Cell-free fetal DNA for genetic evaluation in Copenhagen Pregnancy Loss Study (COPL): a prospective cohort study

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Summary

Background One in four pregnancies end in a pregnancy loss. Although the effect on couples is well documented, evidence-based treatments and prediction models are absent. Fetal aneuploidy is associated with a higher chance of a next successful pregnancy compared with euploid pregnancy loss in which underlying maternal conditions might be causal. Ploidy diagnostics are therefore advantageous but challenging as they require collection of the pregnancy tissue. Cell-free fetal DNA (cffDNA) from maternal blood has the potential for evaluation of fetal ploidy status, but no large-scale validation of the method has been done.

Methods In this prospective cohort study, women with a pregnancy loss were recruited as a part of the Copenhagen Pregnancy Loss (COPL) study from three gynaecological clinics at public hospitals in Denmark. Women were eligible for inclusion if older than 18 years with a pregnancy loss before gestational age 22 weeks (ie, 154 days) and with an intrauterine pregnancy confirmed by ultrasound (including anembryonic sac), and women with pregnancies of unknown location or molar pregnancies were excluded. Maternal blood was collected while pregnancy tissue was still in situ or within 24 h after pregnancy tissue had passed and was analysed by genome-wide sequencing of cffDNA. Direct sequencing of the pregnancy tissue was done as reference.

Findings We included 1000 consecutive women, at the time of a pregnancy loss diagnosis, between Nov 12, 2020, and May 1, 2022. Results from the first 333 women with a pregnancy loss (recruited between Nov 12, 2020, and Aug 14, 2021) were used to evaluate the validity of cffDNA-based testing. Results from the other 667 women were included to evaluate cffDNA performance and result distribution in a larger cohort of 1000 women in total. Gestational age of fetus ranged from 35–149 days (mean of 70·5 days [SD 16·5], or 10 weeks plus 1 day). The cffDNA-based test had a sensitivity for aneuploidy detection of 85% (95% CI 79–90) and a specificity of 93% (95% CI 88–96) compared with direct sequencing of the pregnancy tissue. Among 1000 cffDNA-based test results, 446 (45%) were euploid, 405 (41%) aneuploid, 37 (4%) had multiple aneuploidies, and 112 (11%) were inconclusive. 105 (32%) of 333 women either did not manage to collect the pregnancy tissue or collected a sample classified as unknown tissue giving a high risk of being maternal.

Interpretation This validation of cffDNA-based testing in pregnancy loss shows the potential and feasibility of the method to distinguish euploid and aneuploid pregnancy loss for improved clinical management and benefit of future reproductive medicine and women's health research.

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Introduction

Pregnancy loss is defined as the spontaneous end of a pregnancy before fetal viability[1] and affects approximately one in four pregnancies.[2] Women with a history of pregnancy loss are at an elevated risk for health consequences of a range of diseases both in the short term (eg, obstetric or mental health illness) and long term (eg, mental health disorder, cardiovascular disease, or type 2 diabetes).[3,4] Despite the frequent occurrence of pregnancy loss and the substantial mental and physical health implications, current clinical care focuses on removing the pregnancy tissue from the uterus. Diagnostic work-up is restricted to women who have had recurrent pregnancy loss, who are more likely to lose subsequent euploid pregnancies than women who have had a sporadic pregnancy loss.[5] Diagnostic work-up is also done in rare cases of parental balanced translocations and rare genetic variants that predispose women to recurrent aneuploid pregnancy losses. Diagnostic criteria for, and treatment, prevention, and prediction[6,7] of, recurrent pregnancy loss varies globally, as no international consensus on the definition has been found. The 2021 *Lancet* Series, *Miscarriage Matters*,[8] addressed the discrepancy between the effect, frequency, and consequences of pregnancy loss for the couples and society. Our current knowledge of pregnancy loss is poor, and this was emphasised in the Series. [8]

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Research in context

Evidence before this study

Pregnancy loss is an under-investigated condition without precise prognostic models or evident treatments. From previous studies using karyotype and chromosomal microarray analysis it is well described that approximately half of pregnancy losses are caused by fetal aneuploidy, but international guidelines refrain from recommending routine genetic evaluation of pregnancy loss. Traditional fetal chromosome evaluation is dependent on true pregnancy tissue and therefore entails a risk of contamination with maternal DNA or poor tissue quality resulting in inconclusive results. Consequently, chromosomal investigation of the lost fetus is absent in most literature about pregnancy loss. Moreover, higher risk of maternal cardiovascular, metabolic, endocrine, and immune dysfunction is associated with euploid pregnancy loss. In April, 2022, a systematic search of literature reporting the use of cell-free fetal DNA (cffDNA)-based testing in pregnancy loss was done using the electronic medline database PubMed. The search terms used were: ([Pregnancy loss] OR [Spontaneous abortion] (MeSH) OR [Miscarriage] OR [Missed abortion]) AND ([Next generation sequencing] OR [NGS] OR [Cell-free fetal DNA] OR [cffDNA] or [cfDNA] or [Non-invasive Prenatal Testing] (MeSH) OR [NIPT] OR [NIPD] OR [NIFTY]). The total number of results was 556. Studies were included if they assessed cffDNA-based testing in cases of pregnancy loss or recurrent pregnancy loss up to gestational age 22 weeks plus 0 days and with no language limitations. All titles and abstracts were screened by two independent reviewers. Studies were considered irrelevant if they only described the use of cffDNA-based testing in ongoing pregnancies, in determination of fetal sex, qualitative study designs, or in pre-implementation genetic testing. Only two studies were found to match the inclusion criteria. Both found that cffDNA-based testing is feasible for determining fetal ploidy status and reported sensitivities of 57–82% at a 90% specificity when comparing with cytogenetic results from the pregnancy tissue in small cohorts (n=109 and n=57) of women who had pregnancy loss.

Added value of this study

This study shows that cffDNA-based testing is a robust method for evaluation of fetal chromosome status in women with pregnancy loss. From gestational age 5 weeks it is possible to retrieve a fetal diagnosis and from 7 weeks plus 0 days, the fraction of fetal DNA was sufficiently high in 90% of the samples with a sensitivity of approximately 85% for aneuploidies and a corresponding approximate 93% specificity. In our evaluation of 1000 cffDNA test results in pregnancy loss, we found a 50% frequency of fetal aneuploidy, which corresponds to previous cytogenetic studies based on the pregnancy tissue. We also show the limitations of pregnancy tissue-based fetal diagnosis due to not being able to collect tissue samples in a third of pregnancy losses.

Implications of all the available evidence

Supported by the results of this study, cffDNA-based testing is a valid and applicable tool to determine fetal chromosome status in pregnancy loss, even at young gestational ages, and thereby has the potential to improve clinical management and research in the field of pregnancy loss.

The current clinical practice of no action is probably a consequence of traditional views on pregnancy loss, based on the high incidence of fetal aneuploidy[9,10] that is considered de novo and that is suggestive of a decreased risk of further pregnancy loss.[11] Current clinical practice could also be due to the historical technical challenges related to genetic testing of pregnancy loss. Current practice ignores the association of euploid pregnancy loss with an increased risk of future losses[11] and later maternal health consequences such as diabetes and cardiovascular disease.[4] Also, the development of potential actionable targets from the rapidly evolving genetic and molecular diagnostic fields are not currently taken into account in clinical practice. According to European guidelines[1] microarray-based comparative genomic hybridisation (aCGH) of pregnancy tissue can be used for explanatory purposes in recurrent pregnancy loss, but the method is limited by the collection of the pregnancy tissue, risk of contamination with maternal DNA, and mosaicism, and is not a high-throughput method of investigation.

The detection of cell-free fetal DNA (cffDNA) in maternal plasma has revolutionised prenatal screening and is now widely used in ongoing pregnancies after gestational age of 10 weeks.[12] cffDNA rapidly clears from the maternal blood after delivery,[13] but in the cases of pregnancy loss with pregnancy tissue still in situ we expect cffDNA to remain in maternal blood.[14] Two 2020 studies[15,16] investigated the performance of cffDNA-based testing in pregnancy loss in small cohorts of 109 and 57 women, respectively, and reported sensitivities between 57% and 82% at a 90% specificity when compared with cytogenetic results from pregnancy tissue, but both studies underscore a need for a larger cohort to support the results.

The development of a sensitive, applicable, and scalable method for fetal ploidy evaluation would therefore be a key step in the management of pregnancy loss, as well as in the research of underlying pathophysiology. The prospective Copenhagen Pregnancy Loss (COPL) study is a large and ambitious initiative with the overall aim to explore the causes of pregnancy loss. As a first step, we aim to develop a pipeline for fetal chromosome evaluation in pregnancy loss based on cffDNA in maternal blood, and to validate the method against direct pregnancy tissue sequencing. In this study, we investigated the applicability of cffDNA-based testing in women with pregnancy loss as early as gestational age 5 weeks (ie, 35 days) and to show the potential of the method for future research and clinical application.

Methods

Study design and participants

In this ongoing prospective cohort study, women were referred by a physician (general practitioner, fertility clinic, or gynaecological practice), because of a pregnancy loss or suspicion thereof, to a gynaecological department at one of three hospitals in Denmark: Copenhagen University Hospital Hvidovre, Herlev, or North Zealand. Between Nov 12, 2020, and May 1, 2022, women were consecutively invited to participate and received written and oral information about the project. Information about the project was also available, in Danish, at the Copenhagen Pregnancy Loss study's homepage. The three hospitals are public and free at the point of care.

Women were eligible for inclusion if older than 18 years with a pregnancy loss before gestational age 22 weeks (ie, 154 days) and with an intrauterine pregnancy confirmed by ultrasound (including anembryonic sac). Eligible women were required to be able to understand information given in Danish or English and to give both oral and written informed consent. Women who had pregnancies of unknown location, who had molar pregnancies, and who were unable to make an informed decision were excluded.

This study was done according to the principles of the Declaration of Helsinki. Data and biomaterial collection, and planned analyses (including genetic analyses), were approved by the Health Research Ethics Committees for the Capital Region of Denmark (H-18024745) and by the Videnscenter for Dataanmeldelser (Centre for Data Registration) for the Capital Region of Denmark (P-2020–1019). Personal identifiable information was handled in a REDCAP database according to the requirements of the Capital Region of Denmark. Participants could withdraw from the study at any point, without their treatment and care being affected.

Procedures

Blood samples were drawn from participating individuals before treatment to remove pregnancy tissue was initiated or within 24 h after complete passage of pregnancy tissue. If the pregnancy tissue was removed by surgical management of pregnancy tissue, the tissue was collected from the vacuum system. If medical (mifepristone plus misoprostol) or spontaneous removal occurred, the woman collected the pregnancy tissue at home and brought it into the hospital. In case of a second trimester loss, the induction and delivery took place at the hospital, where the clinical staff collected a biopsy from the fetal foot. 6 weeks after the pregnancy loss, participants were invited for a follow-up visit with a doctor or nurse to receive the cffDNA result. Metadata for the population are listed in the table.

The pregnancy tissue was sent to the University of Copenhagen and stored at 4°C upon arrival. For direct sequencing, each pregnancy tissue sample was washed in sterile phosphate-buffered saline to remove excess maternal blood. The residual sample was inspected under a stereo microscope to identify fetal tissue and chorionic villi from maternal decidua and blood clots.

For more on the **COPL study** see http://www.graviditetstab.dk

	Euploidy (n=446)	Aneuploidy (n=405)	Multiple aneuploidies (n=37)	No-call (n=112)	Total (n=1000)
Maternal age, years	33·2 (5·0)	34·3 (5·1)	37·8 (5·3)	33·9 (5·2)	33·9 (5·2)
p value	..	0·0096	<0·0001	0·21	..
n	446	405	37	112	1000
Paternal age, years	34·8 (5·7)	35·7 (6·0)	40·4 (5·3)	35·6 (6·1)	35·4 (5·9)
p value	..	0·11	<0·0001	0·32	..
n	349	304	26	81	760
BMI (kg/m²)	24·5 (4·6)	24·3 (4·5)	25·1 (3·8)	25·8 (5·2)	24·6 (4·6)
p value	..	0·56	0·27	0·13	..
n	403	377	32	94	906
Gestational age*	71·5 (19·2)	71·4 (12·9)	65·9 (10·5)	64·9 (16·7)	70·5 (16·5)
p value	..	0·040	0·27	0·0042	..
n	439	396	37	108	980
Gestational age†	56·5 (17·7)	52·6 (9·7)	49·1 (7·9)	50·9 (14·5)	54·0 (14·2)
p value	..	0·51	0·041	0·016	..
n	326	343	29	76	774
Conception					
Natural	328 (74%)	305 (75%)	28 (76%)	78 (70%)	739 (74%)
ICSI	30 (7%)	22 (5%)	1 (3%)	7 (6%)	60 (6%)
IUI	18 (4%)	19 (5%)	2 (5%)	7 (6%)	46 (5%)
IVF	49 (11%)	44 (11%)	2 (5%)	17 (15%)	112 (11%)
PGT	2 (<1%)	1 (<1%)	0	0	3 (<1%)
Oocyte donation	7 (2%)	5 (1%)	0	0	12 (1%)
Unknown	12 (3%)	9 (2%)	4 (11%)	3 (3%)	28 (3%)
p value	..	0·97	0·21	0·65	..
Number of pregnancies (including case pregnancy)					
1	132 (30%)	108 (27%)	10 (27%)	31 (28%)	281 (28%)
2	112 (25%)	117 (29%)	7 (19%)	34 (31%)	270 (27%)
3	96 (22%)	88 (22%)	8 (22%)	15 (14%)	207 (21%)
4	44 (10%)	42 (10%)	6 (16%)	14 (13%)	106 (11%)
5	25 (6%)	31 (8%)	3 (8%)	10 (9%)	69 (7%)
6	18 (4%)	13 (3%)	2 (5%)	2 (2%)	35 (4%)
>6	16 (4%)	3 (1%)	1 (3%)	4 (4%)	24 (2%)
p value	..	0·082	0·78	0·26	..
Number of births					
0	234 (52%)	201 (50%)	14 (39%)	62 (56%)	511 (52%)
1	143 (32%)	141 (35%)	14 (39%)	31 (28%)	329 (33%)
2	56 (13%)	49 (12%)	6 (17%)	14 (13%)	125 (13%)
3	7 (2%)	9 (2%)	2 (6%)	2 (2%)	20 (2%)
>3	3 (1%)	3 (1%)	0 (0%)	1 (1%)	7 (1%)
p value	..	0·85	0·19	0·86	..
Number of previous pregnancy losses					
0	249 (56%)	231 (57%)	20 (54%)	60 (55%)	560 (56%)
1	113 (26%)	100 (25%)	7 (19%)	26 (24%)	246 (25%)
2	41 (9%)	40 (10%)	7 (19%)	13 (12%)	101 (10%)
3	18 (4%)	22 (5%)	2 (5%)	8 (7%)	50 (5%)
>3	22 (5%)	10 (2%)	1 (3%)	2 (2%)	35 (4%)
p value	..	0·34	0·36	0·30	..

(Table continues on next page)

	Euploidy (n=446)	Aneuploidy (n=405)	Multiple aneuploidies (n=37)	No-call (n=112)	Total (n=1000)
(Continued from previous page)					
Smoking during pregnancy					
No	306 (95%)	294 (95%)	29 (100%)	74 (93%)	703 (95%)
Yes	15 (5%)	14 (5%)	0 (0%)	6 (8%)	35 (5%)
p value	..	1	0·36	0·23	..
Alcohol use during pregnancy					
No	292 (87%)	280 (88%)	26 (87%)	77 (92%)	675 (88%)
Yes‡	42 (13%)	40 (13%)	4 (13%)	7 (8%)	93 (12%)
p value	..	0·53	0·29	0·78	..

Continuous variables are reported as means (SD). Categorical variables are reported as n (%); percentages may not total 100 due to rounding. Ploidy status was determined by cffDNA-based testing. p values were calculated by Dunn test (numeric variables) or by Fisher's exact test (categorical variables) compared to the euploid group. Alcohol use includes before the pregnancy was recognised. Smoking was at the time of the pregnancy loss. ICSI=intracytoplasmic sperm injection. IUI=intrauterine insemination. IVF=in-vitro fertilisation. cffDNA=cell-free fetal DNA. PGT=preimplantation genetic testing. *Days since last menstrual period. †Days based on crown rump length. ‡Including before positive test.

Table: **Metadata of sample population**

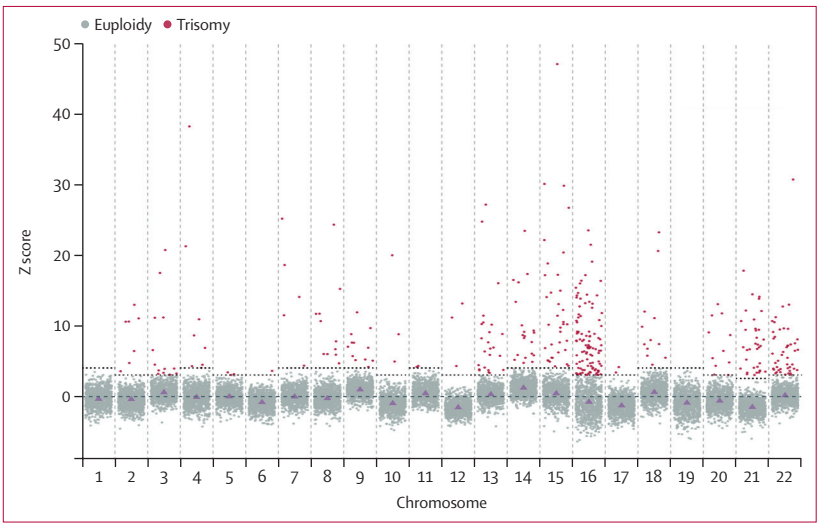

Figure 1: **Plot of *Z* scores for each autosomal chromosome (1–22)**
The dotted black line shows an adjusted cutoff for pregnancy losses between euploid (grey) and aneuploid (red) cases. Cutoff was 4 for chromosome 1, 4, 7, 8, 9, 11, 14, 15, 18, 19. Cutoff was 3 for chromosome 2, 3, 5, 6, 10, 12, 13, 16, 17, 20, 22. Cutoff was 2·5 for chromosome 21. Triangles represent median *Z* scores.

See Online for appendix

The tissue was dissected into pieces of approximately 0·5×0·5 cm if possible, run through three additional phosphate-buffered saline washes, snap frozen in liquid nitrogen in a 2 mL Nunc cryotube vial (Fisher Scientific, Denmark; 10577391), and then stored at –80°C. Fetal tissue samples were bead milled for 1 min at 30 Hz using a 5 mm steel bead on a TissueLyser LT (Qiagen, Italy; 85600). DNA was then extracted using the DNeasy 96 Blood & Tissue Kit (Qiagen, Germany; 69504) and quantified with Quant-iT 1X dsDNA Assay Kits, high sensitivity (ThermoFisher, Netherlands; Q33267) on plate reader FLUOstar OPTIMA (Germany) at excitation of 480 nm and emission of 530 nm. Libraries were generated using Nextera XT DNA Library Prep Kit (Illumina, USA; #FC-131–1096) with 50 ng fetal tissue DNA input. 96 libraries were pooled and sequenced to 0·05–0·1 average depth on NextSeq 550 with NextSeq 500/550 Mid Output Kit version 2.5 (150 cycles) (Illumina, USA; 20024904) with 2×76 cycles paired-end and dual-index (appendix p 3).

Downstream analyses were completed with R (version 4.2.1). First, reference genome bin files were created with the GRCh38.p13 genome. Sequencing reads were aligned onto the GRCh38.p13 genome using Rbowtie2 (2.3.1) to create SAM files and converted into BAM files with Rsamtools (2.13.4). Read counts were binned along the genome (200 kb windows) using the QDNASeq package (1.33.1) and then GC and mappability corrected. Bin counts were also normalised internally within each sample (QDNAseq [1.33.1] normalizeBins) and smoothed (SmoothOutlierBins [1.33.1]) for downstream copy number variation plots using ggplot2 (3.3.6). Chromosome abnormalities such as aneuploidy and partial chromosome copy number variants (≥5 Mbps) were visualised and reported without knowledge about the corresponding cffDNA result. Bioconductor platform references are listed in the appendix (p 12).

We did a short tandem repeat analysis to assess contamination with maternal DNA (appendix p 3). Maternal DNA was isolated from EDTA (edetic acid) blood samples by standard procedure on a QIAsymphony (Qiagen, Hilden, Germany) and compared with the fetal DNA isolated for direct sequencing. The short tandem repeat analyses consisted of six high polymorphic microsatellites, D3S1358 (3p21.31), vWA (12p12), TH01 (11p15.5), D7S820 (7p21.11), FGA (4q28), and CSF1PO (5q33.3), included in AmpFl short tandem repeat Identifiler PCR Amplification Kit (Applied biosystems/Life technologies, Netherlands). Fragments were resolved on an ABI3500xl (ThermoFisher, USA) and analysed by GeneMapper (version 5.1). Primers and conditions are available upon request to the corresponding author.

For the cffDNA-based test (appendix p 3), maternal blood was drawn in 10 mL Streck tubes (Cell-Free DNA BCT CE, Illumina, USA; 15073345) while the pregnancy tissue was still in situ or within 24 h after evacuation. Tubes were stored at room temperature and transferred to Hvidovre Hospitals Non-Invasive Prenatal Testing (NIPT) Center within 1 week from collection, samples were centrifuged twice, and plasma was isolated and stored in 2 mL cryotube vials (In vitro, #GR-122280, Germany) at –20°C until further downstream analysis. The analysis of cffDNA was based on the principles of genome-wide NIPT in ongoing pregnancies as previously described.[17] For data analysis, a modified version of WISECONDOR and DEFRAC software was used.[18–20] Adjustments of cutoff values for the average allowed deviation quality-control scores and *Z* scores for each autosome were determined by using the results from the direct sequencing of the pregnancy tissue

and routine NIPT results from ongoing pregnancies analysed at our in-house platform described in the appendix (p 4).

Aneuploidies were assessed for all chromosomes. Copy number variants, polyploidies, mosaicism, and balanced translocation were not reported. The fetal chromosome fractions were estimated by the bioinformatic data mining method SeqFF, by which small differences of sequencing behaviour for maternal and fetal cell-free DNA and read counts were used for estimation.[20] Fetal sex chromosome status was determined by the fractional ratio between the X and Y chromosomes. Only samples with a minimum of 8 million reads after filtering were reported. Samples with low fetal fractions (SeqFF<0·025) or high average allowed deviation quality scores (average allowed deviation >8·0) were reported as inconclusive (no-calls) except for samples with a fetal fraction between 0·015 and 0·025 and an autosomal *Z* score greater than the threshold, which were reported as aneuploid (figure 1). Samples with a fetal fraction between 0·015 and 0·025 and an autosomal *Z* score below the threshold were reported as no-calls. Further description of the used cutoffs are in the appendix (p 4).

Statistical analysis

For evaluating the diagnostic accuracy of cffDNA-based testing we used the direct sequencing of the pregnancy tissue as the reference standard and compared the results in a 2×2 contingency table by Fisher's exact test. Agreement between the cffDNA-based result and direct sequencing of the pregnancy tissue was calculated by Kappa statistics and reported as Cohen's coefficient (appendix pp 5–6). Concordant results showing fetal aneuploidy were considered true positive and concordant results showing fetal euploidy were considered true negative. Discordant results were considered false positive if the cffDNA-based result was aneuploid and false negative if the cffDNA-based result was euploid. In the initial calculations, instances in which direct sequencing of the pregnancy tissue was unavailable or found to be maternally contaminated, the cffDNA-based test result was considered true positive if aneuploid or true negative if euploid. Sensitivity analyses were added by excluding individuals with no available pregnancy tissue, with short tandem repeat-confirmed contamination with maternal DNA or instances in which pregnancy tissue was classified as unknown tissue, and with direct sequencing showing 46, XX. Further sensitivity analyses were done by excluding individuals with a direct sequencing of the pregnancy tissue showing 46, XX or monosomy X. We used R (version 4.2.1) and Graph Pad Prism (version 9) for statistical analyses and BioRender for graphics.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

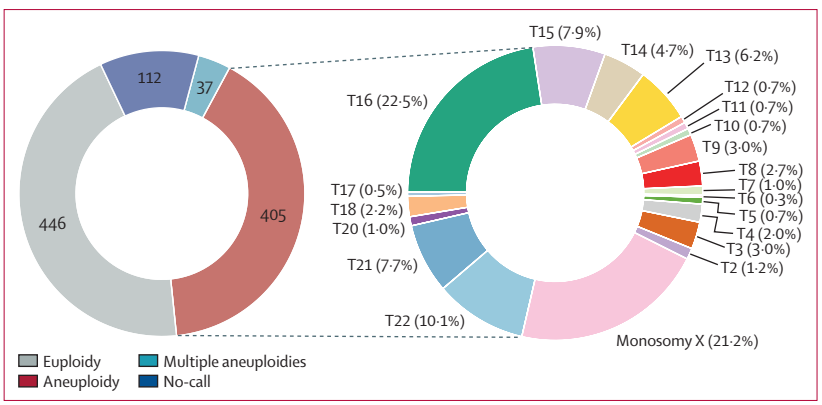

Figure 2: **Distribution of 1000 cell-free fetal DNA-based tests results**

Results

In total, 1000 women diagnosed with pregnancy loss were recruited between Nov 12, 2020, and May 1, 2022. We recruited 333 women from Nov 12, 2020, to Aug 14, 2021, and the sensitivity and specificity of the cffDNA-based tests were assessed by comparing them with the reference test from direct pregnancy tissue sequencing. In 19 (6%) of the 333 women, collection of the pregnancy tissue was not possible for practical or psychological reasons. 137 (44%) of 314 collected the pregnancy tissue themselves after medical or conservative treatment and 177 (56%) had surgical management of pregnancy tissue. DNA was isolated from fetal tissue or chorionic villi in 228 (73%) cases, or from unknown tissue in 86 (27%) cases. As a control for contamination with maternal DNA, a subgroup of pregnancy tissue samples classified as unknown tissue with a euploid female result from the direct sequencing (n=44 [14%]) underwent short tandem repeat analyses to test for contamination with maternal DNA. 31 (70%) of these samples were found to be maternal, six (14%) were confirmed to be fetal, and six (14%) were a mixture of maternal and fetal DNA. In one case (2%) the short tandem repeat analysis was inconclusive. In total, 105 (32%) of 333 women either did not manage to collect the pregnancy tissue (n=19) or collected a sample classified as unknown tissue (n=86) giving a high risk of being maternal (70% according to the STR subgroup control).

The short tandem repeat control did not identify any cases of polyploidy or uniparental disomy. In nine instances, a subchromosomal abnormality was detected by direct sequencing of pregnancy tissue. These cases were included as euploid in the comparison with cffDNA-based testing. Furthermore, we identified two cases of mosaicism in the pregnancy tissue; one case of mosaicism between villi and fetus, but with a cffDNA no-call result, and another in which villi from different parts of the placenta were discordant and the cffDNA analysis showed aneuploidy. The fetus was not available in the latter case and the cffDNA result was classified as true

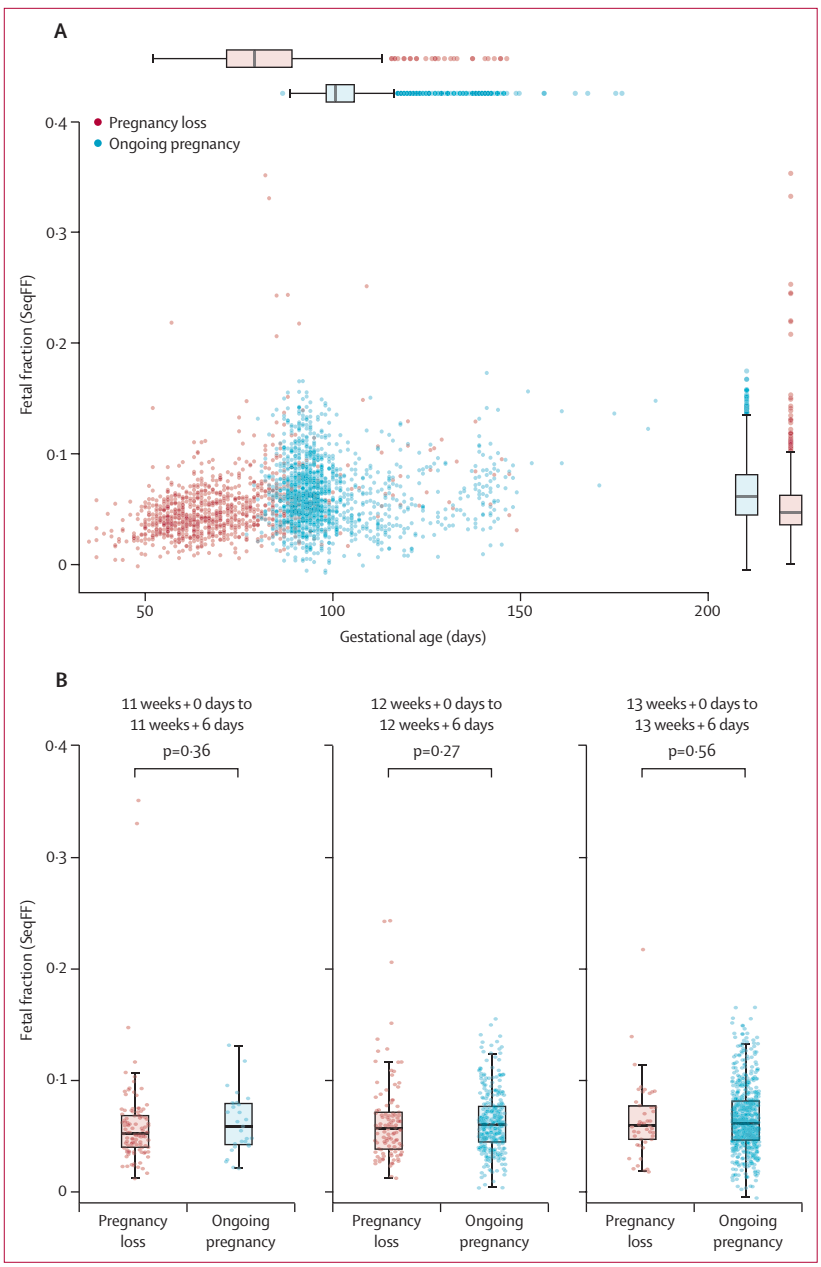

Figure 3: **Fetal fraction of cell-free DNA (estimated by SeqFF) by gestational age based on last menstrual period from pregnancy losses (red) and ongoing pregnancies (blue; A) and in groups of pregnancy loss and ongoing pregnancies matched by gestational week (B)**

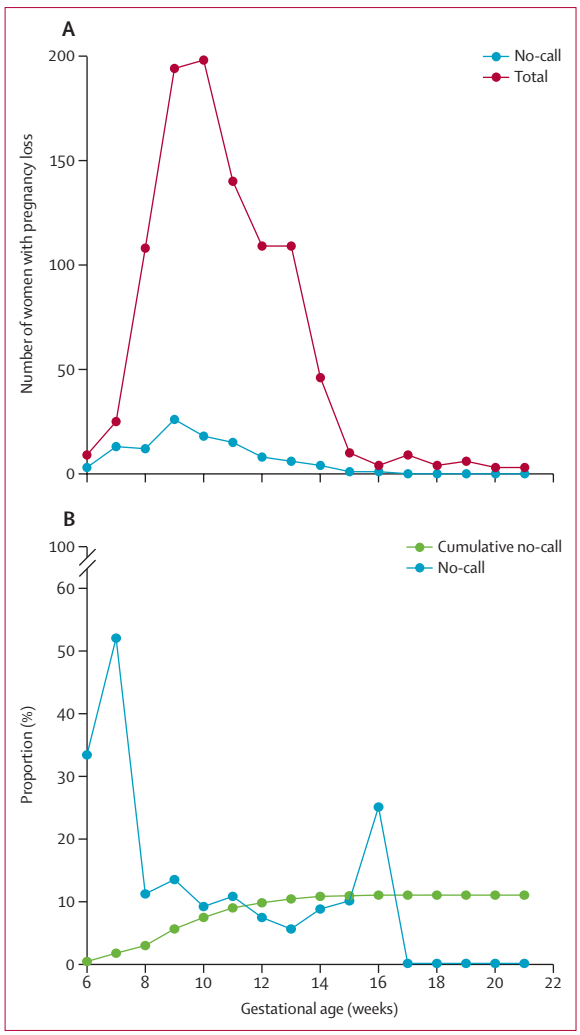

Figure 4: **The association between no-calls and gestational week (measured by last menstrual period) in cases of pregnancy loss, showed by the total numbers of cell-free fetal DNA analyses and the number of no-calls by gestational age in weeks (A), and by the no-call rate and the cumulative no-call rate by gestational age in weeks (B)**

positive. A flowchart of samples is in the appendix (p 2). No maternal malignancies were identified.

The cffDNA result was inconclusive (no-calls) in 31 (9%) of the initial 333 cases due to low fetal fraction (22 [71%] of 31 had a SeqFF<0·015 or <0·025), low sequencing quality (eight [26%] of 31 had an average allowed deviation >8·0), or few reads (one [3%] of 31 had <8 million analysed reads) and were excluded from the sensitivity and specificity calculations. Characteristics of the no-call cases are shown in the appendix (p 9). Based on the cffDNA results of the remaining 302 cases, 149 (49%) were euploid and 153 (51%) aneuploid, with monosomy X (n=34), trisomy 16 (n=22) and trisomy 22 (n=17) being the most common karyotypes.

The sensitivity of cffDNA-based testing compared with pregnancy tissue sequencing was 85% (95% CI 79–90), specificity of 93% (88–96), accuracy of 89% (85–92), and Cohen's coefficient 0·78 (appendix p 6) showing substantial agreement.[21] Excluding cases of unavailable pregnancy tissue and pregnancy tissue classified as 46, XX from unknown tissue (suggestive of a high risk of contamination with maternal DNA) or short tandem repeat-confirmed contamination with maternal DNA, resulted in the sensitivity decreasing to 81% (95% CI 73–87) at a specificity of 91% (84–95) and Cohen's coefficient 0·70. If all female and monosomy X cases

from the direct sequencing (euploid or aneuploid) were excluded to completely avoid the risk of contamination with maternal DNA, the sensitivity was 78% (95% CI 65–87) with a specificity of 94% (85–99) and Cohen's coefficient 0·73.

For further evaluation of the test performance, another 667 women were recruited between Aug 15, 2021, and May 1, 2022 for a consecutive cohort of 1000 women with pregnancy loss. Among 1370 invited candidates the participation rate was 73% (n=1000). The main reasons for women to decline study participation was emotional distress, not wanting to collect the pregnancy tissue, or lack of time for participation. Gestational age of fetuses ranged from 35 days to 149 days measured from last menstrual period with a mean of 70·5 days (SD 16·5), or 10 weeks and 1 day. Gestational age based on crown rump length was 54·0 days (SD 14·2), or 7 weeks and 5 days, and maternal BMI was 24·6 kg/m² (SD 4·6). 233 (23%) of 1000 women conceived after fertility treatment, and three of these women had received preimplantation genetic testing and two had lost euploid fetuses (table).

112 (11%) of 1000 total cffDNA analyses were inconclusive due to low fetal fraction or low sequencing quality. In instances of a conclusive result (n=888), 446 (50%) were euploid, 405 (46%) were aneuploid, and 37 (4%) contained multiple aneuploidies (figure 2). The most common abnormal karyotypes identified in the larger cffDNA sample population were trisomy 16 (n=91), monosomy X (n=86), and trisomy 22 (n=41).

In the total pregnancy loss cohort, the mean fetal fraction estimated by SeqFF was 0·051 (SD 0·030), compared with 0·063 (0·029) in a reference cohort of routine NIPT results from ongoing pregnancies previously used for validation of the platform described in the appendix (p 4; Wilcoxon p<0·0001; figure 3A). In these ongoing pregnancies women had a blood sample taken at a mean gestational age of 13 weeks and 0 days, explaining the higher fetal fraction compared with the pregnancy loss cohort. When comparing the fetal fraction between cases of pregnancy loss and ongoing pregnancies matched by gestational week, we found no significant difference (gestational age 11 weeks plus 0 days to 11 weeks plus 6 days, Wilcoxon test p=0·36; gestational age 12 weeks plus 0 days to 12 weeks plus 6 days, Wilcoxon test p=0·63; gestational age 13 weeks plus 0 days to 13 weeks plus 6 days, Wilcoxon test p=0·56; figure 3B). As expected,[22,23] SeqFF values increased with gestational age and we observed a steeper positive slope for earlier gestational ages that levelled off at around 100 days (figure 3A).

The association between no-calls and gestational age was stable at approximately 10% from gestational age 7 weeks and 0 days (figure 4), whereas the no-call rate was approximately 50% at earlier gestational ages. However, the no-call rate before gestational age 7 weeks and 0 days was based on 16 cases (figure 4). A minor proportion of the blood samples were drawn after the pregnancy tissue was passed (maximum 24 h; n=69; mean gestational age 69·4 days). The SeqFF was significantly lower in this group compared with samples drawn with the pregnancy tissue in situ (Wilcoxon p=0·032): 16 (23%) of 69 blood samples resulted in a no-call. Comparatively, 96 (10%) of 931 samples drawn with pregnancy tissue in situ resulted in a no-call. When dividing the late collected samples by time after the pregnancy tissue passed, we found a significantly lower SeqFF in samples drawn 12–24 h after evacuation of the pregnancy tissue than in the samples drawn less than 6 h after (Wilcoxon test p=0·008; figure 5) and the no-call rate increasing from five (13%) of 38 in the group from which pregnancy tissue was drawn less than 6 h after evacuation to four (40%) of ten in the 6–12 h group and six (35%) of 17 in the 12–24 h group. The decrease in cffDNA after evacuation indicates a slower clearance of cffDNA from maternal blood in case of pregnancy loss than after term delivery.[13]

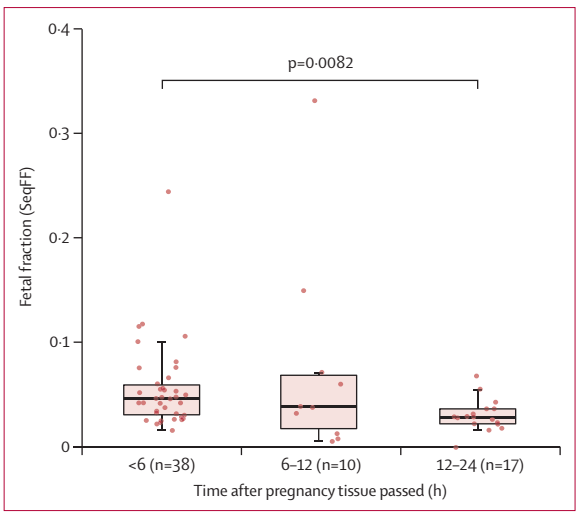

Figure 5: **Fetal fraction of cell-free DNA (estimated by SeqFF) in samples drawn less than 6 h, 6–12 h, and 12–24 h after evacuation**
In four women, the time after the pregnancy tissue had passed the uterus was unknown.

Discussion

There are two main findings in this first large-scale study of cffDNA-based testing in pregnancy loss. First, we found cffDNA-based testing to have high applicability, sensitivity, specificity, and accuracy when compared with direct sequencing of pregnancy tissue, and only 11% of results were inconclusive. Second, almost a third of women were not able to collect the true pregnancy tissue, a rate similar to previous reports.[24,25] This finding illustrates the difficulties of pregnancy tissue collection in early pregnancy loss and why it is relevant to introduce a pregnancy tissue-independent alternative, especially when dealing with early pregnancy loss for which medical treatment is recommended to protect the endometrium and avoid surgical instrumentation and hospitalisation.

The concept of fetal testing in pregnancy loss is fundamentally different from that in ongoing pregnancies, in which screening is widely implemented to identify rare but severe fetal diseases. In pregnancy loss, fetal diagnosis is not recommended by international guidelines despite the high incidence of pregnancy losses, the potential consequences of losing a euploid pregnancy,[4,11] and the psychological burden on patients who desire an explanation for their loss and recognition from the medical community.[26] In daily clinical practice, a history of three or more consecutive pregnancy losses is considered an indicator of euploid pregnancy loss and is used as criteria for further recurrent pregnancy loss work-up. This simplified approach is, in our opinion, outdated and ignores the advancements in genetic testing reached since the beginning of the 2000s. Likewise, a 2022 study recently underscored the importance of separating aneuploid pregnancy loss from miscarriage syndrome,[27] a term suggested to emphasise the unmet need for individualised risk assessment and tailored surveillance following pregnancy loss.

Pregnancy loss and recurrent pregnancy loss have been associated with an increased risk of later-life cardiovascular disease,[28] type 2 diabetes,[3] and mental health disorders among the affected women.[4,29] Consequently, since 2014, the American Heart Association has acknowledged pregnancy loss as a risk factor for stroke and cardiovascular disease in women.[30,31] This increased risk was supported by a study in 2022 that found a stronger association between pregnancy loss and cardiovascular disease in women experiencing pregnancy loss before the age of 30 years and with a history of more than one pregnancy loss.[28] Pregnancy loss at a young maternal age and recurrent pregnancy loss are both indicative of euploid losses, thus suggesting shared underlying pathologic processes between euploid pregnancy loss and cardiovascular disease. Understanding the pathophysiology of euploid pregnancy loss therefore presents an avenue to reduce the risk of further pregnancy losses, as well as later-life disease in women. However, this association represents a substantial knowledge gap in the field of women's health. Based on our results, we consider cffDNA-based testing in pregnancy loss a potential high-throughput method to identify women losing euploid pregnancies after just one or two pregnancy losses without the need to collect pregnancy tissue, and an attractive alternative to the current passive practice of no investigation.

Both cffDNA-based testing and direct pregnancy tissue sequencing used as reference in this study were limited to the detection of trisomies, monosomies, and sex chromosome abnormalities. These aberrations have been found to explain approximately 50% of sporadic first trimester pregnancy losses,[9,10] whereas another approximately 10% are probably caused by polyploidy, uniparental disomy, and copy number variants.[9,10,32] Accordingly, we estimate approximately 10% of aneuploid pregnancy losses are misdiagnosed as euploid with our current platform. Furthermore, as with NIPT in ongoing pregnancies, discordance can occur as a consequence of the rare events of maternal mosaicism, vanished twin, or maternal malignancy.[33] As circulating cffDNA originates mainly from placental trophoblast cells, the cffDNA result will likewise be false positive in the rare instances of high-grade confined placental mosaicism (type 1 and 3) or false negative in instances of true fetal mosaicism (type 5).[34]

High-resolution chromosomal microarray analysis using single-nucleotide polymorphisms has replaced karyotyping in most prenatal diagnostics as it detects aneuploidies, submicroscopic chromosomal imbalances, triploidy, and regions of allelic homozygosity. A 2017 study[35] reported the efficacy and diagnostic power of chromosomal microarray analysis in 6196 fresh pregnancy tissue samples and reported a result in 5725 (92%) samples. Contrary to the COPL cohort, 763 (42%) of 1818 pregnancy tissue samples investigated in this study were collected after gestational age 12 weeks (only 1818 [22%] cases had gestational age reported), which could explain their higher rate of conclusive results than the COPL study. Genetic abnormalities were detected in 3975 (54%) of 7396 samples, of which 94% were considered causal for the pregnancy loss. Applying single nucleotide polymorphism (SNP)-based chromosomal microarray analysis of the pregnancy tissue in the current study would have improved diagnostics of both fetal and maternal conditions. However, SNP-based chromosomal microarray analysis requires successful pregnancy tissue collection, which we were unable to provide in a third of cases. Moreover, improvements to cffDNA sequencing including increased resolution (eg, detection of unbalanced structural aberrations;[36] <7–10 Mbps), imputing and bioinformatic advancements,[14,37] and multiplexing possibilities make it a suitable high-throughput investigation tool for a condition as common as pregnancy loss. And even though cffDNA-based testing is still a relatively expensive method, substantial price reductions are expected within the next few years. In our view, the potential for non-pregnancy tissue-dependent cffDNA-based testing in pregnancy loss outweighs the current approximate 10% risk of a pregnancy loss being incorrectly labelled as euploid.

We recognise the limitation of the narrow time window for blood sampling for cffDNA, and we identified higher no-call rates in blood samples drawn after pregnancy tissue had passed. However, 55% of patients treated for pregnancy loss in a hospital setting experience a missed miscarriage[38] and thereby have an increased window for blood sampling. Additionally, a substantial portion of patients not having a missed miscarrige are in the process of having a miscarriage or have recently had a miscarriage and can have blood drawn for cffDNA-based testing within 24 h after the pregnancy loss.

Another challenge of cffDNA-based methods in pregnancy loss is a lower fetal fraction in early gestational ages, in which the placenta is still small and the

uteroplacental blood flow is low. This challenge was illustrated by no-call rates of approximately 50% in gestational ages below 7 weeks plus 0 days, based on 16 patients. However, more data and research are needed to identify criteria for use of cffDNA-based testing before gestational age 7 weeks and 0 days, as it is of importance for selected patient groups (eg, women who have a pregnancy loss after several in-vitro fertilisation or intracytoplasmic sperm injection attempts), to pursue fetal result even with lower chances of obtaining a test result. We found a stable no-call rate of approximately 10% from gestational age 7 weeks and 0 days and no significant difference in fetal fraction detected in age-matched pregnancy losses and ongoing pregnancies from gestational age 11–13 weeks.

In conclusion, this validation of cffDNA-based fetal chromosome status in pregnancy loss shows the potential and applicability of the method, even at low gestational ages. We believe that adding fetal ploidy assessment for pregnancy loss will lead to improved management and understanding of an overlooked major issue in reproduction and women's health.

Contributors

TSH was responsible for the clinical set-up, patient recruitment, and data collection at the Hvidovre site, analysed and interpreted data, wrote the first draft of this paper, and was a part of the decision to submit the manuscript. LAmb was responsible for the cell-free fetal DNA (cffDNA)-based test set-up and analysed, verified, and interpreted data. JFP was responsible for patient recruitment and data collection at the Herlev and North Zealand sites. TW, MMBSP, and CB were involved in patient recruitment and data collection. JBP was responsible for the systematic review of evidence before this study and for the genetic methods description. LAma was co-primary investigator together with HSN and was in charge of the overall coordination, management, and project economy. JRG and AC-HC did the tissue identification and direct sequencing for pregnancy tissue. BJ was responsible for bioinformatics, statistics, and graphics, and verified the underlying data. MD and IB supported clinical analysis of maternal genetic markers analysis and clinical genetic feedback. MHB-J and LW did the laboratory part of the cffDNA-based tests and evaluated the individual test results. MJH supervised the project establishment, genetics, and statistics. FSJ supervised the cffDNA-based test set-up. ERH was the co-primary investigator together with HSN and was responsible for the direct sequencing set-up and a part of the decision to submit the manuscript. HSN conceptualised and initiated the project, obtained ethical permission and funding, supervised the main author during data collection, data interpretation, and paper writing, and was a part of the decision to submit the manuscript. All authors made substantial contributions to the study set-up, data interpretation and manuscript revision. All authors approved the final manuscript before publication.

Declaration of interests

MJH is part of the leadership of BioInnovation Institute Foundation. ERH has received scientific grants from BioInnovation Institute and Novo Nordic Foundation, and has received personal payment or honoraria for lectures and presentations from Ferring Pharmaceuticals and Merck. ERH is an Executive Board member and Scientific Coordinator in ReproUnion (paid; 5% of full time equivalent). ERH received data from preimplantation genetic testing (in-kind, collaboration) from Igenomix, Natera. HSN has received scientific grants from Freya Biosciences, Ferring Pharmaceuticals, BioInnovation Institute, Ministy of Education, Novo Nordisk Foundation, Augustinus Fonden, Oda og Hans Svenningsens Fond, Demant Fonden, and Ole Kirks Fond. HSN received personal payment or honoraria for lectures and presentations from Ferring Pharmaceuticals, Merck, Astra Zeneca, Cook Medical, and Ibsa Nordic. All other authors declare no competing interests.

Data sharing

All clinical summary data are available in the table. Raw data are available under Danish legal provisions and appropriate material transfer agreements pending The Health Research Ethics Committees for the Capital Region of Denmark (H- 18024745) and General Data Protection Regulation governance. Patient consent sheet and patient information are accessible in Danish and English at the study web page (www.graviditetstab.dk).

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