BMJ Open

Development of a hoRizontal data intEgration classifier for NOninvasiVe early diAgnosis of breasT cancEr: the RENOVATE study protocol

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2021-054256
Article Type:	Protocol
Date Submitted by the Author:	07-Jun-2021
Complete List of Authors:	Ravera, Francesco; Università degli Studi di Genova, Department of internal Medicine Cirmena, Gabriella; Università degli Studi di Genova, Department of internal Medicine Dameri, Martina; Ospedale Policlinico San Martino Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia Gallo, Maurizio; Università degli Studi di Genova, Department of internal Medicine Vellone, Valerio; Università degli Studi di Genova, Department of Surgical Sciences and Integrated Diagnostic Fregatti, Piero; Ospedale Policlinico San Martino Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia Friedman, Daniele; Ospedale Policlinico San Martino Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia Calabrese, Massimo; Ospedale Policlinico San Martino Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia Ballestrero, Alberto; University of Genoa, Department of Internal Medicine Tagliafico, Alberto; Università degli Studi di Genova, Department of Health Sciences Ferrando, Lorenzo; Ospedale Policlinico San Martino Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia Zoppoli, Gabriele; Università degli Studi di Genova, Department of internal Medicine
Keywords:	Breast tumours < ONCOLOGY, Breast imaging < RADIOLOGY & IMAGING, Health informatics < BIOTECHNOLOGY & BIOINFORMATICS, Cancer genetics < GENETICS, Adult oncology < ONCOLOGY

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Development of a hoRizontal data intEgration classifier for NOninvasiVe early diAgnosis of breasT cancEr: the RENOVATE study protocol

Authors: Francesco Ravera[1], Gabriella Cirmena[1], Martina Dameri[2], Maurizio Gallo[2], Valerio Gaetano Vellone[2,4], Piero Fregatti[2,4], Daniele Friedman[2,4], Massimo Calabrese[2], Alberto Ballestrero[1,2] Alberto Tagliafico[2,3,*], Lorenzo Ferrando[2,*], Gabriele Zoppoli[1,2*,#]

[1]Department of Internal Medicine and Medical Specialties DiMI, University of Genoa, 16132, Italy

[2] IRCCS Ospedale Policlinico San Martino, 16132 Genoa, Italy

[3]Department of Health Sciences DISSAL, University of Genoa, 16132, Italy

[4]Department of Surgical Sciences and Integrated Diagnostic DISC, 16132 Genoa, Italy

*These Authors equally contributed to the present manuscript

[#] Correspondence should be addressed to Gabriele Zoppoli: gabriele.zoppoli@unige.it

Keywords: breast cancer, early diagnosis, liquid biopsy, circulating biomarkers, radiomics

Word count: 4459

Abstract

Introduction. Current standards of care for breast cancer (BC) early diagnosis present suboptimal accuracy and imply the execution of invasive and sometimes unnecessary tissue biopsies. The assessment of circulating biomarkers for diagnostic purposes, together with radiomics, is of great potential in BC management.

Methods. This is a prospective translational study investigating the accuracy of the combined assessment of multiple circulating analytes together with radiomic variables for early BC diagnosis. Patients are recruited at their presentation at the Diagnostic Senology Unit of Ospedale Policlinico San Martino (Genoa, IT) for the execution of a diagnostic biopsy after the detection of a suspect breast lesion (t0). Each recruited patient is asked to donate peripheral blood and urine before undergoing biopsy. Blood and urine samples are also collected from a cohort of patients with negative mammography. Only for cases with histological diagnosis of invasive BC, a second sample of blood and urine is collected at the first visit after breast surgery. Circulating tumor DNA, cell-free methylated DNA, RNA from peripheral blood mononuclear cells and circulating proteins will be assessed in samples collected at t0 from patients with stage I-IIA BC at surgery together with those collected from patients with histologically confirmed benign lesions of similar size and from healthy controls with negative mammography. These analyses will be combined with radiomics variables extracted with freeware algorithms applied to cases and matched controls for which digital mammography is available. The overall goal of the present study is to develop a horizontal data integration classifier for the early diagnosis of BC.

Ethics and dissemination. This research protocol has been approved by Regione Liguria Ethics Committee (reference number: 2019/75, study ID: 4452), and is registered in ClinicalTrials.gov with the ID NCT04781062. Results will be published in international peer-reviewed scientific journals.

Strengths and limitations

Strengths

- The study has a prospective design with well-balanced controlled cohorts
- The study assesses the performance of some of the most promising and cutting-edge biomarkers in the field of translational oncology either for diagnostic or predictive purposes in patients affected by BC
- The performance of the combination of multiple circulating biomarkers and radiomics algorithms for BC early diagnosis is assessed
- The study is the first to investigate such biomarkers in early (i.e. stage I-IIA) BC

Limitations

- The study is not designed for the early diagnosis of *in situ* BCs, which are considered as benign lesions and are included in the control group

1.Introduction

Standard of care early diagnosis of breast cancer (BC) relies on the combined use of radiological procedures such as mammography and ultrasound [1,2]. Confirmation biopsy or recall tests are mandatory in case of suspect found during the first examination and bring eventually to a more definite characterization of the radiologically identified lesion. This approach is however burdened by serious issues which include (a) suboptimal sensitivity and positive predictive power respectively for radiological screening and diagnostic approaches, (b) invasiveness of biopsy with discomfort for women undergoing diagnostic tests, along with the risk of drawing non-representative portions of the suspected region considering the genotypic and phenotypic heterogeneity of BCs [3], (c) long turnaround time for recall tests, even in high-level centers. In particular, the suboptimal sensitivity of screening procedures leads to non-diagnosed tumors which become able to advance locally and spread systemically, impacting patients' prognosis, while the suboptimal positive predictive power of diagnostic evaluation implies unnecessary invasive biopsies [1].

The compelling necessity of increasing the accuracy of screening and diagnostic procedures for cancer has brought to a relevant advancement in the pursuit of accurate biomarkers able to efficiently detect and characterize it, in the panorama of liquid biopsies. To date, however, no protocol with effective and recognized clinical validity for the early diagnosis of BC has been developed.

Amongst the most promising biomarkers, circulating tumor DNA (ctDNA) and cell-free methylated DNA (cfMeDNA), exosomes, and microRNA (miRNA) have been the subject of relevant scientific reports [4–9]. Moreover, machine-learning (so called “deep learning”) algorithms have been applied to traditional radiology imaging techniques for diagnosis assistance with exciting results in the field of radiomics [10]. Results in the proteomics field have instead been few, in part due to the cumbersome methodologies often applied to the study of these molecules [11].

Since it is hardly conceivable that a single biomarker is able to achieve 100% accuracy in the early detection of BC, the primary aim of the present study is to merge for this purpose the assessment of multiple biological analytes with the refinement of radiomics algorithms, overcoming the aforementioned limitations in terms of accuracy of the individual biomarkers. The concept of combining different data layers to reach a better classifier compared to the individual analytes is referred to as horizontal data integration (HDI) classification. Therefore, the overall goal of the project is to develop an HDI classifier enabling early noninvasive diagnosis of BC with similar accuracy compared to breast biopsies.

2. Methods

2.1. Study design

This is a prospective translational case-control study with the primary aim of assessing the clinical validity of several biomarkers, individually and combined, in the early detection of BC in a real-life clinical setting (see Figure 1 for study diagram). Patients are recruited at the presentation to the Diagnostic Senology Unit of Ospedale Policlinico San Martino for the execution of the breast biopsy after the radiological detection of a suspected breast lesion ≤ 2

cm (i.e. radiological T1) with no radiological evidence of axillary or distant disease. Blood and urine samples are collected at this time point (t0) from each recruited patient who fulfills the inclusion and exclusion criteria mentioned in paragraph 2.3. Of these, only samples collected from patients with stage I-IIA neoplasia at surgery (T1N0 or T2N0 or T1N1a) or histologically confirmed benign lesions will be analysed. A second sample is obtained from those patients with confirmed histological diagnosis of invasive BC at the first oncologic visit after breast primary surgery (t1) according to normal practice, independently from possible neoadjuvant treatments. No limitations based on tumor stage will be made for the analysis of the latter. Blood and urine samples are also collected and analysed from a cohort of 100 healthy women with two consecutive negative mammograms (BIRADS-1 or BIRADS-0 with negative ecography) as well.

2.2. Candidate biomarkers

2.2.1. Circulating tumor DNA and cell-free Methylated DNA

ctDNA obtained via liquid biopsy has shown serious potential not only in the early diagnosis of cancer but *inter alia* as an effective marker for its recurrence, longitudinal monitoring and response to therapy [12]. Current methods for detecting ctDNA however are mostly based on sequencing somatic mutations from cell-free DNA (cfDNA), a process constrained by relevant limitations in clinical applicability due to (a) expectable low sensitivity in early stage cancers given the limited number of recurrent mutations in ctDNA [8,13], (b) the vast heterogeneity of DNA mutations occurring in a single tumor together with nonspecific mutations profiles along different patients and cancer types [12,13], (c) current cost-prohibitive impact of cfDNA Next Generation Sequencing (NGS) for mutation assessment [14]. On the other hand, the assessment of cfMeDNA may overcome the limitations outlined above. DNA methylation is commonly involved in cellular development, tissue-specific gene expression, and regulation of imprinted alleles, with widespread effects on cellular growth and genomic stability [15]. Changes in methylomes in cancer are usually associated with alterations of the transcriptional outcome and genomic instability, priming or enhancing carcinogenesis. Given the impact of

methylation changes on cellular equilibrium, different methylomes may be associated with specific biological features providing useful information for the early diagnosis of cancer [16]. Shen *et al.* recently developed a sensitive, immunoprecipitation-based protocol to analyze the methylomes of small quantities of cfDNA, providing an efficient method to detect large-scale DNA methylation changes that are enriched for tumour-specific patterns [8]. cfMeDNA immunoprecipitation and high-throughput sequencing (cfMeDIP–seq) was able to find cancer-derived DNA methylation events in cfDNA and to distinguish multiple cancer types from healthy controls, besides effectively discriminating different methylation patterns across diverse cancers.

2.2.2. RNA-based biomarkers

RNA-based biomarkers can refer either to coding or non-coding RNAs. Non-coding RNAs, including miRNAs and long non coding RNAs, have been extensively studied over the last years as promising markers for cancer early diagnosis and monitoring, being of particular interest for their stability and quantity in the bloodstream [17]. On the other hand, coding-RNAs include the cell-free messenger RNA, whose evaluation may allow the non-invasive assessment of the whole-body transcriptome [18] with relevant implications for several clinical purposes.

2.2.3. Circulating proteins

Proteomic analyses in neoplastic patients have been applied to diverse clinical aims with suboptimal results, given the challenging characterization of circulating proteins. The assessment of alterations in protein-mediated signaling networks is of great interest for its possible implications in the early diagnosis and monitoring of cancer, besides the detection of potentially actionable targets for therapeutic purposes [11].

2.3. Patient recruitment and sampling

To conform as much as possible to a real-life clinical setting, patients are recruited from the Diagnostics Senology Unit of Ospedale Policlinico San Martino, one of the highest-level referral centers in Italy. Eligible participants include women with radiologically identified lesions, BIRADS-3/4/5, ≤ 2 cm by radiological assessment (i.e., radiological T1), evaluated at

the Diagnostic Senology Unit of this Institution. These patients are asked, upon completion of an informed written consent, to donate four peripheral blood tubes (30 mL total) and one urine sample (40 mL). Their pseudo-anonymized radiological images as well as demographic and anatomopathological data are collected as well, by using a freeware dedicated, state-of-the-art firewalled data collection system, OpenClinica©, hosted by University of Genoa servers.

All patients are screened with the following inclusion and exclusion criteria. Inclusion criteria include: written informed consent; breast lesions detected by digital bilateral mammography; age ≥ 18 years and ≤ 75 years; eligibility for diagnostic biopsy (tru-cut or Vacuum Assisted Breast Biopsy) as per normal clinical practice for study population, or absence of breast lesions at the digital mammography for healthy controls (BIRADS-1 or BIRADS-0 with negative ecography); ability and willfulness to comply with the protocol requirements.

Exclusion criteria include: previous history of invasive cancer, any type; clinical or radiological suspicion of advanced or metastatic cancer at the time of screening; known history of active or treated autoimmune or manifest chronic or seasonal and active allergic disorders (with the exception of autoimmune thyroiditis); history of major trauma or surgery during the 24 weeks before screening; history of active infectious disease, either chronic or acute occurring during the 8 weeks before screening; history of known acute or chronic cardiac, kidney, or liver disease disorders or acute cardiac events.

2.4. Sample collection

2.4.1. Peripheral blood and urine collection, processing and storage

One investigator, a research nurse, and two biologists are collaborating in the first eighteen months of the project to collect at t0 and t1 two PAXgene® Blood ccfDNA Tubes CE-IVD (PreAnalityX, GmbH), one BD Vacutainer® K2E (EDTA) Plus Blood Collection Tube CE-IVD (BD life Sciences), one Tempus TM Blood RNA tube (Applied Biosystems), and one urine specimen in a sterile container. Blood samples are processed to extract and store cfDNA from plasma, and to collect proteins, exosomes, PBMCs, and total RNA . During sample processing PAXgene tubes are first centrifuged for 15 minutes at 1900 rpm at room temperature (RT)

then the collected plasma is centrifuged for further 10 minutes at 1900 rpm at RT. EDTA tubes are centrifuged for 15 minutes at 1600 rpm at RT and the collected plasma is centrifuged at 1900 rpm for further 10 minutes at RT. Plasma is then aliquoted in cryovials. Tempus tubes for total RNA extraction are immediately stored at -80°C. At the moment of collection, urine is mixed with Cell-Free DNA Urine Preserve (Streck) in order to stabilize cfDNA in samples for up to 7 days at 6 °C to 37 °C. Urine is centrifuged at 2680 rpm for 10 minutes, and the supernatant is aliquoted into 15 mL tubes and stored at -80°C until cfDNA extraction. Samples are stored at -80°C in a dedicated, Eppendorf CryoCube F740hi ULT Freezer, 3 Compartments.

All test tubes, from blood collection to storage, are barcoded to increase traceability and anonymity to external personnel.

2.4.2. Resources

A 150 sqm fully equipped laboratory supports the pivotal work on the samples collected for our project. All procedures of platelet separation and plasma processing, cfDNA and RNA extraction, quality control, amplification, library preparation and sequencing happen in separate, clean environments optimized for such tasks. Every operation from sample processing, to nucleic acid extraction and sequencing is semi-automated in order to minimize cross-sample contamination issues and ensure optimal reproducibility and consistency of operations. A dedicated freezer is available in the laboratories for sample storage. The laboratory is equipped with a ThermoFisher Scientific Ion™ S5 XL sequencer. Data collection and the dry-lab part of the analyses are performed on high performance workstations maintained in high-security, dedicated environments to avoid the risk of data loss and sensible data cyber-theft.

2.5. Analysis and integration of circulating biomarkers with radiomics

2.5.1. Ultrasensitive NGS on ctDNA

We designed a tagged-amplicon NGS panel covering 101 short regions of frequently mutated genes in BC. For this task, we analyzed the GENIE dataset v3.0 [19], which includes 3,269 sequenced primary and metastatic BC. Amongst the more common cancer genes in BC, very few behave as dominant oncogenes (i.e. with frequent mutation in limited regions of the gene, like PIK3CA), whereas several behave as tumor suppressors (i.e., with rare mutations occurring along the whole gene, like CDH1). By dividing all the genes sequenced by GENIE into 9,603 regions shorter than 50 base pairs, we realized that to detect at least one mutation, we would need to sequence only 101 regions to cover 80% of the whole GENIE dataset, but 420 regions to increase such detection rate to 88%. In particular, amplicons covering TP53 (30 regions), CDH1 (50 regions), GATA3 (13 regions), and PIK3CA (7 regions) would optimize the coverage by minimizing the size of our panel. The 101 selected regions will be assessed in plasma ctDNA using a custom design, which leverages the novel, proprietary tagged-amplicon OncoMine™cfDNA methodology by ThermoFisher Scientific Inc. Its use allows for a limit of detection of 0.1% at 20,000x sequencing coverage with 20 ng of circulating DNA, well attainable with two peripheral blood samples collected in PAXgene tubes. cfDNA extraction is performed by using the QIAamp Mini Elute cfDNA Mini or Midi Kit (Qiagen, Hilden, Germany) on the Qiacube system, according to the Manufacturer's instructions. cfDNA is processed on an Ion Chef™ fluidic handler, and sequenced using the tagged-amplicon methodology with our custom-design panel using as per manufacturer's specifications on a Ion S5™ sequencer. Tertiary analysis will be performed on a dedicated Ion Server System available in our laboratory. The library size of our custom panel is half of the commercial ThermoFisher Scientific Ion Torrent™ OncoMine™ Comprehensive cfDNA™ panel, from which the aforementioned performance considerations have been derived. Hence, the use of our custom-design panel appears feasible and should perform better in light of its smaller size and subsequent deeper attainable sequencing coverage.

2.5.2. Methylome profiling of cfDNA

Recent works pointed out astonishing results in terms of accuracy of cfMeDIP-seq in the detection of informative methylation changes of small quantities (1-10 ng) of plasma cfDNA for the diagnosis of renal cell carcinoma and intracranial tumors [20,21], with the former being effectively detected also by cfMeDIP-seq applied to urinary cfDNA. For this study cfDNA will be extracted with the QIAamp Mini Elute cfDNA Mini or Midi Kit (Qiagen, Hilden, Germany) on the Qiacube system from plasma and urine. According to the protocol elaborated by Shen *et al*., cfMeDIP involves four steps: 1) cfDNA end repair, A-tailing and adapter ligation; 2) cfMeDIP immunoprecipitation and enrichment using an antibody targeting 5 methylcytosine; 3) library preparation; 4) high throughput NGS on an Illumina platform for cfMeDNA data [22]. This approach based on immunoprecipitation allows to avoid cfDNA bisulfite treatment, typically used to study DNA methylation but burdened by the high rate of DNA degradation during the process.

2.5.3. Proteomics analysis

The proposed task is based on the relative quantification of blood proteins by a novel, highly multiplexed proteomic assay (SomaScan™) [23,24]. SomaScan™ (SomaLogic™) is considered the most comprehensive protein array available so far for the relative quantification of proteins. Over 7,000 proteins can be simultaneously analyzed starting from 55 µL of plasma. This technology involves a new type of aptamers, which are single stranded DNA molecules able to bind proteins, called SOMAmers. In addition to their high affinity for individual proteins, SOMAmers have a unique 40-nucleotide sequence tag and a fluorescent label that allows their identification and quantification in high-density microarrays. SOMAmers have been successfully assembled in a commercial product allowing the comparative evaluation of proteins in as low as 55µl of serum or plasma (or other biological fluids). The SOMAscan™ assay is a highly multiplexed, sensitive, quantitative, and reproducible proteomic tool for biomarker discovery and development. SomaScan™ can detect proteins within a range of 10 logarithms, allowing for an unmatched sensitivity to detect even femtomolar protein concentrations. The analysis of the SomaScan™ is performed using classic DNA array data

analysis and is based on bioinformatics tools that have been developed for gene array analysis.

2.5.4. Radiomics analyses

A preliminary radiomics classifier has been developed by the Diagnostics Senology team of Ospedale Policlinico San Martino, based on digital breast tomosynthesis (DBT) images of consecutive participants from the ASTOUND trial [2]. Radiomics analyses were performed on all DBT images within manually selected regions of interest (ROIs) including all the dense parts of the breast and excluding the fatty parts. ROIs were selected by a single radiologist, with proven expertise in quantitative image analysis. Descriptors of the preliminary classifier were selected after initial screening of 104 radiomics features to reduce the risk of over-fitting and according to features previously used to associate breast parenchymal patterns with cancer risk [25]. For the present project, image features will be extracted from the same cases and matched controls for whom NGS and proteomics analyses are performed, using an open source software platform for medical image informatics, and will make use of the advanced deep learning methodologies mastered by our External Collaborators (An.B.).

2.5.5. HDI classifier

Data from ctDNA analysis, cfMeDNA tests, proteomics, and radiomics classifiers will be processed and used to generate the HDI model, based on the ensemble learning approach methodology. Ensemble learning combines predictions of multiple individual classifiers obtained by different techniques such as random forest, support vector machine or general linear modeling in order to enhance generalization power [26], avoid overfitting, and increase the strength and reliability of the final outcome [27]. Specifically, the outcomes from ctDNA, cfMeDNA, proteomics and radiomics tests will be combined by using a weighted-majority voting approach implemented in the R environment (caret package).

2.5.6. Experimental validation and other analyses

Upon completion of proteomics experiments, we will seek to validate the possible discovery of novel protein biomarkers and to facilitate their transfer to clinical applicability by performing conventional protein immunoassays. Other promising noninvasive biomarkers will be studied,

thanks to the unique sample set at our disposal. We foresee the possibility to perform exosome-enriched miRNA sequencing, PBMC transcriptome sequencing by using the Ampliseq™ Transcriptome solution on our Ion S5™ XL sequencer. Eventually, such analyses may be integrated into the HDI classifier to reach an even higher accuracy for early BC diagnosis.

2.6. Study outcomes

This study aims to assess the performance of multiple analytes either individually or combined in a HDI classifier in the early detection of BC. The primary outcome is defined upon the histopathological diagnosis of early invasive BC characterized as per SIAPEC/ASCO/CAP criteria, with radiological extension ≤ 2 cm (radiological T1) and stage I-IIA at surgery (T1N0 or T2N0 or T1N1a). Benign lesions are defined upon the detection of radiological lesions ≤ 2 cm without the presence of invasive neoplasia at the first biopsy and at surgery, if performed. *In situ* tumors are included in the latter group.

2.7. Clinical data

Electronic case report forms (eCRF) have been designed for the annotation of patients' clinical data. These include study participants' demographics, biometric parameters such as height, weight and BMI, assumption of alcohol and smoke, information about the endocrine status (pre- or post-menopause, age at menarche, assumption of endocrine therapy, number of pregnancies), familiarity for BC and predisposing mutations, comorbidities. Items included in the eCRF are provided in **Tab. 1**

2.8. Data security and confidentiality

Patients' pseudo-anonymized radiological images as well as demographic and anatomopathological data are collected according to the local ethics committee guidelines using a dedicated, state-of-the-art firewalled data collection system, OpenClinica©, hosted by University of Genova servers.

3. STATISTICAL ANALYSIS

3.1. Projected sample size

The Diagnostics Senology Unit of San Martino Hospital is the highest-level referral center in Italy for a population basin of more than 2,000,000 people. In a single year, approximately 15,000 mammograms are performed (Figure 2). Of these, 1,500 yield a radiologic suspicion of malignancy with an ensuing biopsy. Assuming a 50% refusal rate by our patients to undergo trial-specific blood collection, we foresee to enroll 750 patients with radiologically suspect small breast lesions. Among these patients, we assume diagnosis of invasive BC will be confirmed in one third. Of these 250 bioptic diagnoses of BC, which will undergo primary surgery, as per internal historical records, 40% are assumed to be pT1 and 60% higher than pT1. Assuming another 10% failure rate in sample processing and storage, we will have the potential to collect imaging data and samples from approximately 90 patients with pT1 BC and 180 radiological size-matched lesions in a single year (Figure 2). Patient enrollment and t0 sample collection is projected in a time frame of 12 months from the beginning of the study, whereas t1 sample collection is expected approximately 6 months after the enrollment of the last patient.

3.2. Sample size calculation

The sample size required for our analyses is N = 147, with 49 biopsy-proven stage I-IIA BC cases and 98 biopsy-proven benign lesions of similar radiological size. In particular, assuming that the best non-HDI classifier is, in the end, the one with the highest number of tested variables (worst-case scenario, e.g., transcriptome sequencing on platelets with approximately 20,000 transcripts analyzed), we would need [28,29]:

- N = 87 samples for a training set, in a 1:2 ratio between bioptically proven BCs and

radiological size-matched benign lesions, with a standardized fold change of 1.2, n ~ 20,000 features to be assessed, tolerance = 0.05 from the best possible classifier, as defined by Dobbin *et al. [29]*;

- N = 26 samples for a testing set (70/30 split of samples between training and testing set) with similar ratio of cases and controls;

- N = 34 samples for a validation set (30% of the sum of training and testing sets).

It may be possible that, adopting HDI classification, the actual needed sample size will be smaller than calculated. At present however, we are not aware of well-established statistical approaches to obtain a more robust estimation of the number needed for our experiments other than the one we adopted.

4. Ethics and dissemination

Written informed consents are obtained from each study participant. Participant information sheet includes the main information of the study protocol, the known side effects or any risks implied in the participation to the study, and the contact information of study investigators. All data are deidentified and no patient-related information will be revealed during analysis. The Regione Liguria Ethics Committee c/o Ospedale Policlinico San Martino has approved the study (reference number: 2019/75, study ID: 4452).

All information concerning patients included in this study are covered by strict confidentiality in compliance with the General Data Protection Regulation EU 2016/679 (GDPR) and D.lgs. 30.06.2003, n. 196, as modified from D.lgs. 10.08.2018, n. 101. The study is conducted in accordance with the national law and according to international guidelines for the conduction of clinical trials referred to as “Good Clinical Practice”.

5. Amendments

The original protocol for this study underwent three amendments, mainly aimed at including the assessment of urinary biomarkers and expanding the cohort of analysed patients.

5.1. Amendment #1: collection and analysis of urine samples

The first amendment to this protocol, presented on February 18th 2020, involved the collection and analysis of urine samples in addition to blood samples, which was not included in the original version. An efficient method for the early diagnosis of BC based on the assessment of urinary biomarkers would substantially eliminate the invasiveness of the overall procedure, possibly facilitating the execution of large-scale screening campaigns.

5.2. Amendment #2: healthy controls

The second amendment, presented on February 4th 2021, involved the recruitment and analysis of the cohort of 100 healthy women with negative mammography (BIRADS-1 or BIRADS-0 with adjunct negative ecography) as a healthy control group, in addition to patients undergoing breast biopsies. As the presence of benign breast lesions may determine possible variations in blood and urinary analytes, especially in case of *in situ* tumors, the purpose of the present amendment is to possibly categorize patients upon the presence or absence of non-malignant breast lesions, enhancing the accuracy and of the HDI classifier.

5.3. Amendment #3: redefinition of the study cohort

The third and final amendment, presented on May 27th 2021, involved several substantial points. According to the original protocol, concerning the cohort of patients with histological diagnosis of invasive BC, only samples collected at t0 from patients with T1N0 tumor at surgery would have been analysed. Moreover, the collection and analysis of t1 samples would have involved only this group of patients, with the exclusive aim of distinguishing between tumor- and host-specific molecular alterations in connection with the presence/absence of BC. With the present amendment, samples collected at t0 will be analysed from patients assessed as with stage I-IIA tumor at surgery (T1N0 or T2N0 or T1N1a), allowing an effective expansion of the sample size, while remaining in the setting of early stage BC diagnosis. Furthermore, the collection and analysis of t1 samples will include all patients diagnosed as with invasive BC and sampled at t1, without limitations in terms of tumor stage assessed at surgery. The

analysis of t1 samples, coupled with patients' longitudinal monitoring performed as per normal clinical practice, will allow the assessment of the accuracy of a multi-analyte evaluation for the prediction of BC recurrence, added as exploratory aim of the present study.

6.DISCUSSION

The assessment of circulating biomarkers for clinical purposes is currently burdened by several issues, including suboptimal accuracy and lacking standardization of pre-analytical and analytical procedures. The overcoming of these limitations would allow the achievement of minimally invasive and personalized assays for the management of neoplastic patients, either in the diagnostic setting or in the early detection of recurrence or the prediction of the response to therapy, possibly replacing or implementing current protocols based on traditional tissue biopsies. One of the most effective strategies carried out to enhance the accuracy of liquid biopsies is the contemporary assessment of multiple analytes, which has already shown augmented sensitivity and specificity compared to the evaluation of single biomarkers [30]. Such implementation can occur at different levels of complexity, involving different kinds of integration. A basic integration can be referred to the combination of biomarkers of the same kind, such as DNA-DNA or protein-protein combinations. On the other hand, an advanced integration refers to the combined assessment of different kinds of analytes, such as DNA-protein, or the combination of circulating biomarkers with radiological procedures possibly refined with radiomics [30].

The purpose of this study is to evaluate the performance of the combination of multiple circulating biomarkers, either plasmatic or urinary, and radiomics for BC early diagnosis in patients recruited from a real-life clinical setting. At the enrollment, patients undergo a rigorous selection in order to avoid possible confounding factors that can affect the assessment of tumor-specific cfMeDNA, circulating RNA, and proteins. Only samples collected from patients presenting an early-stage disease (I and IIA) at diagnosis will be analysed for this aim. This selection allows us to assess the effective potential of current techniques for the evaluation of circulating biomarkers in the early diagnosis of the most common cancer worldwide.

Conversely, there will be no limits based on tumor stage for the analysis of circulating biomarkers collected after surgery, this being aimed at the prediction of BC recurrence.

In conclusion, the protocol outlined above presents relevant advantages compared to other studies aimed at the same purpose, including the application of recent and cutting-edge techniques to a selected but realistic cohort of patients, possibly bringing to effective advancements to current protocols for the management of BC patients, and directing future researches in the panorama of translational medicine applied to oncology.

Author statement. GZ conceived the study. FR wrote the manuscript. GC and MD provided a significant contribution in the Methods section of the manuscript. MG, VGV, PF, DF, MC, AB, AT, and LF critically reviewed the manuscript draft.

Funding statement. This trial is entirely supported by a Fondazione AIRC per la Ricerca sul Cancro Investigator Grant ID 21761 to Gabriele Zoppoli.

Competing interests. The authors declare no conflict of interest for the present study.

Figure legends

Figure 1. Study diagram. Blood and urine samples will be collected from patients yielding a radiological breast lesion ≤ 2 cm with no evidence of lymph node disease (radiological T1N0). Images and samples acquired from patients with stage I-IIA (T1N0 or T2N0 or T1N1a neoplasia) BC at surgery will be analysed for diagnostic purposes together with images and samples acquired from patients yielding benign breast lesions and from patients with negative mammography. Blood and urine samples will be re-collected from all patients yielding invasive neoplasia at diagnosis after surgery at the first oncologic visit, and will be analysed for the prediction of BC recurrence.

Figure 2. Sample size diagram. Approximately 1,500 breast biopsies per year are performed at the Diagnostics Senology Unit of San Martino Hospital. Of a projected number of 750 liquid biopsies we foresee to collect samples and acquire mammograms from at least 49 patients with stage I-IIA BC and 98 patients with radiological size-matched lesions, along with those samples and images acquired from 100 healthy women with two consecutive negative mammograms.

Biometrics	**Voluptuous habits**	**Endocrine status**	**Predisposition**	**Comorbidities**
Height	Does/Did the patient smoke?	Is the patient in the pre- or post menopause phase?	Does the patient present predisposing mutations for cancer?	Does the patient present comorbidities?
Weight	If yes, for how many years?	Age at menarche	If yes, which one	If yes, which ones?
BMI	Packets/day	Number of pregnancies	Does the patient have familiarity for breast and/or ovarian cancer?	
	Years since the last cigarette	Does the patient assume sostitutive endocrine therapy?		
	How many alcohol units does the patient assume per day?	If yes, for how many years?		
		Does the patient assume endocrine contraceptive therapy?		
		If yes, for how many years?		

Tab. 1. Items investigated at the recruitment. Along with items concerning the inclusion and exclusion criteria, all patients will be asked about information of interest for the assessment of BC risk and possible interference with the evaluation of circulating biomarkers.

References

1 Sprague BL, Arao RF, Miglioretti DL, *et al.* National Performance Benchmarks for Modern Diagnostic Digital Mammography: Update from the Breast Cancer Surveillance Consortium. *Radiology* 2017;**283**:59–69. doi:10.1148/radiol.2017161519

2 Tagliafico AS, Calabrese M, Mariscotti G, *et al.* Adjunct Screening With Tomosynthesis or Ultrasound in Women With Mammography-Negative Dense Breasts: Interim Report of a Prospective Comparative Trial. *J Clin Oncol* 2016;**34**:1882–8. doi:10.1200/JCO.2015.63.4147

3 Chung W, Eum HH, Lee H-O, *et al.* Single-cell RNA-seq enables comprehensive tumour and immune cell profiling in primary breast cancer. *Nat Commun* 2017;**8**:15081. doi:10.1038/ncomms15081

4 Bettegowda C, Sausen M, Leary RJ, *et al.* Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 2014;**6**:224ra24. doi:10.1126/scitranslmed.3007094

5 Cohen JD, Li L, Wang Y, *et al.* Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* 2018;**359**:926–30. doi:10.1126/science.aar3247

6 Best MG, Sol N, Kooi I, *et al.* RNA-Seq of Tumor-Educated Platelets Enables Blood-Based Pan-Cancer, Multiclass, and Molecular Pathway Cancer Diagnostics. *Cancer Cell* 2015;**28**:666–76. doi:10.1016/j.ccell.2015.09.018

7 Bhome R, Del Vecchio F, Lee G-H, *et al.* Exosomal microRNAs (exomiRs): Small molecules with a big role in cancer. *Cancer Lett* 2018;**420**:228–35. doi:10.1016/j.canlet.2018.02.002

8 Shen SY, Singhania R, Fehringer G, *et al.* Sensitive tumour detection and classification using plasma cell-free DNA methylomes. *Nature* 2018;**563**:579–83. doi:10.1038/s41586-018-0703-0

9 Kalluri R. The biology and function of exosomes in cancer. *J Clin Invest* 2016;**126**:1208–15. doi:10.1172/JCI81135

10 Valdora F, Houssami N, Rossi F, *et al.* Rapid review: radiomics and breast cancer. *Breast Cancer Res Treat* 2018;**169**:217–29. doi:10.1007/s10549-018-4675-4

11 Bhawal R, Oberg AL, Zhang S, *et al.* Challenges and Opportunities in Clinical Applications of Blood-Based Proteomics in Cancer. *Cancers* 2020;**12**. doi:10.3390/cancers12092428

12 Alimirzaie S, Bagherzadeh M, Akbari MR. Liquid biopsy in breast cancer: A comprehensive review. *Clin Genet* 2019;**95**:643–60. doi:10.1111/cge.13514

13 Aravanis AM, Lee M, Klausner RD. Next-Generation Sequencing of Circulating Tumor DNA for Early Cancer Detection. *Cell* 2017;**168**:571–4. doi:10.1016/j.cell.2017.01.030

14 Bennett NC, Farah CS. Next-generation sequencing in clinical oncology: next steps towards clinical validation. *Cancers* 2014;**6**:2296–312. doi:10.3390/cancers6042296

15 Stirzaker C, Taberlay PC, Statham AL, *et al.* Mining cancer methylomes: prospects and challenges. *Trends Genet* 2014;**30**:75–84. doi:10.1016/j.tig.2013.11.004

16 Salomon MP, Orozco JIJ, Wilmott JS, *et al.* Brain metastasis DNA methylomes, a novel resource for the identification of biological and clinical features. *Sci Data* 2018;**5**:180245. doi:10.1038/sdata.2018.245

17 Anfossi S, Babayan A, Pantel K, *et al.* Clinical utility of circulating non-coding RNAs - an update. *Nat Rev Clin Oncol* 2018;**15**:541–63. doi:10.1038/s41571-018-0035-x

18 Koh W, Pan W, Gawad C, *et al.* Noninvasive in vivo monitoring of tissue-specific global gene expression in humans. *Proc Natl Acad Sci U S A* 2014;**111**:7361–6. doi:10.1073/pnas.1405528111

19 AACR Project GENIE Consortium. AACR Project GENIE: Powering Precision Medicine through an International Consortium. *Cancer Discov* 2017;**7**:818–31. doi:10.1158/2159-8290.CD-17-0151

20 Nuzzo PV, Berchuck JE, Korthauer K, *et al.* Detection of renal cell carcinoma using plasma and urine cell-free DNA methylomes. *Nat Med* 2020;**26**:1041–3. doi:10.1038/s41591-020-0933-1

21 Nassiri F, Chakravarthy A, Feng S, *et al.* Detection and discrimination of intracranial tumors using plasma cell-free DNA methylomes. *Nat Med* 2020;**26**:1044–7. doi:10.1038/s41591-020-0932-2

22 Shen SY, Burgener JM, Bratman SV, *et al.* Preparation of cfMeDIP-seq libraries for methylome profiling of plasma cell-free DNA. *Nat Protoc* 2019;**14**:2749–80. doi:10.1038/s41596-019-0202-2

23 Webber J, Stone TC, Katilius E, *et al.* Proteomics analysis of cancer exosomes using a novel modified aptamer-based array (SOMAscan™) platform. *Mol Cell Proteomics* 2014;**13**:1050–64. doi:10.1074/mcp.M113.032136

24 Raffield LM, Dang H, Pratte KA, *et al.* Comparison of Proteomic Assessment Methods in Multiple Cohort Studies. *Proteomics* 2020;**20**:e1900278. doi:10.1002/pmic.201900278

25 Lambin P, van Stiphout RGPM, Starmans MHW, *et al.* Predicting outcomes in radiation oncology--multifactorial decision support systems. *Nat Rev Clin Oncol* 2013;**10**:27–40. doi:10.1038/nrclinonc.2012.196

26 Dietterich TG. Machine-learning research; four current directions. *https://go.gale.com ›*

i.dohttps://go.gale.com › i.do

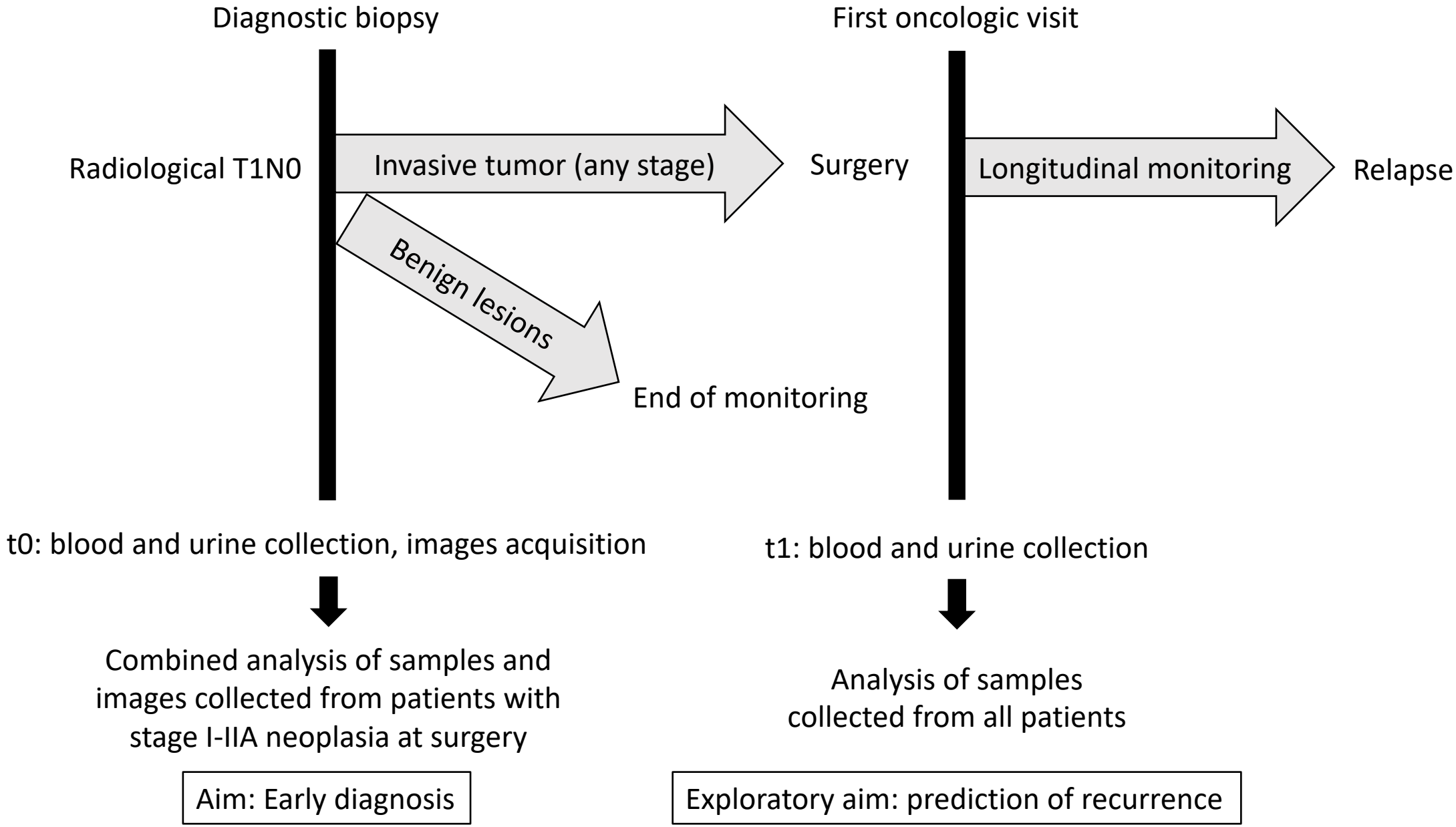
1997;**18**:97+.https://go.gale.com/ps/i.do?id=GALE%7CA20475202&sid=googleScholar&v=2.1&it=r&linkaccess=abs&issn=07384602&p=AONE&sw=w

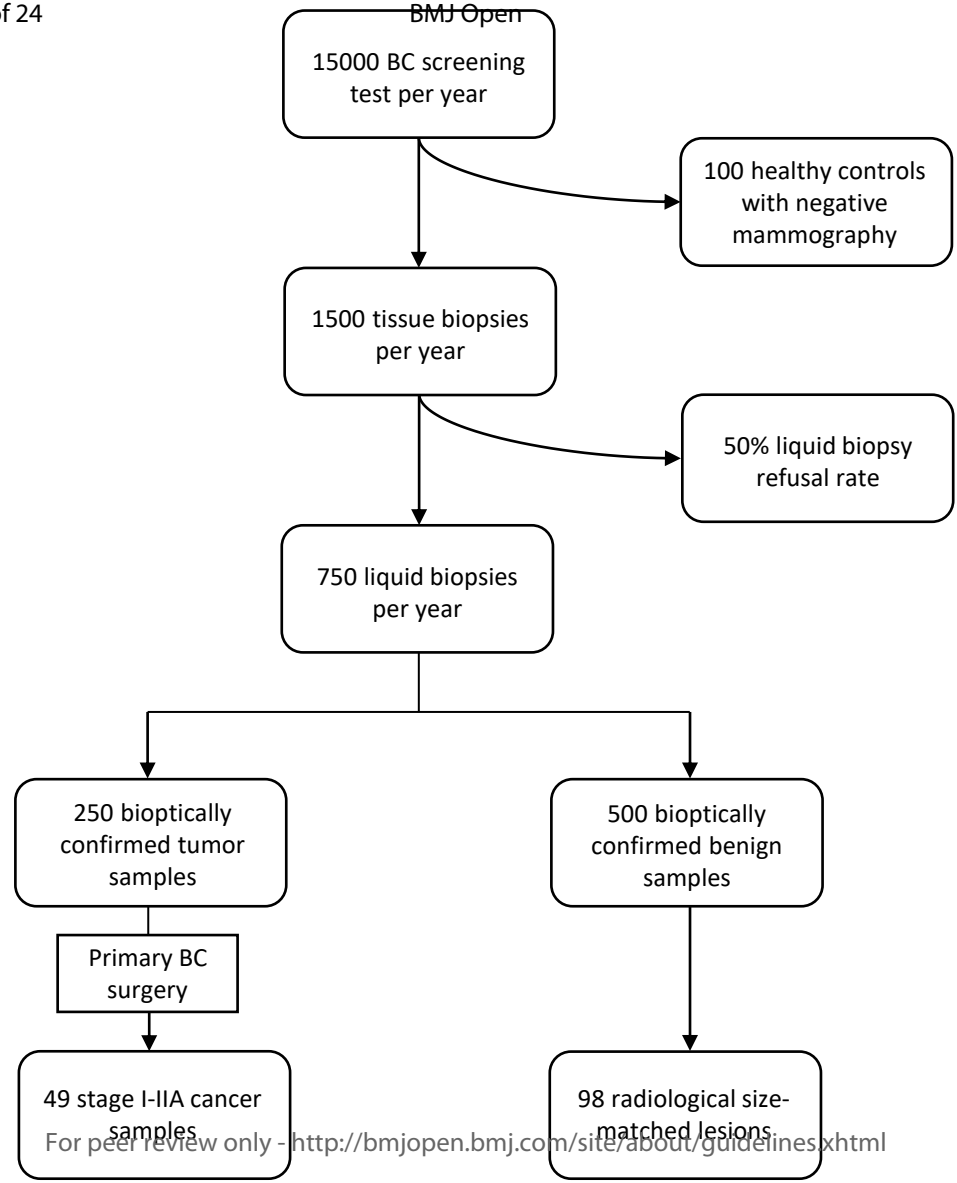
27 Thrun S, Pratt L. Learning to Learn: Introduction and Overview. In: Thrun S, Pratt L, eds. *Learning to Learn*. Boston, MA: : Springer US 1998. 3–17. doi:10.1007/978-1-4615-5529-2_1

28 Dobbin KK, Simon RM. Sample size planning for developing classifiers using high-dimensional DNA microarray data. *Biostatistics* 2007;**8**:101–17. doi:10.1093/biostatistics/kxj036

29 Dobbin KK, Zhao Y, Simon RM. How large a training set is needed to develop a classifier for microarray data? *Clin Cancer Res* 2008;**14**:108–14. doi:10.1158/1078-0432.CCR-07-0443

30 Qiu J, Xu J, Zhang K, *et al.* Refining Cancer Management Using Integrated Liquid Biopsy. *Theranostics* 2020;**10**:2374–84. doi:10.7150/thno.40677


Diagnostic biopsy
First oncologic visit
Radiological T1N0
Invasive tumor (any stage)
Surgery
Longitudinal monitoring
Relapse
Benign lesions
End of monitoring
t0: blood and urine collection, images acquisition
t1: blood and urine collection
Combined analysis of samples and images collected from patients with stage I-IIA neoplasia at surgery
Analysis of samples collected from all patients
Aim: Early diagnosis
Exploratory aim: prediction of recurrence



BMJ Open

Development of a hoRizontal data intEgration classifier for NOninvasiVe early diAgnosis of breasT cancEr: the RENOVATE study protocol

Journal:	*BMJ Open*
Manuscript ID	bmjopen-2021-054256.R1
Article Type:	Protocol
Date Submitted by the Author:	29-Oct-2021
Complete List of Authors:	Ravera, Francesco; Università degli Studi di Genova, Department of internal Medicine Cirmena, Gabriella; Università degli Studi di Genova, Department of internal Medicine Dameri, Martina; Ospedale Policlinico San Martino Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia Gallo, Maurizio; Università degli Studi di Genova, Department of internal Medicine Vellone, Valerio; Università degli Studi di Genova, Department of Surgical Sciences and Integrated Diagnostic Fregatti, Piero; Ospedale Policlinico San Martino Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia Friedman, Daniele; Ospedale Policlinico San Martino Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia Calabrese, Massimo; Ospedale Policlinico San Martino Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia Ballestrero, Alberto; University of Genoa, Department of Internal Medicine Tagliafico, Alberto; Università degli Studi di Genova, Department of Health Sciences Ferrando, Lorenzo; Ospedale Policlinico San Martino Istituto di Ricovero e Cura a Carattere Scientifico per l'Oncologia Zoppoli, Gabriele; Università degli Studi di Genova, Department of internal Medicine
Primary Subject Heading:	Oncology
Secondary Subject Heading:	Diagnostics, Radiology and imaging
Keywords:	Breast tumours < ONCOLOGY, Breast imaging < RADIOLOGY & IMAGING, Health informatics < BIOTECHNOLOGY & BIOINFORMATICS, Cancer genetics < GENETICS, Adult oncology < ONCOLOGY

I, the Submitting Author has the right to grant and does grant on behalf of all authors of the Work (as defined in the below author licence), an exclusive licence and/or a non-exclusive licence for contributions from authors who are: i) UK Crown employees; ii) where BMJ has agreed a CC-BY licence shall apply, and/or iii) in accordance with the terms applicable for US Federal Government officers or employees acting as part of their official duties; on a worldwide, perpetual, irrevocable, royalty-free basis to BMJ Publishing Group Ltd ("BMJ") its licensees and where the relevant Journal is co-owned by BMJ to the co-owners of the Journal, to publish the Work in this journal and any other BMJ products and to exploit all rights, as set out in our licence.

The Submitting Author accepts and understands that any supply made under these terms is made by BMJ to the Submitting Author unless you are acting as an employee on behalf of your employer or a postgraduate student of an affiliated institution which is paying any applicable article publishing charge ("APC") for Open Access articles. Where the Submitting Author wishes to make the Work available on an Open Access basis (and intends to pay the relevant APC), the terms of reuse of such Open Access shall be governed by a Creative Commons licence – details of these licences and which Creative Commons licence will apply to this Work are set out in our licence referred to above.

Other than as permitted in any relevant BMJ Author's Self Archiving Policies, I confirm this Work has not been accepted for publication elsewhere, is not being considered for publication elsewhere and does not duplicate material already published. I confirm all authors consent to publication of this Work and authorise the granting of this licence.

Development of a hoRizontal data intEgration classifier for NOninvasiVe early diAgnosis of breasT cancEr: the RENOVATE study protocol

Authors: Francesco Ravera[1], Gabriella Cirmena[1], Martina Dameri[2], Maurizio Gallo[2], Valerio Gaetano Vellone[2,4], Piero Fregatti[2,4], Daniele Friedman[2,4], Massimo Calabrese[2], Alberto Ballestrero[1,2] Alberto Tagliafico[2,3,*], Lorenzo Ferrando[2,*], Gabriele Zoppoli[1,2*,#]

[1]Department of Internal Medicine and Medical Specialties DiMI, University of Genoa, 16132, Italy

[2] IRCCS Ospedale Policlinico San Martino, 16132 Genoa, Italy

[3]Department of Health Sciences DISSAL, University of Genoa, 16132, Italy

[4]Department of Surgical Sciences and Integrated Diagnostic DISC, 16132 Genoa, Italy

*These Authors equally contributed to the present manuscript

[#] Correspondence should be addressed to Gabriele Zoppoli: gabriele.zoppoli@unige.it

Keywords: breast cancer, early diagnosis, liquid biopsy, circulating biomarkers, radiomics

Word count: 4640

Abstract

Introduction: Standard procedures aimed at the early diagnosis of breast cancer (BC) present suboptimal accuracy and imply the execution of invasive and sometimes unnecessary tissue biopsies. The assessment of circulating biomarkers for diagnostic purposes, together with radiomics, is of great potential in BC management.

Methods and analysis: This is a prospective translational study investigating the accuracy of the combined assessment of multiple circulating analytes together with radiomic variables for early BC diagnosis. Up to 750 patients will be recruited at their presentation at the Diagnostic Senology Unit of Ospedale Policlinico San Martino (Genoa, IT) for the execution of a diagnostic biopsy after the detection of a suspect breast lesion (t0). Each recruited patient will be asked to donate peripheral blood and urine before undergoing breast biopsy. Blood and urine samples will also be collected from a cohort of 100 patients with negative mammography. For cases with histological diagnosis of invasive BC, a second sample of blood and urine will be collected after breast surgery. Circulating tumor DNA, cell-free methylated DNA, and circulating proteins will be assessed in samples collected at t0 from patients with stage I-IIA BC at surgery together with those collected from patients with histologically confirmed benign lesions of similar size and from healthy controls with negative mammography. These analyses will be combined with radiomic variables extracted with freeware algorithms applied to cases and matched controls for which digital mammography is available. The overall goal of the present study is to develop a horizontal data integration classifier for the early diagnosis of BC.

Ethics and dissemination: This research protocol has been approved by Regione Liguria Ethics Committee (reference number: 2019/75, study ID: 4452). Patients will be required to provide written informed consent. Results will be published in international peer-reviewed scientific journals.

Registration: ClinicalTrials.gov, NCT04781062.

Strengths and limitations of this study

- The study has a prospective design with well-balanced controlled cohorts
- The study assesses the performance of some of the most promising and cutting-edge biomarkers in the field of translational oncology either for diagnostic or predictive purposes in patients affected by BC
- The performance of the combination of multiple circulating biomarkers and radiomics algorithms for BC early diagnosis is assessed
- The study is the first to investigate such biomarkers in early (i.e. stage I-IIA) BC
- The study is not designed for the early diagnosis of *in situ* BCs, which are considered as benign lesions and are included in the control group

1. Introduction

Current protocols for the early diagnosis of breast cancer (BC) rely on the combined use of radiological procedures such as mammography and ultrasound [1,2]. Confirmation biopsy or recall tests are mandatory in case of suspect found during the first examination and bring eventually to a more definite characterization of the radiologically identified lesion. This approach is however burdened by serious issues which include (a) suboptimal sensitivity and positive predictive power respectively for radiological screening and diagnostic procedures, (b) invasiveness of biopsy with discomfort for women undergoing diagnostic tests, along with the risk of drawing non-representative portions of the suspected region considering the genotypic and phenotypic heterogeneity of BCs [3], (c) long turnaround time for recall tests, even in high-level centers. In particular, the suboptimal sensitivity of screening procedures leads to non-diagnosed tumors which become able to advance locally and spread systemically, impacting patients' prognosis, while the suboptimal positive predictive power of diagnostic evaluation implies unnecessary invasive biopsies [1].

The compelling necessity of increasing the accuracy of screening and diagnostic procedures for cancer has brought to a relevant advancement in the pursuit of accurate biomarkers able to efficiently detect and characterize it. To date, however, no protocol with effective and recognized clinical validity for the early diagnosis of BC has been developed.

Amongst the most promising biomarkers, circulating tumor DNA (ctDNA) and cell-free methylated DNA (cfMeDNA), exosomes, and microRNA (miRNA) have been the subject of relevant scientific reports [4–9]. Moreover, machine-learning (so called “deep learning”) algorithms have been applied to traditional radiology imaging techniques for diagnosis assistance with exciting results in the field of radiomics [10]. Results in the proteomics field have instead been few, in part due to the cumbersome methodologies often applied to the study of these molecules [11].

Since it is hardly conceivable that a single biomarker is able to achieve 100% accuracy in the early detection of BC, the primary aim of the present study is to merge for this purpose the assessment of multiple biological analytes with the refinement of radiomics algorithms, overcoming the aforementioned limitations in terms of accuracy of the individual biomarkers. The concept of combining different data layers to reach a better classifier compared to the individual analytes is referred to as horizontal data integration (HDI) classification. Therefore, the overall goal of the project is to develop an HDI classifier enabling early noninvasive diagnosis of BC with similar accuracy compared to breast biopsies.

2. Methods and analysis

2.1. Study design

This is a prospective translational case-control study with the primary aim of assessing the clinical validity of several biomarkers, individually and combined, in the early detection of BC in a real-life clinical setting (see Figure 1 for study diagram). Patients are recruited at the presentation to the Diagnostic Senology Unit of Ospedale Policlinico San Martino (Genoa, Italy) for the execution of the breast biopsy after the radiological detection of a suspect breast lesion ≤ 2 cm (i.e. radiological T1, BIRADS-3/4/5) with no radiological evidence of axillary or

distant disease. These patients are asked, upon completion of an informed written consent, to donate four peripheral blood tubes (30 mL total) and one urine sample (40 mL). Of these, only samples collected from patients with stage I-IIA neoplasia at surgery (T1N0 or T2N0 or T1N1a) or histologically confirmed benign lesions will be analysed. A second sample is obtained from those patients with confirmed histological diagnosis of invasive BC at the first oncologic visit after breast primary surgery (t1) according to normal practice, independently from possible neoadjuvant treatments. No limitations based on tumor stage will be made for the analysis of the latter. Blood and urine samples are also collected and analysed from a cohort of 100 healthy women with two consecutive negative mammograms (BIRADS-1 or BIRADS-0 with negative ecography) as well.

All patients are screened with the following inclusion and exclusion criteria. Inclusion criteria include: written informed consent; breast lesions detected by digital bilateral mammography; age ≥ 18 years and ≤ 75 years; eligibility for diagnostic biopsy (tru-cut or Vacuum Assisted Breast Biopsy) as per normal clinical practice for study population, or absence of breast lesions at the digital mammography for healthy controls (BIRADS-1 or BIRADS-0 with negative ultrasound); ability and willfulness to comply with the protocol requirements.

Exclusion criteria include: previous history of invasive cancer, any type; clinical or radiological suspicion of advanced or metastatic cancer at the time of screening; known history of active or treated autoimmune or manifest chronic or seasonal and active allergic disorders (with the exception of autoimmune thyroiditis); history of major trauma or surgery during the 24 weeks before screening; history of active infectious disease, either chronic or acute occurring during the 8 weeks before screening; history of known acute or chronic cardiac, kidney, or liver disease disorders or acute cardiac events.

2.2. Candidate biomarkers

2.2.1. Circulating tumor DNA and cell-free Methylated DNA

ctDNA obtained via liquid biopsy has shown serious potential not only in the early diagnosis of cancer but *inter alia* as an effective marker for its recurrence, longitudinal monitoring and response to therapy [12]. Current methods for detecting ctDNA however are mostly based on

sequencing somatic mutations from cell-free DNA (cfDNA), a process constrained by relevant limitations in clinical applicability due to (a) expectable low sensitivity in early stage cancers given the limited number of recurrent mutations in ctDNA [8,13], (b) the vast heterogeneity of DNA mutations occurring in a single tumor together with nonspecific mutations profiles along different patients and cancer types [12,13], (c) current cost-prohibitive impact of cfDNA Next Generation Sequencing (NGS) for mutation assessment [14]. On the other hand, the assessment of cfMeDNA may overcome the limitations outlined above. DNA methylation is commonly involved in cellular development, tissue-specific gene expression, and regulation of imprinted alleles, with widespread effects on cellular growth and genomic stability [15]. Changes in methylomes in cancer are usually associated with alterations of the transcriptional outcome and genomic instability, priming or enhancing carcinogenesis. Given the impact of methylation changes on cellular equilibrium, different methylomes may be associated with specific biological features providing useful information for the early diagnosis of cancer [16]. Shen *et al.* recently developed a sensitive, immunoprecipitation-based protocol to analyze the methylomes of small quantities of cfDNA, providing an efficient method to detect large-scale DNA methylation changes that are enriched for tumor-specific patterns [8]. cfMeDNA immunoprecipitation and high-throughput sequencing (cfMeDIP–seq) was able to find cancer-derived DNA methylation events in cfDNA and to distinguish multiple cancer types from healthy controls, besides effectively discriminating different methylation patterns across diverse cancers.

2.2.2. RNA-based biomarkers

RNA-based biomarkers can be referred either to coding or non-coding RNAs. Non-coding RNAs, including miRNAs and long non coding RNAs, have been extensively studied over the last years as promising markers for cancer early diagnosis and monitoring, being of particular interest for their stability and quantity in the bloodstream [17]. On the other hand, coding-RNAs include the cell-free messenger RNA, whose evaluation may allow the non-invasive assessment of the whole-body transcriptome [18] with relevant implications for several clinical purposes.

2.2.3. Circulating proteins

Proteomic analyses in neoplastic patients have been evaluated in regard to diverse clinical purposes with suboptimal results, given the challenging characterization of circulating proteins. The assessment of alterations in protein-mediated signaling networks is of great interest for its possible implications in the early diagnosis and monitoring of cancer, besides the detection of potentially actionable targets for therapeutic purposes [11].

2.3. Sample collection

2.3.1. Peripheral blood and urine collection, processing and storage

One investigator, a research nurse, and two biologists are collaborating in the first eighteen months of the project to collect at t0 and t1 two PAXgene® Blood ccfDNA Tubes CE-IVD (PreAnalityX, GmbH), one BD Vacutainer® K2E (EDTA) Plus Blood Collection Tube CE-IVD (BD life Sciences), one Tempus TM Blood RNA tube (Applied Biosystems), and one urine specimen in a sterile container. Blood samples are processed to extract and store cfDNA from plasma, and to collect proteins, exosomes, PBMCs, and total RNA. PAXgene tubes are first centrifuged for 15 minutes at 1900 rcf at room temperature (RT), then the collected plasma is further centrifuged for 10 minutes at 1900 rcf at RT. EDTA tubes are centrifuged for 15 minutes at 1600 rcf at RT and the collected plasma is further centrifuged at 1900 rcf for 10 minutes at RT. Plasma is then aliquoted in cryovials. Tempus tubes for total RNA extraction are immediately stored at -80°C. At the moment of collection, urine is mixed with Cell-Free DNA Urine Preserve (Streck) in order to stabilize cfDNA in samples for up to 7 days at a temperature ranging from 6 °C to 37 °C. Urine is centrifuged at 2680 rcf for 10 minutes, and the supernatant is aliquoted into 15 mL tubes and stored at -80°C until cfDNA extraction. Samples are stored at -80°C in a dedicated, Eppendorf CryoCube F740hi ULT Freezer, 3 Compartments.

All test tubes, from blood collection to storage, are barcoded to increase traceability and anonymity to external personnel.

2.3.2. Resources

A 150 sqm fully equipped laboratory supports the pivotal work on the samples collected for our project. All procedures of platelet separation and plasma processing, cfDNA and RNA

extraction, quality control, amplification, library preparation and sequencing happen in separate, clean environments optimized for such tasks. Every operation, from sample processing, to nucleic acid extraction and sequencing, is semi-automated in order to minimize cross-sample contamination and ensure optimal reproducibility and consistency of operations. A dedicated freezer is available in the laboratories for sample storage. The laboratory is equipped with a ThermoFisher Scientific Ion™ S5 XL sequencer. Data collection and the dry-lab part of the analyses are performed on high performance workstations maintained in high-security, dedicated environments to avoid the risk of data loss and sensible data cyber-theft.

2.4. Analysis and integration of circulating biomarkers with radiomics

2.4.1. Ultrasensitive NGS on ctDNA

We designed a tagged-amplicon NGS panel covering 101 short regions of frequently mutated genes in BC. For this task, we analyzed the GENIE dataset v3.0 [19], which includes 3,269 sequenced primary and metastatic BC. This dataset was chosen for its size and homogeneity. Since our purpose was looking at the most frequent, non-overlapping (i.e. mutually exclusive) mutations, the sample size of GENIE was deemed as adequate for the panel design. Amongst the most common cancer genes in BC, very few behave as dominant oncogenes (i.e. with frequent mutation in limited regions of the gene, like PIK3CA), whereas several behave as tumor suppressors (i.e., with rare mutations occurring along the whole gene, like CDH1). By dividing all the genes sequenced by GENIE into 9,603 regions shorter than 50 base pairs, we realized that to detect at least one mutation, we would need to sequence only 101 regions to cover 80% of the whole GENIE dataset, but 420 regions to increase such detection rate to 88%. In particular, amplicons coveringTP53 (30 exonic regions), CDH1 (50 exonic regions), GATA3 (13 exonic regions), and PIK3CA (7 exoic regions) would optimize the coverage by minimizing the size of our panel. TP53 is planned to be analyzed, together with CDH1, for all its exons. On the other hand, PIK3CA presents mostly hotspot mutations in exons 9 and 20. Hence, only those two exons are going to be covered by this design. The 101 selected regions will be assessed in plasma ctDNA using a custom design, which leverages the novel, proprietary tagged-amplicon OncoMine™cfDNA methodology by ThermoFisher Scientific Inc.

Its use allows for a limit of detection of 0.1% at 20,000x sequencing coverage with 20 ng of circulating DNA, well attainable with two peripheral blood samples collected in PAXgene tubes. ThermoFisher defines ctDNA positivity as the detection of three molecular families. However, this threshold is arbitrary and will be adapted depending on the actual class the samples fall in, in order to optimize the accuracy of the multiomic HDI. cfDNA extraction is performed by using the QIAamp Mini Elute cfDNA Mini or Midi Kit (Qiagen, Hilden, Germany) on the Qiacube system, according to the Manufacturer's instructions. cfDNA is extracted from at least 4 mL of plasma collected in the EDTA tubes and 2 mL of plasma collected in the PAXgene tubes, managing to obtain the requested amount of cfDNA for cfMeDIP-seq (~ 5 ng) and the assessment of ctDNA mutations (~15 ng). cfDNA is processed on an Ion Chef™ fluidic handler, and sequenced using the tagged-amplicon methodology with our custom-design panel using as per manufacturer's specifications on a Ion S5™ sequencer. Tertiary analysis will be performed on a dedicated Ion Server System available in our laboratory. The library size of our custom panel is half of the commercial ThermoFisher Scientific Ion Torrent™ OncoMine™ Comprehensive cfDNA™ panel, from which the aforementioned performance considerations have been derived. Hence, the use of our custom-design panel appears feasible and should perform better in light of its smaller size and subsequent deeper attainable sequencing coverage.

2.4.2. Methylome profiling of cfDNA

Recent works pointed out encouraging results in terms of accuracy of cfMeDIP-seq in the detection of informative methylation changes of small quantities (1-10 ng) of plasma cfDNA for the diagnosis of renal cell carcinoma and intracranial tumors [20,21], with the former being effectively detected also by cfMeDIP-seq on urinary cfDNA. According to the protocol elaborated by Shen *et al*., cfMeDIP involves four steps: 1) cfDNA end repair, A-tailing and adapter ligation; 2) cfMeDIP immunoprecipitation and enrichment using an antibody targeting 5 methylcytosine; 3) library preparation; 4) high throughput NGS on an Illumina platform for cfMeDNA data [22]. This approach based on immunoprecipitation allows to avoid cfDNA

bisulfite treatment, typically used to study DNA methylation but burdened by the high rate of DNA degradation during the process.

2.4.3. Proteomics analysis

The proposed task is based on the relative quantification of blood proteins by a novel, highly multiplexed proteomic assay (SomaScan™) [23,24]. SomaScan™ (SomaLogic™) is considered the most comprehensive protein array available so far for the relative quantification of proteins. Over 7,500 proteins can be simultaneously analyzed starting from 55 µL of plasma. This technology involves a new type of aptamers, which are single stranded DNA molecules able to bind proteins, called SOMAmers. In addition to their high affinity for individual proteins, SOMAmers have a unique 40-nucleotide sequence tag and a fluorescent label that allows their identification and quantification in high-density microarrays. SOMAmers have been successfully assembled in a commercial product allowing the comparative evaluation of proteins in as low as 55µl of serum or plasma (or other biological fluids). The SOMAscan™ assay is a highly multiplexed, sensitive, quantitative, and reproducible proteomic tool for biomarker discovery and development. SomaScan™ can detect proteins within a range of 10 logarithms, allowing for an unmatched sensitivity to detect even femtomolar protein concentrations. The analysis of the SomaScan™ is performed using classic DNA array data analysis and is based on bioinformatics tools that have been developed for gene array analysis.

2.4.4. Radiomics analyses

A preliminary radiomics classifier has been developed by the Diagnostics Senology team of Ospedale Policlinico San Martino, based on digital breast tomosynthesis (DBT) images of consecutive participants from the ASTOUND trial [2]. Radiomics analyses were performed on all DBT images within manually selected regions of interest (ROIs) including all the dense parts of the breast and excluding the fatty parts. ROIs were selected by a single radiologist, with proven expertise in quantitative image analysis. Descriptors of the preliminary classifier were selected after initial screening of 104 radiomics features to reduce the risk of over-fitting and according to features previously used to associate breast parenchymal patterns with

cancer risk [25]. For the present project, image features will be extracted from the same cases and matched controls for whom NGS and proteomics analyses are performed, using an open source software platform for medical image informatics, and will make use of advanced deep learning methodologies.

2.4.5. HDI classifier

Sensitivity and specificity of ctDNA analysis, cfMeDNA tests, proteomic analysis, and radiomics for the early detection of BC will be assessed. Data from these classifiers will be subsequently processed and used to generate the HDI model, based on the ensemble learning approach methodology. Ensemble learning combines predictions of multiple individual classifiers obtained by different techniques such as random forest, support vector machine or general linear modeling in order to enhance generalization power [26], avoid overfitting, and increase the strength and reliability of the final outcome [27]. Specifically, the outcomes from ctDNA, cfMeDNA, proteomics and radiomics tests will be combined by using a weighted-majority voting approach implemented in the R environment (caret package). We do not envisage RNA-based classifiers to be initially included in our multianalyte model.

2.4.6. Experimental validation and other analyses

Upon completion of these experiments, we will seek to validate the possible discovery of novel biomarkers and to facilitate their transfer to clinical applicability. Other promising noninvasive biomarkers will be studied, thanks to the unique sample set at our disposal. We foresee the possibility to perform exosome-enriched miRNA sequencing, PBMC transcriptome sequencing by using the Ampliseq™ Transcriptome solution on our Ion S5™ XL sequencer. Eventually, such analyses may be integrated into the HDI classifier to augment the accuracy of BC early diagnosis.

2.5. Study outcomes

This study aims at assessing the performance of multiple analytes, individually and combined in a HDI classifier, in the early detection of BC. The primary outcome is defined upon the histopathological diagnosis of early invasive BC characterized as per SIAPEC/ASCO/CAP criteria, with radiological extension ≤ 2 cm (radiological T1) and stage I-IIA at surgery (T1N0

or T2N0 or T1N1a). Benign lesions are defined upon the detection of radiological lesions ≤ 2 cm without the presence of invasive neoplasia at the first biopsy and at surgery, if performed. *In situ* tumors are included in the latter group.

2.6. Clinical data

Electronic case report forms have been designed for the annotation of patients' clinical data. These include study participants' demographics, biometric parameters such as height, weight and BMI, assumption of alcohol and smoke, information about the endocrine status (pre- or post-menopause, age at menarche, assumption of endocrine therapy, number of pregnancies), familiarity for BC and predisposing mutations, comorbidities. Items included in the eCRF are provided in **Tab. 1.** Tumor histology and immunohistochemical features will be annotated as well.

2.7. Data security and confidentiality

Patients' pseudo-anonymized radiological images as well as demographic and anatomopathological data are collected according to the local ethics committee guidelines using a dedicated, state-of-the-art firewalled data collection system, OpenClinica©, hosted by University of Genova servers.

2.8. Statistical analysis

2.8.1. Projected sample size

The Diagnostics Senology Unit of San Martino Hospital is the highest-level referral center in Italy for a population basin of more than 2,000,000 people. In a single year, approximately 15,000 mammograms are performed (Figure 2). Of these, 1,500 yield a radiologic suspicion of malignancy with an ensuing biopsy. Assuming a 50% refusal rate by our patients to undergo trial-specific blood collection, we foresee to enroll 750 patients with radiologically suspect small breast lesions. Among these patients, we assume diagnosis of invasive BC will be confirmed in one third. Of these 250 bioptic diagnoses of BC, which will undergo primary surgery, as per internal historical records, 40% are assumed to be pT1 and 60% higher than pT1. Assuming another 10% failure rate in sample processing and storage, we will have the potential to collect imaging data and samples from approximately 90 patients with pT1 BC and

180 radiological size-matched lesions in a single year (Figure 2). Patient enrollment is projected in a time frame of 12 months from the beginning of the study, whereas samples collection at t1 is expected to end approximately 6 months after the enrollment of the last patient.

2.8.2. Sample size calculation

The sample size required for our analyses is N = 147, with 49 biopsy-proven stage I-IIA BC cases and 98 biopsy-proven benign lesions of similar radiological size. In particular, assuming that the best non-HDI classifier is, in the end, the one with the highest number of tested variables (worst-case scenario, e.g., transcriptome sequencing on platelets with approximately 20,000 transcripts analyzed), we would need [28,29]:

- N = 87 samples for a training set, in a 1:2 ratio between histologically proven BCs and benign lesions matched for radiological size, with a standardized fold change of 1.2, n ~ 20,000 features to be assessed, tolerance = 0.05 from the best possible classifier, as defined by Dobbin *et al. [29]*;
- N = 26 samples for a testing set (70/30 split of samples between training and testing set) with similar ratio of cases and controls;
- N = 34 samples for a validation set (30% of the sum of training and testing sets).

It may be possible that, adopting HDI classification, the actual needed sample size will be smaller than calculated. At present however, we are not aware of well-established statistical approaches to obtain a more robust estimation of the number needed for our experiments other than the one we adopted.

2.9. Amendments

The original protocol for this study underwent three amendments, mainly aimed at including the assessment of urinary biomarkers and expanding the cohort of analysed patients.

2.9.1. Amendment #1: collection and analysis of urine samples

The first amendment to this protocol, presented on February 18th 2020, involved the collection and analysis of urine samples in addition to blood samples, which was not included in the original version. An efficient method for the early diagnosis of BC based on the assessment

of urinary biomarkers would substantially eliminate the invasiveness of the overall procedure, possibly facilitating the execution of large-scale screening campaigns.

2.9.2. Amendment #2: healthy controls

The second amendment, presented on February 4th 2021, involved the recruitment and analysis of a cohort of 100 healthy women with negative mammography (BIRADS-1 or BIRADS-0 with adjunct negative ultrasound) as a healthy control group, in addition to patients undergoing breast biopsy. As the presence of benign breast lesions may determine possible variations in blood and urinary analytes, especially in case of *in situ* tumors, the purpose of the present amendment is to possibly categorize patients upon the presence or absence of non-malignant breast lesions, enhancing the accuracy of the HDI classifier.

2.9.3. Amendment #3: redefinition of the study cohort

The third and final amendment, presented on May 27th 2021, involved several points. According to the original protocol, concerning the cohort of patients with histological diagnosis of invasive BC, only samples collected at t0 from patients with T1N0 tumor at surgery would have been analysed. Moreover, the collection and analysis of t1 samples would have involved only this group of patients, with the exclusive aim of distinguishing between tumor- and host-specific molecular alterations in connection with the presence/absence of BC. With the present amendment, samples collected at t0 will be analysed from patients assessed as with stage I-IIA tumor at surgery (T1N0 or T2N0 or T1N1a), allowing an effective expansion of the sample size, while remaining in the setting of early stage BC. Furthermore, the collection and analysis of t1 samples will include all patients diagnosed as with invasive BC and sampled at t1, without limitations in terms of tumor stage assessed at surgery. The analysis of t1 samples, coupled with patients' longitudinal monitoring performed as per normal clinical practice, will allow the assessment of the accuracy of a multi-analyte evaluation for the prediction of BC recurrence, added as exploratory aim of the present study.

2.10. Study current status

The recruitment phase of the research project started on January 18th 2021. To date (October 21st 2021), 183 patients undergoing diagnostic biopsy were recruited. Of these, 98 presented

a benign lesion, while 51 presented a malignant lesion. Thirty-four patients were ruled out. Of the recruited patients with the histological diagnosis of BC, 35 underwent breast surgery. Of these, 23 patients had a stage I-IIA breast tumor. One hundred and ten patients with negative mammography were recruited as healthy controls. Of these, 20 patients were ruled out. The recruitment phase of the study will end by March 2022. Proteomics, ctDNA, and cfMeDIP-seq experiments will be performed during the second year of the research project. The third year of the research project will be dedicated to assess the performance of the individual classifiers, including the one based on radiomics. During the fourth year we will proceed to the wet lab validation, besides performing the transcriptomic experiments. The fifth and final year of the research project will be dedicated to build and optimize the HDI classifier.

2.11. Patient and Public Involvement

There was no patient or public involvement in the design of this study.

3. Ethics and dissemination

Written informed consents are obtained from each study participant. Participant information sheet includes the main information of the study protocol, the known side effects and any risk implied in the participation to the study, beside the contact information of study investigators. All data are deidentified and no patient-related information will be revealed during analysis. The Regione Liguria Ethics Committee c/o Ospedale Policlinico San Martino has approved the study (reference number: 2019/75, study ID: 4452).

All information concerning patients included in this study are covered by strict confidentiality in compliance with the General Data Protection Regulation EU 2016/679 (GDPR) and D.lgs. 30.06.2003, n. 196, as modified from D.lgs. 10.08.2018, n. 101. The study is conducted in accordance with the national law and according to international guidelines for the conduction of clinical trials referred to as “Good Clinical Practice”.

Results will be published in international peer-reviewed scientific journals.

4. DISCUSSION

The assessment of circulating biomarkers for clinical purposes is currently burdened by several issues, including suboptimal accuracy and the lack of standardized pre-analytical and analytical procedures. Overcoming these limitations would allow the achievement of minimally invasive and personalized assays for the management of neoplastic patients, either in the diagnostic setting or in the early detection of recurrence or the prediction of the response to therapy, possibly replacing or implementing current protocols based on radiology and traditional tissue biopsies. One of the most effective strategies carried out to enhance the accuracy of liquid biopsies is the contemporary assessment of multiple analytes, which has already shown augmented sensitivity and specificity compared to the evaluation of the individual biomarkers [30]. Such implementation can occur at different levels of complexity, involving different kinds of integration. A basic integration can be referred to the combination of biomarkers of the same kind, such as DNA-DNA or protein-protein combinations. On the other hand, an advanced integration refers to the combined assessment of different kinds of analytes, such as DNA-protein, or the combination of circulating biomarkers with radiological procedures possibly refined with radiomics [30].

The purpose of this study is to evaluate the performance of the combination of multiple circulating biomarkers, either plasmatic or urinary, and radiomics for BC early diagnosis in patients recruited from a real-life clinical setting. At the enrollment, patients undergo a rigorous selection in order to avoid possible confounding factors that can affect the assessment of tumor-specific cfMeDNA, circulating RNA, and proteins. Only samples collected from patients presenting an early-stage disease (I and IIA) at diagnosis will be analysed for this aim. This selection allows us to assess the effective potential of current techniques for the evaluation of circulating biomarkers in the early diagnosis of the most common cancer worldwide. Conversely, there will be no limits based on tumor stage for the analysis of circulating biomarkers collected after surgery, this being aimed at the prediction of BC recurrence.

This study is not designed for the detection of *in situ* BCs, which are considered as benign lesions and will be included in the control group. This represents indeed the main limitation of

this protocol, given the typical transition of *in situ* BC to invasive cancer and the consequent necessity of its early detection and eradication.

However, the protocol outlined above presents relevant advantages compared to other studies aimed at the same purpose, including the application of recent and cutting-edge techniques to a selected but realistic cohort of patients, possibly bringing to effective advancements in current standards BC patients management, besides directing future researches in the panorama of translational medicine applied to oncology.

Author statement. GZ conceived the study. FR wrote the manuscript. GC and MD provided a significant contribution in the section “Methods and analysis” . MG, VGV, PF, DF, MC, AB, AT, and LF critically reviewed the manuscript draft.

Funding statement. This trial is entirely supported by a Fondazione AIRC per la Ricerca sul Cancro, Investigator Grant ID 21761 to Gabriele Zoppoli.

Competing interests. The authors declare no conflict of interest for the present study.

Figure legends

Figure 1. Study diagram. Blood and urine samples will be collected from patients yielding a radiological breast lesion ≤ 2 cm with no evidence of lymph node dissemination (radiological T1N0). Images and samples acquired from patients with stage I-IIA (T1N0 or T2N0 or T1N1a neoplasia) BC at surgery will be analysed for diagnostic purposes together with images and samples acquired from patients yielding benign breast lesions and from patients with negative mammography. Blood and urine samples will be re-collected from all patients yielding invasive neoplasia at diagnosis after surgery at the first oncologic visit, and will be analysed for the prediction of BC recurrence.

Figure 2. Sample size diagram. Approximately 1,500 breast biopsies per year are performed at the Diagnostics Senology Unit of San Martino Hospital. Of a projected number of 750 liquid

biopsies, we foresee to collect samples and acquire mammograms from at least 49 patients with stage I-IIA BC, and 98 patients with radiological size-matched lesions, along with those samples and images acquired from 100 healthy women with two consecutive negative mammograms.

Biometrics	**Voluptuous habits**	**Endocrine status**	**Predisposition**	**Comorbidities**
Height	Does/Did the patient smoke?	Is the patient in the pre- or post menopause phase?	Does the patient present predisposing mutations for cancer?	Does the patient present comorbidities?
Weight	If yes, for how many years?	Age at menarche	If yes, which one	If yes, which ones?
BMI	Packets/day	Number of pregnancies	Does the patient have familiarity for breast and/or ovarian cancer?	
	Years since the last cigarette	Does the patient assume substitutive endocrine therapy?		
	How many alcohol units does the patient assume per day?	If yes, for how many years?		
		Does the patient assume endocrine contraceptive therapy?		
		If yes, for how many years?		

Tab. 1. Items investigated at the recruitment. Along with items concerning the inclusion and exclusion criteria, information of interest for the assessment of BC risk and possible interference with the evaluation of circulating biomarkers will be annotated.

References

1 Sprague BL, Arao RF, Miglioretti DL, *et al.* National Performance Benchmarks for Modern Diagnostic Digital Mammography: Update from the Breast Cancer Surveillance Consortium. *Radiology* 2017;**283**:59–69. doi:10.1148/radiol.2017161519

2 Tagliafico AS, Calabrese M, Mariscotti G, *et al.* Adjunct Screening With Tomosynthesis or Ultrasound in Women With Mammography-Negative Dense Breasts: Interim Report of a Prospective Comparative Trial. *J Clin Oncol* 2016;**34**:1882–8. doi:10.1200/JCO.2015.63.4147

3 Chung W, Eum HH, Lee H-O, *et al.* Single-cell RNA-seq enables comprehensive tumour and immune cell profiling in primary breast cancer. *Nat Commun* 2017;**8**:15081. doi:10.1038/ncomms15081

4 Bettegowda C, Sausen M, Leary RJ, *et al.* Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci Transl Med* 2014;**6**:224ra24. doi:10.1126/scitranslmed.3007094

5 Cohen JD, Li L, Wang Y, *et al.* Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* 2018;**359**:926–30. doi:10.1126/science.aar3247

6 Best MG, Sol N, Kooi I, *et al.* RNA-Seq of Tumor-Educated Platelets Enables Blood-Based Pan-Cancer, Multiclass, and Molecular Pathway Cancer Diagnostics. *Cancer Cell* 2015;**28**:666–76. doi:10.1016/j.ccell.2015.09.018

7 Bhome R, Del Vecchio F, Lee G-H, *et al.* Exosomal microRNAs (exomiRs): Small molecules with a big role in cancer. *Cancer Lett* 2018;**420**:228–35. doi:10.1016/j.canlet.2018.02.002

8 Shen SY, Singhania R, Fehringer G, *et al.* Sensitive tumour detection and classification using plasma cell-free DNA methylomes. *Nature* 2018;**563**:579–83. doi:10.1038/s41586-018-0703-0

9 Kalluri R. The biology and function of exosomes in cancer. *J Clin Invest* 2016;**126**:1208–15. doi:10.1172/JCI81135

10 Valdora F, Houssami N, Rossi F, *et al.* Rapid review: radiomics and breast cancer. *Breast Cancer Res Treat* 2018;**169**:217–29. doi:10.1007/s10549-018-4675-4

11 Bhawal R, Oberg AL, Zhang S, *et al.* Challenges and Opportunities in Clinical Applications of Blood-Based Proteomics in Cancer. *Cancers* 2020;**12**. doi:10.3390/cancers12092428

12 Alimirzaie S, Bagherzadeh M, Akbari MR. Liquid biopsy in breast cancer: A comprehensive review. *Clin Genet* 2019;**95**:643–60. doi:10.1111/cge.13514

13 Aravanis AM, Lee M, Klausner RD. Next-Generation Sequencing of Circulating Tumor DNA for Early Cancer Detection. *Cell* 2017;**168**:571–4. doi:10.1016/j.cell.2017.01.030

14 Bennett NC, Farah CS. Next-generation sequencing in clinical oncology: next steps towards clinical validation. *Cancers* 2014;**6**:2296–312. doi:10.3390/cancers6042296

15 Stirzaker C, Taberlay PC, Statham AL, *et al.* Mining cancer methylomes: prospects and challenges. *Trends Genet* 2014;**30**:75–84. doi:10.1016/j.tig.2013.11.004

16 Salomon MP, Orozco JIJ, Wilmott JS, *et al.* Brain metastasis DNA methylomes, a novel resource for the identification of biological and clinical features. *Sci Data* 2018;**5**:180245. doi:10.1038/sdata.2018.245

17 Anfossi S, Babayan A, Pantel K, *et al.* Clinical utility of circulating non-coding RNAs - an update. *Nat Rev Clin Oncol* 2018;**15**:541–63. doi:10.1038/s41571-018-0035-x

18 Koh W, Pan W, Gawad C, *et al.* Noninvasive in vivo monitoring of tissue-specific global gene expression in humans. *Proc Natl Acad Sci U S A* 2014;**111**:7361–6. doi:10.1073/pnas.1405528111

19 AACR Project GENIE Consortium. AACR Project GENIE: Powering Precision Medicine through an International Consortium. *Cancer Discov* 2017;**7**:818–31. doi:10.1158/2159-8290.CD-17-0151

20 Nuzzo PV, Berchuck JE, Korthauer K, *et al.* Detection of renal cell carcinoma using plasma and urine cell-free DNA methylomes. *Nat Med* 2020;**26**:1041–3. doi:10.1038/s41591-020-0933-1

21 Nassiri F, Chakravarthy A, Feng S, *et al.* Detection and discrimination of intracranial tumors using plasma cell-free DNA methylomes. *Nat Med* 2020;**26**:1044–7. doi:10.1038/s41591-020-0932-2

22 Shen SY, Burgener JM, Bratman SV, *et al.* Preparation of cfMeDIP-seq libraries for methylome profiling of plasma cell-free DNA. *Nat Protoc* 2019;**14**:2749–80. doi:10.1038/s41596-019-0202-2

23 Webber J, Stone TC, Katilius E, *et al.* Proteomics analysis of cancer exosomes using a novel modified aptamer-based array (SOMAscan™) platform. *Mol Cell Proteomics* 2014;**13**:1050–64. doi:10.1074/mcp.M113.032136

24 Raffield LM, Dang H, Pratte KA, *et al.* Comparison of Proteomic Assessment Methods in Multiple Cohort Studies. *Proteomics* 2020;**20**:e1900278. doi:10.1002/pmic.201900278

25 Lambin P, van Stiphout RGPM, Starmans MHW, *et al.* Predicting outcomes in radiation oncology--multifactorial decision support systems. *Nat Rev Clin Oncol* 2013;**10**:27–40. doi:10.1038/nrclinonc.2012.196

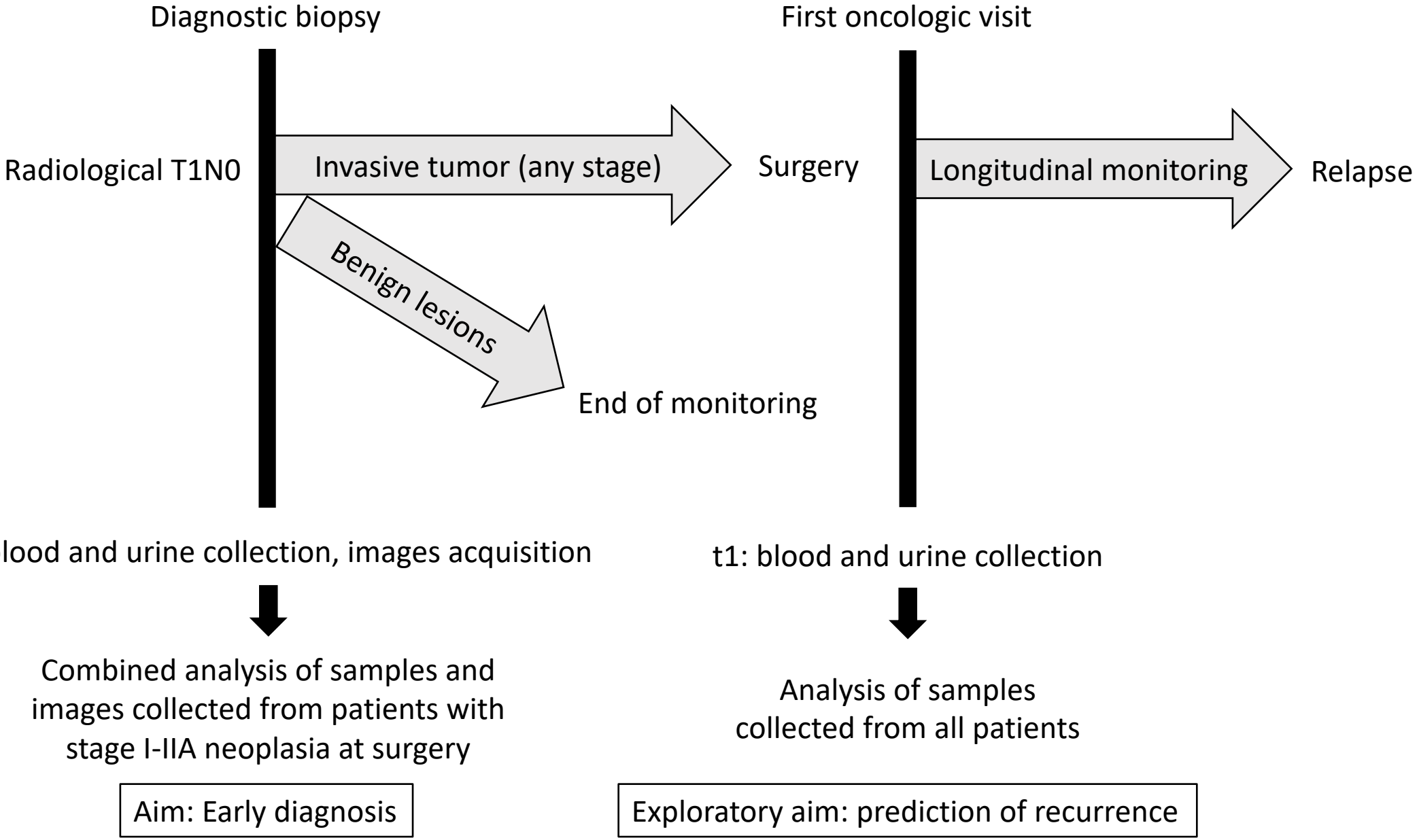
26 Dietterich TG. Machine-learning research; four current directions. *https://go.gale.com › i.dohttps://go.gale.com › i.do* 1997;**18**:97+.https://go.gale.com/ps/i.do?id=GALE%7CA20475202&sid=googleScholar&v=2.1&it=r&linkaccess=abs&issn=07384602&p=AONE&sw=w

27 Thrun S, Pratt L. Learning to Learn: Introduction and Overview. In: Thrun S, Pratt L, eds. *Learning to Learn*. Boston, MA: : Springer US 1998. 3–17. doi:10.1007/978-1-4615-5529-2_1

28 Dobbin KK, Simon RM. Sample size planning for developing classifiers using high-dimensional DNA microarray data. *Biostatistics* 2007;**8**:101–17. doi:10.1093/biostatistics/kxj036

29 Dobbin KK, Zhao Y, Simon RM. How large a training set is needed to develop a classifier for microarray data? *Clin Cancer Res* 2008;**14**:108–14. doi:10.1158/1078-0432.CCR-07-0443

30 Qiu J, Xu J, Zhang K, *et al.* Refining Cancer Management Using Integrated Liquid Biopsy. *Theranostics* 2020;**10**:2374–84. doi:10.7150/thno.40677


Diagnostic biopsy
First oncologic visit
Radiological T1N0
Invasive tumor (any stage)
Surgery
Longitudinal monitoring
Relapse
Benign lesions
End of monitoring
t0: blood and urine collection, images acquisition
t1: blood and urine collection
Combined analysis of samples and images collected from patients with stage I-IIA neoplasia at surgery
Analysis of samples collected from all patients
Aim: Early diagnosis
Exploratory aim: prediction of recurrence