



4. Practical and Ethical Issues in Establishing a Collection of Normal Breast Tissue Biopsies—Part of the NOWAC Post-Genome Cohort

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Abstract For tissue-based studies of breast cancer, getting access to truly normal, well-annotated tissue can be a challenge. To address that need, we collected 368 breast tissue biopsies and buffered blood samples from healthy postmenopausal women. Volunteers were part of the Norwegian Women and Cancer (NOWAC) Post-genome cohort, recruited through the national mammography screening program. The NOWAC normal breast tissue biobank for gene expression analysis will provide a correct basis for comparison in case-control studies.

Keywords normal breast tissue | biobank | breast cancer

BACKGROUND

Epidemiology and risk factors of breast cancer

Breast cancer is the most frequent type of cancer among females worldwide. The latest GLOBOCAN report estimated approximately 2.1 million newly diagnosed breast cancers in 2018 (Bray et al. 2018). The incidence of breast cancer varies significantly around the world, but is increasing in most countries (Bray et al. 2018). The high incidence in developed countries has to some extent been counterbalanced by a reduction in mortality. Early diagnosis due to mammographic screening, improved treatment, secondary prophylaxis and follow-up have improved the outcome for breast cancer patients. The 5-year survival rate in Norway is 90.4%—yet breast cancer is the leading cause of cancer-related deaths among females

(Cancer Registry of Norway 2017). The increasing incidence and improved survival rate results in high prevalence of the disease. Since the treatment is associated with severe side effects over a long period, the burden of the disease is large.

The current body of evidence suggests that genetic structure and internal and external risk factors, as well as their interactions, combine to constitute the causes of breast cancer. Two major risk factors are gender and age. Other causal factors relate to the levels of endogenous hormones determined by age at the first menstruation, age at menopause, age at first birth, and number of births, as well as use of oral contraceptives and hormone therapy (HT) (Kaminska et al. 2015). Lifestyle factors regarded as risk factors include lack of physical activity, obesity, alcohol consumption, smoking, night shift work, exposure to radiation, and possibly diet (Sun et al. 2017). Hereditary breast cancer accounts for 5–10% of cases (Apostolou and Fostira 2013), making non-hereditary risk factors the major drivers of incidences of breast cancer.

Breast cancer characteristics

Breast cancer is a heterogeneous disease both etiologically and genetically. It consists of several sub-types with different molecular profiles, and biological and clinical behavior. Different sub-groups are associated with different risk profiles and present a big challenge for clinical management. In clinical practice, an array of methods is used to determine which sub-type the patient has: tumor-node-metastasis (TNM) staging, histological sub-typing, tumor grade, tumor invasion in lymphatic and vascular tissue, axillary lymph node status, immune-histochemical staging providing estrogen and progesterone receptor status, presence of human epidermal growth factor receptor 2 (HER2) receptor, and Ki67 marker. These factors describe the tumor biology regarding hormone sensitivity and tumor aggressiveness, guide decision-making for treatment, and predict the prognosis.

Today there are efficient surgical and medical treatments available, but we are unable to determine specifically which type of treatment the individual patient needs, often implying overtreatment. There is a need for better prognostic and predictive markers to individualize the treatment in order to provide the best treatment for patients with high-risk profiles, and to avoid overtreatment of patients with a low risk profile.

Normal breast tissue histology and development

The human breast is an apocrine gland designed to produce milk, and breast tissue is heterogeneous and complex in composition. The breast consists of three main

components: the skin, containing areola and nipple, the subcutaneous adipose tissue (white fat tissue), and the glandular tissue (functional tissue of the breast) including both parenchyma and stroma. The parenchyma is divided into 15–25 lobes, each made up of 20–40 lobules. The structure is based on a branching duct system that leads from the collecting ducts to the terminal duct-lobular units (TLUs). The TLUs are the functional unit of the breast tissue and sites of milk production. The terminal collecting ducts drain the milk from TDLUs into 4–18 lactiferous ducts, which drain to the nipple. The inter- and perilobular connective tissue surrounding the TLUs and lobules contain fibrovascular tissue and white adipose tissue. Fibrous stroma provides the background architecture for the glandular tissue, as well as nutrition and protection. The proportion of adipose and fibrous tissues varies from one woman to another and changes in the same person over time.

Breast tissue development occurs in defined stages: embryonic, pre-pubertal, pubertal, pregnancy, lactation and involution. The tissue only reaches its final level of development during the last stages of pregnancy, and if pregnancy does not occur, it is never reached. During menopause, the glandular tissue is progressively atrophied. The lobules decrease in size and number, mainly through progressive involution of the milk-producing acini. Fibrous tissue is also replaced by adipocytes. However, the extensive use of hormonal replacement therapy has considerably changed the appearance of this postmenopausal breast tissue.

Biobanking of normal breast tissue for research

Tissue-based studies of breast carcinogenesis utilize breast cancer tissue and different types of non-cancerous breast tissue, sometimes called normal breast tissue, as control for comparison. Most commonly used non-cancerous breast tissue is derived from reduction mammoplasty either from breast cancer patients, of unaffected breast for symmetry in breast cancer patients, or from healthy women operated for cosmetic purposes. Other sources of non-malignant breast tissue used in research include tissue from prophylactic mastectomy, neighboring breast tissue from women with benign breast lesions, excess tissues with benign histological appearance collected from surgical procedures, or unaffected ipsilateral or contralateral breast tissue from patients with breast cancer.

Usually there is a medical reason to surgically remove tissue—for example in prophylactic mastectomy for high risk of breast cancer due to gene mutations, or removal of benign lesions due to pathological features. Therefore, this type of tissue is not suitable for use as “normal” tissue. Breast tissue collected by reduction

mammoplasty, selected on the basis of convenience, may be the best representative of normal tissue. It is plentiful and removed for cosmetic reasons, not because of clinical abnormalities or high-risk profiles. However, none of these tissues have been found suitable as a substitute for truly normal breast tissue in studies of breast cancer carcinogenesis (Ambaye et al. 2009, Graham et al 2010, Degnim et al. 2012, Tadler et al. 2014, Acevedo et al. 2019).

Today there are several breast cancer tissue biobanks around Europe, North and South America, Asia and Australia, but to our knowledge the only biobank that collects truly normal breast tissue is the Susan G Komen for the Cure Tissue Bank (KTB) at Indiana University Simon Comprehensive Cancer Center in the USA (Sherman et al. 2012). There, tissue has been collected from volunteers of all ethnicities aged 18 and upward. Several articles have been published using this material. Radovic et al. 2014 concluded that breast tissue from healthy volunteers acts as a superior normal breast tissue control. The same source of tissue has been used in Pardo et al. 2014, where the author analyzed the transcriptome of normal, healthy, pre-menopausal breast tissue using next-generation sequencing.

In order to move breast cancer research forward, there is a need for well-annotated collections of breast tissue from healthy women (Thompson et al. 2008, Eccles et al. 2013). Adequate control tissue will help shed light on pre-clinical molecular events, and provide the correct basis for comparison in case-control studies. The overall goal of this study was to establish a biobank of normal breast tissue biopsies. The biobank was established for the purpose of describing baseline gene expression patterns in normal breast tissue of postmenopausal women. We will also explore the variation of gene expression in normal breast tissue following exposure to known breast cancer risk factors (smoking, alcohol consumption, HT use, obesity and parity), and finally, we will use the normal breast tissue in future case-control studies.

METHODS

The normal breast tissue biopsy study, part of the NOWAC Postgenome cohort

This study is part of the Norwegian Women and Cancer (NOWAC) Postgenome cohort. NOWAC is a national, prospective study started in 1991, where breast cancer is the most important endpoint (Lund et al. 2008). The study included 150 000 women born 1943–1957, who to date have answered between one and three questionnaires. During the period 2003–2006 we built a unique biobank by collecting

blood samples, buffered to protect the mRNA gene expression profile, from 50 000 NOWAC participants. These samples constitute the major part of the NOWAC Postgenome cohort. Furthermore, starting in 2006 and in collaboration with 11 Norwegian hospitals, we collected buffered blood samples and tissue samples from 400 women with breast cancer tumors at the time of diagnosis. These women were also participants in NOWAC, they were born between 1943–1957, and were diagnosed with breast cancer during the period 2006–2011. Until that time, there was no suitable tissue material available that expressed the normal pattern of variation in gene expression in the relevant age group. To address that need, during the period 2010–2012 we collected breast tissue and buffered blood samples from 368 healthy women. Volunteers for this part of the study were recruited from the NOWAC cohort through the national mammography screening program, which they were participating in at the time.

Recruitment of study participants

Recruitment to the study and the tissue collection took place at the Breast Diagnostic Center at the University Hospital of Northern Norway (UNN), Tromsø, Norway. Inclusion criteria were as follows: enrolled in the NOWAC cohort, born between 1943 and 1957, and consent given. The radiographer (not affiliated with the NOWAC study) asked women, when presenting at the mammography screening unit, if they would consider participating in this study. If answering positively, the candidate would meet after the screening procedure for written and oral information and to get answers to any questions they may have had. The women who agreed to participate were asked to sign a written, informed consent form. All participants completed a two-page questionnaire regarding menopausal status, weight and height, exposure to smoking and alcohol consumption, use of HT and other medication. Exclusion criteria included previous history of breast cancer, positive mammogram, other relevant malignant diseases, and use of anticoagulation therapy with Coumadin (Marevan), Heparin, Persantine, or Plavix. Use of acetylsalicylic acid was not an exclusion criterion.

Procedures for tissue and blood sampling

Core biopsies of normal breast tissue were obtained immediately after mammography, from the gland tissue of the upper lateral quadrant of the left breast. The tissue biopsy was taken with the women in declined position on the examination bed. The skin was disinfected with chlorhexidine solution in alcohol prior to incision.

Intradermal local anesthesia was applied using 2 ccl of 1% Lidocaine. A 3 mm skin incision was performed with a scalpel. With ultrasound guidance, a cylinder biopsy was taken with a needle size 14 gauge in a biopsy pistol, by an experienced radiologist. Compression bandage was placed at the biopsy site, which was to be kept in place until the next day. No further activity restriction was advised. During the study, no systematic follow-up has been undertaken. The biopsy was immediately placed in RNAlater for RNA stabilization (Qiagen, Hilden, Germany), and kept at room temperature for <24 hours until storage in a freezer at -70°C .

Two vials of blood were taken by standard venipuncture (phlebotomy) with hypodermic butterfly needle on a closed system to the vacuum test tubes. One of the blood samples was taken using the PAXgene Blood RNA collection system (Pre-analytix/Qiagen, Hombrechtikon, Switzerland), which contains a buffer for stabilizing the mRNA gene expression profile during long-term storage. The other blood sample was mixed with standard citrate solution. Blood samples were kept at -70°C until further use. The blood sampling was performed before the tissue sampling.

RESULTS

We collected 368 biopsies of normal breast tissue from postmenopausal women. The rate of inclusion of all women invited to participate was 64%. A linkage to the Norwegian Cancer Registry 3 years after the sampling period ended resulted in five biopsies being excluded due to breast cancer diagnosis within 3 years after the biopsy was taken, and one due to a prior lymphoma diagnosis with unknown treatment. We used 16 biopsies for testing of different laboratory methods. A total of 311 biopsies were included for further analysis, which matched the number of cancer biopsies in our biobank collected for a comparative study.

All participants were advised to contact a physician in case of any suspicion of adverse reaction or complication such as hematoma, infection, or pain. No case of allergic reaction to the local anesthesia was registered. One participant directly reported a hematoma at the biopsy site. She was examined by a surgeon, who found a 3 cm hematoma, but no treatment or follow-up was considered necessary.

Characteristics of women included in this study

Characteristics of the 311 women included in the final study sample is summarized in Table 4.1. All participants were post-menopausal, and the average age was 60 years. The population, as a whole, were slightly overweight after WHO standard, with average BMI 26,2. Most of the women had given birth (have completed

full term pregnancy), and the average number of children was 1,9. The highest number of children was 8. A majority of the women (79%) had consumed alcohol during the week before sampling, and 21 % had been smoking during the week prior to biopsy sampling. Very few participants (8,4 %) used HT for menopausal symptoms. The majority of participants (70 %) used different types of medication in the week prior to blood sampling, either alone or in combination. The most frequent types were blood pressure medication, anti-cholesterol drugs, and synthetic thyroid hormone, followed by ASA (aspirin) and NSAIDs.

Table 4.1. Characteristics of the study population (n=311)

| Characteristics | Mean/Frequency | Missing |
|--|----------------|---------|
| Age, mean (SD) | 60,1 (3,9) | 0 |
| BMI, mean (SD) | 26,2 (4,5) | 4 |
| Parity (n, %) | | 0 |
| Yes | 256 (82,3) | |
| No | 55 (17,7) | |
| N children (mean, SD) | 1,9 (1,2) | 0 |
| Smoking (n, %) | | 0 |
| Yes | 66 (21,2) | |
| No | 245 (78,8) | |
| HT use (n, %) | | 1 |
| Yes | 26 (8,4) | |
| No | 284 (91,6) | |
| Alcohol (n, %) | | 6 |
| Yes | 241 (79) | |
| No | 64 (21) | |
| Medication use (n, %) | | |
| Any medication | 216 (70,8) | 6 |
| Blood pressure alone or in comb. with antiarrhythmic | 56 (18,4) | |
| Anti-cholesterol | 36 (11,8) | |
| Levaxin (synthetic thyroid medications) | 30 (9,8) | |
| Asthma/allergy | 23 (7,5) | |
| NSAIDs alone or in combination with Paracetamol | 22 (7,2) | |
| Albyl (acetylsalicylic acid) | 19 (6,2) | |
| Other | 30 (9,8) | |

Abbreviations: BMI, body mass index; HT, hormone therapy; NSAID, non-steroidal anti-inflammatory drugs; SD, standard deviation.

DISCUSSION

Above we have described the process of establishing a biobank of normal breast tissue biopsies from 311 postmenopausal women. In the following we discuss practical aspects of establishing the biobank, as well as ethical considerations, and highlight some factors that enabled the successful establishment of the NOWAC normal breast tissue biobank.

Where to find volunteers and how to recruit them?

The process of recruiting healthy volunteers for an invasive procedure may, if not planned properly, render the final study sample heavily affected by selection bias, subsequently reducing the generalizability of any findings. To reduce selection bias, our starting point was the nationally representative NOWAC study, as well as the national mammography screening program. The screening program invites all Norwegian women aged 50–69 years to mammography every other year, free of charge. Hence, an important success factor for this study was the use of the local screening facility, which enabled us to contact all eligible women in the region.

Prior to our work, the same facility had completed two small surveys (unpublished) to start the process of assessing the feasibility of collecting tissue biopsies from healthy women. The first was conducted to register discomfort and possible complications associated with the biopsy procedure and was based on interviews with 100 women who had undergone this procedure. The women were asked about pain, bleeding, hematoma, and infections. The result was consistent with the impression from the clinical work that biopsy taking is virtually painless and there is a very low risk for complications associated with the procedure. The second survey aimed to determine whether it would be possible to collect breast tissue biopsies from healthy women. We asked 81 women who participated in the mammography screening program if, hypothetically, they would be willing to have a breast biopsy taken to be used for research purposes. After receiving written and oral information, 12% answered no, 14% needed more information, and 74% answered yes. These results gave important cues on feasibility.

Collaboration with clinicians

The local mammography screening facility handles about 40 invitations every day. The NOWAC study has been collaborating with the facility since March 2002, when approximately 2 000 blood samples were collected for a different NOWAC project. The facility also played an active role in recruiting partners for a cancer

biopsy study at eleven of the country's hospitals. This close and long-standing collaboration is another important success factor for the present project. The screening facility already had valuable experience in contributing to research during their clinical everyday setting. Though the environment was familiar with research, it was necessary to make a detailed plan and spend time to figure out the most feasible way to complete all the steps with the clinical personnel involved. This included having the same person involved every day, who was familiar with the hospital environment and the department's work, as well as being involved in the research project.

The biopsy procedure involved is virtually painless, with a very low complication rate, and was performed by an experienced radiologist within the well-established framework of the screening facility, minimizing the risk of unforeseen incidents. All women were given information on actions to be taken in the case of complications. Since the procedures took place in the hospital setting, any complication or injury would be reported as a patient injury according to established national guidelines. Women were encouraged to contact the screening facility if a suspicion of a complication should arise after leaving the department. Complications requiring immediate treatment outside opening hours would be attended by the staff in the emergency room. These actions were largely comparable to actions to be taken in case of complications after any breast tissue biopsy procedure, and put no extra burden on the clinical staff.

Ethical aspects

In accordance with legal requirements for research on human biological material and personal data (The Health Research Act, Chapters 3-7), the Regional Committee for Medical and Health Research Ethics of Northern Norway (REC North) approved the protocol for the present study, and the Data Protection Authority granted a license for the use of health-related data. However, the project was planned some years ago, before the European Union issued the new General Data Protection Regulation (GDPR) in 2018. In Norway, GDPR was implemented at the national level through a new Personal Data Act, also in 2018. The risk of misuse of personal information, or the risk of loss of control of the personal information, is present in the current project, but this risk is by no means greater here than in comparable projects. These aforementioned risks are the focus of GDPR, and after its implementation, data-handling procedures have also been improved for the NOWAC project.

The need for close regulation of biomedical research dates back to atrocities during the Second World War, which led to the emphasis on human rights in the

Nuremberg Code of 1947. A main point in the Code stated that participation in research must be voluntary. Furthermore, the World Medical Association's Declaration of Helsinki (1964) focused on obligations of the researchers and the research institutions, and stressed the concept of informed consent (Fisher 2006). That the consent must be voluntary or free means that the individual included in the research shall not decide his/her position through a process characterized by coercion or pressure. Likewise, situations that do not include direct coercion can mean an unacceptable weakening of the consent that was given. Our participants were already part of the NOWAC study when they were invited for the biopsy study. Potentially, this could contribute to a feeling of pressure to participate in the biopsy study. We, the researchers, regarded this project as a continuation of the ongoing NOWAC study, and this backdrop may have put an indirect pressure on the women at the point of invitation. Still, the option to decline participation was always clearly communicated, both orally and in writing, hence we conclude that the principle of voluntary participation was never challenged.

The principle of informed consent entails that the individual being subjected to research must be aware of the study's methodology/procedures, purposes, and the type of results expected. The information given to participants must include a description of any expected inconvenience, discomfort, or risk that may be inflicted. This principle may be regarded as particularly important when performing an invasive procedure on healthy volunteers who would not otherwise undergo such procedures. Further, as the material collected in our study will be used for genomic profiling (mRNA gene expression analysis and potentially DNA profiling), care must be taken to ensure that participants understand the information that was given. The participants may have different experiences and assumptions when they internalize and interpret the information. We did not undertake any evaluation of the participants' understanding of the scientific content of the project, but each woman spoke personally to our radiologist, with ample opportunity to ask questions. Legislation on this topic focuses only on groups of people that may be non-competent to give consent (e.g. persons under the age of 18, or for medical reasons). Hence, some questions may be ethically interesting, but will not have any practical consequences for our project. As examples, one might ask if it would be ethically acceptable to include participants if we discovered that they had not understood the information correctly. In addition, what about individuals who did not want to read the information that was given, but nonetheless wished to participate in the project?

One of our pre-study surveys assessed the healthy women's willingness to donate a breast tissue biopsy. The majority (74%) were willing to donate, and many women expressed a high degree of motivation to continue contributing to research

on breast cancer. Contributing biological sample material to research may be viewed in different ways. The biopsy may be viewed as a gift or a donation, with no expectation of receiving anything in return. It may also be viewed as a transaction. In that case, the regional ethical committee would act as the real estate agent, looking out for the donors' rights, and the consent form may be regarded as the contract between the two parties in the transaction. Viewed as a transaction, there is an expectation of receiving something in return, in this case somewhat distant "payments" such as knowledge of breast cancer, and better treatment. Another option for how to view the act of contributing a biopsy would be as an act of reciprocity. Modern-day medicine is an empirical science which has been built on the knowledge generated from the general population and from patients. Patients today expect to receive the latest treatments that are developed on the basis of this knowledge, and as such, they are morally obliged to contribute to that same knowledge base. In this normative ethics setting, the consent may be viewed as an expression of gratitude toward previous sample donors, of acknowledgment of the moral obligation to contribute, of the will to contribute, and of trust in that the donated material will be used as intended.

We do not have information on each woman's motivation to contribute to the study, but some external factors may also be at play. The city of Tromsø is small, with only 72 000 inhabitants. The city's one university is young and was founded in 1968 during a period of strong growth for the city, and, naturally, its foundation contributed to this growth. Today, the university is one of the city's two largest workplaces, along with the university hospital. These aspects contribute to the fact that the university is a strong part of the city's identity and the inhabitants are well known for contributing to research (Jacobsen et al. 2012). Hence, the feeling of reciprocity, grounded in normative ethics, and supported by favorable local conditions, may be important aspects for the high participation rates in the present study.

There is an ongoing debate on whether researchers should be obliged to return information on health-related aspects to research participants (Klingstrom et al. 2018). However, the present study and its analytical methodology is purely explorative in nature. No clinical relevance of potential findings based on our chosen analytical methods has been established (low clinical validity), and any findings would be non-actionable (i.e. the participant or clinicians could not take action to improve the risk or progression of a potential disease) (Klingstrom et al. 2018). Based on the limited clinical relevance of any findings in this project, any results were unlikely to affect the patient's need for further information, or for their consent. Hence, in this project giving feedback to participants was not considered as relevant, and this was stated in the information given to participants.

Strength and weaknesses

Firstly, the women were recruited from the mammography screening program, not referred from a physician due to symptoms or suspicion of breast pathology. Their biopsies are therefore representative of truly normal breast tissue, and the women have the same risk of developing breast cancer as any other women in the same age group. Since all women were NOWAC participants, extensive information on exposures in the past can be retrieved from questionnaires answered prior to the initiation of the biopsy study. Further strengths of the study include the high inclusion rate (64%) and the high number (368) of biopsies sampled via a standard procedure, which ensures low technical variability. The blood samples were taken at the same time as the biopsies, enabling a valid comparison of gene expression profiles in two different tissues.

One weakness of the study pertains to the risk of selection bias. Our participants were recruited at the mammography screening facility in Tromsø, hence, at one single location. As a consequence, there is the possibility of geographical differences compared to the average Norwegian population regarding the gene expression in relation to different types of exposures. It should be mentioned that the blood and tissue samples were collected by random and continuous invitation during the whole 2-year period, so we expect minimal influence of seasonal biorhythms.

Due to heterogeneity of breast tissue, one single biopsy is not representative of the entire breast. Studies have shown intra-individual variability in composition of breast biopsies, and its impact on gene expression (Chollet-Hinton et al. 2018). This fact has important implications for studies based on normal breast tissue, including our own study. Since our inclusion rate was high and the complication rate turned out to be almost nil, we could have chosen to sample several biopsies from different areas of the same breast via the same skin incision. This can be considered for future trials, taking the varying biopsy composition into account. On the same note, our biopsies are whole tissue biopsies containing multiple cell types which may confound gene expression results. The biopsies were not histologically controlled/evaluated, so we do not have information on the ratio between different cell types. The biopsies were taken from the upper lateral area of the breast, known for a higher density of glandular tissue, in order to reduce the amount of adipocytes and increase mRNA output amounts. However, the biopsies were collected from postmenopausal women. The quantity of glandular tissue decreases with age, and our biopsies likely contain a higher proportion of fat and less glandular tissue compared to samples taken from younger women.

CONCLUSION

The work presented shows that establishing a collection of normal breast tissue samples is feasible and doable. Enabling factors for the present study included largely unbiased access to eligible participants, and close collaboration with clinicians during all steps of the sampling procedures. Furthermore, the source population of the present study has a high degree of health literacy and willingness to participate in research, which contributes to a high participation rate. The NOWAC normal breast tissue biobank for gene expression analysis will provide much-needed information on pre-clinical molecular events and a correct basis for comparison in case-control studies.

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