microarray analysis was performed in case 6 to characterize the anomaly.

eFigure. Tipping Point Analysis



The figure above shows all the possible combinations of replacement of missing outcomes with miscarriages in the two treatment groups, along with corresponding p-values. As expected, the higher is the number of miscarriages in the cfDNA group, the more the p-value of the test increases and gets closer to 1 (in dark green). Tipping points that modify the conclusion on the primary outcome (i.e. make the miscarriage rates in the two groups become significantly different) occur when the number of miscarriages is increasing in the women lost to follow-up of the invasive group. For example, if there is no miscarriage among the 19 patients lost to follow up in the cfDNA group, the test would only become significant if at least 8 miscarriages occur in the 35 patients lost to follow up in the invasive group (23%), which seems implausibly high.