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The Circulating Proteome of Ultra-Early Breast Cancer: A Comparative Study with Benign Lesions and Healthy Controls

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1. BACKGROUND AND RATIONALE

1.1 Breast Cancer Epidemiology

Breast cancer (BC) is the most frequently diagnosed type of cancer worldwide, with approximately 2.3 million new cases in 2020 (11.7%), and the fifth cause of cancer death worldwide, with a mortality rate of 6.9% (1).

The situation in Italy is not different: in 2023 the most diagnosed tumor has been BC with almost 56000 new cases. When considering the prevalence, 800.000 women, the majority of whom are over the age of 75, are currently living with a diagnosis of breast cancer in our country (2, 3). BC is by far the most diagnosed neoplasm in women, as it represents almost one-third of the new malignant tumor diagnosis, as well as the primary cause of death from cancer in females. Despite the modest increase in incidence (+0,3% per year), BC mortality has decreased of 6% when comparing data from 2015 to that of 2020. This is bound both to the wider spread of screening programs and to the improvements that the available therapies have seen in these years (3).

These advances have also been possible thanks to the better understanding of how specific types of cancer develop and grow, and to the progresses in the strive to tailor therapies to each patient and to the particular subtype of tumor they bear. BC is no exception: classifications are used to categorize each patient's tumor. These reflect the histology, the anatomical involvement, and the molecular characteristics of the neoplasm and represent an attempt to better categorize the complexity and diversity of this disease in order to foster the shift towards an even more personalized medical approach.

1.2 Histological Classification

The last World Health Organization’s histological classification of breast tumors has been published in 2019 (4).

BC histology is classified as follows:

1. **Epithelial tumors**
    1.1. Microinvasive carcinoma.
2. **Invasive breast carcinoma**
    2.1. Invasive breast carcinoma of no special type (NST).
    2.2. Invasive lobular carcinoma.
    2.3. Tubular carcinoma.
    2.4. Cribriform carcinoma.
    2.5. Mucinous carcinoma.
    2.6. Carcinoma with medullary features.
    2.7. Carcinoma with apocrine differentiation.
    2.8. Carcinoma with signet-ring-cell differentiation.
    2.9. Invasive micropapillary carcinoma.
    2.10. Metaplastic carcinoma.
    2.11. Rare types:
        2.11.1. Carcinoma with neuroendocrine features.
3. **Precursor lesions**
    3.1. Ductal carcinoma in situ.
    3.2. Lobular neoplasia.
    3.3. Lobular carcinoma in situ.
    3.4. Atypical lobular hyperplasia.

4. **Intraductal proliferative lesions**
    4.1. Usual ductal hyperplasia.
    4.2. Columnar cell lesions including flat epithelial atypia.
    4.3. Atypical ductal hyperplasia.
5. **Papillary lesions**
    5.1. Intraductal papilloma.
    5.2. Intraductal papillary carcinoma.
    5.3. Encapsulated papillary carcinoma.
    5.4. Solid papillary carcinoma.

The most frequent form of infiltrating BC is the NST, with a prevalence between 70% and 80%. This histotype is defined by the presence of a considerable difference in cellular morphology as cells are organized in tubular or glandular structures. The second most diagnosed form of BC is lobular carcinoma (10%), which features little variety of cancer cells, frequent expression of steroid receptors, and very rare HER-2 receptor overexpression (5). The histological type is important in determining the tumor's prognosis. In this regard the tubular, papillary, and medullary subtypes are associated to a better prognosis.

Moreover, grading is also to be accounted for: tumors can be divided into three grades based on cellular characteristics:

- Grade 1: more of the 75% of tumoral cells form glands, are uniform and have small nuclei with a size similar to that of normal breast cells. Less than 7 mitoses are visible per 10 high power fields.
- Grade 2: 10 to 75% of the tumor has a glandular differentiation, its cells are larger than the normal ones, are more variable in size and shape and have visible nucleoli. 8 to 15 mitoses are visible per 10 high power fields.

- Grade 3: less than 10% of the tumor is differentiated, cells have a lot of atypia with vesicular nuclei, prominent nucleoli and important shape and size differences. More than 18 mitoses are visible per 10 high power fields.

Considering NST and lobular cancer, the prognosis is greatly influenced by their grading: G3 negatively impacts the overall survival of patients.

Despite the aforementioned importance of the histological classification, the use of biotechnological techniques has made it possible to develop a molecular classification, based on cancer specific immunohistochemistry characteristics, which is also useful as far as targeted treatment is concerned (6).

1.3 Molecular Classification

This classification is better suited to describe the behaviour of BC, keeping in mind that the most frequent histological subtype of invasive BC is the NST. Using immunohistochemistry (IHC) and fluorescent in situ hybridization (FISH), BC can be subdivided into 4 classes:

1. Luminal A (ER+, PR ≥20%, HER2-, Ki67low).
2. Luminal B (ER+, PR <20% or HER2+ or Ki67high) which are subdivided into HER2- and HER2+.
3. HER2-Enriched (ER-, PR-, HER2+).
4. Triple negative (ER-, PR-, HER2-).

The subtype division is based on the immunohistochemistry (IHC) phenotype of the tumor and takes under consideration the expression of the Estrogenic Receptor (ER), the Progesterone receptor (PR), and the cell proliferation index using the fraction of Ki-67 positive cells while FISH is used to confirm the Human Epidermal Growth Factor Receptor 2 (HER2) positivity.

Luminal A represents the most frequent subtype (50-60%), followed by Luminal B (15-20%), triple negative (15-20%), and HER2 positive (10%). Luminal A neoplasms are usually characterized by lower grade and better prognosis compared to the other subclasses. Luminal B tumors on the other hand are more often higher grade and therefore have a worse prognosis. HER2-Enriched (or overexpressing) and basal like (or triple negative) neoplasms tend to be high grade and aggressive (3), (6).

The therapeutic approach varies depending on tumor subtype: hormonal therapy alone can yield clinical benefit for luminal A tumors while an association with chemotherapy is recommended for luminal B neoplasms. Both these types respond to hormonal therapy because of the expression of ER and PR receptors, however, considering the higher Ki67 positivity and the eventual HER2 expression in the luminal B subtype, chemotherapy plays an additional therapeutic role.

HER2 is an oncogene located on chromosome 17 that codes the Human Epidermal Growth Factor tyrosine kinase receptor. In overexpressing HER2 neoplasms, the development of target therapies such as Trastuzumab, a monoclonal antibody that binds an extracellular domain of HER2, has greatly improved the clinical outcomes of such subtype. Recently, new therapies specific for this subtype of BC have been unveiled: Pertuzumab, another monoclonal antibody targeting a different extracellular domain of HER2, Lapatinib and Neratinib, tyrosine kinase inhibitors, Kadcyla and Enhertu, the conjugation between Trastuzumab and, respectively, Emtansine and Deruxtecan (7).

The basal like subtype, which represents between 15% and 20% of all BC, sees limited benefit from molecular-targeted therapy and standard postoperative adjuvant chemoradiotherapy, which, considering its high aggressiveness and greater metastatic potential, accounts for the shorter relapse time and higher mortality rate it is associated with (8).

1.4 TNM Classification

The TNM based staging, the common language used in cancer staging, is issued by two main sources: the Union for International Cancer Control (Union Internationale Contre le Cancer; UICC) and the American Joint Committee on Cancer (AJCC). The latest TNM classification, which has reached the 8th edition, has been published by these two sources respectively in 2017 and 2018. The AJCC-TNM8 includes a dual stage designation: one anatomical, and one prognostic, which combines the T (tumor), N (node), and M (metastasis) categories but also considers data regarding the histological grade, ER, PR, and HER2 statuses and gene expression profiles (9).

The T category considers the size of the tumor and its locoregional invasion and ranges from Tis to T4:

- T0 No evidence of primary tumor.
- Tis (DCIS) Ductal carcinoma in situ.
- Tis (Paget's) Paget's disease of nipple NOT associated with invasive carcinoma and/or carcinoma in situ (DCIS) in underlying breast parenchyma. Carcinomas in breast parenchyma associated with Paget disease are categorized based on size and characteristics of parenchymal disease, although the presence of Paget disease should still be noted.
- T1 Tumor $\leq$ 20 mm in greatest dimension.
- T2 Tumor $>$ 20 mm but $\leq$ 50 mm in greatest dimension.
- T3 Tumor $>$ 50 mm in greatest dimension.
- T4 Tumor of any size with direct extension to chest wall and/or to skin (ulceration or macroscopic nodules); invasion of dermis alone does not qualify as T4.

The N category considers the locoregional lymph nodes involvement. It is divided into a clinical and a pathologic classification and ranges from N0 to N3 The clinical classification is based on imaging or clinico-radiological evaluation of the nodal basins, while the pathologic one is based on the number of involved lymph nodes on surgical pathology (10).

Clinical classification:

- cN0 No regional lymph node metastases.
- cN1 Metastases to movable ipsilateral level I, II axillary lymph node.
- cN2 Metastases in ipsilateral level I, II axillary lymph nodes that are clinically fixed or matted; or in clinically detected ipsilateral internal mammary nodes in absence of clinically evident axillary lymph node metastases.
- cN3 Metastases in ipsilateral infraclavicular (level III axillary) lymph node(s) with or without level I, II axillary lymph node involvement; or in clinically detected ipsilateral internal mammary lymph node(s) with clinically evident level I, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph node(s) with or without axillary or internal mammary lymph node involvement.

Pathologic classification:

- pN0 No regional lymph node metastasis identified or ITCs only (malignant cell clusters no larger than 0.2 mm).
- pN1 Micro metastases; or metastases in 1-3 axillary lymph nodes; and/or clinically negative internal mammary lymph nodes with micro metastases or macro metastases by sentinel lymph node biopsy.
- pN2 Metastases in 4-9 axillary lymph nodes; or positive ipsilateral internal mammary lymph nodes by imaging in absence of axillary lymph node metastases.
- pN3 Metastases in 10 or more axillary lymph nodes; or in infraclavicular (level III axillary) lymph nodes; or positive ipsilateral internal mammary lymph nodes by imaging in presence of one or more positive level I and II axillary lymph nodes; or in more than 3 axillary lymph

nodes and micro metastases or macro metastases by sentinel lymph node biopsy in clinically negative ipsilateral internal mammary lymph nodes; or in ipsilateral supraclavicular lymph nodes.

The M category classifies the presence of metastases as follows:

- M0 No distant metastases.
- M1 Metastatic disease present.

The most frequently involved organs by breast cancer metastases are bones, lungs, brain, and liver. As far as prognostic staging is concerned, the addition of biomarkers into the AJCC-TNM8 may bring an upstaging or downstaging of the tumor depending on its biology; therefore, the prognostic stage may differ from the anatomic stage. For example, patients with triple-negative tumors, regardless of grade, have survival rates comparable with those of patients with diseases ranked one stage higher in the anatomical classification (10).

1.5 Current screening, diagnosis methods, and main limitations

The adoption of population wide screening programs is associated with a significant reduction of BC mortality, due to the higher number of BC cases diagnosed at an early stage (11). Consensus guidelines suggest that the screening for women considered to have an average-risk of developing BC should be performed between 40 and 74 years (12).

As far as Italy is concerned, screening programs instruct to perform a mammography every two years in women aged between 50 and 69 (in some regions up to 74).

Mammography is considered to be the most effective screening test (3), and, although its association to ultrasonography seems to increase the accuracy in BC detection when compared to mammography alone, to this day there is no evidence that combining ultrasonography to mammography lowers mortality from BC nor all-cause mortality (13).

The most recent European guidelines regarding BC have been issued by the European Commission Initiative on Breast Cancer (ECIBC) in 2019. These guidelines suggest to screen differently women with no risk factors such as genetic predisposition or previous thoracic radiotherapy, namely average-risk women, who represent 80% of those diagnosed with BC (14):

- 40-45 years old women: no screening recommended.
- 45-49 years old women: a mammography every 2 or 3 years is recommended.
- 50-59 years old women: a mammography every 2 years is recommended.
- 70-74 years old women: a mammography every 3 years is recommended.

Notably, among women between 50 and 69 years old, those who attended mammographic screening had a reduction in risk of death by BC up to 40% (14).

Many technological advances have been made in BC screening, one of which has been the transition from film screen to digital mammography, which yielded an improvement in accuracy in women under 50 years of age, women with dense breasts, and pre or perimenopausal women (16). Another progress was represented by the introduction of digital breast tomosynthesis, a pseudo 3D mammography that has improved detection rates by 33-53% while reducing false positives by 30-40%, also thanks to the diminished tissue overlap and structure noise in comparison to 2D mammography (14) (17). The introduction of ultrasound in the screening process has also represented an important advance: as mammographic sensitivity significantly declines when breast density increases, a frequent scenario in younger women, ultrasound can help avoid false negatives. In younger women with dense breasts and negative mammographies, ultrasound has proven its importance as a complementary examination to mammography: it has led to an increase from 0.78 to 0.91 in diagnostic accuracy when compared to mammography alone (18).

In women at high risk of developing BC for family history of BC or for being carrier of the mutation of *BRCA1* and/or 2, it is recommended to begin the screening at 25 years of age or 10 years before the age in which the youngest relative was diagnosed with BC (3).

Moreover, in women with BRCA1 or 2 mutation, Li-Fraumeni, Cowden or Bannayan-Riley-Ruvalcaba syndromes or who underwent thoracic radiotherapy between 10 and 30 years of age, contrast-enhanced breast MRI is indicated (3). In particular, MRI is better at identifying early-stage diseases when compared to mammography and the combination of the two techniques has improved survival rates (19). Furthermore, the indication for MRI is also extended to:

- Preoperative staging of newly diagnosed BC.
- Evaluation of the response to neoadjuvant chemotherapy (NACT).
- Differential diagnosis of peri-cicatricial lesions.
- Carcinoma of unknown primary (CUP) syndrome.
- Equivocal results at mammography or ultrasonography.
- Clinical or instrumental suspect in women with breast prostheses.

BI-RADS Classification

The breast imaging-reporting and data system (BI-RADS) classification was developed with the aim of standardizing the risk evaluation for mammography and providing a common language to interpret radiological reports. The first version was released in 1993 by the American College of Radiology (ACR) using literature review to elaborate descriptors associated with benign or malignant disease. Then, it has been updated to include new technical such as the adoption of ultrasound (2003) and MRI (2006) in the diagnostic process. The latest edition is the fifth one, which was issued in 2013 and includes seven categories for lesions.

Firstly, to describe a mammographic report using this classification, one must stage the breast density using the terms provided: fatty, scattered, heterogeneously dense, and extremely dense. In case of identification of a breast lesion, the radiologist must specify its shape, which can be round, oval or irregular, its margins, that can be circumscribed, obscured, micro-lobulated, indistinct, and spiculated, and finally its density, which can be higher, equal, or lower when compared to the breast density and can also be described as fat-containing. Breast lesions are more likely to be malign when of irregular shape, with spiculated margins, and high density. When present, calcifications must be adequately described: benign calcifications are typically large rod-like, popcorn or coarse while suspicious ones are usually be amorphous, fine pleomorphic, and fine-linear branching. The possible grouping of calcifications also has to be described as it might be diffuse, regional, grouped, linear, and segmental (20).

The seven categories of the BI-RADS final assessment are hereby described:

1. BI-RADS 0: incomplete evaluation with further imaging required such as additional mammographic views and or ultrasound.
2. BI-RADS 1: negative examination as there are no masses, suspicious calcifications, or areas of architectural distortion.

3. BI-RADS 2: benign findings such as secretory calcifications, simple cysts, fat-containing lesions, calcified fibroadenomas, implants, and intramammary lymph nodes.
4. BI-RADS 3: probably benign, should have short interval follow-up to determine stability as the risk of malignancy is below 2%. Findings belonging in this category include: a non-palpable, circumscribed mass on a baseline mammogram; a focal asymmetry, which becomes less dense on spot compression images, or a solitary group of punctate calcifications.
5. BI-RADS 4: suspicious abnormality. This category is further grouped in:
   a) 4a (2% to 10% chance of malignancy).
   b) 4b (10% to 50% chance of malignancy).
   c) 4c (50% to 95% chance of malignancy).
6. BI-RADS 5: highly suggestive of malignancy with a probability higher than 95%.
7. BI-RADS 6: histologically proven malignancy.

The definitive diagnosis of BC relies on the biopsy of the suspect lesion. Breast biopsy can be performed using three image-guided techniques: stereo-guided, ultrasonography-guided, and MRI-guided biopsy. The ultrasonography-guided biopsy technique can be used for a wide variety of lesions while the MRI-guided technique is generally used when the lesion is only detectable with MRI. The stereo-guided technique is used for lesions associated to calcifications (21).

Limitations of current screening approaches

Despite the proven clinical utility of screening campaigns, current techniques used in the secondary prevention of BC are characterized by suboptimal accuracy, with frequent false negative and false positive results. Table 1 summarizes the sensitivity and specificity of currently approved screening techniques for the early diagnosis of BC.

TABLE 1	SENSITIVITY	SPECIFICITY
MMG	**91%**	**74% (22)**
USG	**96%**	**93% (24)**
MRI	**97%**	**69% (23)**

Table 1. Sensitivity and specificity of current imaging techniques uses in BC screening. MMG: mammography, USG: ultrasonography, MRI: Magnetic Resonance Imaging

False negatives are especially frequent with certain types of BC, with particular regard to lobular carcinoma with a lepidic pattern of growth, and in patients with dense breast tissue (14). In these patients, some of the current screening technologies struggle to maintain a high sensitivity level: MMG in particular, shows the lowest sensitivity in this condition. Therefore, additional USG is recommended in case of dense breast (BIRADS-D). MRI represents the most accurate technique for the detection of breast lesions but is burdened by higher cost and, in certain conditions, lower specificity (22).

False positives, on the other hand, lead to an increased number of unnecessary biopsies representing a significant burden for both patients as it brings supplementary stress, and the national health care system in terms of increased costs. Almost 80% of the suspicious breast lesions identified with MMG only require additional imaging like USG or supplementary MMG to rule out BC, while the other 20% require further confirmation through tissue biopsy (14). Of these biopsies, only 30-50% actually diagnose malignant lesions (14) (25). Although evaluated as a safe and minimally invasive technique, with the most frequent complication being mild/moderate hematoma (26), breast biopsy is not a complications-free procedure: vasovagal reactions, infections, and massive bleeding, although rare, can occur (26) (27). Moreover, the tumor mass isn't homogeneous in all its area: spatial heterogeneity impacts the expression of crucial biomarkers used in BC molecular classification, such as ER, PR, and HER2, and does not allow therefore to capture BC overall biology (28). This heterogeneity has been also associated with intra-tumoral therapeutic resistance, which the foundation for disease relapse (29).

Another problem affecting screening is over diagnosis, namely the diagnosis of a cancer that would have never caused a problem in the patient's lifetime, thus resulting in an unneeded treatment. When considering breast cancer, the literature shows an estimation that ranges from 0% to more than 50%, even though the benefits clearly overtake the risks (14) (27) (28).

1.6 Liquid biopsy

General Characteristics

The suboptimal accuracy and the invasiveness of the procedures currently adopted for BC early diagnosis represent an unmet need. In such context, the availability of accurate, non-invasive biomarkers for BC would be of extreme relevance, allowing to reduce the rate of unnecessary tissue biopsies. One of the approaches that is showing potential to fulfil this need is Liquid biopsy. In oncology, the concept of liquid biopsy refers to a non-invasive test performed on biological samples collected non-invasively or minimally invasively, such as blood, urine, or saliva, to detect and assess molecules released from the tumor into the bloodstream or other body fluids.

As previously stated, nowadays the molecular and the genetic alterations in cancer are crucial to shape patients' treatment. To obtain tumor samples viable to identify such alterations, tissue biopsies are generally performed, and, considering the limitations associated to them, the availability of a procedure that makes possible to obtain tumor samples more accessibly, allows real time monitoring of its cancer molecular alterations and could provide data that might unfold information otherwise not detected by tissue genotyping (31) is even more important.

The advantages of liquid biopsies when compared to standard tissue biopsies are numerous: they are not invasive, less expensive, they allow to analyse the overall *secretome* of cancer, better assessing tumor heterogeneity, they can be performed serially, possibly allowing for longitudinal time series amenable for the non-invasive assessment of the response to therapy (32).

Biomarkers

Circulating tumor cells (CTC) represent the first biomarker studied in the context of liquid biopsy. CTC are released by the primary tumor or by its metastases in two forms, as single cells or as clusters. CTC assessment has a great clinical potential, including the possibility to perform cytological and molecular analysis or to develop patient-specific CTC cultures (33). In addition, these cells have a significance in both metastatic and non-metastatic BC: their detection through the FDA-approved CellSearch® System leads to a worse prognosis for the affected patients (34). Another component proposed are tumor educated platelets (TEPs), which play a role in systemic and local reactions to the tumor as they are influenced by the neoplastic cells (35).

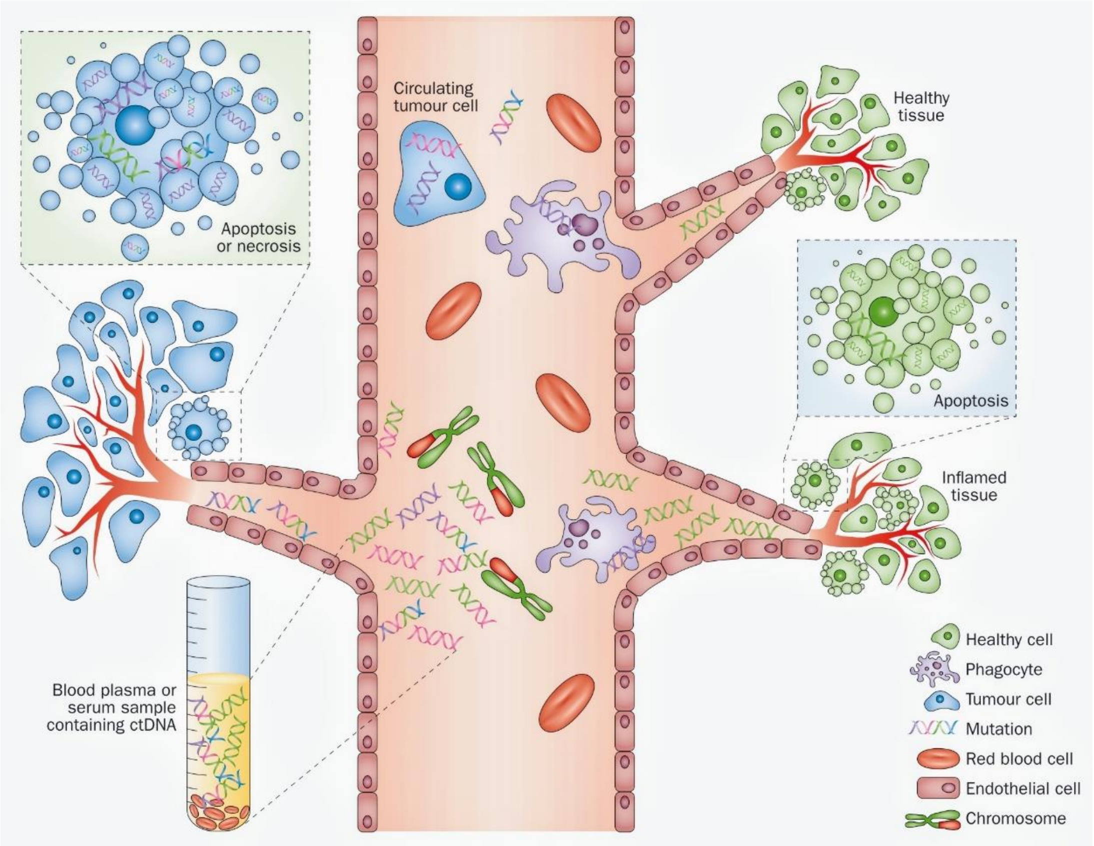

Figure 1. Release and extraction of cfDNA from the blood (36).

Currently, circulating cell-free nucleic acids (cfNAs) are regarded with great interest in literature for the useful insights they can provide on tumor molecular landscape. These are fragments of DNA or RNA molecules released from apoptotic or necrotic cancer cells or through active release Among these, one the more promising is cfDNA (37).

cfDNA has been identified as a possible cancer biomarker but, due to the variety of both para-physiological and pathological factors that have an effect on its levels, such as inflammation, only the fraction directly deriving from tumoral cells (ctDNA) has been proven to possess adequate sensitivity and specificity for such a purpose (38, 39). CtDNA is composed by small fragments of nucleic acids, the size of which is also influenced by the release mechanism used, it represents only a small fraction of total cfDNA, although in some circumstances, such as advanced cancer, it can constitute the majority of it, its blood concentration has been proven to be higher in patients with metastatic cancer and its blood half-life is quite short (15 min-2,5 h) (40).

CtDNA reflects tumor genomic and epigenomic aberrations. To date, ctDNA is measured in case of advanced cancer, with the aim of identifying actionable mutations in plasma of to monitor the emergence of resistance to therapy. However, sequencing somatic mutations from plasma does not have adequate accuracy in case of early-stage cancer. Moreover, ctDNA can reflect intratumoral heterogeneity, the sample’s mutation levels (41), and its examination can also unveil mutations not discovered by traditional biopsy samples (42).

Another interesting aspect of cfDNA is epigenomics, in particular methylation: it was demonstrated that DNA methylation can be used to identify the tissue from which cfDNA originated (43) as methylation patterns specific to a certain tissue are retained in cfDNA fragments (44). Methylation is a highly preserved epigenetic modification among vertebrates and the most frequent one occurs on cytosine, especially on CpG dinucleotides. This is believed to be one of the main regulators of chromatin accessibility, as it silences retrotransposon elements, prevents transcription, and regulates binding of transcription factors. On the other hand, regions rich with CpGs, also called CpG islands, are usually hypomethylated. These islands are often within promoters and, due to their low methylation levels, are believed to foster transcription of the related genes. In addition, the correlation between methylation dysregulation and the development of cancer has been highlighted by many studies: this corresponds to both a hypomethylation of intergenic regions and a hypermethylation of CpG islands (45). As far as cfDNA analysis is concerned, two of the most promising epigenetic markers are 5-methylcytosine (5mC) and its hydroxylated analogue. Considering the low cfDNA concentration and that ctDNA represent a lesser part of this already minimal quantity of material in early-stage cancer, the analysis of methylation on these samples requires a technique that allows the use of exceptionally low quantities of input DNA. The most promising one is cfMeDIP-seq: this is a high-throughput sequencing method that, after immunoprecipitating with monoclonal antibodies specific for 5mC previously denatured DNA, uses filler DNA enriching to lower the needed input cfDNA up to 1-10ng (46).

Plasma proteomics

Even considering the key role of DNA mutations in cancer development and growth, the primary actors in the cellular metabolism and function in general are proteins and, furthermore, they are also directly targeted by many therapies against cancer. Therefore, one additional major implementation of liquid biopsies, aimed at filling the gap between genomic information and cell behaviour, involves proteomics. Analysing the proteome means systematically studying the set of proteins included in a given biological sample, assessing their concentration, structure, and function. This can provide insight into the complex process that is oncogenic transformation as well as provision new targets against which implementing novel drugs and, given the dynamicity of proteomes, provide information on treatment response (47). A catalogue that contains some of the available biomarkers discoverable with liquid biopsies can be found at 48 and features markers intended for, among others, determining treatment and prognosis, such as EGFR for NSCLC and colorectal cancer, helping in diagnosis, as c-kit for GISTs and monitoring the therapeutic response, like Alpha-fetoprotein for liver and ovarian cancer.

Various companies provide assays to identify new promising proteic biomarkers. One of these is Somalogic® through the SomaScan® platform, which makes possible to quantify up to 10k unique human proteins utilizing a new type of aptamer, i.e. a single stranded DNA molecule able to bind proteins. Another prominent company active in the proteome analysis field is Olink®, which is based on high multiplex immunoassays. It allows simultaneous analysis of ninety-two protein biomarkers using Proximity Extension Assay® (PEA) technology, which is based on antibodies bound to oligonucleotides hybridized to each other to be uniquely associated.

Given the various techniques that can be applied to samples obtained via liquid biopsy, its potential uses are quite vast.

One of such purposes is early detection, both of primary tumors and relapses. In particular, the analysis of plasma cfDNA methylation pattern as well as the detection of circulating miRNAs have been found to be important tools to early detect NSCLC when combined with imaging to better identify pulmonary nodules (49). As far as cholangiocarcinoma is concerned, the proteomics analysis of extracellular vesicles derived from the tumoral cells have allowed the precise diagnosis of this subtle neoplasm even in early phases (50). Both these tumoral types are considered exceedingly difficult to diagnose early as they initially exhibit a mostly silent growth pattern, thus precluding patients' accessibility to certain treatments.

When taking care of a patient neoplasm, the importance of the diagnostic iter is undeniable as it is unquestionable the need to understand what the best treatment to utilize against his specific cancer type is. Liquid biopsy can also aid in this field: the Food and Drug Administration (FDA) has approved various companion diagnostic tests to assist in therapy selection: ctDNA analysis in NSCLC via the Cobas EGFR mutation Test v2 to identify patients eligible to receive target therapy (also approved by the European Medicines Agency), therascreen® PIK3CA test in breast cancer, and a test based on NGS to identify BRCA1 or 2 mutation on ctDNA of patients with ovarian cancer (51).

Another crucial point is assessing in this path is assessing treatment response and, eventually, resistance. This can be obtained yet again analysing ct and cfDNA using targeted, broad panels or genome wide approaches depending on the spectrum of mutations that must be examined. Data suggest that monitoring cfDNA in colorectal cancer to identify lethal clones during treatment with EGFR-targeted therapies could be useful to decide the optimal timing for an anti-EGFR re-challenge and measuring circulating BRAF mutation levels in cutaneous melanoma can help to determine when to switch from target therapy to immunotherapy (52).

Finally, it is vital for patients to be followed-up in order to identify a possible disease relapse. Given that the mutations in cancer cells are dynamic as their genes are more prone to accumulate new alterations due to the lessening of control systems such as oncosuppressors and to the increased number of mitosis occurring, considering that disease relapses and metastases are bound to the selection of factors that favour the survival of cells making them resistant to therapies, the ability to profile these undergoing mutations is very important during follow-up (53). Once again, the analysis of ctDNA proves itself useful: for example, it can help clinicians evaluate whether to administer adjuvant chemotherapy, thus effectively reducing the number of recurrences and decreasing the number of superfluous therapies (54).

The RENOVATE study

Today, the clinically useful proteomic biomarkers in cancer screening are in a limited number. These include CA 125 for ovarian cancer, CA 19-9 for Pancreatic cancer, CA 15-3 for BC, CEA for Colorectal cancer, Alpha fetoprotein in Hepatocellular and Germ cell line cancers, and PSA Prostate cancer (55).

Research in this field is constantly active, with a significant number of studies trying to broaden this list. One of these, focused specifically on BC, is the RENOVATE trial (Clinicaltrials.gov ID: NCT04781062), whose purpose is to evaluate the diagnostic and predictive performance of several non-invasive biomarkers and to estimate the efficiency of their combination with radiomics algorithms in diagnosing early BC. Considering the small probability for a single marker to be sufficiently accurate, sensitive, and specific to be used alone for this scope, this study will use horizontal data integration, which is to say the combination of several analytes and radiomic algorithms to build a classifier able to accurately diagnose early non-invasive BC. This will be possible thanks to the analysis of peripheral blood samples and urine samples obtained from a cohort of 750 patients followed by the Diagnostics Senology Unit of San Martino Hospital.

Taking into account the enormous amount of different cancer types, the various detection methods or biomarkers that can be analysed in liquid biopsy samples can also be integrated with each other in order to further improve sensibility and specificity and to ease early diagnosis and treatment selection. This process can be categorized in three main groups depending on the complexity of the types of data that are being integrated:

1. Elementary integration: embedding of methods or biomarkers of the same kind as DNA-DNA or protein-protein.
2. Intermediate integration: embedding of two or more different classes as protein-DNA.
3. Advanced integration: embedding of results obtained both from liquid biopsy and from other methods such as cDNA and medical imaging.

The elementary integration has already seen a quite broad implementation into the clinical processes, while the other two methods have yet to be widely applied even though they seem very promising (56).

1.7 Study rationale and scopes

Considering the benefits of liquid biopsy when compared to the current standard screening procedures and keeping in mind how the analysis of proteomics can reflect the inner mechanisms underlying carcinogenesis, the research towards its implementation is flourishing. Unfortunately, as of today there aren't any approved biomarkers that can be used to detect breast cancer in an early stage as the ones employed in the management of this tumor are mainly used to help determine treatment, like the expression of ER, PR and HER2/neu among others, and to detect metastasis or recurrence after treatment, like CA 15-3 and 27.29 (47).

Therefore, the aim of this work is to identify novel circulating biomarkers of ultra-early BC by dissecting its plasma proteome. This will help improving current diagnostic protocols of BC, potentially generating innovative knowledge on the physiopathology of BC onset.

2. MATERIALS AND METHODS

2.1 Study Design

Patients were selected among women whose radiological screening process identified breast lesions less than 2cm big (T1, BIRADS-3/4/5), that had no evidence of axillary or distant involvement. These women where then recruited to donate, after having signed a written informed consent, four peripheral blood samples (30mL total volume) and one urine specimen (40mL). Subsequently, only patients with T1N0/T2N0/T1N1a surgically staged neoplasms or with histologically benign lesions have been enrolled. A cohort of healthy women, i.e. women with two consecutive mammograms resulting BIRADS-0 or 1, was selected to obtain the same blood and urine samples to be used as negative control. Other data collected includes pseudo-anonymized radiological images, anatomopathological data, demographics, BMI, smoking and alcohol consumption habits, number of pregnancies, age at menarche, menopausal status, eventual undergoing endocrine therapies, comorbidities, and familiarity for BC.

The patients who were diagnosed with invasive BC histologically were asked to donate a second blood and urine sample during the first follow-up oncological visit after surgery (t1).

This data will be used to fulfil the scopes of this prospective case-control translational trial.

2.2 Patients

The patients participating in this study have been enrolled according to the following criteria.

Inclusion criteria:

- Written informed consent.
- Breast masses identified by digital bilateral mammography.
- Patients between 18 and 75 years old.
- Patients eligible for tru-cut or VA breast biopsy as per normal clinical practice for study population.
- Absence of breast lesion during screening in healthy controls.
- Patients who are able to willingly comply with the study requirements.

Exclusion criteria:

- Previously diagnosed invasive cancer of any type.
- Suspicion, either clinical or radiological, of advanced cancer or of metastases during screening.
- Patients with active or treated autoimmune disorders (except autoimmune thyroiditis) or with either chronic or seasonal and active allergies or with history of such diseases.
- Patients who underwent a major trauma or surgery within 24 weeks prior to screening.
- Patients with a chronic or acute active infectious disease during the 8 weeks before screening.
- Patients with a diagnosis of acute or chronic cardiac, kidney or liver disease or that suffered acute cardiac events.

2.3 Sample collection, processing, and storage

The team behind this trial is composed of a dedicated Research Nurse, whose roles are to undertake venipuncture and to curate sample collection, a Clinical Research fellow, dealing with patients selection, informed written consent collection, who also records outcomes and curates data, and an Early Research fellow in molecular biology, who handles the processing, storage and shipment of blood and urine samples, records outcomes and curates data.

A unique code has been assigned to each sample collected in order for anonymization of the patients to be guaranteed. This consists in the term UPN (Univocal Person Number) followed by three numbers identifying the case number.

The containers utilized for the collection of fluid samples have been: K2EDTA tube in the first place, then Tempus™ Blood RNA tube and, lastly, PAXgeneBlood ccfDNA tube for the blood samples and a sterile cup for urine. These specimens have been collected at the senology laboratory, have been coded with date of collection, patient name, and their identifier number and have then been moved to the Translational Genomics laboratory where they have been processed within two hours from the collection.

The processing is comprised of various steps. To extract cfDNA from plasma and to collect exosomes, proteins and other markers to be assessed, blood and urine samples must be processed. PAXgene tubes are first centrifuged for 15 min at 1900 Relative Centrifugal Force (rcf) at room temperature (RT), then the collected plasma is further centrifuged for 10 min at 1900 rcf at RT. EDTA tubes are centrifuged for 15 min at 1600 rcf at RT and the collected plasma is further centrifuged at 1900 rcf for 10 min at RT. After being collected, urine is mixed with Cell-Free DNA Urine Preserve (Streck) to stabilise cfDNA for up to 7 days at temperatures between 6°C to 37°C. Urine is then centrifuged at 2680 rcf for 10 min, and the supernatant is aliquoted into 15 mL tubes and stored until cfDNA is extracted.

2.4 Proteomics

The analysis of the proteome has been brought on via the SomaScan® 7k platform using 55 µL plasma samples. To pursue this assessment, this platform makes use of a new kind of aptamers, called SOMAmers®, which possess high affinity for individual proteins. To make them identifiable and quantifiable in high-density microarrays, they possess a unique sequence tag of forty nucleotides and a fluorescent label, which is then used to execute the proteins' quantification, then expressed in relative fluorescent units (RFU). It has an extremely high sensitivity, allowing to identify proteins in the order of femtomoles contained in volumes of various biological fluids, such as serum and urine.

The Olink® Target 96 Oncology Panel® has also been used to study the proteome. This assay is based on oligonucleotides with unique DNA sequences linked to antibodies, which are used to bind plasmatic proteins. The quantification of these is executed using real-time polymerase chain reaction (PCR) with high sensitivity and specificity. The measurement unit used to meter the proteins' quantity is the Normalized Protein eXpression® (NPX), an arbitrary unit based on threshold cycles (Ct) values. This method uses only 1 µL of biological sample.

2.5 Statistical analysis

Statistical analyses were conducted in the R environment. Proteomics data from SomaLogic® were loaded and studied with the SomaDataIO® package. Each aptamer was associated with its unique UNIPROT ID. Data were subsequently normalized according to SomaLogic® pipeline. Differential analysis was conducted by using limma. Significance threshold was set at false discovery rate < 0.1 for the discovery phase and at p-value 0.05 for the testing phase. The agreement of HPGDS abundance by SomaLogic® and Olink® was assessed using Spearman correlation.

3. RESULTS

3.1 Demographics

A total of eighty-four patients have been enrolled in this part of the RENOVATE trial. They have been subdivided in two different cohorts: a discovery cohort and a testing cohort.

Discovery cohort

This cohort was composed by a group of twenty healthy women, i.e. women with two consecutive mammograms resulting BIRADS-0 or 1 and another composed of nineteen patients with distinct types of malignant BC. Data regarding this cohort can be viewed in Tables 2 and 3.

Table 2	No-cancer (N = 20)	Cancer (N = 19)	p value
Age, median (IQR)	57 (48.75 – 66.25)	56 (49 – 65.5)	0.91
Menopausal status, n (%)	Pre: 6 (30%) Post: 14 (70%)	Pre: 6 (31.5%) Post: 13 (68.5%)	1
BMI, median (IQR)	23.4 (21.8 – 27.1)	23.28 (20.9 – 28.4)	0.85
Smoke, n (%)	Ever: 6 (30%) Currently: 0 (0%)	Ever: 10 (52.6 %) Currently: 4 (21%)	0.2 0.047

Table 2. General characteristics of the discovery cohort. IQR: interquartile range, i.e. ages of the 25th and 75th percentiles, N: total number of patients, n: number of patients with that specific characteristic, Pre: premenopausal status, Post: postmenopausal status.

Table 3	
Histotype, n (%)	NST: 17 (89.5%) Lobular: 1 (5.25%) ST: 1 (5.25%)
Molecular subtype, n (%)	Luminal A: 8 (42.1%) Luminal B HER2-: 6 (31.6 %) Luminal B HER2+: 4 (21%) Triple negative: 1 (5.2%)
Stage, n (%)	0: 1 (5.3%) I: 13 (68.4%) IIA: 5 (26.3%)

Table 3. Characteristics of patients in the discovery cohort bearing cancer. n: number, NST: no special type, ST: special type. The stage is based on the TNM staging system.

Testing cohort

This cohort was composed of twenty-nine patients who were biopsied after the screening phase and were diagnosed with benign neoplasms and of 16 patients with different types of malignant BC. Data regarding this cohort can be found in Tables 4 and 5.

Table 4	No-cancer (N = 29)	Cancer (N = 16)	p-value
Age, median (IQR)	58 (49 – 64)	63 (54.5 – 69)	0.067
Menopausal status, n (%)	Pre: 9 (31%) Post: 20 (69%)	Pre: 3 (18.8%) Post: 13 (81.2%)	0.49
BMI, median (IQR)	22.5 (20.8 – 25.7)	24.7 (21.7 – 26.4)	0.27
Smoke, n (%)	Ever: 13 (44.8%) Currently: 6 (20.7%)	Ever: 10 (62.5%) Currently: 3 (18.7%)	0.35 1

Table 4. General characteristics of the testing cohort. IQR: interquartile range, i.e. ages of the 25th and 75th percentiles, N: total number of patients, n: number of patients with that specific characteristic, Pre: premenopausal status, Post: postmenopausal status

Table 5	
Histotype, n (%)	NST: 12 (75%) Lobular: 2 (12.5%) ST: 2 (12.5%)
Molecular subtype, n (%)	Luminal A: 8 (50%) Luminal B HER2-: 4 (25 %) HER2+: 1 (6.25%) Triple negative: 3 (18.75%)
Stage, n (%)	0: 1 (6.25%) I: 13 (81.25%) IIA: 2 (12.5%)

Table 5. Characteristics of patients in the testing cohort bearing cancer. n: number, NST: no special type, ST: special type. The stage is based on the TNM staging system.

The Fisher test and the Student's *t*-test tests have been applied respectively to categorical and continuous variables to ensure that the differences in age, BMI, menopausal status, and smoking habits between groups of patients in the same batch were not statistically significative. Only Age in testing patients and Smoke currently in discovery patients showed a potential influence on statistical analyses, therefore both univariate and multivariate tests have been performed on the results of this study to assess statistical significance. In particular, there were no patients smoking at time of data collection in the healthy controls of the discovery cohort and the patients diagnosed with BC in the testing cohort were significantly older when compared to patients bearing benign lesions.

3.2 Hematopoietic prostaglandin D synthase (HPGDS) is significantly lower in BC patients compared to cancer-free controls

The SomaScan 7k platform was used to analyse the proteome of the two cohorts, a high-throughput assay based on aptamers that enables the simultaneous assessment of a total of 7596 aptamers per kit used, simultaneously and with a wide variety of plasmatic concentrations. To obtain the desired results, mouse proteins had to be filtered and internal calibrations had to be performed. This reduced the total number of proteins measured to 7057.

The analysis of the results obtained shows a notable difference in the variance of the circulating proteome between the BC cohort and the cancer-free controls.

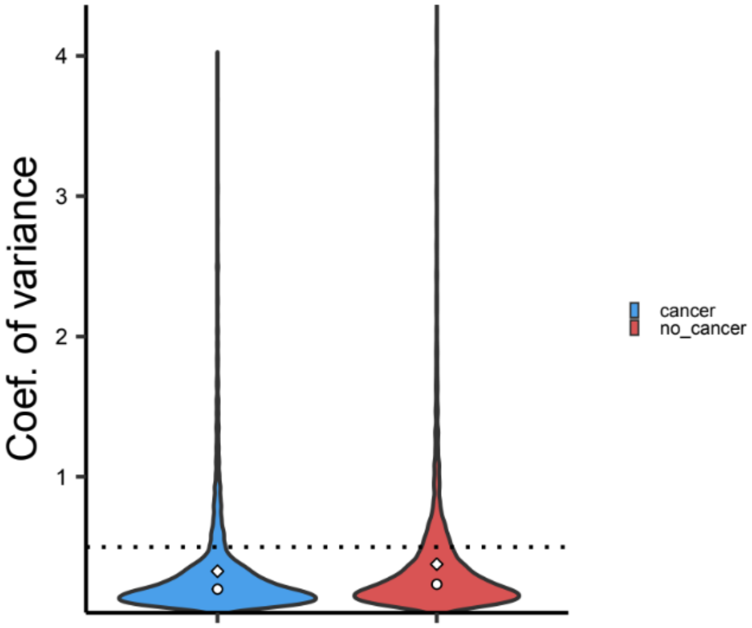

Figure 2. Violin plot comparing the variance in protein expression between the cancer and no_cancer group.

Figure 2 highlights the difference in protein expression between the two groups of patients and how its variance in women bearing cancer is lower when compared to healthy women ($p=2.2e^{-16}$). This suggests the presence of some kind of factor hypothetically stabilizing the variance of protein expression in cancerous cells.

Results in the discovery cohort

The results obtained in the discovery cohort allow to discriminate the different expression of each protein between healthy women and patients diagnosed with BC.

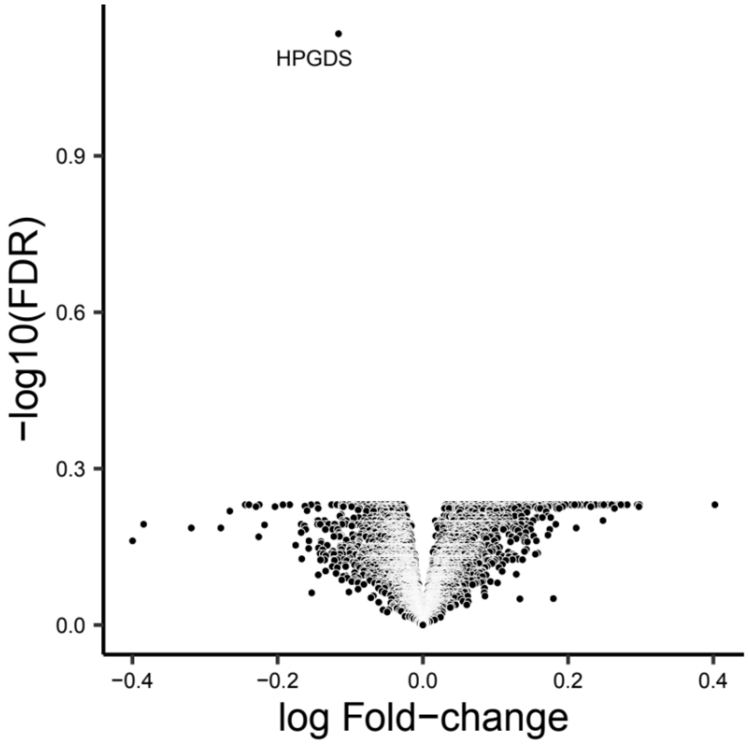

Figure 3. Volcano plot of expressed proteins obtained from the Somalogic® platform. On the y axis logarithm to base 2 of false discovery rate (FDR) plotted against the logarithm to base 2 of fold change in cancer (patients with BC) vs no_cancer (healthy women) on the x axis. HPGDS: hematopoietic prostaglandin D synthase.

Figure 3 depicts the distribution of the different proteins using fold change (FC), i.e. the ratio of the quantity of each protein between the two aforementioned groups. False discovery rate is used to express the significance of the results: it is used to prevent data from appearing as statistically significant even if they are not. This proves that the group of patients diagnosed with BC has a decreased amount of circulating hematopoietic prostaglandin D synthase (HPGDS), an enzyme which catalyses the conversion of prostaglandin H2 to prostaglandin D2 (PGD2) (57). The pathway linked to PGD2 is involved in various cancer and immunology related processes: it inhibits cancer cell growth, their proliferation, and their migrating ability by interacting with the tumoral micro-environment altering vascular permeability, tampering with important signalling pathways such as JAK/STAT3 and inhibiting mRNA expression of proteins such as NANOG and OCT4 (58).

Two different analyses have been performed to verify the statistical significance of this results: a univariate analysis (logFC=-0.116, adjusted p=0.073) and a multivariate analysis (adjusted p=0.088), which took into account age, menopausal status, BMI, and smoking habits differences between the two subjects' groups.

Results in the testing cohort

This result was then verified upon the testing cohort. The assays performed on this cluster of patients further confirmed the lower expression of HPGDS in individuals bearing cancer relative to women diagnosed with benign breast neoplasms.

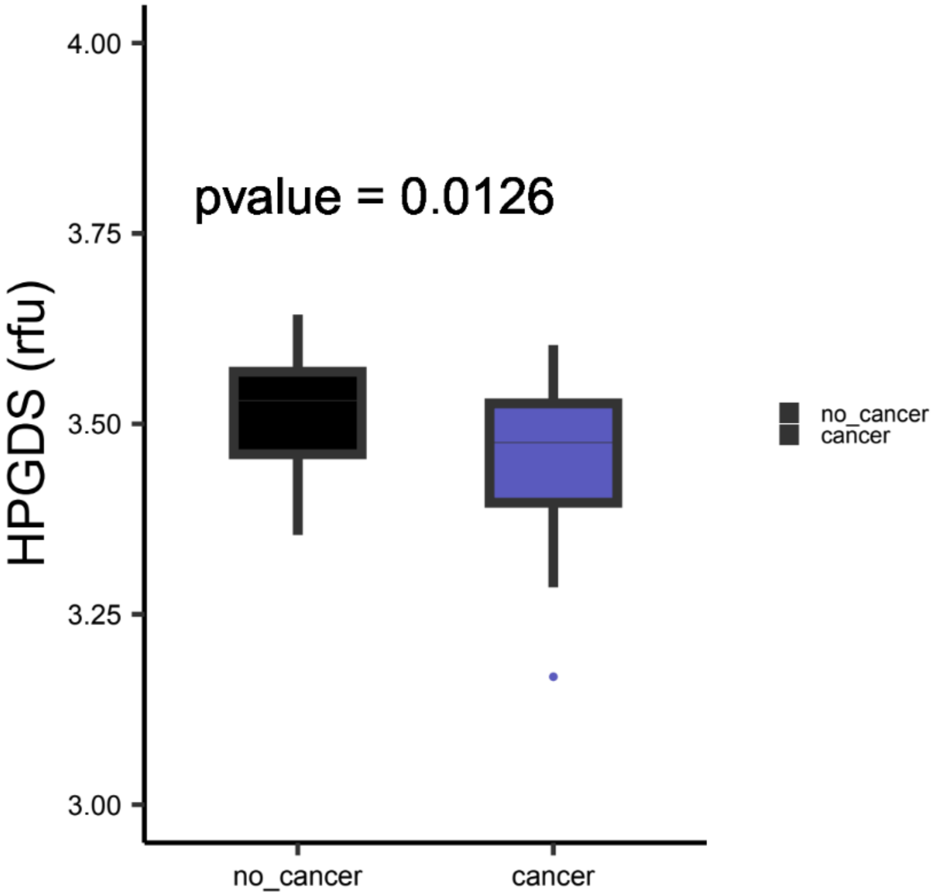

Figure 4. Box plot, on the y axis the quantity of HPGDS measured in relative fluorescent units (RFU), on the x axis the two groups of patients. The boxes contain values between the 25th and the 75th percentiles, the line inside the boxes represents the median value.

Figure 4 depicts more thoroughly the lower expression of HPGDS in the cancer group. The two analyses performed in the discovery cohort to verify the significance of the results have been performed on this cohort as well. Both the univariate (logFC=-0.101, p=0.0126) and the multivariate (p=0.04) tests confirmed how the difference in expression is statistically significant.

Reliability assessment

To assess the reliability of the Somalogic® platform, a cross-validation between the resulted HPGDS abundancy obtained with it and with the Olink® Target 96 Oncology Panel® has been performed. This validation was executed on data obtained from eighty of the eighty-four patients involved in the study.

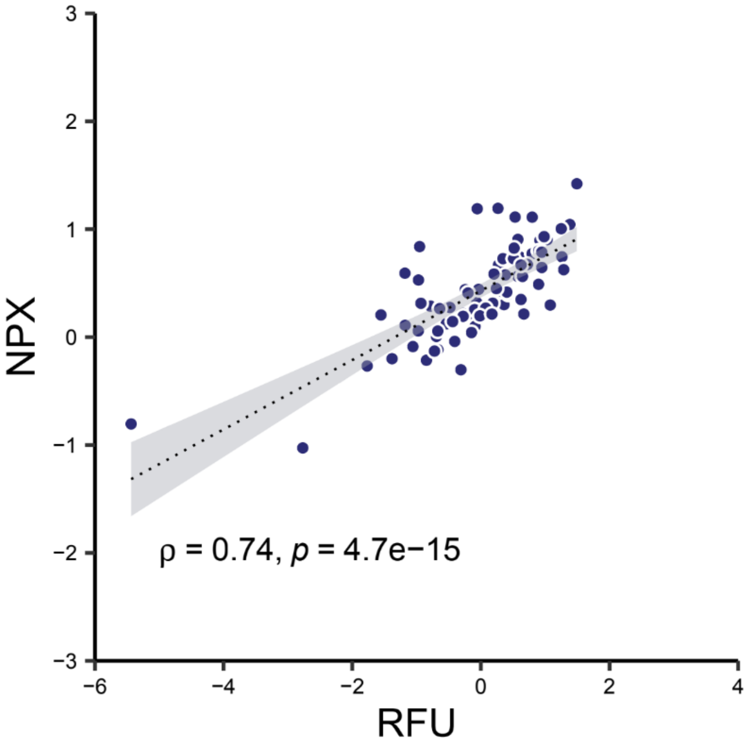

Figure 5. Scatter plot showing the correlation between NPX values obtained with the Olink® platform on the y axis and the RFU values obtained with the Somalogic® platform on the x axis via a linear regression function. ρ: coefficient of correlation.

Figure 5 demonstrates the statistically significant similarity between the results that the two yield. As the ρ is >0, the correlation between the results is direct. The *p* is calculated utilizing the Spearman correlation.

The HPGDS quantity assessment performed with the Olink® panel showed statistical significance both in the univariate (p=0.0032) and in the multivariate (p=0,0024) analyses.

4. DISCUSSION

Researching literature available on PubMed, it is clear that various teams have been focusing their studies on different and novel omics technologies to identify which diagnostic biomarkers for BC might be assessed via liquid biopsy.

Olmedo C. G. et al. have discovered new biomarkers to be detected in liquid biopsies via untargeted hydrophilic interaction liquid chromatography−mass spectrometry analysis (HILIC-HRMS). More specifically, this group has been focusing on assessing this strategy as a new mean to be used in further studies to dissect metabolomics and identify biomarkers useful in BC early diagnosis (59).

Another technique assessed involves detecting integrin α6β4 deriving from extracellular vesicles via silver-coated gold nanorods SERS probes based on DNA aptamers. This has been found useful to detect BC progression with high sensitivity and specificity in mice models (60).

Among others, transcriptomics and epigenomics have been evaluated and have been proven to be rich sources of biomarkers with vast potentialities when coupled with liquid biopsy. When considering transcriptomics, single cell DNA/RNA sequencing could be utilized to identify biomarkers for BC, such as polyadenylation, a potential predictive biomarker for early BC. On the other hand, it has permitted the development of tests to diagnose early stage BC, such as the MammaPrint test, based on microarrays used to quantify 70 genes involved in carcinogenesis. Epigenomics can provide insight on innovative biomarkers too: the methylation of genes is considered an indicator of cancer presence. The potential of several genes' methylation status in early BC detection has been unveiled by different studies (61).

Although present, research on plasmatic proteomic biomarkers able to detect early-stage BC is limited. Thus, this study's aim is to provide the groundwork to stimulate the growth of this specific field, focusing on this issue. Thanks to the powerful proteomic platform utilized, we have been able to unveil a relation between the presence of BC and the expression of a specific enzyme, HPGDS.

More precisely, in patients who have been diagnosed with BC via biopsy, HPGDS is significantly less abundant when compared to both healthy women and females with benign neoplasms. HPDGS is one isoform of an enzyme (the other being the brain isoform, found in the nervous system, in the epididymis and in the heart) catalysing the conversion of prostaglandin H2 to PGD2, a well-studied regulatory factor for many physiological and para-physiological functions such as sleep cycles, platelet aggregation, inflammatory response, contraction of smooth muscles and constriction of bronchi. It also bears a role in cancer development as a lower concentration of this PG has been associated to more metastatic foci of melanoma whereas higher levels inhibit cultural leukemic cells growth. Moreover, mice with heterozygous mutations in the adenomatous polyposis coli gene (Apc) and HPGDS deficiency have been observed to develop 50% more small bowel adenomas and 200% more colonic adenomas when compared to non-deficient ones (62). The results of our study further foster the hypothesis of an involvement of HPGDS and PGD2 in carcinogenesis, even in early stages.

Due to a narrow sample size and to the utilization of meso-scale proteomic platforms, the results of this study cannot yet be utilized in clinical practice. To this end, a larger number of assessed patients would be needed to confirm the reliability of the discovered data. In addition, more research is needed to uncover whether HPGDS can effectively be a viable biomarker to be used in BC screening in clinical practice, to develop a usable test to assess its levels in patients, and to identify its possible link to specific subtypes of BC. Furthermore, considering its plausible involvement in the physiopathology of BC onset, additional studies should be conducted to investigate its precise role in the intricate environment of carcinogenesis.

5. REFERENCES

1. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global Cancer Statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *Ca*, *71*(3), 209–249. https://doi.org/10.3322/caac.21660
2. https://www.aiom.it/tumori-nel-2023-in-italia
3. https://www.aiom.it/wp-content/uploads/2021/11/2021_LG_AIOM_Neoplasie_Mammella_11112021.pdf.pdf
4. https://tumourclassification.iarc.who.int
5. Smolarz, B., Nowak, A. Z., & Romanowicz, H. (2022). Breast Cancer—Epidemiology, Classification, Pathogenesis and Treatment (Review of Literature). *Cancers*, *14*(10), 2569. https://doi.org/10.3390/cancers14102569
6. Tsang, J. Y., & Tse, G. M. (2019). Molecular Classification of breast Cancer. *Advances in Anatomic Pathology*, *27*(1), 27–35. https://doi.org/10.1097/pap.0000000000000232
7. Kay, C., Martínez-Pérez, C., Meehan, J., Gray, M., Webber, V., Dixon, J. M., & Turnbull, A. K. (2021). Current trends in the treatment of HR+/HER2+ breast cancer. *Future Oncology*, *17*(13), 1665–1681. https://doi.org/10.2217/fon-2020-0504
8. Lu, B., Natarajan, E., Raghavendran, H. R. B., & Markandan, U. D. (2023). Molecular Classification, Treatment, and Genetic Biomarkers in Triple-Negative Breast Cancer: a review. *Technology in Cancer Research & Treatment*, *22*, 153303382211452. https://doi.org/10.1177/15330338221145246
9. Cserni, G., Chmielik, E., Cserni, B., & Tot, T. (2018). The new TNM-based staging of breast cancer. *Virchows Archiv*, *472*(5), 697–703. https://doi.org/10.1007/s00428-018-2301-9

10. Teichgraeber, D. C., Guirguis, M. S., & Whitman, G. J. (2021). Breast cancer staging: Updates in the AJCC Cancer Staging Manual, 8th edition, and Current Challenges for radiologists, from the AJR Special Series on Cancer Staging. *American Journal of Roentgenology*, *217*(2), 278–290. https://doi.org/10.2214/ajr.20.25223

11. Moss, S. M., Wale, C., Smith, R., Evans, A., Cuckle, H., & Duffy, S. W. (2015). Effect of mammographic screening from age 40 years on breast cancer mortality in the UK Age trial at 17 years' follow-up: a randomised controlled trial. *Lancet Oncology/Lancet. Oncology*, *16*(9), 1123–1132. https://doi.org/10.1016/s1470-2045(15)00128-x

12. Ren, W., Chen, M., Qiao, Y., & Zhao, F. (2022). Global guidelines for breast cancer screening: A systematic review. *Breast*, *64*, 85–99. https://doi.org/10.1016/j.breast.2022.04.003

13. Gartlehner, G., Thaler, K., Chapman, A., Kaminski-Hartenthaler, A., Berzaczy, D., Van Noord, M. G., & Helbich, T. H. (2013). Mammography in combination with breast ultrasonography versus mammography for breast cancer screening in women at average risk. *Cochrane Library*. https://doi.org/10.1002/14651858.cd009632.pub2

14. Seely, J., & Alhassan, T. (2018). Screening for Breast Cancer in 2018—What Should We be Doing Today? *Current Oncology*, *25*(11), 115–124. https://doi.org/10.3747/co.25.3770

15. https://cancer-screening-and-care.jrc.ec.europa.eu/en/ecibc/european-breast-cancer-guidelines.eu

16. Pisano, E. D., Gatsonis, C., Hendrick, E., Yaffe, M., Baum, J. K., Acharyya, S., Conant, E. F., Fajardo, L. L., Bassett, L., D'Orsi, C., Jong, R., & Rebner, M. (2005). Diagnostic Performance of Digital versus Film Mammography for Breast-Cancer Screening. *New England Journal of Medicine/˜ the œNew England Journal of Medicine*, *353*(17), 1773–1783. https://doi.org/10.1056/nejmoa052911

17. Svahn, T., Andersson, I., Chakraborty, D., Svensson, S., Ikeda, D., Fornvik, D., Mattsson, S., Tingberg, A., & Zackrisson, S. (2010). The diagnostic accuracy of dual-view digital

mammography, single-view breast tomosynthesis and a dual-view combination of breast tomosynthesis and digital mammography in a free-response observer performance study. *Radiation Protection Dosimetry, 139*(1–3), 113–117. https://doi.org/10.1093/rpd/ncq044

18. Guo, R., Lu, G., Qin, B., & Fei, B. (2018). Ultrasound Imaging Technologies for Breast Cancer Detection and Management: A review. *Ultrasound in Medicine & Biology*, *44*(1), 37–70. https://doi.org/10.1016/j.ultrasmedbio.2017.09.012

19. Mann, R. M., Cho, N., & Moy, L. (2019). Breast MRI: state of the art. *Radiology*, *292*(3), 520–536. https://doi.org/10.1148/radiol.2019182947

20. Liberman, L., & Menell, J. H. (2002). Breast imaging reporting and data system (BI-RADS). *Radiologic Clinics of North America/˜ the œRadiologic Clinics of North America*, *40*(3), 409–430. https://doi.org/10.1016/s0033-8389(01)00017-3

21. Nakano, S., Imawari, Y., Mibu, A., Otsuka, M., & Oinuma, T. (2018). Differentiating vacuum-assisted breast biopsy from core needle biopsy: Is it necessary? ˜ *the œBritish Journal of Radiology/British Journal of Radiology*, *91*(1092), 20180250. https://doi.org/10.1259/bjr.20180250

22. De Oliveira Pereira, R., Da Luz, L. A., Chagas, D. C., Amorim, J. R., De Jesus Nery-Júnior, E., Alves, A. C. B. R., De Abreu-Neto, F. T., Da Conceição Barros Oliveira, M., Silva, D. R. C., Soares-Júnior, J. M., & Da Silva, B. B. (2020). Evaluation of the accuracy of mammography, ultrasound and magnetic resonance imaging in suspect breast lesions. *Clinics*, *75*, e1805. https://doi.org/10.6061/clinics/2020/e1805

23. Pötsch, N., Vatteroni, G., Clauser, P., Helbich, T. H., & Baltzer, P. a. T. (2022). Contrast-enhanced Mammography versus Contrast-enhanced Breast MRI: A Systematic Review and Meta-Analysis. *Radiology*, *305*(1), 94–103. https://doi.org/10.1148/radiol.212530

24. Yang, L., Wang, S., Zhang, L., Sheng, C., Song, F., Wang, P., & Huang, Y. (2020). Performance of ultrasonography screening for breast cancer: a systematic review and meta-analysis. *BMC Cancer*, *20*(1). https://doi.org/10.1186/s12885-020-06992-1

25. Park, V. Y., Kim, E., Moon, H. J., Yoon, J. H., & Kim, M. J. (2018). Evaluating imaging-pathology concordance and discordance after ultrasound-guided breast biopsy. *Ultrasonography, 37*(2), 107–120. https://doi.org/10.14366/usg.17049

26. Pansa, E., Guzzardi, G., Santocono, S., & Carriero, A. (2023). Vascular Complications following Vacuum-Assisted Breast Biopsy (VABB): A Case Report and Review of the Literature. *Tomography*, *9*(4), 1246–1253. https://doi.org/10.3390/tomography9040099

27. Chaltiel, D., & Hill, C. (2021). Estimations of overdiagnosis in breast cancer screening vary between 0% and over 50%: why? *BMJ Open*, *11*(6), e046353. https://doi.org/10.1136/bmjopen-2020-046353

28. Marmot, M., Altman, D., Cameron, D., Dewar, J., Thompson, S., & Wilcox, M. (2012). The benefits and harms of breast cancer screening: an independent review. *Lancet, 380*(9855), 1778–1786. https://doi.org/10.1016/s0140-6736(12)61611-0

29. Allott, E. H., Geradts, J., Sun, X., Cohen, S. M., Zirpoli, G. R., Khoury, T., Bshara, W., Chen, M., Sherman, M. E., Palmer, J. R., Ambrosone, C. B., Olshan, A. F., & Troester, M. A. (2016). Intratumoral heterogeneity as a source of discordance in breast cancer biomarker classification. *Breast Cancer Research*, *18*(1). https://doi.org/10.1186/s13058-016-0725-1

30. Dagogo-Jack, I., & Shaw, A. T. (2017). Tumour heterogeneity and resistance to cancer therapies. *Nature Reviews. Clinical Oncology, 15*(2), 81–94. https://doi.org/10.1038/nrclinonc.2017.166

31. Thierry, A., Messaoudi, S. E., Mollevi, C., Raoul, J. L., Guimbaud, R., Pezet, D., Artru, P., Assenat, E., Borg, C., Mathonnet, M., De La Fouchardière, C., Bouche, O., Gavoille, C., Fiess, C., Auzemery, B., Meddeb, R., Lopez-Crapez, E., Pastor, B., Ychou, M., & Sanchez, C. (2017). Clinical utility of circulating DNA analysis for rapid detection of actionable mutations to select metastatic colorectal patients for anti-EGFR treatment. *Annals of Oncology*, *28*(9), 2149–2159. https://doi.org/10.1093/annonc/mdx330

32. Nikanjam, M., Kato, S., & Kurzrock, R. (2022). Liquid biopsy: current technology and clinical applications. *Journal of Hematology & Oncology*, *15*(1). https://doi.org/10.1186/s13045-022-01351-y

33. Siravegna, G., Marsoni, S., Siena, S., & Bardelli, A. (2017). Integrating liquid biopsies into the management of cancer. *Nature Reviews. Clinical Oncology*, *14*(9), 531–548. https://doi.org/10.1038/nrclinonc.2017.14

34. Millner, L. M., Linder, M. W., & Valdes, R., Jr (2013). Circulating tumor cells: a review of present methods and the need to identify heterogeneous phenotypes. *Annals of clinical and laboratory science*, *43*(3), 295–304. 26525104 RNA-Seq of Tumor-Educated Platelets Enables Blood-Based Pan-Cancer, Multiclass, and Molecular Pathway Cancer Diagnostics

35. Best, M. G., Sol, N., Kooi, I., Tannous, J., Westerman, B. A., Rustenburg, F., Schellen, P., Verschueren, H., Post, E., Koster, J., Ylstra, B., Ameziane, N., Dorsman, J., Smit, E. F., Verheul, H. M., Noske, D. P., Reijneveld, J. C., Nilsson, R. J. A., Tannous, B. A., . . . Wurdinger, T. (2015). RNA-SeQ of Tumor-Educated platelets enables Blood-Based Pan-Cancer, multiclass, and molecular pathway cancer diagnostics. *Cancer Cell*, *28*(5), 666–676. https://doi.org/10.1016/j.ccell.2015.09.018

36. Crowley, E., Di Nicolantonio, F., Loupakis, F., & Bardelli, A. (2013). Liquid biopsy: monitoring cancer-genetics in the blood. *Nature Reviews. Clinical Oncology*, *10*(8), 472–484. https://doi.org/10.1038/nrclinonc.2013.110

37. Szilágyi, M., Pös, O., Márton, É., Buglyó, G., Soltész, B., Keserű, J., Penyige, A., Szemes, T., & Nagy, B. (2020). Circulating Cell-Free Nucleic Acids: Main characteristics and clinical Application. *International Journal of Molecular Sciences*, *21*(18), 6827. https://doi.org/10.3390/ijms21186827

38. Pös, Z., Pös, O., Styk, J., Mocova, A., Strieskova, L., Budis, J., Kadasi, L., Radvanszky, J., & Szemes, T. (2020). Technical and methodological aspects of Cell-Free nucleic Acids

analyzes. *International Journal of Molecular Sciences*, *21*(22), 8634. https://doi.org/10.3390/ijms21228634

39. Poulet, G., Massias, J., & Taly, V. (2019). Liquid Biopsy: General Concepts. *Acta Cytologica*, *63*(6), 449–455. https://doi.org/10.1159/000499337

40. Caputo, V., Ciardiello, F., Della Corte, C. M., Martini, G., Troiani, T., & Napolitano, S. (2023). Diagnostic value of liquid biopsy in the era of precision medicine: 10 years of clinical evidence in cancer. *Exploration of Targeted Anti-tumor Therapy*, 102–138. https://doi.org/10.37349/etat.2023.00125

41. De Mattos-Arruda, L., Weigelt, B., Cortes, J., Won, H., Ng, C., Nuciforo, P., Bidard, F., Aura, C., Saura, C., Peg, V., Piscuoglio, S., Oliveira, M., Smolders, Y., Patel, P., Norton, L., Tabernero, J., Berger, M., Seoane, J., & Reis-Filho, J. (2014). Capturing intra-tumor genetic heterogeneity by de novo mutation profiling of circulating cell-free tumor DNA: a proof-of-principle. *Annals of Oncology*, *25*(9), 1729–1735. https://doi.org/10.1093/annonc/mdu239

42. Murtaza, M., Dawson, S., Pogrebniak, K., Rueda, O. M., Provenzano, E., Grant, J., Chin, S., Tsui, D. W. Y., Marass, F., Gale, D., Ali, H. R., Shah, P., Contente-Cuomo, T., Farahani, H., Shumansky, K., Kingsbury, Z., Humphray, S., Bentley, D., Shah, S. P., . . . Caldas, C. (2015). Multifocal clonal evolution characterized using circulating tumour DNA in a case of metastatic breast cancer. *Nature Communications*, *6*(1). https://doi.org/10.1038/ncomms9760

43. Chim, S. S. C., Tong, Y. K., Chiu, R. W. K., Lau, T. K., Leung, T. N., Chan, L. Y. S., Oudejans, C. B. M., Ding, C., & Lo, Y. M. D. (2005). Detection of the placental epigenetic signature of the maspin gene in maternal plasma. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(41), 14753–14758. https://doi.org/10.1073/pnas.0503335102

44. Bronkhorst, A. J., Ungerer, V., Diehl, F., Anker, P., Dor, Y., Fleischhacker, M., Gahan, P. B., Hui, L., Holdenrieder, S., & Thierry, A. R. (2020). Towards systematic nomenclature for cell-free DNA. *Human Genetics*, *140*(4), 565–578. https://doi.org/10.1007/s00439-020-02227-2

45. Li, Y., Chen, X., & Lu, C. (2021). The interplay between DNA and histone methylation: molecular mechanisms and disease implications. *EMBO Reports*, *22*(5). https://doi.org/10.15252/embr.202051803

46. Cirmena, G., Dameri, M., Ravera, F., Fregatti, P., Ballestrero, A., & Zoppoli, G. (2021). Assessment of circulating nucleic acids in cancer: from current status to future perspectives and potential clinical applications. *Cancers*, *13*(14), 3460. https://doi.org/10.3390/cancers13143460

47. Tan, H. T., Lee, Y. H., & Chung, M. C. (2012). Cancer proteomics. *Mass Spectrometry Reviews*, *31*(5), 583–605. https://doi.org/10.1002/mas.20356

48. https://www.cancer.gov/about-cancer/diagnosis-staging/diagnosis/tumor-markers-list

49. Casagrande, G. M. S., De Oliveira Silva, M., Reis, R. M., & Leal, L. F. (2023). Liquid Biopsy for Lung Cancer: Up-to-Date and Perspectives for Screening Programs. *International Journal of Molecular Sciences*, *24*(3), 2505. https://doi.org/10.3390/ijms24032505

50. Lapitz, A., Azkargorta, M., Milkiewicz, P., Olaizola, P., Zhuravleva, E., Grimsrud, M. M., Schramm, C., Arbelaiz, A., O'Rourke, C. J., La Casta, A., Milkiewicz, M., Pastor, T., Vesterhus, M., Jimenez-Agüero, R., Dill, M. T., Lamarca, A., Valle, J. W., Macias, R. I., Izquierdo-Sanchez, L., . . . Banales, J. M. (2023). Liquid biopsy-based protein biomarkers for risk prediction, early diagnosis, and prognostication of cholangiocarcinoma. *Journal of Hepatology*, *79*(1), 93–108. https://doi.org/10.1016/j.jhep.2023.02.027

51. Markou, A., Tzanikou, E., & Lianidou, E. (2022). The potential of liquid biopsy in the management of cancer patients. *Seminars in Cancer Biology*, *84*, 69–79. https://doi.org/10.1016/j.semcancer.2022.03.013

52. Kilgour, E., Rothwell, D. G., Brady, G., & Dive, C. (2020). Liquid Biopsy-Based Biomarkers of Treatment response and Resistance. *Cancer Cell*, *37*(4), 485–495. https://doi.org/10.1016/j.ccell.2020.03.012

53. Pessoa, L. S., Heringer, M., & Ferrer, V. P. (2020). ctDNA as a cancer biomarker: A broad overview. *Critical Reviews in Oncology/Hematology*, *155*, 103109. https://doi.org/10.1016/j.critrevonc.2020.103109

54. Tie, J., Cohen, J. D., Lahouel, K., Lo, S. N., Wang, Y., Kosmider, S., Wong, R., Shapiro, J., Lee, M., Harris, S., Khattak, A., Burge, M., Harris, M., Lynam, J., Nott, L., Day, F., Hayes, T., McLachlan, S., Lee, B., . . . Gibbs, P. (2022). Circulating Tumor DNA analysis guiding adjuvant therapy in stage II colon cancer. *The New England Journal of Medicine*, *386*(24), 2261–2272. https://doi.org/10.1056/nejmoa2200075

55. Duffy, M. J. (2012). Tumor markers in Clinical practice: A review focusing on common solid cancers. *Medical Principles and Practice*, *22*(1), 4–11. https://doi.org/10.1159/000338393

56. Qiu, J., Xu, J., Zhang, K., Gu, W., Nie, L., Wang, G., & Luo, Y. (2020). Refining cancer management using Integrated liquid biopsy. *Theranostics*, *10*(5), 2374–2384. https://doi.org/10.7150/thno.40677

57. https://www.uniprot.org/uniprotkb/O60760/entry

58. Tian, H., Ge, K., Wang, L., Gao, P., Chen, A., Wang, F., Guo, F., Wang, F., & Zhang, Q. (2024). Advances in PGD2/PTGDR2 signaling pathway in tumors. *Biomolecules & Biomedicine*. https://doi.org/10.17305/bb.2024.10485

59. Olmedo, C. G., Beltrán, L. D., García, V. M., Ferrer, J. L. P., Jiménez, A. C., Cubero, R. U., Del Palacio, J. P., Díaz, C., Vicente, F., & Rovira, P. S. (2024b). Assessment of untargeted

metabolomics by hydrophilic interaction liquid Chromatography−Mass spectrometry to define breast cancer liquid Biopsy-Based biomarkers in plasma samples. *International Journal of Molecular Sciences*, *25*(10), 5098. https://doi.org/10.3390/ijms25105098

60. Lei, H., Wang, H., Wang, X., Xiao, Z., Tian, T., & Cui, K. (2024). Surface-enhanced Raman scattering-based identification of breast cancer progression using extracellular vesicles-derived integrin α6β4. *Talanta*, *275*, 126092. https://doi.org/10.1016/j.talanta.2024.126092

61. Orsini, A., Diquigiovanni, C., & Bonora, E. (2023). Omics Technologies Improving Breast Cancer Research and Diagnostics. *International Journal of Molecular Sciences*, *24*(16), 12690. https://doi.org/10.3390/ijms241612690

62. Park, J. M., Kanaoka, Y., Eguchi, N., Aritake, K., Grujic, S., Materi, A. M., Buslon, V. S., Tippin, B. L., Kwong, A. M., Salido, E., French, S. W., Urade, Y., & Lin, H. J. (2007). Hematopoietic prostaglandin D Synthase Suppresses intestinal adenomas INAPCMin/+ mice. *Cancer Research*, *67*(3), 881–889. https://doi.org/10.1158/0008-5472.can-05-3767