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Liquid biopsies and patient-reported outcome measures for integrative monitoring of patients with early-stage breast cancer: a study protocol for the longitudinal observational Prospective Breast Cancer Biobanking (PBCB) study

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ABSTRACT

Introduction Breast cancer is still the most common malignancy among women worldwide. The Prospective Breast Cancer Biobank (PBCB) collects blood and urine from patients with breast cancer every 6 or 12 months for 11 years from 2011 to 2030 at two university hospitals in Western Norway. The project aims to identify new biomarkers that enable detection of systemic recurrences at the molecular level. As blood represents the biological interface between the primary tumour, the microenvironment and distant metastases, liquid biopsies represent the ideal medium to monitor the patient's cancer biology for identification of patients at high risk of relapse and for early detection systemic relapse.
Including patient-reported outcome measures (PROMs) allows for a vast number of possibilities to compare PROM data with biological information, enabling the study of fatigue and Quality of Life in patients with breast cancer.

Methods and analysis A total of 1455 patients with early-stage breast cancer are enrolled in the PBCB study, which has a one-armed prospective observational design. Participants consent to contribute liquid biopsies (i.e., peripheral blood and urine samples) every 6 or 12 months for 11 years. The liquid biopsies are the basis for detection of circulating tumour cells, circulating tumour DNA (ctDNA), exosomal micro-RNA (miRNA), miRNA in Tumour Educated Platelet and metabolomic profiles. In addition, participants respond to 10 PROM questionnaires collected annually. Moreover, a control group comprising 200 women without cancer aged 25–70 years will provide the same data.

Ethics and dissemination The general research biobank PBCB was approved by the Ministry of Health and Care Services in 2007, by the Regional Ethics Committee (REK) in 2010 (#2010/1957). The PROM (#2011/2161) and the biomarker study PerMoBreCan (#2015/2010) were approved by REK in 2011 and 2015 respectively. Results will be published in international peer reviewed journals. Deidentified data will be accessible on request.

Trial registration number NCT04488614.

Strengths and limitations of this study

- Prospective Breast Cancer Biobank (PBCB) is a population-based observational study of more than 1000 breast cancer patients with a follow-up of 11 years.
- PBCB collects information from ten patient-related oucome measures yearly, including fatigue and health-related quality of life scores.
- PBCB provides liquid biopsies every 6 months, allowing for analysis of an extensive repertoire of liquid biomarkers
- Some patients will retract from the project due to study attrition, which may introduce a bias.
- Not all variables have undergone statistical power analysis.

INTRODUCTION

Breast cancer remains the world's most frequent female malignancy. The last 50 years, the incidence has been more than doubled leading to 2 088 849 new cases registered worldwide.[1] Likewise, among the 5.3 million population in Norway 3726 women were diagnosed with breast

cancer in 2019.[2] Furthermore, due to both early and late relapses (>5 year after diagnosis), 626 679 global deaths and 650 deaths in Norway occurred in 2019.[2] Moreover, in 2019, there was a prevalence of 51 192 of breast cancer survivors in Norway.

Hence, the classical prognostic and predictive factors such as tumour (T),lymph nodes(N) and metastasis(M) stage, oestrogen receptor (ER) and progesterone receptor (PR) status, histological grade, the proliferation marker Ki67 and human epidermal growth factor receptor-2 (HER-2) amplification seem to be insufficient for optimal treatment decisions to cure systemic disease.[3 4] This is due to primary tumour heterogeneity or evolutionary changes in residual tumour cells during therapy.[5] Clearly, new biomarkers for detection of systemic relapse are urgently needed for more accurate diagnostics to guide treatment and to detect patients with systemic spread at the earliest possible time point.[6]

As blood represents the biological interface between the primary tumour, the microenvironment and the distant metastases it forms an ideal medium as liquid biopsies to monitor the patient's cancer biology.[7] Until a clinical overt metastasis is detectable, blood is the only real intermediate medium that can be investigated. It is therefore a paradox that current practice does not include searches for early systemic relapse in blood in early-stage / operable breast cancer patients. In light of this, we have designed this study protocol with the aim to establish methods and biomarkers to detect relapses on the molecular level. Such approaches are circulating tumour cells (CTC), circulating tumour DNA (ctDNA), micro-RNA (miRNA) and metabolomics, which are further described below.

Circulating tumour cell

CTCs are tumour cells that have detached from the primary tumour either by passive tumour cell shedding, or by an active mechanism involving the epithelial-to-mesenchymal transition.[8] CTC count provides independent prognostic information both in operable and metastatic breast cancer.[9 10]

ctDNA

ctDNA is cell-free tumour-derived DNA present in the plasma.[11] It may originate from apoptotic and necrotic tumour cells in the primary tumour, metastases or circulation, as well as being directly released from viable cells.[12]Substantial evidence supports the idea that ctDNA detection may provide therapeutically relevant, predictive and prognostic information in breast cancer.[13 14] Recent evidence suggests that residual tumour cell material can be found in peripheral blood samples and function as possible biomarkers. When comparing genetic aberration from primary tumours with metastases from the same patient reports show that many of the acquired driver mutations detected in the metastases cannot be found in the primary tumour.[15 16]

miRNA, extracellular vesicles and platelets

miRNAs are small, noncoding RNA molecules that control gene expression. They are remarkable stable in plasma and serum.[17] Recently, miRNAs have been found in exosomes,[18–20] which are small extracellular vesicles (sEV) with a diameter of 40–150 nm.[21] Data also suggest that sEVs may act as messengers of metastasis and assist in the formation of metastasis by 'priming' the metastatic site to create a microenvironment that is conducive for cancer cells (The premetastatic niche hypothesis).[22] Analysis of miRNAs in patients with blood of breast cancer can provide an novel way for long-term monitoring of tumour related miRNAs.[21] Recently the use of exosomal miRNAs from urine for the detection of breast cancer has been described as well.[23]

More recently, platelets have emerged as central players in the systemic and local responses to tumour growth.[24] Confrontation of platelets with tumour cells via transfer of tumour-associated biomolecules ('education") is an emerging concept and results in the term tumour educated platelets (TEPs). It has become apparent that TEPs absorb protein and RNA molecules from tumours, possibly playing a role in tumour growth and metastasis.[25]

Metabolomics

Metabolomics is the study of small molecules comprising substrates, intermediates and end- products of cellular metabolism, such as amino acids, sugars and small organic acids. The metabolic state of cancer cells is substantially altered compared with normal cells,[26 27] a fact that can be used for diagnostic purposes. Specific metabolic signatures from tumour tissue provide additional information for determination of breast cancer subtypes and prediction of outcome.[26 28–30] Recently, we have shown that systemic lactate and pyruvate levels predict inferior outcome in patients with operable ER-positive breast cancers.[31] Metabolomic analyses of primary tumours have also demonstrated predictive value in relation to neoadjuvant treatment of patients with locally advanced disease.[28] So, there is proof of principle showing the use of preoperative blood and urine samples for metabolic profiling in patients with breast cancer.[25 32]

Concept of detection of molecular relapse in breast cancer

Based on the described molecular biomarkers, we propose to establish a method for early detection of systemic relapse in patients with early-stage breast cancer. We recommend calling this type of recurrence a 'molecular relapse' as it is only detectable by molecular biological methods. The model in figure 1 presents our main hypothesis in the Prospective Breast Cancer Biobank (PBCB). We propose that it is possible to find a molecular relapse in the blood before the recurrence is clinically or radiologically detectable.

Patients-reported outcome measures

Healthcare has shifted towards an increasingly value-based framework for quality-of-care improvement during recent

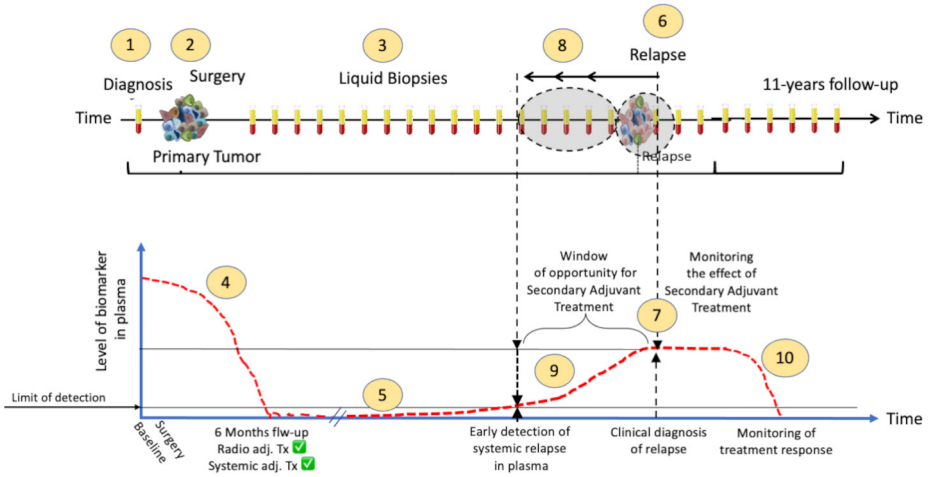


Figure 1 Overview of the concept of molecular systemic relapse detection by analysis of liquid biopsies in the PBCB project. Blood and tumour samples are biobanked at diagnosis (1) and surgery (2). Blood samples continue to be drawn every 6 months for 11 years (3). The blood samples will allow for CTC, ctDNA, miRNA andmetabolite analysis (4). Following surgery and treatment, the serum level of ctDNA is too low for detection (5). At the time of macroscopic clinical or radiological detected relapse (6) the liquid biopsies (7) are carefully examined for various biomarkers. The findings are validated by examination of the primary tumour (2). Then, the previously drawn liquid biopsies (8) are carefully examined to find how many months ahead of the macroscopic relapse molecular biomarkers may be found in the liquid biopsies (9). The biomarkers in the liquid biopsies may be used to monitor the effect of secondary adjuvant treatment (10). Red dotted line: plasma level of the biomarker indicating systemic relapse in liquid biopsies. Green check mark, indicating that all systemic adjuvant treatments are initiated. CTC, circulating tumour cell; ctDNA, circulating tumour DNA; miRNA, micro-RNA; PBCB, Prospective Breast Cancer Biobank.

decades. As a consequence, more attention has been paid to patient-reported outcomes.[33] The information gained from patient-reported outcome measures (PROMs) comes directly from the patients without intermediate processing.[34] Although PROMs are increasingly collected in breast cancer care, studies reporting on the implementation of systematic PROM administration, including methods and challenges, are less than frequent.[35] PROMs consist of questionnaires that may be single item, unidirectional or multidirectional.[36] They may be collected by several methods, locations and at repeated time points. Systematic PROM collection in breast cancer care is feasible and has a promising results in multiple outcome areas (eg, adherence, satisfaction and clinical decision making) on multiple levels (patients, care providers and care processes).[35] Thus, PROMs may add valuable information in monitoring of psychosocial disease burden in breast cancer survivors.

METHODS AND ANALYSIS

Aims

The primary aim of the PBCB is to develop a diagnostic tool to detect systemic relapses in patients with early-stage breast cancer on the earliest possible level (molecular level). The secondary aim is to map the long-term psychosocial disease burden in breast cancer survivors.

Design and setting

The PBCB project is a one-armed prospective long-term observational study of patients with early-stage breast cancer in Western Norway. The follow-up time is 11 years. The present paper is a protocol paper outlining the plan of the PBCB study and follows the guidelines for preparation outlined by the BMC. We have performed some analyses, and the results are pending for publication.

Characteristics of patients and normal controls

The PBCB project is a regional collaboration between Haukeland (HUH) and Stavanger University Hospitals (SUH), in Western Norway. Approximately 550 patients with breast cancer are diagnosed annually at HUH and SUH, approximately 310 and 220, respectively. Women diagnosed with ductal carcinoma in situ (DCIS) or early-stage breast cancer (pT1-pT2/pN0pN1/M0) at HUH and SUH were consecutively invited to participate in the PBCB-project. The clinic-pathological features of our study population are shown in table 1. Norwegian citizens have unique personal social security numbers allowing coupling of information to various nationwide registries including the Norwegian Cancer Registry, the Cause of Death Registry, Norwegian Prescription Database,[37] the Norwegian Labour and Welfare Administration Database.[38] Therefore, a complete follow-up of the patients is facilitated, creating a robust population-based prospective biobank of women with early-stage breast cancer.

The enrolment of the control cohort of women without breast cancer commenced at 1 September 2011 at SUH and is estimated to last until 1 September 2032. From these women, blood will be sampled once, and they will fill in the questionnaires once (see Section 3.2).

Inclusion and exclusion criteria

Inclusion criteria for participation in the PBCB project are women of all ages, who are diagnosed preoperatively with early-stage breast cancer. Some of these patients

Table 1 Clinical and pathological features of the PBCB population

Clinical pathological Feature	No HUH	HUH (%)	No SUH	SUH (%)	No overall	Overall (%)
Enrolled	1104	75.9	351	24.1	1455	100
Age (mean)	57.9		58.6		58.3	
Age (median)	58.0		59.0		58.5	
Age: range	23–86		24–90		23–90	
<35 years	11	1.0	3	0.9	14	0.96
36–55 years	417	37.8	123	35.0	540	37.1
55–69 years	537	48.6	181	51.6	717	49.3
≥70 years	139	12.6	44	12.5	183	12.6
Missing	0	0	0	0.0	0	0.0
DCIS	20	1.9	30	8.5	50	3.4
pT1mic	2	0.2	3	0.9	5	0.3
pT1a	43	3.9	8	2.3	51	3.5
pT1b	147	13.3	52	14.8	199	13.6
pT1c	430	38.9	141	40.2	571	39.2
pT2	304	27.5	110	31.3	414	28.5
pT3	71	6.4	2	0.6	79	5.4
PT4a	10	0.9	0	0.0	10	0.7
pT4b	2	0.18	0	0.0	2	0.1
Missing*	75	6.8	5	1.4	80	5.5
pN0	766	69.4	238	67.8	1004	69.0
pN1mic	2	0.2	6	1.7	8	0.5
pN1	213	19.2	60	17.1	273	18.8
pN2	29	2.6	17	4.8	46	3.2
pN3	13	1.2	4	1.1	17	1.2
pN positive	257	23.2	87	24.8	344	25.9
Missing*	81	7.3	26	7.4	107	7.4
ER pos	916	83.0	271	77.2	1187	81.6
ER neg	124	11.2	14	4.0	138	9.5
Missing*	64	5.8	66	18.8	130	8.9
PR pos	794	71.9	229	65.2	1020	70.1
PR neg	229	20.7	84	23.9	313	21.5
Missing	81	7.3	38	10.8	119	12.2
HER2 neg	917	83.1	285	81.2	1202	82.6
HER2 pos	121	11.0	28	8.0	149	10.2
Missing*	66	6.0	38	10.8	104	7.2
Ki65 <15%	171	15.5	72	20.5	243	16.7
Ki67 15%–29%	280	25.4	102	29.1	382	26.3
Ki67 ≥30%	263	23.8	156	44.4	419	28.8
Missing*	390†	35.3	21	6.0	411	28.2
MAI<10‡	–	–	210	59.8	210	59.8
MAI≥10	–	–	91	25.9	91	25.9
Missing*	–	–	50	14.2	50	14.2
Grade 1	251	22.3	69	19.7	320	22.3
Grade 2	420	38.0	117	33.3	537	37.3

Continued

Table 1 Continued

Clinical pathological Feature	No HUH	HUH (%)	No SUH	SUH (%)	No overall	Overall (%)
Grade 3	254	23.0	124	35.3	378	26.3
Missing	179	16.2	41	6.8	203	14.1
BCT	430	38.9	237	67.5	667	45.6
Mastectomy	648	58.7	112	31.9	760	52.0
Missing	26	2.4	9	2.6	35	2.4

*The high number of missing data is due to inclusion of DCIS cases.
†Ki-67 was not implemented in the pathology reports until 2013.
‡MAI is not used at HUH.
BCT, breast conservative treatment; DCIS, Ductal Carcinoma in situ; ER, oestrogen receptor; Grade, Histological Grade; HER2, human epidermal growth factor receptor-2; HUH, Haukeland University Hospital; MAI, Mitotic Activity Index; PBCB, Prospective Breast Cancer Biobank; pN, pathological node status; PR, progesterone receptor; pT, pathological tumour status; SUH, Stavanger University Hospital.

turn out to be diagnosed with DCIS. However, they are offered the same follow-up in the PBCB study as the invasive cancers. Interestingly, these patients will represent an internal control group for the effect of surgery and radiation. Likewise, some pT3 and pT4 tumours are included as their clinical tumour stage was cT2.

Exclusion criteria are patients with a prior history of malignancy. Furthermore, patients with cT3, cT4 tumours or any other reason for preoperative downstaging will be omitted from inclusion. Also, patients unable to communicate due to diseases (eg, Alzheimer) or language barriers (eg, immigrants) are also excluded. Moreover, long travel distance or patient flow that exceed the logistic capacity of the research team are reasons for exclusions from participation in the study. The general enrolment into PBCB is finalised and recruitment is now by invitation only (mastectomies at SUH). The drop-out is approximately 14% and 21% at SUH and HUH, respectively. An overview of the patient flow is shown in figure 2.

The patient and tumour characteristics in the PBCB-cohort were compared with the corresponding characteristics for all patients with breast cancer diagnosed in the same time period the PBCB-patients were enrolled. The mean age of the PBCB patients was 58 years while the average age of patient with breast cancer in Western Norway was 60 years (p=0.012). However, there were no differences in tumour size (p=0.68), nodal status(p=0.31), histological grade (p=0.14), ER (p=0.09), PR (p=0.42), HER-2 (p=0.57) between the PBCB cohort and the treated patients in Western Norway in the same time period as the PBCB material was collected. However, when it comes to several tumour related parameters, we observe a heterogeneity between the study sites. For example, there is more DCIS at SUH than at HUS. Moreover, the frequencies of histological grade 3, PR negativity, HER-2 negativity and triple negative cancer are higher in SUH than in HUH (table 1). Interestingly, when comparing these differences with national data on breast cancer from 2014[39] and 2020[40] and we find a concordant heterogeneity between HUH and SUH on differences of the above-mentioned biomarkers in this study. Despite some missing data, this distribution supports a population-based patient accrual in the PBCB study.

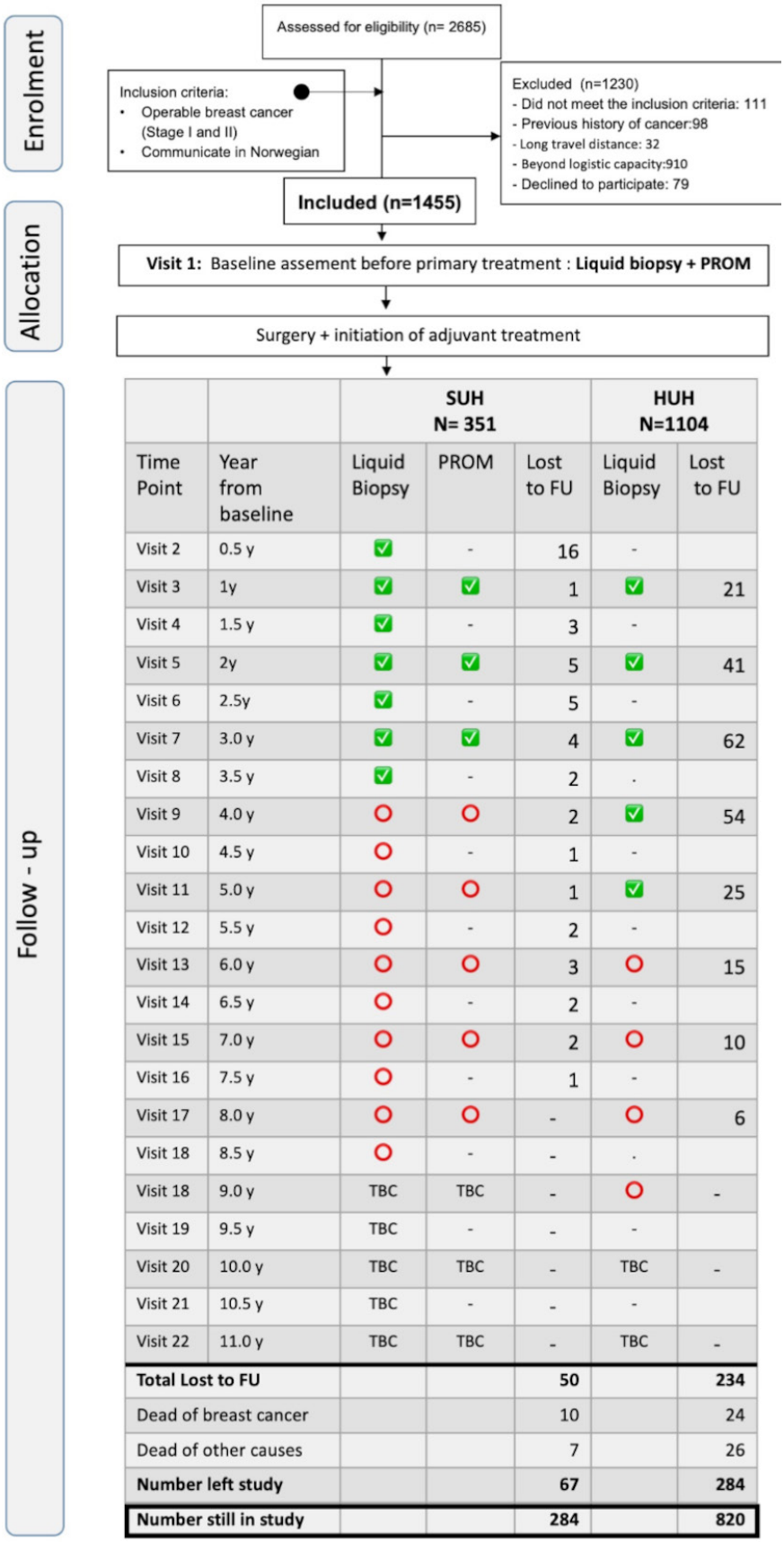
Characteristics of the control group

In the PBCB project, we will accrue up to 200 healthy women (now 87) without breast cancer from the Norwegian mammography screening programme who will serve as a control group. The enrolled women will be between 25 and 70 years of age. In this cross-sectional study, these women will also provide the same package of liquid biopsies as the patients with breast cancer participating in PBCB. Moreover, they will fill out an adapted questionnaire package with the same PROM-questions as the patients with breast cancer provide. Thus, these healthy women will serve as important reference points.

Patients and public involvement

When the study started, the use of patient in planning studies was not very well established in Norway. Later, However, the patients were involved in study planning including patient priorities. Furthermore, the PBCB group at SUH maintains an active collaboration with the regional breast cancer society, which represents all patients with breast cancer in our region. Two society representatives are included in regular PBCB meeting as active partners of the user involvement programme and have provided valuable input into several aspects of the PBCB project including recruitment and conduct of the study.

Dissemination of the results from this project towards the scientific and medical communities will include publication of scientific papers in international peer-reviewed scientific journals. Project results will also be presented at international conferences, as well as communication to the public through national and regional newspapers and websites like www.forskning.no and our home page (www.sus.no). Results will also be presented at scenes of patient enquiry, such as the Norwegian Cancer Society and the National Network of Breast Cancer Research, to strengthen the breast cancer research, inspire user



		SUH N= 351			HUH N=1104	
Time Point	Year from baseline	Liquid Biopsy	PROM	Lost to FU	Liquid Biopsy	Lost to FU
Visit 2	0.5 y	✅	-	16	-	
Visit 3	1y	✅	✅	1	✅	21
Visit 4	1.5 y	✅	-	3	-	
Visit 5	2y	✅	✅	5	✅	41
Visit 6	2.5y	✅	-	5	-	
Visit 7	3.0 y	✅	✅	4	✅	62
Visit 8	3.5 y	✅	-	2	.	
Visit 9	4.0 y	○	○	2	✅	54
Visit 10	4.5 y	○	-	1	-	
Visit 11	5.0 y	○	○	1	✅	25
Visit 12	5.5 y	○	-	2	-	
Visit 13	6.0 y	○	○	3	○	15
Visit 14	6.5 y	○	-	2	-	
Visit 15	7.0 y	○	○	2	○	10
Visit 16	7.5 y	○	-	1	-	
Visit 17	8.0 y	○	○	-	○	6
Visit 18	8.5 y	○	-	-	.	
Visit 18	9.0 y	TBC	TBC	-	○	-
Visit 19	9.5 y	TBC	-	-	-	
Visit 20	10.0 y	TBC	TBC	-	TBC	-
Visit 21	10.5 y	TBC	-	-	-	
Visit 22	11.0 y	TBC	TBC	-	TBC	-
Total Lost to FU				**50**		**234**
Dead of breast cancer				10		24
Dead of other causes				7		26
Number left study				**67**		**284**
Number still in study				**284**		**820**

Figure 2 Overview of the study design (one armed observational study) and sampling protocol in the PBCB project. Number of patients at each study site lost to follow-up at each time point. ✅=Completed; ○=Ongoing; FU, follow-up; HUH, Haukeland University Hospital; PBCB, Prospective Breast Cancer Biobank; PROM, patient-reported outcome measure; SUH, Stavanger University Hospital; TBC, to be conducted.

involvement and provide research dissemination at an approachable level for the public.

Study period and follow-up

At HUH, patients with early-stage breast cancer were included from 25 January 2011 to 31 January 2017. Thus, the proportion of included patients in this period was 1104/1860 = 59%. At SUH, 351 patients were included into PBCB from 7 November 2013 to 25 August 2016 giving a proportion of 53% inclusion of total eligible patients. Average for both study sites is 54% inclusion. Follow-up at both sites will last until 31 January 2030 regarding questionnaires and blood samples. Clinical follow-up (ie, relapses) will last until 2050. Recruitment of healthy women included in the control group will be from 2 September 2020 to 2 September 2022 (n=200).

PROTOCOLS OF ANALYSES

Fresh frozen and paraffin-embedded tissue samples

At SUH, fresh frozen tumour tissue is obtained from the surgical specimens from tumours larger than 10 mm in diameter. Otherwise, paraffin embedded tumour tissue is available from the diagnostic biobanks at both HUH and SUH for all patients. DNA from primary tumours and available metastatic lesions will be isolated using the Allprep FFPE kit (Qiagen). The resulting DNA profile will be analysed by next-generation sequencing using the Oncomine Comprehensive Assay V.3 (ThermoFisher). This is an amplicon-based approach, which detects commonly occurring single nucleotide variants, insertions and deletions, and copy number variations in the tumour genome, especially in ER, p53 and other known oncogenes. These analyses will provide us with a biological understanding and background knowledge for each of the individual tumours.

Liquid biopsies

The sampling protocol of liquid biopsies is detailed in table 2. In brief, 60 mL blood and 20 mL urine samples are collected from each patient. The blood samples are divided and preserved into 31 aliquots while the urine samples are divided into 6 aliquots. All sample aliquots are stored at −80°C. Importantly, plasma is sampled at +4°C for assessment of cytokines and Cell Partitian Tube (CPT) tubes makes DNA isolation from blood lymphocytes possible. Isolation of CTCs and ctDNA is performed from blood and plasma samples collected and processed at room temperature.

Circulating tumour cells

CTCs will be enriched from peripheral blood samples by density centrifugation and subsequent immunomagnetic depletion of leucocytes using a new EpCAM-independent

Table 2 Sampling protocol of liquid biopsies in the PBCB project

Sample	Supplier	Volume	No of tubes	Centrifuge	Aliquots for storage
Serum	BD Vacutainer CAT	10 mL	2	1500g×25 min at RT	6×0.5 mL and 4×1.5 mL, stored at −80°C
EDTA-Whole Blood	Vacuette greiner bio-one	3 mL	1	–	2×1.5 mL, stored at −80°C
EDTA-plasma 'cold' (kept on ice)	Vacuette greiner bio-one	9 mL	1	2500g×10 min at 4°C	6×0.5 mL, 2×1.0 mL, 1×0.5 mL and buffy coat, stored at −80°C
EDTA-plasma (RT)	Vacuette greiner bio-one	9 mL	2	2200g×10 min at room temperature	6×0.5 mL and 2×1.5 mL, stored at −80°C
EDTA-platelets (kept on ice)	Vacuette greiner bio-one	9 mL	1	1. ii) 200g×20 min /10 min at 4°C *with no brake applied*. 2. 1000g×10 min at 4°C	1×0.5 mL, stored at −80°C
EDTA-CTC	Vacuette greiner bio-one	9 mL	1	CTCs enrichment by MINDEC negative depletion according to Lapin et al 2016.[27]	one tube with lysed CTCs in 350 µL RLT buffer, stored at −80°C
Vacutainer CPT	BD Vacutainer CPT Mononuclear Cell Preparation Tube	8 mL	1	i. 1500g×25 min at room tempetature ii. 1000g×10 min at 4°C	1×0.5 mL, stored at −80°C
PAX-gene blood RNA tube	PAXgene PreAnalytix	2.5 mL	1	–	Room temperature at 2–72 hours; −20°C for 24 hours; stored at −80°C
Urine		20 mL	1	–	6×1.5 mL, stored at −80°C

CAT, Clot Activator Tube; CPT, Cell Partitian Tube; PBCB, Prospective Breast Cancer Biobank; RLT, RNeasy Lysis cells and Tissue; RT, room temperature.

depletion strategy, termed MINDEC, developed previously.[41] This method has superior recovery and enrichment rates,[41] and enables isolation of both mesenchymal-like and epithelial-like CTCs.[42] The CTCs are lysed and subsequently detected by multimarker mRNA quantification.

Tumour educated platelets

miRNA in blood will be isolated from blood platelets, which have been in contact with tumour cells. This contact have resulted in uptake of miRNAs from the tumour into the TEPs.[43] Several studies have also shown that miRNAs from TEPs can be transferred and mediate biological effects in recipient cells.[43] An efficient protocol for miRNA isolation from platelets by using the using the miREASY kit (Qiagen) has been established. Preliminary data show that we are able to detect 10 tested miRNAs.

Circulating tumour DNA

In the PBCB project, we isolate cell-free DNA (cfDNA), will be isolated from 1 to 2 mL room temperate plasma samples by QIAamp circulating nucleic acid kit (Qiagen). The cfDNA samples are stored at stored at −80°C for subsequent sequencing of ctDNA.

Determination of miRNA in sEVs

Total RNA will be separated from sEVs in plasma samples using the exoRNeasy Serum/Plasma kit (Qiagen), which isolates sEVs and sEV-contained RNA in a two-step process. In a first phase, miRNA profiling will be performed on the isolated RNA with the Ion total RNA-seq kit V2 (Thermo Fisher) on our Ion Proton deep sequencing instrument. Preliminary results show that miRNAs are present in sEVs at measurable levels.

Although there are few studies that have analysed circulating exosomal miRNAs and TEPs in blood from patients with breast cancer, a recent study[44] reported on 11 exosomal miRNAs that were differentially expressed in patients with breast cancer with and without recurrence.

Thermolabile biomarkers

Certain biomarkers decay rapidly at room temperature and must be preserved by immediately cooling of the drawn blood sample on water ice, cooled centrifugation at 4°C, followed by aliquotation and storage in −80°C freezer without any delay.

Metabolomics

Serum will be used for the analysis of small molecules, hormones (eg, estradiol), drugs (eg, tamoxifen, aromatase inhibitors) and vitamins (eg, vitamin D) by using ELISA and liquid chromatography-tandem mass spectrometry methodology. Moreover, metabolites will be measured by MR spectroscopy at the MR Core Facility, Norwegian University of Science and Technology. Approximately 30 metabolites and 105 lipoprotein subfractions can be quantified in the serum.[45] The MR Core Facility collaborates with the vendor (Bruker BioSpin GmbH, Germany) to ensure efficient and validated acquisition protocols, making the method feasible for high throughput analysis and implementation into the clinic. Modelling biological pathways will be conducted by integrating multivariate statistics with biological databases (Human Metabolome Database, KEGG), using available online tools (Metaboanalyst).

Urine samples

Urine samples are aliquoted into 1.5 mL tubes and preserved in −80°C freezer for later analyses of various analytes excreted into the urine.

Biobanking

All samples are registered in a regional tracking system for biobank samples, Lab vantage. Several different parameters related to sampling are logged, for example, time of sampling, centrifugation and freezing, discrepancies, number of aliquots, etc. These data are available at all times. All biomarkers will be analysed in batches to avoid systematic errors and an unpredictable day-to-day variation. Long term storage of all analytes exerts a challenge of stability at −80°C.[46] For example, some miRNAs show a decay of 12% after 5 years of storage,[47] while others have shown only minor changes in micro-RNA at −80°C storage levels.[48] Moreover, metabolomics analytes like amino acids, sugars carnitine show a decay of 15% and increment of 14% during long term storage at −80°C during 5-year storage.[49] In this regard, thermolabile biomarkers like cytokines will be analysed first to avoid too much decay of the analytes. In contrast, ctDNA is more stable to long-term storage.[50] For analytes known to degrade over time, we will consider the effect of storage in our analyses.

PROM data (table 2) and clinical data (table 1) are all registered in an Access database, made and kindly provided by TB.

Preanalytical conditions and centralisation of analysis

To minimise differences in preanalytical condition between the two study sites the PBCB steering committee meet several times a year to monitor the collection protocol of the various liquid biopsies. Due to the heterogeneity in the PBCB-material between SUH and HUH, all analysis of the various biomarkers will we centralised to Stavanger. This will enhance control of the data quality.

MONITORING OF PSYCHOSOCIAL DISEASE BURDEN IN BREAST CANCER SURVIVORS

To enable identifying patients under high load of side effects and reduced quality of life (QoL), PROMs are collected from participants at SUH at baseline and yearly for 11 years. The PROM-data consists of (1) Basic health and clinical data; (2) health-related QoL (HRQoL) instruments (EORTC QLQ-C30, EORTC QLQ-BR23 and Functional Assessment of Cancer Therapy–Breast (FACT)); (3) Hospital Anxiety and Depression Scale, HADS; (4) Fatigue instruments=Fatigue Impact Scale; Fatigue Severity Scale and fatigue-Visual Analogue Scale; (5) Side effect questionnaires; (6) Joint-pain questionnaire; (7)

The Mishel Uncertainty in Illness Scale; (8) Diet questionnaire and (9) ROMA III questionnaire for irritable bowel complaints. Some of the PROMs require trained staff to guide the patient in their completion. At HUH, PROM data are obtained annually from a cohort of 160 patients. At SUH, 351 patients have completed the PROM data at baseline. Figure 2 shows how many patients that are lost during PROM data follow-up. PROM questionnaires are further described in table 3.

Scores of the questionnaires

We applied mean sum scores from SHC-Inventory and its five subscales, and the satisfaction with the quality of patient information given by the healthcare professionals. Sum scores of FACT-Endocrine Subscale and FACIT-F were used following the FACIT Organisation's scoring guidelines. We performed the reversals as indicated in the guidelines and summed individual items to obtain a score. The subscale scores were produced by multiplying the sum of each item score by the number of items in the subscale, and then divided by the numbers of items answered. In the FACIT scales, a high score means better social well-being, or fewer symptoms of fatigue or endocrine symptoms. As an example of the utilisation of the PROM-data we have shown that fatigue and depression overrule side effects of oncological treatment in predicting self-reported health complaints in breast cancer survivors.[51]

STATISTICAL AND POWER ANALYSIS

We will use univariate (Kaplan Meier) and multivariabel (Cox) for survival analysis for both molecular and ordinary relapses. If we anticipate that 30% of the patients are high-risk patients, that is, receive chemotherapy and with our 34 endpoints (relapse) pr. 1 January 2021 (figure 2), we will have an 80% power to detect a survival difference of HR=2.9 (alpha=0.05 and beta=0.80). Presently, we have a median follow-up of 5 years. During further follow-up, the number of endpoints will increase and consequently we will be able to detect a lower survival difference. As appropriate, we will use simple and multivariable linear regression, and logistic regression analysis.

THE PBCB STUDY GROUP ACRONYM

Research members of the PBCB study group are represented collectively under the acronym ' The PBCB Study Group', which can be used as a co-author in research papers where material from the PBCB biobank or PROM data are used. These researchers are:

Prof. Håvard Søiland MD, PhD, Prof. Gunnar Mellgren MD, PhD, Tone Hoel Lende MD, PhD, Anette Heie MD, Prof. Jørn V. Sagen MD, PhD, Prof. Emiel AM Janssen PhD, Kristin Jonsdottir PhD, Ann Cathrine Kroksveen MSc, Linda Sleire MSc, Thomas Helland PhD, Einar Gudlaugsson, MD, PhD, Prof. Oddmund Nordgård PhD, Kjersti Tjensvoll PhD, Satu Oltedal PhD, Bjørnar Gilje MD, PhD, Prof. Jan Terje Kvaløy PhD, Assoc. Prof. Kirsten Lode RN PhD, Prof. Birgitta Haga Gripsrud, PhD, Kari Britt Hagen RN, PhD, Marie Austdal, PhD, Siri Lunde MSc, Nina Gran Egeland PhD, Prof. & Chair Timothy L Lash, DSc, MPH, Finn Magnus Eliassen MD and Prof. emeritus Ernst A Lien MD, PhD

DISCUSSION

The overall aim of the PBCB is to better capture patients with early-stage breast cancer at high risk of systemic relapse and monitor the patients in order to identify the relapse at the molecular level.[52] With our planned follow-up time of 11 years, we expect to identify patients experiencing both early and late relapse. Previous studies have followed patients with breast cancer with PROM data for 2–3 years at most[53] It is reasonable to anticipate that detection of molecular relapses in liquid biopsies will give a novel opportunity to initiate secondary adjuvant treatment at an earlier time point than with the clinical approach of today.[6] Others have shown that ctDNA may be detected up to 10 months prior to the clinical relapse in patients with early breast cancer irrespective of molecular subtypes.[54] We will investigate CTC, ctDNA, TEPs, miRNA and metabolomics in both serum/plasma and urine. Thus, we combine five biomarker modalities to detect molecular relapses, which has not been done before. We anticipate that we will have a good chance of detecting molecular relapses in the earliest possible way. Considering that most relapses appear after 2–3 years[55] the best approach may be to sample blood every 4 months for 10 years. This would enable detection of molecular relapses treatable by secondary adjuvant treatment, including immune therapy[6 56] However, there are still several issues such as standardisation of protocols, which needs to be resolved before translation into clinical practice.[57] We aim to increase our knowledge regarding liquid biopsies in patients with early breast cancer.

Furthermore, PBCB is ideal for therapeutic drug monitoring. Recently, we have shown a reduced breast cancer specific and overall survival in patients with low levels of active tamoxifen metabolites.[58 59] The PBCB protocol will allow for measurement of drug metabolites every 6 months, allowing for monitoring of adherence and drug discontinuation.

The focus on disease burden in breast cancer survivors during an 11-year follow-up is to our knowledge the longest prospective observational study of patients with breast cancer using PROM data. The long follow-up creates a unique opportunity to join circulating biomarker data and PROM-data to the extensive Norwegian public patients' registries.[37 38 60] Although this long follow-up may produce bias by attrition,[61] we do not anticipate that attrition will become a problem in our study due to close follow-up by our dedicated study nurses.

In summary, the PBCB-project represents an interdisciplinary platform for future research projects on

Table 3 Overview of the various questionaires in the PBCB study

Questionnaire (abbrev)	Full name	Items	Scoring/scale	Remarks	References
EORTC-QLQ-C30	European Organisation for Research and Treatment of Cancer- Quality of Life Questionnaire-Cancer 30	30 All items address health issues during the last week	four point rating scale 0=not at all 1=a little 2=some 3=very much Total score range: 0–90	Cronbach's alpha-coefficient for multi-item scales ranged from 0.56 to 0.85, with emotional functioning having the highest Cronbach's alpha-coefficient. General health/QoL subscale was correlated significantly with all other subscales.	62
EORTC-QLQ BR23	European Organisation for Research and Treatment of Cancer-Quality of Life Questionnaire-Breast 23	23 All items address health issues during the last week	four point rating scale 0=not at all 1=a little 2=some 3=very much Total score range: 0–69	Cronbach's alpha ranged from 0.78 to 0.83 for the EORTC-BR23 questionnaire.	63 64
FACT-B	Functional assessment of Cancer Therapy - Breast	A 37-item instrument designed to measure five domains of HRQOL in patients with breast cancer: Physical, social, emotional, functional well-being as well as a breast-cancer subscale	five point rating scale 0=not at all 1=a little 2=some 3=a lot 4=very much Range: 0–148 According to the guidelines, all the questionnaires are drafted in a way that higher scores represent improved quality of life	Cronbach's alpha for the various constructs is behind 0.66 and 0.85	65
FACT-ES	Functional Assessment of Cancer Therapy-Endocrine Subscale	Contains 19 statements examining self-reported menopausal and sexual symptoms related to breast cancer endocrine therapy during the last week	five point rating scale 0=not at all 1=a little 2=some 3=a lot 4=very much High scores=low symptom complaints	Cronbach's alpha for the Norwegian version of the 19 items endocrine subscale was 0.86 in patients and 0.83 in healthy controls	66–70
FACIT-F version 4	Functional Assessment of Chronic Illness Therapy-Fatigue	Consists of 13 items measuring physical and mental fatigue affecting daily life during the last week	five point rating scale 0=not at all 1=a little 2=some 3=a lot 4=very much High scores=low symptom complaints	Cronbach's alpha for the questionnaire was 0.84 in patients and 0.81 in healthy controls	51 66 71
SHC	Subjective Health Complaints	The instrument measures 29 subjective, somatic and psychological complaints experienced during the last month 1. 'Musculoskeletal pain' (back pain, low back pain, arm pain, shoulder pain, migraine and leg pain, range 0–24), 2. 'Pseudoneurology' (tiredness, sleep problems, anxiety, sadness/depression, extra heartbeats, heath flushes and dizziness, range 0–21), 3. 'Gastrointestinal problems' (gas discomfort, stomach discomfort, diarrhoea, constipation, gastritis/ulcer, heartburn and stomach pain, range 0–21), 4. 'Allergy' (allergies, breathing difficulties, eczema, and asthma, range 0–15) and 5. 'Influenza' (cold/influenza and coughing, range 0–6)	four point rating scale 0=no complaints 1=a little complaint 2=some complaints 3=severe complaints Total score range: 0–87	The instrument has a satisfactory validity and reliability in both the general Norwegian population, and Norwegian patients with irritable bowel syndrome The Cronbach's alpha for Subjective Health Complaints Inventory in patients with breast cancer and healthy controls were 0.89 and 0.87 respectively	62–66

Continued

Table 3 Continued

Questionnaire (abbrev)	Full name	Items	Scoring/scale	Remarks	References
HADS	Hospital Anxiety and Depression Scale Two subscales: Anxiety (A) and Depression (D)	HADS is a self-assessment mood scale consisting of 14 items, 7 for HADS-A (anxiety) and seven for HADS-D (depression	four point rating scale 0–3 Total score range: A=48 D=56 Sum A+D = 104 Cut-off scores for HADS, HADS-A ≥8 and HADS-D ≥8, indicate the presence of cases with clinically significant depressive or anxiety disorders	The Cronbach alpha for HADS-A ranged from 0.68 to 0.93 (below adequate to excellent) with a mean of 0.83 (very good). Cronbach alpha for HADS-D ranged from 0.67 to 0.90 (below adequate to excellent) with a mean of 0.82 (very good).	72–75
FSS	Fatigue Severity Scale	9 items Each item is scored from 1 to 7.	The total score is divided by 9 Total score range from 1.00 to 7.00.		
fVAS	Fatigue – Visual Analogue Scale	Single item Addresses the intensity of fatigue during the last week	The scale is a 100 mm line where the far left is 0 mm = 'no problems with fatigue and exhaustion', and the far right is 100 mm = 'so much fatigue and exhaustion that is possible to experience', during the last week	Intra Class Correlation values for the fVAS is between 0.846 and 0.888	72 76 77
Muscle – Joint Questionairre	Muscle and joint pains	three items	100 mm VAS scale		
SF-MUIS	Short-form Mishel Uncertainty in Illness Scale	The SF-MUIS covers five statements from the modified 33 questions MUIS, examining the uncertainty in illness in hospitalised adults asking the participants to reflect and report how they agreed to the following five statements; 'I have a lot of questions without answers', 'I understand everything explained to me', 'The doctors say things to me that can have many meanings', 'There are so many different types of staff; it's unclear who is responsible for what', and 'The purpose of each treatment is clear to me'.	five point rating scale: 1 'strongly disagree', 2 'disagree', 3'uncertain', 4.'agree', 5 'strongly agree' Range: 5–25	The MUIS-BT measures four constructs: ambiguity/inconsistency, unpredictability of disease prognosis, unpredictability of symptoms and other triggers, and complexity. The ordinal coefficient alpha for the SF-MUIS was 0.70 in Norwegian patients with breast cancer.	78 79
Side effects endocrine therapy	Aromatase Inhibitors (AI)	9 organ systems	yes/no/don't know	This questionnaire is based on knowledge on side effects of AIs. Not validated.	–
Side effects endocrine therapy	Tamoxifen	6 organ systems	yes /no /don't know	This questionnaire is based on knowledge on side effects of tamoxifen. Not validated	–
Adherence to endocrine treatment	Self-reported adherence	One question: the patients were asked if they take Tamoxifen or Aromatase inhibitor every day?	yes or no		
Diet questionnaire	Nordic Nutrition Recommendations 2012	The patients were asked whether they had made any changes in their diet 2 years after they were diagnosed with breast cancer. If the answer was 'yes', they were asked about the major changes they had made in relation to food items excluded or included in their diet. Avoidance of 36 different food items over six clusters: Cereals, fish/shellfish, fruit and vegetables, dairy products, processed food and others Finally, the participants were asked to report their daily and weekly use of milk and other dairy products, and whether they consumed alcohol or not	The response options: 'yes', 'no' or 'partially' Further, the respondents who replied positively to consuming alcohol, reported their consumption (daily, every second day, once or twice per week, once per month, or more rarely), and the number of glasses during 1 week.	Cronbach's alfa=0.83 (0.71–0.97)	80 81

HRQoL, health-related quality of life; PBCB, Prospective Breast Cancer Biobank; SF-MUIS, Short Form of the Mishel Uncertainty in Illness Scale.

early-stage breast cancer. Providing a method for molecular detection of systemic relapse will be one small step for the development of biomarkers but one giant leap for the treatment of patients with early-stage breast cancer.

ETHICS

The general research biobank PBCB was approved by the Ministry of Health and Care Services in 2007, by the Regional Ethics Committee (REK) of Northern Norway in 2010 (#2010/1957). The PROM (#2011/2161) and the biomarker study PerMoBreCan (#2015/2010) were approved by REK in 2011 and 2015, respectively. All patients have been informed by study nurse or a research coordinator both certified in Good Clinical Practice, prior to consenting to take part in the study. This took place in the time window between diagnosis and the operation. The patients have given written consent to the general research biobank PBCB and HR-QoL studies (ie, two written consents) from 2010 onward. Results from the PBCB-studies will be published in international conventions, in international peer-reviewed scientific journals, in mass media and in social media. At the end of the study, deidentified data will be curated in-house according to European guidelines, and will be available for researchers on request. Requests should describe the nature of the proposed research and scope of the requirements, and will be reviewed by the PBCB board.

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Contributors HS: study idea, design, enrolment of patients, building database, writing of paper; EAMJ: study design, analysis of micro RNA, primary tumour and TEPs, writing of paper; TH: enrolment of patients, writing of paper; FME: analysis of drug concentrations in serum; MH: building database; ON, analysis of ctDNA, CTCs, next-generation sequencing, writing of paper; SL: enrolment of patients; handling of blood samples, biobanking, building database; THL: study idea, study design, enrolment of patients, writing of paper; JVS: building database; KT, analysis of ctDNA, CTCs and next-generation sequencing, writing of paper; BG: enrolment of patients and building av database, writing of paper; KJ: biobanking, analysis of microRNA and TEPs, writing of paper; EG: microscopic analysis of tissue section of the patients, KL: PROM data, writing of paper, KBH, PROM data, writing of paper, BHG; PROM data, writing of paper, RL, PROM data, writing of paper; AH: enrolment of patients, TA: enrolment of patients, MA: next-generation sequencing of primary tumour and metastases, microRNA, TEPs and writing of paper, NGE, enrolment of patients, analysis of micro-RNA, writing of paper, TB: establishing database for PROM data, TLL: providing instrumental ideas to planning of the PBCB protocol, LS: biobanking; ACK, Biobanking; SO: handling of blood samples, biobanking; JTK: statistics; EAL: Study idea, design, LS: biobanking and GM, study idea, study design, writing of paper.

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