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Breast Cancer Detection and Treatment Monitoring Using a Noninvasive Prenatal Testing Platform: Utility in Pregnant and Nonpregnant Populations

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BACKGROUND: Numerous publications have reported the incidental detection of occult malignancies upon routine noninvasive prenatal testing (NIPT). However, these studies were not designed to evaluate the NIPT performance for cancer detection.

METHODS: We investigated the sensitivity of a genome-wide NIPT pipeline, called GIPSeq, for detecting cancer-specific copy number alterations (CNAs) in plasma tumor DNA (ctDNA) of patients with breast cancer. To assess whether a pregnancy itself, with fetal cell-free DNA (cfDNA) in the maternal circulation, might influence the detection of ctDNA, results were compared in pregnant (n = 25) and nonpregnant (n = 25) cancer patients. Furthermore, the ability of GIPSeq to monitor treatment response was assessed.

RESULTS: Overall GIPSeq sensitivity for detecting cancer-specific CNAs in plasma cfDNA was 26%. Fifteen percent of detected cases were asymptomatic at the time of blood sampling. GIPSeq sensitivity mainly depended on the tumor stage. Also, triple negative breast cancers (TNBC) were more frequently identified compared to hormone-positive or HER2-enriched tumors. This might be due to the presence of high-level gains and losses of cfDNA or high ctDNA loads in plasma of TNBC. Although higher GIPSeq sensitivity was noted in pregnant (36%) than in nonpregnant women (16%), the limited sample size prohibits a definite conclusion. Finally, GIPSeq profiling of cfDNA during therapy allowed monitoring of early treatment response.

CONCLUSIONS: The results underscore the potential of NIPT-based tests, analyzing CNAs in plasma cfDNA in a genome-wide and unbiased fashion for breast cancer detection, cancer subtyping and treatment monitoring in a pregnant and nonpregnant target population.

Introduction

The co-existence of cancer and pregnancy is a relatively rare phenomenon with an estimated incidence rate of 1 in 1000 to 2000 pregnancies *(1)*. However, as women in developed countries tend to delay childbearing, the incidence rate is expected to increase. Pregnant women are at high risk of not being diagnosed at early cancer stages because cancer symptoms (such as fatigue, nausea, or abdominal discomfort) can be misinterpreted as physiologic gestational symptoms *(2)*. In particular for breast cancer, normal physiological changes in the breast during gestation increase the challenges in rendering a correct pathological evaluation, which may lead to a delayed cancer diagnosis and a negative impact on clinical outcome *(3)*. On the other hand, accumulating evidence indicates that initiating cancer treatment during pregnancy has no adverse effect on pediatric outcome *(4)*. Instead, an early start of chemotherapy may improve the prognosis of the mother similar to that of nonpregnant women *(5, 6)*. Together, these points underscore the need for appropriate tests for timely cancer detection. Lately, a growing number of articles have

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reported the incidental finding of an occult maternal malignancy upon routine noninvasive prenatal testing (NIPT) *(7–11)*. In these cases, NIPT, originally designed to screen placenta-derived cell-free DNA (cfDNA) in the bloodstream of pregnant women for the presence of fetal aneuploidies, also detected the presence of cancer-specific copy number alterations (CNAs) in tumor-derived circulating DNA (ctDNA). This incidental cancer detection via NIPT prompted investigations into the association between a deviating NIPT outcome and detection of an occult maternal malignancy, mainly through retrospective assessment of files from routine NIPT screening. However, as these studies were not designed to evaluate the ability of NIPT for cancer screening, deductions about the test's diagnostic specificity and sensitivity for this purpose could not be made *(12)*. Hence, to investigate the sensitivity of NIPT for cancer detection, we set up a study in pregnant women having a known diagnosis of breast cancer, the most commonly diagnosed tumor type during pregnancy *(13)*. In this population, we evaluated the performance of our unbiased genome-wide cfDNA analysis pipeline, coined Genomic Imbalance Profiling from cfDNA SEQuencing (GIPSeq), with regard to the detection of cancer-derived signals *(14)*. GIPSeq was originally developed for clinical NIPT and enabled (i) incidental yet highly accurate identification of occult maternal malignancies during routine NIPT *(7, 8)* and (ii) presymptomatic cancer detection upon screening of a large asymptomatic, nonpregnant cohort *(15)*.

Patients and Methods

OVERVIEW

On plasma cfDNA of pregnant women with a known breast cancer diagnosis, we applied our GIPSeq pipeline to evaluate its performance for detecting tumor-specific CNAs in ctDNA. To examine whether a pregnancy by itself, characterized by steadily increasing plasma volumes and rising fetal cfDNA concentrations in the circulation *(16)*, may impact the detection of ctDNA, nonpregnant breast cancer cases were included as well. Finally, as cancer detection during pregnancy would enable an early start of cancer therapy, we also explored the potential of GIPSeq to monitor treatment response.

PARTICIPANTS

Pregnant and nonpregnant premenopausal patients with a confirmed diagnosis of invasive breast adenocarcinoma of no special type (IBC-NST) (unilateral, bilateral, unifocal, and/or multifocal) and who had not received surgery or neo-adjuvant chemotherapy at the time of enrollment, were eligible for inclusion in this study. Patients with in situ carcinomas or with a prior cancer history were excluded. All patients were recruited via University Hospitals Leuven. Pregnant breast cancer cases (n = 25) and nonpregnant women with a breast cancer diagnosis (n = 25) were frequency-matched to obtain a comparable distribution of molecular subtypes, low (I, II) and high (III, IV) stages and histological tumor grades across both groups (Table 1). At cancer diagnosis a pretreatment peripheral blood sample (8–9 ml) was sampled in Streck or Roche Diagnostics tubes. In case of an aberrant cfDNA profile at baseline, consecutive blood samples were taken in the course of the treatment. All samples were collected between August 2014 and July 2019. The study was approved by the Ethics Committee of University Hospitals Leuven (S57197). Written informed consent was obtained from all participants.

TUMOR PATHOLOGY ASSESSMENT

Clinical/pathological tumor stage (TNM, eighth edition), tumor grade, and receptor status were retrieved from the patient's medical record. Estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) status were assessed by immunohistochemistry according to current American Society of Oncology/College of American Pathologists (ASCO/CAP) guidelines. Additional fluorescent in situ hybridization (FISH) was used to check HER2 amplification in case of score 2+ or 3+ immunohistochemistry, according to national guidelines. Depending on the immunohistochemical phenotype, the tumors were stratified into 3 clinically and therapeutically meaningful categories further referred to as triple negative breast cancers (ER-, PR-, and HER2-negative; designated as TNBC), ER-positive/HER2-negative tumors (ER+HER2-) or HER2-overexpressing tumors (HER2+). Mitotic count was performed by a board-certified breast pathologist according to the Nottingham grading system on a formalin-fixed paraffin-embedded (FFPE) and hematoxylin-eosin stained slide of the diagnostic core needle biopsy *(17)*. The average number of mitoses per mm^2 was calculated to obtain the mitotic activity index.

GENOMIC IMBALANCE PROFILING FROM CFDNA SEQUENCING (GIPSEQ)

Extraction of plasma cfDNA and DNA from matching FFPE tumor specimens, preparation of DNA sequencing libraries, and whole-genome sequencing was done as described previously *(15)*. The mean number of read counts per sample, after filtering and removal of duplicates, was 9.9×10^6 reads. Whole-genome sequencing data were then subjected to GIPSeq, our previously described NIPT bioinformatics analysis pipeline, using genome-wide parameters (quality score, QS) and

Table 1. Patient and tumor characteristics in the pregnant and nonpregnant breast cancer groups.

	Pregnant breast cancer cases (n = 25)		Nonpregnant breast cancer cases (n = 25)		*P*-value
Median age at diagnosis (years; range)	**33**	**(28 to 42)**	**38**	**(26 to 47)**	**0.002**
Clinical presentation at diagnosis[a]					
Symptomatic	22	(88.0 %)	20	(80.0 %)	0.70
Asymptomatic	3	(12.0 %)	5	(20.0 %)	
Immunophenotype					
ER+ HER2-	10	(40.0 %)	12	(48.0 %)	0.78
HER2+[b]	6	(24.0 %)	8	(32.0 %)	0.75
TNBC	9	(36.0 %)	5	(20.0 %)	0.35
TNM stage					
I	3	(12.0 %)	3	(12.0 %)	1.00
II	13	(52.0 %)	14	(56.0 %)	1.00
III	7	(28.0 %)	6	(24.0 %)	1.00
IV	2	(8.0 %)	2	(8.0 %)	1.00
Tumor size (T)					
1	4	(16.0 %)	9	(36.0 %)	0,20
2	12	(48.0 %)	10	(40.0 %)	0,78
3	5	(20.0 %)	3	(12.0 %)	0.70
4	4	(16.0 %)	3	(12.0 %)	1.00
Number of positive lymph nodes (N)					
0	10	(40.0 %)	5	(20.0 %)	0.22
1	8	(32.0 %)	14	(56.0 %)	0.15
2	2	(8.0 %)	1	(4.0 %)	1.00
3	5	(20.0 %)	5	(20.0 %)	1.00
Metastasized status (M)					
yes	2	(8.0 %)	2	(8.0 %)	1.00
no	23	(92.0 %)	23	(92.0 %)	
Histological grade					
I	0	(0.0 %)	1	(4.0 %)	1,00
II	4	(16.0 %)	9	(36.0 %)	0.20
III	21	(84.0 %)	15	(60.0 %)	0.11

[a]Symptomatic refers to the presentation of clinical symptoms or the presence of a palpable nodulus; asymptomatic patients were diagnosed via preventive screening modalities. TNM: tumor, node, metastasis (Eighth edition). When available, the pathological pTNM staging at primary surgical intervention was used; in neo-adjuvant chemotherapy setting, we refer to the clinical cTNM stage. Breast cancers were either categorized as triple negative breast cancers [negative for estrogen receptor (ER), progresteron receptor (PR), and human epidermal growth factor receptor 2 (HER2); designated as TNBC), ER-positive/HER2-negative breast cancers (ER+HER2-), or HER2-overexpressing breast cancers (HER2+)].

[b]Except for 2 cases (having a ER-PR-HER2+ tumor), all cases designated as HER2+ were having a triple positive breast tumor. *P*-values were determined using two-sided Fisher Exact testing.

chromosomal parameters (*z*- and *zz*-score) *(14)*. QS was calculated as the standard deviation of all the autosomal *z*-scores following removal of the highest and lowest scoring chromosomes, as outlined in *(14)*. A GIPSeq profile was scored 'normal' when QS < 2 and no significant gains or losses were present across one of the chromosomes (i.e., chromosomal |*z*-score|<3.0 and |*zz*-score|<3.0). The GIPSeq profile was called 'aberrant' (i.e., suggestive of an underlying malignancy) when QS ≥ 2 and arm-level or subchromosomal gains and/or losses were present across multiple chromosomes, or when QS < 2 but one or more individual chromosomes had |*z*-score|≥3.0 and |*zz*-score|≥3.0 (i.e., arm-level or subchromosomal gains and/or losses on a single

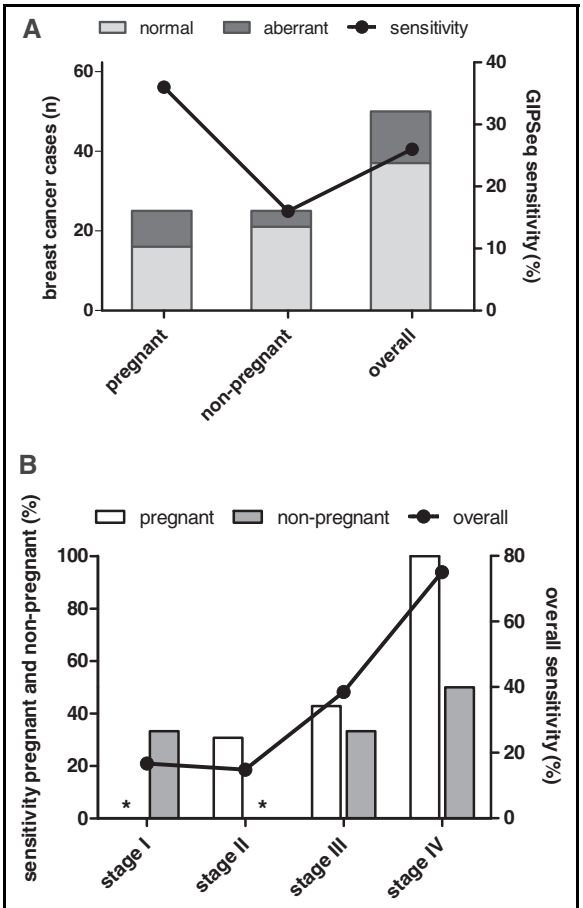

Fig. 1. a) GIPSeq sensitivity for detection of breast cancer-derived CNAs in pregnant and nonpregnant breast cancer cases. Sensitivity was higher in pregnant (36%) than in nonpregnant (16%) cases, though not statistically significant ($P = 0.196$, Fisher Exact test). b) Correlation between tumor TNM stage and GIPSeq sensitivity for breast cancer detection. *, values are 0%. The left y-axis shows GIPseq sensitivity in pregnant (white bars) and nonpregnant (gray bars) cases; the right y-axis shows overall GIPSeq sensitivity (black dots) in combined pregnant and nonpregnant breast cancer populations, per tumor TNM stage (x-axis). There was a trend towards higher breast cancer detection by GIPSeq in later stages ($P = 0.012$; chi-square test for trend).

chromosome) *(15)*. The GIPSeq pipeline makes use of a standard reference set of 100 cfDNA samples from normal pregnancies *(14)*. Exploration, using a reference set of cfDNA from a nonpregnant cohort *(15)*, indicated that the pregnancy status of the reference cfDNA samples did not affect the outcome of the GIPSeq analyses presented herein (Supplemental Table 1). Constitutional copy number variations were identified using the CNV caller SeqCBS and filtered out in the scoring process *(18)*.

CLINICAL IMAGING INVESTIGATIONS

Pregnant breast cancer patients with a deviating GIPSeq profile at baseline were invited for regular whole-body diffusion-weighted MRI (WB-DWI MRI) evaluation at clinically relevant time points to correlate with the results from cfDNA profiling. WB-DWI MRI was chosen because of its nonradiation modality and proven efficacy for detection of cancer lesions, lymphadenopathies, and metastases in pregnancy *(19)*. In cases for whom no WB-DWI MRI was available, imaging data from routine clinical follow-up examinations (using MRI, PET-CT, and/or CT scans) were retrieved from the patient's medical record for correlation with cfDNA data.

STATISTICAL ANALYSES

Principal Component Analysis (PCA) was performed to identify patient and tumor characteristics that were associated with GIPSeq sensitivity for breast cancer detection. For each patient, 23 GIPSeq variables were used as input (i.e., the 22 chromosomal *z*-scores and the genome-wide QS-value). Two-tailed *t*-testing assuming equal variances in unpaired samples was used to compare the number of mitoses/mm^2 and QS-scores among study groups. The Fisher exact test was applied to assess the tumor parameter distribution in pregnant versus nonpregnant patients and in cases with a normal versus an aberrant GIPSeq profile. The chi-square test for trend was done to examine the relationship between GIPSeq sensitivity and cancer stage. Multivariate logistic regression analysis was performed to evaluate the associations between clinical characteristics and GIPSeq outcome.

Results

SENSITIVITY OF GIPSEQ FOR BREAST CANCER DETECTION DEPENDS ON TUMOR STAGE, MOLECULAR SUBTYPE AND PREGNANCY STATUS

Nine of 25 pregnant (36%) and 4 of 25 (16%) nonpregnant breast cancer cases had an aberrant CNA profile in cfDNA ($P = 0.196$; Fig. 1A), resulting in an overall GIPSeq sensitivity of 26% for breast cancer detection. In the remaining 16 pregnant (64%) and 21 nonpregnant (84%) breast cancer patients, no chromosomal imbalances could be observed in cfDNA.

PCA analyses indicated that the major determinants of GIPSeq sensitivity for detection of cancer-related CNAs were tumor stage and immunophenotype

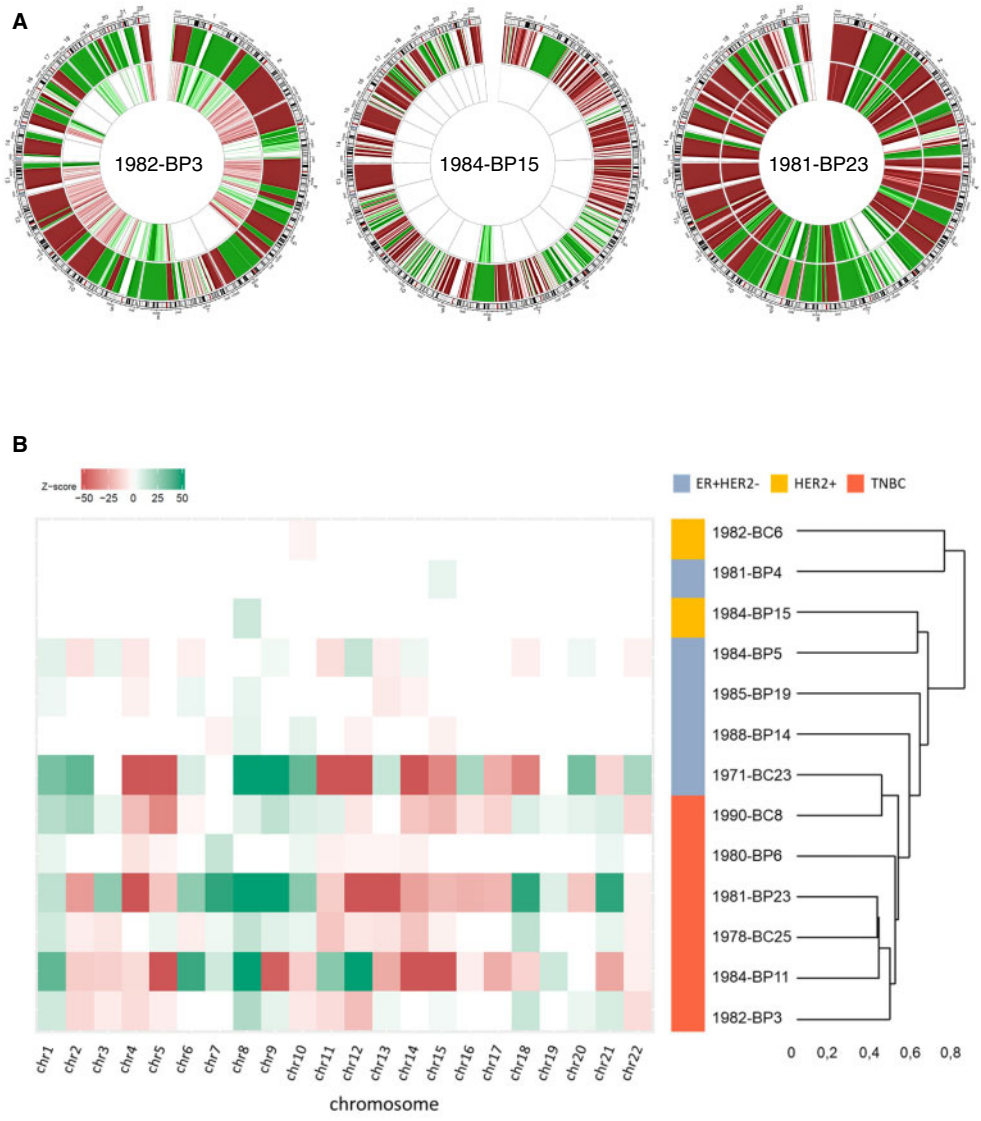

Fig. 2. a) Representative circos plots showing high congruency between copy number alterations (CNAs) detected in plasma cfDNA and matched tumor DNA in pregnant breast cancer cases (n = 3). The genomic representation profile of the autosomal chromosomes is shown in clockwise order, aligned with chromosomal ideograms (outer circle). Chromosomal anomalies with a chromosomal *z*-score $\geq$3.0 (suggesting gain) are indicated in green; those with a *z*-score ≤ -3.0 (suggesting loss) are shown in red. For each case, the outer circle shows the copy number profile of tumor DNA extracted from tumor biopsy (whole-genome low-pass sequencing, 0.1× coverage). The inner circle depicts the matched genome-wide GIPSeq profile in plasma cfDNA in the pretreatment sample. For case 1982-BP3 (stage II, TNBC), genome-wide copy number gains and losses are observed in cfDNA, being highly congruent with those present in the tumor DNA. For case 1984-BP15 (stage IV, HER2+ breast cancer), an isolated amplification of 8q was observed in plasma cfDNA at diagnosis, corresponding to the copy number gain observed in tumor DNA. For case 1981-BP23 (stage III, TNBC), highly similar genome-wide gains and losses are observed in cfDNA at baseline and tumor biopsy DNA. Circos plots, depicting matched cfDNA: tumor DNA profiles of additional breast cancer cases with an aberrant GIPSeq profile are given in Supplemental Fig. 6. b) Heatmap of chromosomal imbalances observed in cfDNA of the pregnant (n = 9) and nonpregnant (n = 4) breast cancer cases with an aberrant cfDNA profile. Each horizontal line represents one breast cancer case. *z*-Scores of each individual chromosome in cfDNA are depicted vertically. Similarly as in Fig. 2A, chromosomal anomalies with a chromosomal *z*-score $\geq$3.0 (suggesting gain) are indicated in green in the heatmap; those with a *z*-score ≤ -3.0 (suggesting loss) are shown in red. *z*-Scores that were lower than −50 or greater than 50 were set to −50 or 50. The vertical color bar on the right shows the molecular breast cancer subtype of each included case. On the right side is the dendrogram that is generated by Agnes clustering, in which the similarities of two GIPSeq profiles are calculated by Spearman correlation coefficient using GIPSeq *z*-scores. As such it shows, in a genome-wide manner, whether there is a similar type of aberration (gain, indicated by a positive *z*-score or loss, indicated by a negative *z*-score), regardless of the magnitude of the *z*-score. In general, the triple negative breast cancer cases tended to have more similar z-score profiles (dendogram) and higher chromosomal *z*-scores (heatmap) than the other breast cancer subtypes. BC, nonpregnant breast cancer cases; BP, pregnant breast cancer cases.

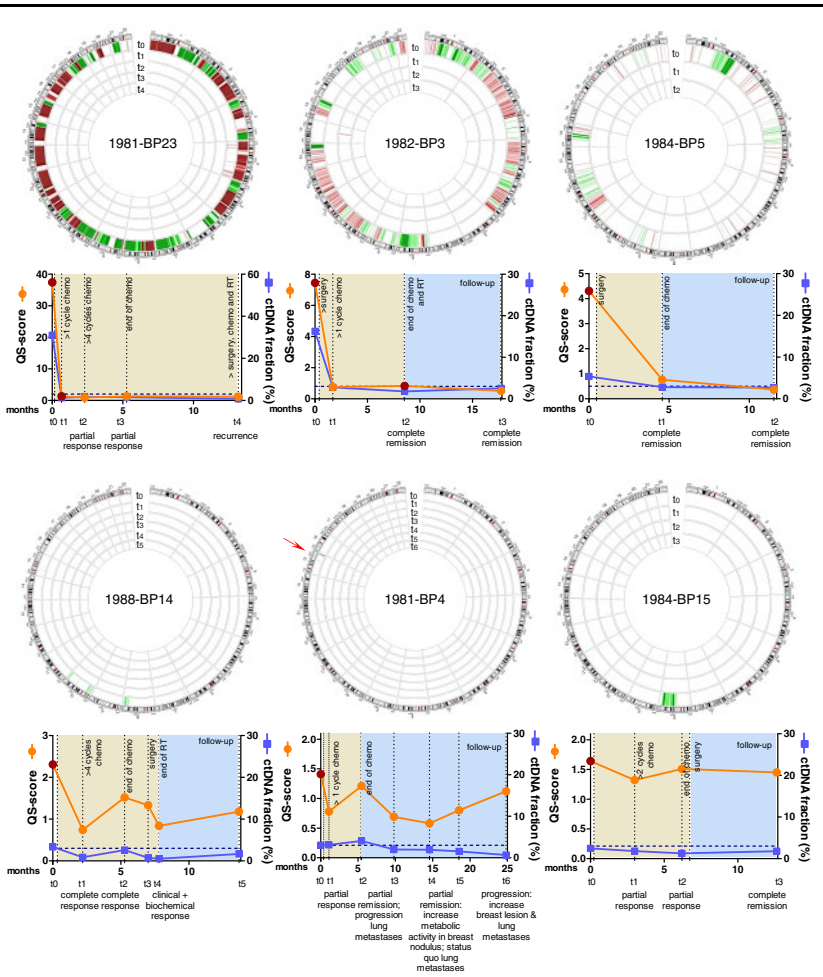

Fig. 3. Evolution of GIPSeq profiles and ctDNA fractions during treatment of 6 pregnant breast cancer patients. For each patient, the circos plot shows the spectrum of CNAs detected in plasma cfDNA via GIPSeq: the outer circle depicts the cfDNA profile in a pretreatment sample (t0); the inner circles show the GIPSeq profiles during treatment and/or a follow-up period after ending therapy (t1–t6). Visualization of chromosomal anomalies is done as in Fig. 2A. The linear graph below each circos plot, depicts the longitudinal evolution of the GIPSeq QS-scores (left y-axis, orange dots). QS-scores of GIPSeq profiles that were scored as aberrant are indicated in dark red. Estimated tumor-derived fractions in cfDNA (right y-axis), using ichorCNA are indicated by blue squares. Colored shading indicates therapy (brown) or follow-up (blue) periods. Vertical dotted lines indicate the therapy status at the time of each plasma sampling (time points correspond to those indicated in the circos plots): chemo, chemotherapy; RT, radiotherapy. The disease status (ascertained on MRI, PET-CT and/or CT) is indicated below the x-axis. The horizontal blue dotted line points to the ichorCNA ctDNA detection limit [3% as defined in *(20)*]. For case 1981-BP23, after 1 cycle of epirubicin-cyclophosphamide (EC) therapy (t1) only loss of chromosome 4 is detected in cfDNA (cfr. circos plot and red dot in QS-scoring). After 4 cycles of EC (t2), the cfDNA profile is normalized. Clinically, the patient shows partial response to therapy. A normal cfDNA profile is observed at the end of neo-adjuvant chemotherapy treatment (EC-paclitaxel; t3). Three months after ending treatment (surgery, adjuvant chemotherapy and RT), the patient relapses with metastases to the brain. Additional plasma cfDNA profiling (while under eribulin therapy for breast cancer, and RT for brain metastases; t4) shows a normal cfDNA profile. For case 1982-BP3, the aberrant CNA profile at baseline (t0) normalizes after surgery and 1 cycle of adjuvant EC (t1). At t2, after completing chemotherapy treatment (EC-paclitaxel) and RT, a focal deletion of chromosome 14q appears in cfDNA (cfr. circos plot and red dot in QS-scoring). A follow-up sample taken 9 months after ending of therapy (t3) shows again a normal plasma cfDNA profile. Corresponding imaging data point to complete remission at t2 and t3. For case 1984-BP5, CNAs observed in plasma cfDNA at diagnosis (t0) are not detectable any more in a plasma sample taken at the end of adjuvant chemotherapy treatment (5FU-EC-docetaxel; t1) when the patient is in complete remission. A similar picture is seen 7 months after ending of chemotherapy (t2). For case 1988-BP14, the aberrant cfDNA profile at baseline (t0) normalizes after 4 cycles of neo-adjuvant EC (t1), corresponding to the clinical observation of a complete response. No chromosomal imbalances are observed in plasma cfDNA samples taken after ending of chemotherapy (EC-paclitaxel; t2), after mastectomy (t3), after radiotherapy (t4), nor at 6 months after ending treatment (t5). For case 1981-BP4, at baseline an isolated focal amplification on chromosome 15q is detectable in cfDNA (indicated by the red arrow). After treatment with 1 cycle EC (t1), the cfDNA profile normalizes. No chromosomal imbalances are detectable in plasma cfDNA samples taken at the end of the chemotherapy treatment (t2), nor at 4 months (t3), 9 months (t4), 13 months (t5), or 19 months (t6) after ending chemotherapy. Corresponding imaging data point to a partial remission at t1, but to the progression of the lung metastases at t2 and an increase of the breast lesion at t6. For case 1984-BP15, after 2 cycles neo-adjuvant EC (t1) the cfDNA profile normalizes. Clinical evaluation points to a partial response. A normal cfDNA profile is also observed at the end of the chemotherapy treatment (EC-paclitaxel; t2). Six months after ending treatment (t3), the patient is clinically in complete remission and no chromosomal imbalances are observed in plasma cfDNA.

of the breast tumor (Supplemental Fig. 1). For tumor TNM stage, there was a link between stage and overall GIPSeq sensitivity for breast cancer detection (Fig. 1B and chi-square test for trend, $P = 0.012$). Stage III and stage IV tumors had the highest chance to be detected upon genome-wide CNA profiling of cfDNA [Odds Ratio (OR) = 2.226 and OR = 10.800, with $P = 0.281$ and $P = 0.049$, respectively, whereas ORs were 0.533 and 0.271 for stage I and stage II, respectively; Supplemental Table 2]. There was a contribution of the tumor's size (T; $P = 0.002$), the number of affected lymph nodes (N; $P = 0.050$) as well as the metastatic state (M; $P = 0.049$). Furthermore, there was a tendency to detect more aberrant cfDNA profiles in plasma of patients with TNBC; whereas patients with ER+HER2- or HER2+ tumors were more often associated with a normal cfDNA profile (OR = 3.107; $P = 0.149$; Supplemental Table 2 and Fig. 2A). This was not due to any disproportional representation of TNBC tumors in late stage diseases (Supplemental Fig. 3). Although the highest QS-scores, pointing to the presence of genome-wide copy number alterations in cfDNA, were almost exclusively observed in poorly differentiated tumors, no correlation was observed between GIPSeq sensitivity for breast cancer detection and tumor proliferation when examining the mitotic index (Supplemental Fig. 4).

Pregnant breast cancer patients had a nonsignificantly increased rate of having an aberrant GIPSeq profile compared to nonpregnant cases (OR 2.953; $P = 0.196$). When adjusting for the tumor characteristics that were shown to mainly determine GIPSeq sensitivity (i.e. T, M), pregnant breast cancer patients still tended to be more frequently detected with cancer-like plasma cfDNA profiles compared to nonpregnant cases (adjusted OR = 4.502; 95% CI [0.718–28.229]; $P = 0.108$; multivariate logistic regression analysis).

COPY NUMBER ALTERATIONS DETECTED IN PLASMA CFDNA VIA GIPSEQ ARE TUMOR-SPECIFIC

For patients for whom a breast tumor biopsy specimen was available (n = 24 pregnant; n = 22 nonpregnant cases) low-pass genome-wide sequencing revealed that all these patients had detectable CNAs in their tumor genome, indicating that cases with a normal plasma cfDNA profile were in fact false negatives (Supplemental Fig. 5). The CNAs observed in plasma cfDNA of cases with an aberrant GIPSeq were found to be tumor-specific (Fig. 2A and Supplemental Fig. 6 A). To achieve high specificity in classifying imbalances in cfDNA identified via GIPSeq as being cancer-specific, our scoring parameters were made stringent. As a consequence, some chromosomal gains and losses in cfDNA, though shown to be derived from tumor DNA, were not classified as aberrant (Supplemental Fig. 6B), thereby restraining GIPSeq sensitivity for breast cancer detection. Finally, since the aberration observed in cfDNA of case 1982-BC6 marginally exceeded the predefined *z*- and *zz*-threshold, and as this case was classified among the normal cfDNA cases in PCA analyses and supervised clustering (Supplemental Figs. 2A and 6A), the meaning of this signal may be uncertain. If we were to assume that this was a false positive case, then sensitivity in the nonpregnant breast cancer group would drop from 16% to 12%.

The majority (77%) of patients with an aberrant cfDNA profile had detectable (sub)chromosomal anomalies on multiple chromosomes which was reflected in QS scores higher than 2 (Fig. 2 and Supplemental Fig. 6). These aberrant cfDNA profiles (of all breast cancer subtypes) tended to cluster apart from the normal GIPSeq profiles (Supplemental Fig. 2A). Among those aberrant GIPSeq profiles, highest genome-wide correlations were observed for the profiles of TNBC cases (Agnes clustering, Fig. 2B). Also, cfDNA of the TNBC cases with an aberrant GIPSeq tended to have higher-level gains and losses of cfDNA, being reflected in elevated *z*- and QS-scores (heatmap here presented and Supplemental Fig. 7). In 3 cases (23%), an isolated aberration on only one chromosome arm or chromosomal segment was observed (QS < 2). Overall, most of the detected aberrancies in cfDNA were arm-level imbalances. Most significantly affected chromosomes were 1q (gain), 4 (loss), 5q (loss), 8q (gain), and 14q (loss) and 17p (loss) (Supplemental Fig. 8A).

GIPSEQ PROFILING ALLOWS MONITORING OF EARLY TREATMENT RESPONSE

For all pregnant breast cancer patients with an aberrant pretreatment cfDNA profile (n = 9), serial plasma follow-up samples were taken in the course of their treatment. For 6 cases, matching cfDNA and clinical imaging data were available, allowing evaluation of GIPSeq profiling for monitoring of treatment response. As shown in Fig. 3, for all but one case (1981-BP23) chromosomal imbalances, observed in a pretreatment plasma cfDNA sample of these cases, resolved early during therapy (first follow-up time point, t1). This was accompanied by a normalization of the genome-wide QS-scores, for cases with multiple detectable CNAs and an initial QS > 2 (1982-BP3, 1984-BP5 and 1988-BP14) and a normalization of chromosomal parameters (*z*-and *zz*-score) for cases with a single chromosomal cfDNA aberration at baseline and QS < 2 (cases 1981-BP4 and 1984-BP15). For 4 of the 6 cases (1982-BP3, 1984-BP5, 1988-BP14, and 1984-BP15), the evolution of the GIPSeq profile (and QS-score) was consistent with the clinical response. In parallel, we estimated the fraction

of tumor-derived cfDNA in plasma of these cases using the ichorCNA algorithm *(20)*. For those cases with detectable ctDNA levels at baseline (i.e., all cases except 1984-BP15), these fractions dropped below the detection limit early during therapy (t1) (except for case 1981-BP4). Despite initial therapy response however, patient 1981-BP23 relapsed with metastases to the brain 3 months after ending treatment (adjuvant chemotherapy and radiotherapy). Additional plasma cfDNA profiling (while under eribulin therapy for breast cancer and radiotherapy for brain metastases,t4) showed a normal GIPSeq profile. Likewise, patient 1981-BP4 experienced disease progression 4 months after ending therapy. Although QS-scores also started to increase, no chromosomal aberrations were observed in the GIPSeq profile in any of the follow-up plasma samples. Finally, for one case (1982-BP3), a focal deletion of chromosome 14q appeared in plasma cfDNA after completing chemo- and radiotherapy (t2). This was not visible in a follow-up plasma sample taken 9 months later. No post-treatment tumor biopsy was available to verify the cancer-specificity of the 14q- signal in cfDNA.

Discussion

Proper identification of cancer through clinical routine NIPT screening may have a major impact on the management of pregnant women. As the worldwide use of NIPT is likely to expand, an increased number of atypical results might be expected. Therefore, improved knowledge of the test's ability to detect specific cancer types is a prerequisite.

In the cohort of pregnant and nonpregnant breast cancer patients presented here, our unbiased genome-wide GIPSeq test was able to pick up breast cancer-derived CNAs in plasma cfDNA of 26% of all cases. In 2 cases, cancer-specific CNAs were detected in asymptomatic women that were diagnosed with breast cancer via other screening programs. Highest sensitivities (38.5%–75%) were reached in the subset of advanced-stage cancers. No breast cancer-specific signal was observed in cfDNA from the remaining 74% of patients, although their tumor genomes displayed CNAs. Either these tumors did not shed ctDNA into the circulation or the load of ctDNA was too low to detect tumor-specific CNAs via GIPSeq, the latter being especially plausible for early-stage tumors *(21)*. IchorCNA estimations of the ctDNA fraction in the total plasma cfDNA pool in our study participants were also in favor for the latter hypothesis (Supplemental Fig. 9A) *(20)*. So far, only a limited number of studies investigated the potential of cfDNA CNA profiling for breast cancer detection, the focus of these studies being predominantly on the metastatic and relapse setting *(22)*. In their efforts to develop a liquid biopsy pan-cancer detection test, GRAIL (a cancer diagnostics company) reported an average sensitivity of 30% for breast cancer detection using whole-genome deep sequencing for somatic CNA detection in plasma cfDNA with the highest sensitivities being found in patients with a high tumor stage or with TNBC *(23)*. These findings are in line with the detection rates we found through low-pass sequencing of cfDNA. In our dataset, the tendency to a higher GIPSeq sensitivity for TNBC was accompanied by the observation that the aberrant TNBC cfDNA profiles shared similar high-level gains and losses across the genome. Though a partial resemblance of the TNBC cfDNA profile cluster was observed in the tumor genomes of TNBC cases analyzed in our cohort, the higher amplitude of copy number gains and losses observed in cfDNA of these TNBC patients, was not reflected in the tumor DNA (Supplemental Figs 2B and 8B). Estimation of the tumor fraction, pointed to a higher ctDNA load in plasma of TNBC patients compared to the other breast cancer types, which also could explain the higher GIPSeq sensitivity observed for this subtype (Supplemental Fig. 9B). A link between the tumor's receptor status and plasma ctDNA levels in patients with localized breast cancer was recently reported by Moss et al. *(24)*. We observed no correlation between the BRCA mutation status of TNBC tumors and GIPSeq positivity (data not shown). Interestingly, in retro- and prospective studies on the detection of occult malignancies in asymptomatic pregnant women following routine NIPT, about 14% of identified cancers were breast cancers [own analysis of data reported in *(7, 9–11, 25, 26)*]. However, according to data from our International Network on Cancer in Pregnancy, breast cancer is the most frequently encountered cancer type in pregnancy, accounting for 39% of registered cases *(13)*. This discrepancy suggests that a substantial number of breast cancers are not detected upon routine NIPT, which is in agreement with what we find here. Applying the GIPSeq thresholds set out here on about 88.000 routine diagnostic NIPT tests performed in our hospital, 14 cancer-like results were observed and, upon further clinical examination, 13 out of these 14 asymptomatic pregnant women were finally diagnosed with cancer. For each identified cancer case, the aberrant CNA profiles in plasma cfDNA were confirmed to be derived from the malignancy. This result amounts to a PPV of 92.8%.

Remarkably, even after correcting for tumor characteristics that were largely determining the sensitivity of our pipeline for breast cancer detection, the detection rate remained higher in the pregnant population than in their nonpregnant counterparts although the difference was not significant. Although there is no strong clinical evidence that disease stage-specific prognosis differs between pregnant and nonpregnant cancer patients, pregnancy is associated with hormonal and immunological changes that may affect tumor biology *(27)*. However, the sample size of the present study is too limited to

draw a firm conclusion. Larger sufficiently-powered studies could evaluate whether and how pregnancy-associated factors influence the ctDNA detection rate. Due to the rare co-occurrence of cancer and pregnancy, achieving large sample sizes might be challenging.

Lastly, our data show the ability of the GIPSeq pipeline to monitor early treatment response to cancer therapy. A noninvasive manner for cancer treatment monitoring would be especially valuable in the pregnant setting where soluble protein markers show moderate diagnostic sensitivity and are less reliable *(28)*. For all 6 evaluated pregnant breast cancer cases, the initial therapy response was accompanied by a drop in the GIPSeq's QS-score, and for 4 of them, the evolution of the QS-score was consistent with the clinical outcome of the patients. Two patients showed disease progression without detectable ctDNA signals in plasma. In this regard, a recent report specifically investigating breast cancer relapse suggested that there exist 'dark' relapse sites that are less readily detectable by ctDNA mutation analysis *(29)*.

In conclusion, as a relatively low-cost method, our genome-wide GIPSeq pipeline enables the unbiased detection of breast cancer-specific CNAs in plasma cfDNA, as opposed to currently investigated mutation detection methods requiring high sequencing depth, foreknowledge about the tumor's mutation profile, and costly tailor-made cfDNA mutation panels. Further enhancement of the GIPSeq sensitivity is warranted to ultimately allow detection of minimal ctDNA amounts in a sea of normal cfDNA in early-stage breast cancers. This may be achieved by combining the analyses of multiple biomarkers in cfDNA. Finally, the performance of such a noninvasive unbiased genome-wide plasma profiling tool for early cancer detection would need evaluation in an asymptomatic population, such as high-risk groups with a genetic predisposition to develop breast cancer.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: cfDNA, , cell-free DNA; CNA, copy number alteration; ctDNA, circulating tumor DNA; ER, estrogen receptor; FFPE, formalin-fixed paraffin-embedded; GIPSeq, Genomic Imbalance Profiling from cfDNA SEQuencing; NIPT, noninvasive prenatal testing; PCA, principal component analysis; PR, progesterone receptor; QS, quality score; TNBC, triple negative breast cancer; WB-DWI MRI, whole-body diffusion-weighted MRI.

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