ctDNA in Breast Milk for Early Detection of Pregnancy Associated Breast Cancer

Cristina Saura (✉ csaura@vhio.net)

Vall d'Hebron Institute of Oncology (VHIO)

Carolina Ortiz

Vall d'Hebron Institute of Oncology

Enrique Arenas Lahuerta

Vall d'Hebron Institute of Oncology (VHIO) <https://orcid.org/0000-0001-7076-2666>

Judit Matito

Vall d'Hebrón Institut d'Oncologia (VHIO)

Anna Suñol

Vall d'Hebron Institute of Oncology

Octavi Córdoba

Hospital Universitari Son Espases <https://orcid.org/0000-0002-9591-7430>

Alex Martínez-Sabadell Aliguer

Vall d'Hebron Institute of Oncology <https://orcid.org/0000-0002-9477-1396>

Itziar García-Ruiz

Vall d'Hebron Hospital Universitari

Ignacio Miranda

Vall d'Hebron Hospital Universitari <https://orcid.org/0000-0002-4813-5946>

Clara Morales-Comas

Vall d'Hebron University Hospital

Estela Carrasco

Vall d'Hebron Institute of Oncology

Cristina Viaplana

Vall d'Hebron Institute of Oncology

Vicente Peg

Universidad Autónoma de Barcelona, Barcelona; Department of Pathology in Vall d'Hebron University Hospital, Barcelona; Spanish Biomedical Research Network Centre in Oncology (CIBERONC)

<https://orcid.org/0000-0002-5203-6166>

Paolo Nuciforo

Vall d'Hebron Institute of Oncology <https://orcid.org/0000-0003-1380-0990>

Neus Bayó-Puxan

Vall d'Hebron Institute of Oncology

Josep Miquel

Vall d'Hebron Institute of Oncology (VHIO), Barcelona

Marina Gómez-Rey

Vall d'Hebron Institute of Oncology (VHIO)

Guillermo Villacampa

Vall d'Hebron Institute of Oncology (VHIO)

Silvia Arévalo

Vall d'Hebron Hospital Universitari

Martin Espinosa-Bravo

Vall d'Hebron Hospital Universitari

Judith Balmaña

Vall d'Hebron Institut d'Oncologia <https://orcid.org/0000-0002-0762-6415>

Rodrigo Dienstmann

Vall d'Hebron Institute of Oncology, Vall d'Hebron University Hospital, Autonomous University of Barcelona

Joaquín Arribas

Vall d'Hebron Institute of Oncology <https://orcid.org/0000-0002-0504-0664>

Josep Tabernero

Vall d'Hebron Institute of Oncology (VHIO) <https://orcid.org/0000-0002-2495-8139>

Miriam Sanso (✉ msanso@vhio.net)

Vall d'Hebron Institute of Oncology (VHIO)

Ana Vivancos

Vall d'Hebrón Institut d'Oncologia (VHIO)

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**ctDNA IN BREAST MILK FOR EARLY DETECTION OF PREGNANCY ASSOCIATED BREAST
CANCER**

C. Saura^{*,1,2}, C. Ortiz^{*1,2}, EJ. Arenas^{3,4}, J. Matito⁵, A. Suñol^{1,2}, O. Córdoba^{6,7,8}, A. Martínez-Sabadell³, I. García-Ruiz⁹, I. Miranda¹⁰, C. Morales-Comas¹¹, E. Carrasco^{2,12}, C. Viaplana¹³, V. Peg^{4, 14,15,16}, P. Nuciforo¹⁷, N. Bayó-Puxan¹⁸, JM. Miquel¹⁸, M. Gómez-Rey⁵, G. Villacampa¹³, S. Arévalo⁹, M. Espinosa-Bravo¹¹, J. Balmaña^{2,12}, R. Dienstmann¹³, J. Arribas^{3,4,19,20,21}, J. Tabernero²², M. Sanso^{# 5}, A. Vivancos⁵

*co-first authors

#co-corresponding authors

1. Breast Cancer and Melanoma Group, Vall d'Hebron Institute of Oncology (VHIO), Vall d'Hebron Barcelona Hospital Campus, C/ Natzaret, 115-117, 08035 Barcelona, Spain
2. Medical Oncology Department, Vall d'Hebron University Hospital, Vall d'Hebron Barcelona Hospital Campus, Passeig Vall d'Hebron 119-129, 08035 Barcelona, Spain
3. Preclinical & Translational Research Program, Vall d'Hebron Institute of Oncology (VHIO), Vall d'Hebron Barcelona Hospital Campus, C/ Natzaret, 115-117, 08035 Barcelona, Spain
4. Centro de Investigación Biomédica en Red en Cáncer (CIBERONC), Instituto de Salud Carlos III, Avenida de Monforte de Lemos 3-5, 28029 Madrid, Spain
5. Cancer Genomics Group, Vall d'Hebron Institute of Oncology (VHIO), Vall d'Hebron Barcelona Hospital Campus, C/ Natzaret, 115-117, 08035 Barcelona, Spain
6. Servicio Obstetricia y Ginecología, Hospital Universitari Son Espases, Carretera de Valldemosa 79, 07120, Palma, Spain
7. IdISBa. Institut de Investigació de les Illes Balears, Carretera de Valldemosa 79, 07120, Palma, Spain
8. Universitat de les Illes Balears, Carretera de Valldemosa 79, 07120, Palma, Spain

- 27 9. Maternal Fetal Medicine Unit, Department of Obstetrics. Vall d'Hebron Hospital
28 Universitari, Vall d'Hebron Barcelona Hospital Campus, Passeig Vall d'Hebron 119-129,
29 08035 Barcelona, Spain
- 30 10. Unitat Diagnòstic per la Imatge de la Dona, Servei de Radiodiagnòstic. Vall d'Hebron
31 Hospital Universitari, Vall d'Hebron Barcelona Hospital Campus, Passeig Vall d'Hebron
32 119-129, 08035 Barcelona, Spain
- 33 11. Breast Surgical Unit. Breast Cancer Center. Gynecology Department, Hospital
34 Universitari Vall d'Hebron, Vall d'Hebron Barcelona Hospital Campus, Passeig Vall
35 d'Hebron 119-129, 08035 Barcelona, Spain
- 36 12. Hereditary Cancer Genetics Group, Vall d'Hebron Institute of Oncology (VHIO), Vall
37 d'Hebron Barcelona Hospital Campus, C/ Natzaret, 115-117, 08035 Barcelona, Spain
- 38 13. Oncology Data Science Group, Vall d'Hebron Institute of Oncology (VHIO), Vall d'Hebron
39 Barcelona Hospital Campus, C/ Natzaret, 115-117, 08035 Barcelona, Spain
- 40 14. Servei d'Anatomia Patològica, Vall d'Hebron Hospital Universitari, Vall d'Hebron
41 Barcelona Hospital Campus, Passeig Vall d'Hebron 119-129, 08035 Barcelona, Spain
- 42 15. Grup de Patologia Molecular, Vall d'Hebron Institut de Recerca (VHIR), Vall d'Hebron
43 Hospital Universitari, Vall d'Hebron Barcelona Hospital Campus, Passeig Vall d'Hebron
44 119- 129, 08035 Barcelona, Spain
- 45 16. Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
- 46 17. Molecular Oncology Group, Vall d'Hebron Institute of Oncology (VHIO), Vall d'Hebron
47 Barcelona Hospital Campus, C/ Natzaret, 115-117, 08035 Barcelona, Spain
- 48 18. Scientific Development Area, Vall d'Hebron Institute of Oncology (VHIO), Vall d'Hebron
49 Barcelona Hospital Campus, C/ Natzaret, 115-117, 08035 Barcelona, Spain
- 50 19. Department of Biochemistry and Molecular Biology, Universitat Autònoma de
51 Barcelona, Campus de la UAB, 08193, Bellaterra, Spain.
- 52 20. Cancer Research Program, IMIM (Hospital del Mar Medical
53 Research

Institute), Barcelona, Spain.

21. Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010, Barcelona, Spain.

22. Vall d'Hebron Hospital Campus and Institute of Oncology (VHIO), UVic-UCC, IOB-Quiron
Barcelona, SPAIN

ABSTRACT

Pregnancy associated breast cancer (PABC) is an overall poor prognosis group compared to non-PABC breast cancer (BC), mainly due to late diagnosis. Effective screening approaches for early detection would likely improve its outcome. For the first time, we demonstrate the presence of cell-free tumor DNA (ctDNA) in the breast milk serum (sBM) of PABC in patients with early-stage disease and its ability to recapitulate somatic mutations present in the primary tumor. Moreover, our results demonstrate that sBM robustly surpasses plasma for tumor genetic profiling due to a prevalent shedding of ctDNA into the sBM in localized disease, as well as increased total cell-free DNA (cfDNA) abundance and integrity. Thus, we propose sBM as a potential new non-invasive liquid biopsy for a prompt detection of PABC.

When BC is diagnosed during pregnancy, lactation, or within one year after delivery, it is known as PABC¹. PABC is the most common malignancy and cancer-related death diagnosed during pregnancy and lactating patients, occurring in 1 out of every 3.000 to 10.000 pregnancies^{1,2}. The exact incidence is unknown, but considering that BC risk increases with age and the current trend of delaying pregnancy in developed countries, PABC cases are expected to rise in years to come^{3,4}.

PABC confers a significantly higher risk of death compared to the non-pregnancy related BC, with an even poorer outcome when the diagnosis is postpartum⁵. The main factor behind

the poor prognosis is a delay in diagnosis more than the pregnancy-increased hormonal state⁶. During pregnancy, breast density, volume and water content increase, impairing both clinical and radiologic evaluation¹. Screening for BC relies mainly in imaging technics focused on postmenopausal women, leaving the population in fertile age with the only option of clinical- and self-examination⁷. Since localized BC detection is critical in reducing mortality, and diagnosis is particularly challenging in pregnant and lactating women, highly sensitive and non-invasive new methods are eagerly awaited for early detection of PABC¹.

In the era of precision oncology, liquid biopsy arises as a solid candidate for early cancer detection as cfDNA fragments present in the blood or other body fluids may be used as diagnostic biomarkers⁸. Malignant cells release DNA into the bloodstream, known as ctDNA, a small fraction of the total cfDNA and where somatic mutations, methylation and copy number alterations are potentially traceable⁹. Nowadays, ctDNA analysis is well established in the metastatic BC setting, where up to 85% of the patients release enough ctDNA into the bloodstream for successful detection and its clinical utility has been already defined¹⁰. As an inflection point of liquid biopsy adoption in BC, the FDA approved the analysis of *PIK3CA* mutations in both plasma or tissue to select HR+/HER2- advanced BC patients for administration of the PI3K inhibitor alpelisib¹¹. *ESR1* mutations, a marker of acquired resistance to endocrine therapies, are also robustly identified in plasma and considered for clinical decisions¹². In contrast, localized tumors release less ctDNA into the blood, challenging its detection with current techniques¹³. This obstacle is especially relevant in early BC, where only up to 35 to 50% of the patients present detectable ctDNA in plasma^{14,15} and this may dramatically drop to only 16% in stages I and II¹⁶.

Breast milk (BM) originates from the lobular tissue distributed throughout the mammary gland and mammary ducts carry the milk from the lobules during breastfeeding. Upon maturation, the Erk1/2 pathway regulates an apical/basal polarization of the mammary epithelial cells to ensure directional secretion of milk into the lumen of the mammary gland.

Tight junction protein complexes build a blood-milk barrier that partly isolates the breast microenvironment from systemic circulation¹⁷. BM has proven to be a non-invasive source for studying mature gland cellularity and its microenvironment¹⁸, genomic promoter methylation of specific CpGs¹⁹, cancer-associated miRNAs²⁰, cancer stem-like cells²¹ or dysregulation of proteins as possible biomarkers of BC risk²². These previous findings, the physical properties of the mammary gland, its partial isolation from the systemic circulation and the intimate contact with the tumor, led us to hypothesize that BM might contain detectable ctDNA, probably before than in blood-derived plasma.

To test this hypothesis, during a period of 36 months we enrolled 16 PABC patients diagnosed at different stages of the disease who had available tumor tissue (Table 1 and Supp. Table 1). DNA obtained from FFPE-derived tissues was analyzed by NGS with a custom 432-gene hybrid capture-based panel (VHIO-300 panel, Supp. Table 2) in all cases except for patient 1, whose primary tissue was analyzed by NGS with a similar panel after enrollment in the AURORA (NCT02102165) clinical trial (411 genes, Ion torrent, see Supp. Table 3)²³. Genetically, all tumors showed single nucleotide variants, frameshifts and/or copy number alterations (Figure 1A-B and Supp. Tables 4, 5). Consistently with previous observations, a high prevalence of mutations in *GATA3* was observed, probably due to the young median age (36 years, range 25-42) and high proportion of luminal tumors of the patient cohort (81%)²⁴ (Table 1), followed by *PIK3CA*, *TP53* and *KMT2C*. The data was also analyzed for homologous recombination deficiency status (HRD). 43,75% of the tumors where HRD-high according to Marquard *et al.*²⁵ HRD scoring ≥ 42 (Figure 1A and Supp Table 1). This result correlates with a previous study in an European cohort of 70 BC samples from young patients, with high representation of TNBC tumors²⁶, a known subtype for its enrichment in HR pathway deficiency²⁷. Interestingly, our set of cases is biased towards luminal PABC tumors and still with high percentage of HR defective phenotype.

Out of the 16 patients enrolled in the study, 4 cases were excluded for further analysis. Briefly, 3 cases were discarded due to tumor surgery before childbirth (patients 13, 15 and

16), and in 1 case there was no production of BM, probably due to neoadjuvant chemotherapy treatment (case 14) (Supp. Table 1). The remaining 12 cases, with available BM sample, were considered for further analysis, although the first two had no synchronous plasma collected (Supp. Figure 1). In order to study the presence of ctDNA in BM, we fractionated the sample by centrifugation to isolate the cellular fraction and the milk fat, analyzing only the sBM. We chose to test the presence of ctDNA in sBM with ddPCR, the most sensitive and quantitative technique available at that time. Custom assays were successfully validated in the FFPE-derived DNA (Figure 1C, Supp. Table 1). Strikingly, 10 out of 12 of the sBM samples were positive for the presence of the mutant alleles matching the tumor tissues. To assess whether sBM is a superior source of ctDNA than plasma, we performed the same analysis in the plasma samples synchronously collected with the BM. Only 1 out of 10 samples presented ctDNA, in a patient diagnosed with locally advanced BC in stage IIIC, the highest stage in the cohort (Supp. Table 1). This result is in agreement with the lower rates of plasma ctDNA in localized BC¹⁰. But more importantly, it confirms expeditious detectability and superior sensitivity of ctDNA in sBM versus plasma, even in a patient with a stage IA tumor.

Interestingly, relative abundance of ctDNA in sBM did not appear to correlate with disease (tumor, nodal status nor stage), in a similar fashion to previous reports analyzing blood-derived plasma (Figure 1D). Importantly, the two samples that resulted negative for mutated alleles in sBM were isolated from colostrum, collected the first day after delivery. These result lead us to speculate whether maturity of the milk affects the sensitivity of the assay. Thus, we classified all samples as colostrum, collected between day 1 and 5 after delivery; transitional, collected from day 6 to 15 of lactation; and mature, after day 16 until the end of lactation (Supp. Table 1)²⁸. We split immature milk samples, including colostrum and transitional, from mature ones, and observed that the milk collection day from delivery is crucial for increasing ctDNA detection, since the lowest MAFs were observed in immature milk specimens in a statistically significant manner (Figure 1D). Therefore, we hypothesize that restricting collection of samples to mature BM specimens might increase sensitivity of

the protocol to a 100% (in fact, the actual sensitivity of samples classified as transitional/mature collected in our project). Other factors that correlated with sBM MAF detection were the molecular subtype (luminal A versus luminal B), and the diagnosis of an invasive lobular carcinoma (ILC) versus a ductal one (Figure 1D). The last observation may be explained due to the origin of ILC in the milk-producing glands of the breast, where cells experience a higher pressure for milk extraction compared to the cells from the ducts, which simply transport the BM. Moreover, ILC is characterized by small non-cohesive neoplastic cells invading the stroma due to dysregulation of cell-cell adhesion, primarily driven by lack of E-cadherin (*CDH1*)²⁶. Indeed, both ILC cases in our cohort had a truncating mutation in *CDH1*, classified as likely pathogenic (Figure 1A and Supp. Table 4). Such loss of tightness between cells might also influence the higher DNA shedding capacity of ILC subtype into the sBM.

One of the most exciting results came from case 1, whose milk sample was not synchronous to BC diagnosis. Instead, the patient was diagnosed during her third pregnancy, and provided a sample from the previous lactation of her second child. Thus, the sample was collected 18 months before diagnosis, but still a 4,05% of *PIK3CA* alleles harbored the p.H1047R mutation detected in the tumor (Figure 1C and Supp. Table 1). This result, along with a high sensitivity for ctDNA detection reported, strongly makes us postulate that sBM genomic profiling may be a powerful tool for early detection of PABC.

Interestingly, case 4, corresponding to a patient with a luminal A stage IA tumor showed a 15% of *CBFB* p.W113X allelic frequency and 1460 ng/ml of cfDNA in sBM, showing the second highest detected MAF and also one of the highest DNA concentrations in the cohort (and Supp. Figure 2A and Supp. Table 1). A plausible explanation for this interesting result is not only its lobular tumor origin but also that the patient was suppressing breastfeeding of a previous child at the time of BC diagnosis and sBM collection coincided with the involution of the mammary gland, a stage where a dramatic remodeling and intensive cellular apoptosis occurs²⁹.

A main limitation of liquid biopsy is the scarce concentration of cfDNA and high dilution of ctDNA. Even though sBM volumes varied considerably among patients, we obtained on average 486,5 ng of cfDNA from each ml of sBM. This was approximately 100-fold that of blood, with a median of 5,7 ng of cfDNA obtained per ml (p-val <0.0001) in the PABC cohort of patients, or 5,2 ng/ml obtained from 56 metastatic BC plasma samples previously analyzed in our laboratory (Supp. Figure 2A). Therefore, the number of genome equivalents available for analysis in the sBM is also superior than in plasma, a very convenient feature for an early detection screening approach.

Another trait of cfDNA is its pattern of fragmentation³⁰. To study the fragmentome of sBM cfDNA we performed lab-on-a-chip assay on the samples from each patient, and compared to healthy controls of plasma and sBM (Supp. Figure 2B). For patients 1 and 2 we used asynchronous plasma samples collected during response to treatment. Importantly, most sBM samples presented a wide length distribution of DNA molecules, ranging from 100 until far beyond 10380 bp (the upper limit of resolution). The expected peak of 167 bp corresponding to mononucleosomal DNA, a common feature in plasma samples, was not always prominent or the major size peak (Supp. Figure 2B right panel). Finally, no significant difference in amount of cfDNA nor in fragmentome was observed between the affected and the healthy breast, when both samples were available (data not shown) or when compared to a healthy control sBM sample (Supp. Figure 2B right panel). More detailed studies are required to understand the nature of the sBM cfDNA fragmentome. For instance, it would be interesting to clarify whether a different mixture of nucleases present in the milk or composition of extracellular vesicles protecting the DNA are responsible for the observed pattern, but the present work clearly revealed a more variable fragmentome with a generalized higher DNA integrity compared to plasmatic cfDNA. Indeed, contrary to blood-derived plasma, it has been reported that the fat layer of the BM is a good source of RNA to profile gene expression of the breast epithelium, due to the protection of the molecules inside of milk fat globules³¹.

The number of cases in this study is limited, mainly due to the difficulty to recruit PABC patients in the clinic with synchronous collection of BM and blood samples. That being said, the results among the cohort are quite compelling and demonstrate for the first time: I) the presence of ctDNA in 83,33% of the sBM samples from patients diagnosed with PABC (100% if only transitional and mature milk samples considered). II) ctDNA in sBM may be detected in localized disease even in stage IA. III) ctDNA is detectable in sBM prior to plasma in 91% of the synchronous analyzed samples. IV) ctDNA shedding in BM could anticipate PABC diagnosis, according to results from case 1. V) Maturation of the milk sample is crucial for the sensitivity of the sBM genomic analysis of ctDNA. VI) Sensitivity of the sBM as liquid biopsy might be higher in ILC tumor type since it liberates more ctDNA into the sBM than IDC. VII) cfDNA concentration in sBM is 100 times higher than in blood. VIII) cfDNA integrity from BM is superior when compared to cfDNA from plasma.

To our knowledge this is the first study reporting the presence of ctDNA in BM. Moreover, in the light of the results obtained from sBM demonstrating the higher sensitivity, cfDNA concentration and fragmentome integrity compared to blood-derived plasma, we propose BM genomic analysis as the most appropriate approach for PABC early detection. This could be the first specific PABC early detection method for a population that lacks of any other opportunity for early screening of BC, where established protocols are generally based on imaging and performed exclusively in postmenopausal women.

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349 **DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

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378 **AUTHORSHIP**

379 Conception and design of the work: CS, CO, EA, OC, NB, JM, RD, JA, JT, MS, AV. Development of
380 methodology: CS, CO, EA, JM, AMS, JA, MS, AV. Acquisition of data (acquired and managed patients,
381 provided facilities, etc.): CS, CO, EA, JM, AS, AMS, IG, IM, CM, EC, VP, PN, SA, ME, JB, MS, AV. Analysis
382 and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): CS, CO, EA,
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385 of databases): CO, EA, AS, CV, NB, JM, RD. Study supervision: CS, CO, JT, MS, AV.

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395 **Figures and Tables**

		Total	%
<i>N</i>		16	100
<i>Age</i>	<i>Median (range)</i>	36 years	(25-42)
<i>pT</i>	1	4	25%
	2	7	44%
	3	4	25%
	4	1	6%
<i>pN</i>	0	7	44%
	≥1	9	56%
<i>M</i>	0	15	94%
	1	1	6%
<i>Stage</i>	I	1	6%
	II	9	56%
	III	5	31%
	IV	1	6%
<i>HR</i>	Positive	14	88%
	Negative	2	13%
<i>HER2</i>	Positive	1	6%
	Negative	15	94%
<i>Subtype*</i>	Luminal A	4	25%
	Luminal B	9	56%
	HER2+	1	6%
	TNBC	2	13%
<i>Diagnosis</i>	Trimester 1	3	19%
	Trimester 2	3	19%
	Trimester 3	4	25%
	Lactancy	6	38%

*Subtype based on Immunohistochemistry.

Luminal A is defined as ER or PR positive, HER2 negative and Ki67<20%

Luminal B is defined as ER or PR positive, HER2 negative and Ki67>=20%

HER2+ is defined as HER2 positive, regardless ER, PR and Ki67 status

TNBC is defined as ER, PR and HER2 negative

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397 **Table 1.** Clinical-pathological characteristics of the cohort.

Figure 1. A. Oncoprint of somatic mutations detected by NGS in PABC tumors at least in 2 samples out of 16, and HRD score defined as high when ≥ 42 . **B.** Oncoprint of CNA alterations detected by NGS in PABC tumors occurring at least in 2 samples out of 16. **C.** MAF % detected by ddPCR in solid tissue, plasma and sBM samples, all performed for the most abundant somatic mutation detected in the solid tissue by previous NGS. **D.** Statistical significance and correlations between MAF % and milk maturation, histology, breast cancer subtype, nodal status, disease stage, and tumor size. Mann-Whitney Wilcoxon t-test analysis was performed for the different features (* depicts statistical significance with $p\text{-val} < 0.05$).

Supplementary Files

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