Early detection of breast cancer by organ-specific and circulatory liquid biopsies

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Early detection of breast cancer by organ-specific and circulatory liquid biopsies

Vroege opsporing van borstkanker door gerichte analyse van lichaamsvloeistoffen

(met een samenvatting in het Nederlands)

Deteção precoce do cancro da mama utilizando biópsias líquidas

(com um resumo em Português)

Proefschrift

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Contents

INTRODUCTION

CHAPTER 1

General introduction and thesis outline

Early detection of breast cancer

Breast cancer is the most common type of cancer in women, representing approximately one-fourth of all cancers diagnosed in women, both worldwide and in the Netherlands (1, 2). The most recent numbers from the Netherlands show that in 2019, a total of 17,114 women were diagnosed with primary ductal carcinoma *in situ* (DCIS) or invasive breast cancer (3). The incidence increased by 2.5% between 2015 and 2019 and, according to the Health Council of the Netherlands (Gezondheidsraad), this trend is expected to continue or increase even further, leading to a surge of 10-17% new cases between 2020 and 2040 (4).

This high incidence of breast cancer led to the implementation of population screening programs in several countries in order to detect breast tumors at an early stage. Early detection of breast cancer leads to less extensive and less mutilating treatments (e.g., a lumpectomy instead of a mastectomy) and lower mortality (5-11). In 1989, the Netherlands was one of the first European countries to establish a mammography-based breast cancer screening program for women between 50-74 years of age at a population risk (12, 13). Participation is now temporarily triennial after having been every other year until the end of 2020 (14, 15). Women with an increased risk of breast cancer due to mutations in breast cancer susceptibility genes or a familial history of breast cancer undergo a customized imaging-based screening program beginning in their early 20s or 30s, depending on their risk category (16-20). These programs are continuously evaluated and qualitatively improved to keep up with technological advances while managing costs.

A disadvantage of current programs that rely on imaging is the fact that imaging only detects breast cancer when a tumor can be visualized. In view of the surge of liquid biopsy research (i.e., research focused on biofluids as sources of tumor-derived material shed by (pre)cancerous cells), it is not unlikely that a combination of imaging and measurement of biomarkers could be an approach used in the near future. Liquid biopsies can be collected minimally invasively or non-invasively and allow an easy and regular (even serial; e.g., every half year) measurement of cancer biomarkers. A liquid biopsy of special interest for early detection of breast cancer is nipple aspirate fluid, on account of its breast-specific source for biomarkers.

Nipple aspirate fluid

Nipple aspirate fluid (NAF) refers to a fluid that is present in the ductal tree of the breasts, released by luminal cells that surround the lining, and that can be obtained non-invasively using adapted manual or electrical breast pumps (21). Given its ductal (and possibly lobular) origin, which is where breast tumors develop, the acquisition of NAF and evaluation of its biomarker content are

hypothesized to provide relevant information about early changes inside the breast. Another advantageous characteristic of NAF is the ability to synchronously acquire matched pairs of bilateral NAF samples, which provides intra-patient control samples for unilateral disease. NAF samples are a feasible source for the evaluation of biomarkers. The focus of investigation in this thesis was the biomarker class of microRNAs (miRNAs).

MicroRNAs in nipple aspirate fluid samples

MiRNAs are short, non-coding RNAs of typically 22 nucleotides in length, which regulate gene expression at the post-transcriptional level by binding to messenger RNA (mRNA), in this way exerting many regulatory functions such as cell proliferation, cell differentiation, and cell death (22). A single miRNA may regulate over a hundred target mRNAs and approximately one-third of protein coding genes in humans are regulated by miRNAs (23, 24). Many reported characteristics of miRNAs, such as their low complexity, tissue specificity, stability, resistance to long-term storage, easy quantification and amplification, and pivotal role in biological processes, make them promising biomarkers for disease (22, 25, 26).

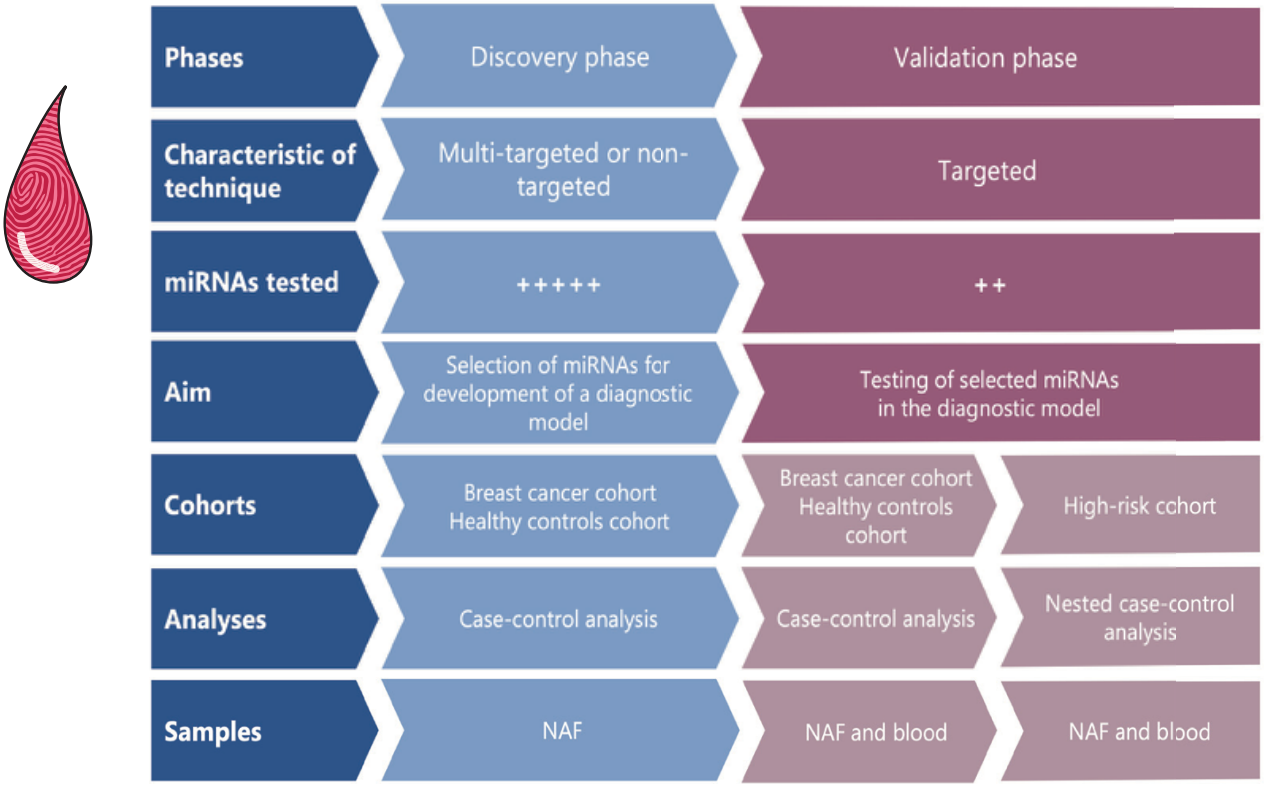
Clinically, miRNAs are biomarkers of interest because, in combination, they can form a signature or fingerprint for oncological diseases. Furthermore, miRNAs can be of value as biomarkers to understand the biology of disease or disease-related risk factors (such as high breast density for breast cancer (27)). Identification of their mRNA targets and investigation of the pathways in which they are involved by using online tools such as miRPathDB (28) could set in motion the development of targeted therapies or risk-reducing drugs (29) such as synthetic miRNA mimics (30, 31). So far, miRNA signatures for breast cancer have mostly been identified in blood, breast tissue, and urine samples, but not yet in NAF.

Design of the NAF study cohorts

To investigate the potential of miRNAs in NAF and blood as biomarkers for early breast cancer detection, NAF, serum, and plasma samples were collected from three cohorts of women: a longitudinal high-risk cohort with repeated sample collection, a healthy cohort, and a cohort of women with established invasive breast cancer. Blood was also collected to compare the diagnostic value of biomarkers found in each fluid. All samples were immediately biobanked after collection.

The setup of the biobank of samples from these three cohorts allows two study designs: a multicenter, cross-sectional study and a multicenter, longitudinal, prospective study. The first compares the healthy control cohort and the breast cancer cohort to allow for a case-control analysis. The second allows for a nested case-control analysis. Moreover, the two traditional stepwise approaches in biomarker studies, that is, the discovery phase and the validation phase, are integrated in this study. The discovery phase aims to select, among a wide range of miRNAs, the miRNAs that have a significantly differential expression profile in breast cancer patients compared to healthy controls. The selected miRNAs are subsequently validated in a similar cohort and in other cohorts. Figure 1 illustrates the overall study design.

Figure 1. Study design. NAF: nipple aspirate fluid.



Thus, the established biobank opens the way for discovering and validating miRNAs in liquid biopsies for the early detection of breast cancer in the future. NAF-miRNA research in the samples from this biobank is ongoing with the first results presented in this thesis.

Outline of the thesis

The first part of this thesis provides background information about NAF research, summarizes the practical aspects of creating the biobank, and describes the NAF collection process from the perspective of participants. As an introduction to NAF research, in the narrative review in **Chapter 2**, we give an overview of the NAF studies published to date. In **Chapter 3**, we summarize the views and reflections of the researchers on the practical aspects of conducting a longitudinal prospective study within a cohort of high-risk women. These challenges were summarized in eleven "lessons learned" and are shared between two independent research groups conducting similar clinical studies. In **Chapter 4**, we describe how women experience the nipple fluid aspiration (NFA) technique and their willingness to undergo this procedure repeatedly. We compare the tolerability of the NAF procedure with self-reported discomfort experienced by mammography and breast MRI.

The second part reports the first results of miRNAs analyses in NAF. In **Chapter 5**, we assess whether miRNAs can be detected in NAF, using samples from healthy women. We report the most highly expressed miRNAs in the healthy state and investigate the overlap between the most highly expressed miRNAs in NAF and other samples (serum, plasma, breast milk, and breast tissue) as described in the literature. **Chapter 6** evaluates the influence of the NAF sample color on NAF-miRNA expression levels, by establishing seven classes of NAF colors coupled with the appearance of cloudiness to identify significant differential miRNA expression levels between the color-cloudiness classes.

In **Chapter 7**, we summarize the data obtained. Moreover, we discuss their relevance to future projects for which this thesis sets the foundation and discuss the possible future integration of liquid biopsies in the context of early breast cancer detection.

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PART 1

CHAPTER 2

Nipple Aspirate Fluid at a Glance

Cancers, December 2021; 14(1):159.

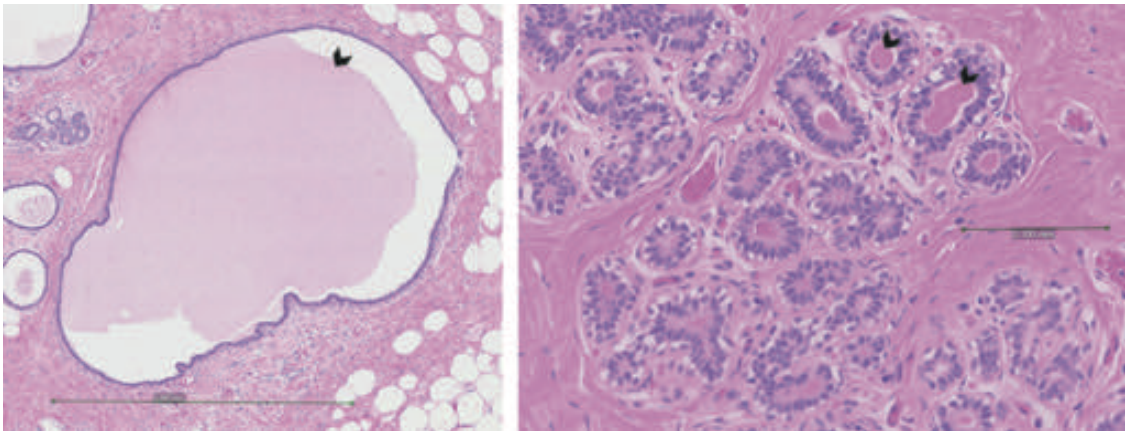
Susana I.S. Patuleia, Karijn P.M. Suijkerbuijk, Elsken van der Wall, Paul J. van Diest, and Cathy B. Moelans

Abstract

Nipple aspirate fluid (NAF) is an intraductal mammary fluid that, due to its close proximity to and origin from the tissue from which breast cancer originates, is a promising source of biomarkers for early detection of breast cancer. NAF can be non-invasively acquired via the nipple by aspiration using a suction device; using oxytocin nasal spray helps increase yield and tolerability. The aspiration procedure is generally considered more tolerable than the currently used breast imaging techniques mammography and breast magnetic resonance imaging. Future applications of NAF-derived biomarkers include their use as a tool in the detection of breast carcinogenesis at its earliest stage (before a tumor mass can be seen by imaging), or as a supporting diagnostic tool for imaging, such as when imaging is less reliable (to rule out false positives from imaging) or when imaging is not advisable (such as during pregnancy and breastfeeding). Ongoing clinical studies using NAF samples will likely shed light on NAF's content and clinical potential. Here, we present a narrative review and perspectives of NAF research at a glance.

1. What is nipple aspirate fluid?

NAF can be defined as the physiological fluid that is present in the breast ductal system (Figure 1). As indicated by the name, NAF can be acquired via the nipple by aspiration using a suction device and, as such, non-invasively. This collection procedure differs from ductal lavage, a more invasive procedure in which a microcatheter is used to cannulate and flush the ducts (1). Also of relevance is the difference in definition between NAF and pathological nipple discharge (PND). PND is defined as spontaneous, unilateral nipple discharge that can be an indication of benign (and rarely malignant) breast disease (2, 3). In contrast, NAF, which is fluid physiologically present in the breast ducts, in normal circumstances does not spontaneously leave the breast.



(a) (b)

Figure 1. Breast tissue of a non-lactating breast. (**a**) Cross-section of breast ducts filled with nipple fluid on the inside (see arrowhead). The scale bar indicates 1.00 mm. (**b**) Cross-section of breast lobules with acini filled with eosinophilic nipple fluid on the inside (arrowheads indicate two examples). The scale bar indicates 0.100 mm.

2. How is NAF produced?

The backbone of the breast is the lobular–ductal system which branches into the breast from the nipple as has elegantly been shown in studies using contrast-injection in the nipple followed by mammography (galactography) (4) and in three-dimensional computer reconstructions (5, 6). The lobular–ductal system contains two main cell types, the luminal cells, and the myoepithelial (basal) cells (Figure 2), and is surrounded by fatty tissue and extracellular matrix (7, 8).

NAF is believed to be produced by luminal epithelial cells (Figure 2), which are also responsible for the production of breast milk during lactation (9). More specifically, the components present in NAF are thought to be synthesized

within the breast epithelium and directly released into the ducts, and/or to be transferred from the circulation into the breast fluid (10). The first theory is based on studies that have shown that components such as estrogen, which can be produced in breast epithelial cells and stromal breast cells, have a higher concentration NAF compared to serum (11). The latter theory is based on studies that have found exogenously derived substances in the ductal fluid, such as nicotine in the NAF of smokers (12-15). The physiology of NAF also includes a re-absorption mechanism to the blood or lymphatics. This mechanism has been shown in studies in which India ink intraductal injections traveled through the ductal walls to the lymphatics (14) and also by the fact that cisplatin intraductal injections in mice caused systemic side effects (16).

NAF is (in theory) present in every breast. The success percentages of obtaining NAF are very high (77–95%) (17-20). Unsuccessful aspiration can be explained by factors like the collection procedure, the number and duration of attempts, the use of oxytocin to release NAF, and whether existing NAF is able to reach the nipple. In addition to the procedure itself, it is believed that characteristics of women, such as younger age (21, 22), history of breastfeeding (21, 23), not taking oral contraception (24), and soy consumption (25) could be good predictors of successful NAF collection; however, other studies do not confirm these data (23, 26-28). The presence of nipple fluid can be seen microscopically in breast tissues, as shown in Figure 1.

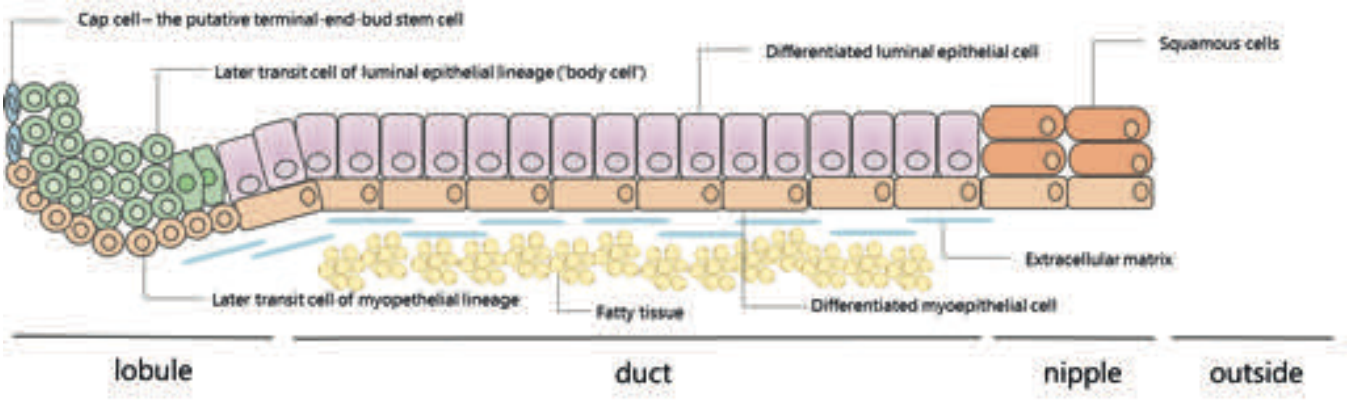


Figure 2. The mammary lobular–ductal system: cellular composition of the lobule, duct, and nipple (half linear representation). The breast contains around 5–12 mammary lobulo–ductal systems that start at the terminal end-bud and reach the nipple. Several lobulo–ductal structures can come together, leading to branching of this system into common ducts that end in the nipple. The mammary duct is composed of an outer layer of myoepithelial cells and an inner layer of luminal epithelial cells. At the end, near the nipple, there is a transition to squamous cells. Nipple aspirate fluid is probably produced by the inner luminal layer. Cap cells can take on either a myoepithelial or luminal epithelial lineage and are therefore thought to be multipotent stem cells. This scheme is adapted from Smalley and Ashworth (29) and Jakub et al. (30).

3. How is NAF collected?

NAF can be obtained by nipple fluid aspiration (NFA) with a (manual or electrical) suction device that creates negative pressure around the nipple. The manual suction device is comparable to a breast pump and consists of a cup that is placed on the breast around the nipple, a plastic tube, and a syringe, which are connected (Picture S1a). The negative pressure is created manually by drawing the plunger of the syringe (at one end of the plastic tube) back and forth (Picture S1b). In our experience, this movement can be facilitated by the use of a handle that fits the plunger of the syringe, which makes this more ergonomic (Picture S1c). The aspiration cup used can be a Sartorius cup or a commercially available breast pump (e.g., FirstCyte aspirator; Cytyc Corporation, Marlborough, MA (24, 31, 32).

NFA is a non-invasive procedure that can be performed repeatedly and that allows obtaining a liquid biopsy from both breasts. Common steps of the procedure include the use of a scrubbing gel followed by cleaning the surface of the nipple with alcohol to remove keratin plugs that clog the orifices of the nipple ducts. Several pre-procedural and per-procedural additions have been proposed to improve the NFA success rate, such as the application of warm compresses or heat pads to the breast (28), breast massage before and/or during the procedure (33-35), or repeated suction attempts (36-38). Other approaches such as the application of an anesthetic cream on the nipple and intranasal administration of oxytocin have been suggested to reduce vacuum-induced pain and increase the success rate of NAF collection, respectively (17, 38-40). NFA requires a short training and can be mastered after 2 to 5 try-outs. Most studies collect NAF in the outpatient clinic setting, with the patient sitting upright, but there are also a few studies that describe the collection of NAF from breast cancer patients directly before surgery under general anesthesia (41, 42).

4. Discomfort associated with NFA

The tolerability of the NFA procedure has only been reported by a few studies. On a scale of 0 to 10, the discomfort of NFA has been rated with a mean score of 0.6 to 2.4 (17, 26, 39, 40) in healthy female volunteers and women at high risk for breast cancer (Table 1). These scores are comparable to the scores given to a pap smear for cervical cancer screening and are generally lower than mammography, breast magnetic resonance imaging (MRI), physical breast examination, and breastfeeding (see Table 1 for reported means). It is very encouraging to see that healthy women and women at high risk for breast cancer experience low discomfort with NFA, given that these are the women in whom a NAF test would be especially applicable.

Table 1. Reported mean discomfort rates (on a scale of 0–10) for NFA (nipple fluid aspiration) compared to pap smear, mammography, breast magnetic resonance imaging (MRI), breastfeeding, and breast examination. Abbreviations: N.R., not reported; H, healthy female volunteers; HR: women at high risk of developing breast cancer.

First Author (Year) (Reference)	NFA	Pap Smear	Mammo-graphy	MRI	Breast-Feeding	Breast Exami-nation	Cohort	Number of Women
Klein et al. (2001) (26)	2.4	2.2	4.6	N.R.	6.8	N.R.	H	25
Suijkerbuijk et al. (2007) (37)	1.3	N.R.	4.3	N.R.	1.9	N.R.	H	67
Suijkerbuijk et al. (2010) (17)	0.6	N.R.	4.9	2.6	1.8	1.1	HR	90
De Groot et al. (2015) (38)	0.7	N.R.	5.2	3.55	2.5	1.15	HR	451

5. Why investigate NAF?

It is assumed that biomarkers in body fluids that are closer to the tumor or its target organ have more potential as a disease biomarker because they represent local pathological processes better than the more distant fluids such as serum and plasma that also receive cellular debris and other components from other body parts. Many biomarker classes have been investigated in NAF, such as hormones (e.g., estrogen, testosterone), tumor markers (e.g., carcinoembryonic antigen and prostate-specific antigen (PSA)), and biochemical components (e.g., aluminum). Furthermore, NAF contains molecular markers, such as DNA (43, 44), RNA, microRNA (45) and proteins (46, 47). These findings have been summarized in two reviews by Edward Sauter and Ferdinando Mannello et al. (46, 47). In Table S1, we provide an overview of the molecules and molecular changes found in NAF and the corresponding techniques used. Regarding cellular composition, NAF samples are known to be quite acellular or paucicellular, with reports of the presence of foam, white, red blood, and/or (atypical) epithelial cells (46, 48-50). Given that breast tumors develop from the ductal and/or lobular epithelium, investigating NAF as a biofluid secreted by breast cells provides the opportunity to evaluate biomarkers directly originating from the breast. Moreover, the NAF procedure provides an intra-patient control to compare an affected breast with the other healthy breast of the same woman. As such, NAF is a potentially very valuable source of biomarkers that can serve as a diagnostic tool for breast disease, including breast cancer.

6. How could NAF be of added value as an early breast cancer detection tool?

With current image-based breast cancer screening tools, breast cancer can only be detected once it has formed a mass. Liquid biopsy approaches such as NAF could be of added value by detecting breast cancer at an earlier or even pre-invasive stage.

Three recent studies have suggested how NAF (or other liquid biopsies) could be integrated into an early detection pathway for breast cancer (1, 51, 52). Shaheed et al. suggest using NAF collection as a first step in breast cancer screening, followed by mass spectroscopy sample analysis (1). Another suggestion by Shaheed et al. is the use of an easy-to-interpret NAF self-collection kit (1). These elements are also included in the recent publication of Jiwa et al., which describes a possible workflow for the management of breast screening that integrates NAF (51). A more specific potential application of NAF could be, as Zubor et al. suggest, the use of liquid biopsies as an adjunct to supplemental MRI for women with dense breasts or (additional) breast cancer-related risk factors (52).

These proposed roles of NAF or other liquid biopsies for the early detection of breast cancer, together with our views, are summarized as shown in Figure 3. In general, there are three possible roles for a new biomarker test in an already existing detection pathway, namely as a triage, add-on, or replacement test (53). A triage test is used before the existing imaging method; only women with an abnormal biomarker test result would continue in the detection pathway. Such a triage test may be less accurate, but it should provide advantages such as simplicity and low costs. An add-on test could be positioned after or together with imaging to identify false-positives or false-negatives and hence, avoid unnecessary breast biopsies and missing tumors, respectively. The third role, namely a replacement test, could be of value if there are actionable consequences to halt further tumor development or when imaging as a screening or diagnostic tool is not advisable, such as during pregnancy and breastfeeding. In the end, the true application of a NAF test will depend on the precision of biomarkers for disease detection. Furthermore, it will depend on the characteristics of the cohorts used in clinical studies to test the NAF samples. Last but not least, involvement of medical decision-making experts and patient advocate groups is of utmost importance to discuss the translational applications.

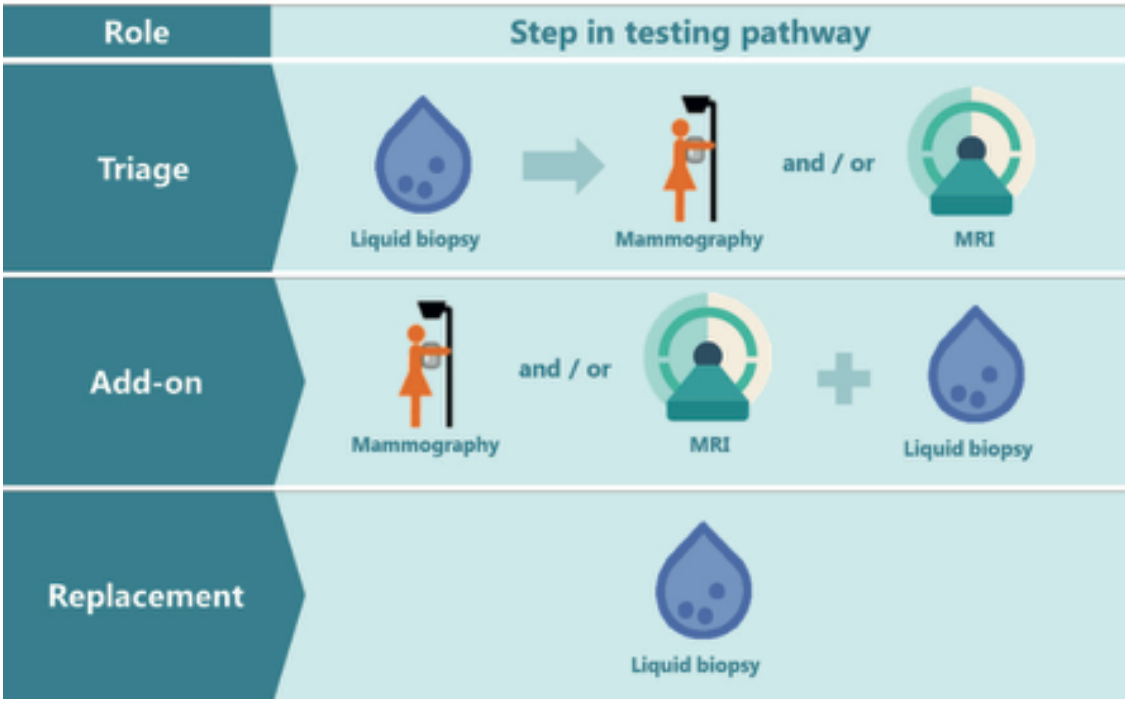


Figure 3. The possible roles of a liquid biopsy test in the breast cancer detection pathway: triage, add-on, or replacement test. MRI: Magnetic resonance imaging. The mammography schematic icon is adapted from the Dutch information folder for participation in the National Program for Breast Cancer Screening (54).

7. Challenges in using NAF as a biomarker source

Although NAF holds promise as a biomarker source for the detection of breast cancer, some challenges emerge when handling NAF. An important limitation is the small volume (ranging from 1 to 500 µL (55)). Furthermore, viscosity and different colors have been shown to affect biomarker analyses (10, 56). In our experience, volume and viscosity are an issue when using fluorescence-activated cell sorting, spectrophotometry, and microfluidics. Ways to render viscous, heterogeneous NAF samples to a homogeneous state could be the use of mechanical liquefaction, such as vortexing with the aid of glass beads or sonication and/or chemical liquefaction with the use of mucolytic buffers and centrifugation through a QIAshredder homogenizer prior to nucleic acid extraction (57). Since pipetting is also hampered by viscous samples, a requirement would be to have automatic positive displacement pipettes to ensure accuracy and reproducibility. Still, relevant current biomarker techniques such as reverse transcription quantitative real-time PCR (RT-qPCR) (45, 58) and mass spectrometry (15, 47) have been shown to be feasible for NAF samples.

Another limitation of NAF is that it is unclear from which of the 5–12 ducts within the breast the NAF droplets originate, which comes with the risk of not acquiring

NAF from the duct where carcinogenesis occurs. Furthermore, it could be that larger tumors may occlude the ducts distally and hence obstruct NAF flow for collection; from our experience, the success percentages of NFA from affected and unaffected breasts in women with breast cancer are comparable (data not shown). Still, if NAF is to be used in the context of early breast cancer detection by making use of its biomarkers to signal carcinogenesis before an obstructing tumor mass has formed, this should not be a matter of concern.

8. Historical overview of studies focusing on NAF

NAF was first described by Papanicolaou et al. (59) and Fleming (60) in the mid-1950s. In 1977, Sartorius et al. developed a "Sartorius" suction cup for NFA which consisted of a small plastic cup attached to a 10-mL syringe by a short plastic tube (61). Sartorius and his colleagues pioneered epidemiological research using NAF samples by building up a cohort of more than 3000 women of whom they attempted NFA (24). Data from these cohorts were used for several publications, especially by himself, Eileen King, Nicholas Petrakis, and Margaret Wrensch. So far, more than 200 publications have been written about NAF. To give a historical overview of research related to NAF, we briefly describe the work of selected researchers that have especially dedicated themselves to NAF, namely Otto Sartorius, Nicholas Petrakis, Ferdinando Mannello, Edward Sauter, and Paul van Diest and Elsken van der Wall. The search strategy that led to this selection comprised a PubMed search for all articles that included the terms "nipple aspirate fluid" or "nipple aspiration", together with snowballing. This was followed by a selection of articles by authors who had published more than four articles as first or last author, not including reviews. When co-authorship occurred often, selection was performed for the most senior author. A timeline with their list of publications is represented in Figures S1–S4.

The work of Petrakis et al. (Figure S1) on NAF was first published in 1975 and consisted of investigations on the success percentage of NFA, the cytological analysis of NAF, and the association of both with risk factors for breast cancer. Initial work focused mainly on the success percentages of acquired NAF and the relation to age, ethnicity, menopausal status, and cerumen type. A higher success rate of NAF yield was observed in Caucasian women younger than 50 years of age and with wet type cerumen (62, 63), which was later confirmed in another study (64). Additional variables with a positive influence on the success rate of NFA were higher fat consumption (65), history of parity, and breastfeeding (66). Another scope of research was the relation between cells found in NAF and the risk of developing breast cancer (67, 68). In one publication, it was shown that, when adding cellular atypia found in NAF, a minimal improvement of the Gail model for risk prediction was obtained (69). In another publication, they reported

that atypical cells could be found in healthy women, women with benign breast disease, and women with breast cancer, with increasing percentages per group (15%, 32%, and 64%, respectively) (70). Later, for the first time, an association was made between cellular atypia in NAF and high mammographic density (>50% density) (50). Moreover, foam cells were characterized for the first time in NAF (71). An interventional study investigating the effect of soy protein intake showed that this led to increased NAF secretion and the appearance of hyperplastic epithelial cells in NAF in premenopausal women (25). Lastly, they analyzed the biochemical content of NAF for cholesterol (as it could be a potential carcinogen) (72) and lactose (as it could be considered a measure of secretory activity) (73). In 1993, Petrakis gave a distinguished award lecture summarizing his work on NAF until then (74).

The work of Mannello et al. (Figure S2) started with publications at the beginning of the 21st century and was mainly focused on the biochemical constituents of nipple aspirate such as P-cadherin (75), proteins involved in the lipid peroxidation and oxidative stress (76-78), C-reactive protein (79), and aluminum (80-82). Methodologically, for these investigations, Mannello et al. usually compared NAF from cohorts of healthy women with cohorts of women with breast cancer; comparing biomarker concentrations between NAF, plasma, and serum. Most of his studies were observational, but he also conducted an interventional study investigating the levels of urokinase-type plasminogen activator (uPa) in NAF after oral administration of celecoxib, a non-steroidal anti-inflammatory drug (83). He also wrote four reviews summarizing current knowledge about proteins, hormones, and aluminum detected in NAF of breast cancer patients compared to healthy controls (47, 84-86). His latest publication was a comment reflecting on the potential of investigating biomarkers in NAF (87).

Another researcher with NAF as a clear line of investigation is Sauter, who has compiled an impressive number of publications on NAF over almost 30 years (Figure S3). Sauter focused on the levels of markers in NAF, especially prostate-specific antigen (PSA) (PSA is produced by breast tissue) (88-90), and PSA's association with hormones such as progesterone (91) and testosterone (92) and insulin-like growth factor binding protein-3 (93). Specifically, PSA levels in NAF were inversely associated with the presence of breast cancer, suggesting a possible role for PSA to help establish breast cancer risk (88). Moreover, the levels of PSA were investigated in breast cancer patients, and shown to decrease with advanced breast cancer stages, and to be able to better predict disease in pre-menopausal women compared to post-menopausal women (94). In addition to his focus on the biochemical composition of NAF (44, 75, 77, 78, 90, 95-105), he also investigated the cellular components of NAF. In one study, he established that there was no cellular variation in NAF throughout the menstrual cycle of

15 healthy women by evaluating weekly acquired NAF samples (106). He also conducted interventional studies where it was investigated whether celecoxib had an effect on prostaglandin E2 concentrations in NAF from women at increased risk of breast cancer (107). His latest publication is an editorial about a publication of our group (108).

The work of our group (Figure S4) on NAF started in 2007 with publications focused on the use of oxytocin nasal spray prior to the NFA procedure, which proved to produce a higher success rate, safe, and well accepted by the participating women (17, 39, 40). This was followed by a publication on methylation levels of tumor suppressor genes in NAF samples from women with breast cancer compared to healthy women. This study showed that cancerous nipple fluid contained higher levels of methylation biomarkers compared to healthy nipple fluid, although with an area under the curve that was lower than current imaging methods (109, 110). We also focused on NAF microRNA analysis, based on the fact that these biomarkers can be detected in several liquid biopsies and can constitute a signature of oncological disease. We have shown that microRNAs (miRNAs) are measurable in NAF by RT-qPCR (45) and are currently investigating the feasibility of small RNA sequencing for miRNA detection in NAF samples. Now that our group has piled up a large sample biobank with blood and NAF from healthy women, women with breast cancer, and serial samples from women at increased risk of developing breast cancer, more publications will soon follow (111-113). These data will reveal whether early detection before mammography can be reliably achieved in NAF by means of miRNAs. The practical aspects of building these cohorts have been described in a recent publication (114), followed by a publication reporting the technical aspects of analyzing biomarkers in NAF samples of different appearance (56). We are currently investigating the role of miRNAs in the development of high breast mammographic density and the consequently increased risk of breast cancer by using NAF as a surrogate for the breast microenvironment.

The latter three publications comprise the most recent publications on NAF, together with publications by George et al. (115) and Jiwa et al. (48, 51, 116). The first reported a proteomic evaluation of NAF (115) while the latter pointed out, in a systematic review, that the diagnostic accuracy of nipple smear cytology is limited by poor sensitivity (48, 116) and, in a questionnaire-based study, that there is a great readiness of women to undergo NFA (51). Based on ongoing clinical studies using NAF samples (see Table 2), future data on NAF's content and clinical potential will be generated and reported. The state of the art of NAF research is summarized in Box 1 and a list of past reviews on NAF is shown in Table S2.

Table 2. List of registered trials on nipple aspiration fluid identified by searches in https://clinicaltrials.gov/, accessed on 25 December, 2021. Trialregister.nl, ISRCTN, and PubMed. The following syntax was used for a search on PubMed: ("protocol"[Title]) AND (nipple aspirate fluid OR nipple aspirate*)).

Title of the Clinical Study	Characteristics of Participants	Aims Related to Nipple Aspirate Fluid Samples	Status	Source	Reference
Nipple Aspirate Fluid in Detecting Breast Cancer	Participants are healthy volunteers >40 years who undergo nipple aspirate fluid collection from both breasts.	Nipple aspiration fluid samples will be compared between breast cancer participants and healthy participants. will perform the logistic regression model for each biomarker showing any difference between breast cancer patients and healthy individuals. Then we will include multiple biomarkers in one model while controlling for confounders.	Recruiting	Clinicaltrials.gov	(117)
Early detection of Hereditary Breast Cancer by Monitoring MicroRNA expression in Nipple Aspirate Fluid	Women at high risk of developing breast cancer	- To establish biomarker profiles in nipple aspirate fluid, follow them in time and establish a correlation with breast cancer development. - To determine threshold values of these biomarkers that point to a significant risk of imminent breast cancer development thereby indicating the right time of prophylactic breast surgery (Trial NL8661).	Recruitment stopped in 2021. Lab analyses ongoing.	trialregister.nl	(111)
Breast Cancer Biomarkers in Nipple Aspirate Fluid and Blood in Healthy Women	Participants are healthy volunteers ≥45 years	The main study aim is to evaluate microRNA expression patterns in the NAF and blood of healthy women, and to compare this with the pattern of women with breast cancer (ORNAMENT study, Trial NL6031).	Recruitment stopped in 2021. Lab analyses ongoing.	trialregister.nl	(112)
The ORNAMENT study: A multicenter, crOss-sectional study to assess microRNA expression in Nipple Aspirated Fluid, blood and tuMor material in women with primary brEast caNcer compared with healthy conTrols.	Women with pathologically established non-metastasized invasive breast carcinoma	To assess the microRNA expression levels in the nipple aspiration fluid obtained just before primary surgery. These will be compared to the microRNA expression levels in NAF obtained from healthy controls (Trial NL8987).	Recruitment stopped in 2021. Lab analyses ongoing.	trialregister.nl	(113)

Title of the Clinical Study	Characteristics of Participants	Aims Related to Nipple Aspirate Fluid Samples	Status	Source	Reference
Physical activity and dietary counseling and supervised group exercise for first-time pregnant women—a feasibility study of a controlled trial	Women who gave birth and stopped breastfeeding	Secondary outcome: Levels of selected breast cancer risk markers (hormones, growth factors) in blood and nipple aspirate fluid (only in postpartum women).	Active, but not recruiting	ISRCTN registry	(118)
Phase II study of metformin for the reduction of obesity-associated breast cancer risk: a randomized controlled trial protocol	Eligible participants will be randomized to receive metformin 850 mg BID (*n* = 75) or placebo (*n* = 75) for 12 months.	Exploratory outcomes: changes in metabolomic profiles in plasma and nipple aspirate fluid.	Not recruiting	PubMed	(119)

Box 1. Summary of the state-of-the-art of NAF research: what we know, advantages, hurdles to be aware of, and recommendations and needs in NAF studies.

Box 1 | Summary

What we know

- Nipple aspirate fluid (NAF) is a physiological fluid that is produced by the luminal layer of the breast lobules and ducts
- Nipple fluid aspiration (NFA) is well tolerated by women
- NAF can be acquired in the majority of women
- Many biomarkers can be found in NAF, such as DNA, RNA, microRNA, and proteins
- Cytology assessment in NAF has low diagnostic accuracy

Advantages

- NAF originates from the location where breast cancer arises
- NAF can be acquired repeatedly, easily, and non-invasively
- Bilateral NAF samples allow intra-patient control analyses

Hurdles to be aware of

- NAF samples are of low volume, can be viscous, have low cellularity, and have different colors, which may affect biomarker analysis
- It is unclear which duct NAF derives from

Recommendations and needs in NAF studies

- For the NFA procedure, use oxytocin nose spray to increase success rate and tolerability for the woman, and use an ergonomic handle for the research nurse performing the procedure
- Combine nipple fluid research with imaging results and include anthropomorphic measures and risk factors for breast cancer
- Develop technologies that are feasible for detection and interpretation of biomarkers in samples that are viscous and of low volume
- To reduce sample viscosity, use mechanical and/or chemical liquefaction in sample processing steps
- Report NAF biomarker results in diagnostic accuracy values in order to be able to interpret their translational role
- Involve medical decision making experts and patient advocate groups to discuss the potential use of liquid biopsies in early detection
- Develop a self-test for NFA collection and interpretation

9. Conclusions

NAF is a physiological fluid that rests in the ductal tree of the breast and can easily be obtained by non-invasive aspiration. It is an established source of biomarkers that deserves to be investigated for its potential application in breast cancer management such as in screening or as a confirmatory additive tool in imaging diagnostics. As research on the role of NAF is still actively being conducted by many research groups, including our own, more data on NAF and its potential clinical value is expected to be reported in the near future.

Supplementary Materials

Picture S1. Pictures of materials used for manual nipple fluid aspiration procedure.
Table S1. Summary of molecules or molecular changes found in NAF and used techniques, their advantages, and limitations.
Table S2. List of reviews about nipple aspirate fluid or where nipple aspirate fluid is mentioned amongst other approaches in the manuscript.
Figures S1–S4. Timeline of published articles of Petrakis (S1), Mannello (S2), Sauter (S3), and Van Diest/Van der Wall (S4).

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Supplementary Material

(**a**) (**b**) (**c**)

Picture S1. Pictures of materials used for manual nipple fluid aspiration procedure. (**a**) Interconnection with a plastic tube between the cup that is placed on the breast around the nipple and a syringe; (**b**) figure of the research nurse showing how to grasp the plunger of the syringe to create vacuum. (**c**) Ergonomic handle that we developed to attach to the plunger of the syringe.

Table S1. Summary of molecules or molecular changes found in nipple aspirate fluid (NAF) and used techniques, their advantages, and limitations. This information was retrieved from the reviews of Sauter (1) and Mannello et al. (2).

Molecules or Molecular Changes Detected in NAF	Technique Used in NAF	References
DNA mutagens	Ames test	[26,51,52]
Methylation changes in DNA	Methylation-specific PCR (MSP) technique	(3)
Mutations in mitochondrial DNA		(4)
Microsatellite markers in DNA to investigate loss of heterozygosity or microsatellite instability alterations	Polymerase chain reaction (PCR)	(4, 5)
Mutations in the mitochondrial genome (mtgenome)	Sequencing the entire mitochondrial genome and mitochondrial resequencing array 2.0 (MCv2)	(6)
RNA and microRNA	Reverse transcription quantitative real-time PCR (RT-qPCR)	(7, 8)
Proteome	Mass spectrometry Surface-enhanced laser desorption/ ionization (SELDI)-TOF technique and Matrix-Assisted Laser Desorption Ionization (MALDI)-TOF mass spectrometry	(9-12)
	2D polyacrylamide gel electrophoresis (PAGE)	(13)
	Liquid chromatography	(14)
	Enzyme-linked immunosorbent assay (ELISA)	(13)
Biochemical substances (e.g., α-lactalbumin, immunoglobulins, lipids, fatty acids, proteins, cholesterol, and cholesterol oxidation products)	Gas liquid chromatography (GLC, for lipids)	(15)
	Fluorescence technique of Tappel (for lipid peroxidation)	(15)
	Immunoelectrophoresis (for alpha-lactalbumin) and rocket immunoelectrophoresis (RIE, for immunoglobulins)	(15)
Hormones (e.g., estrogens, androgens, progesterone, dehydroepiandrosterone Sulfate (DHEAS), prolactin, growth hormone and leptin, and the growth factors epidermal growth factor, transforming growth factor-α, vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF))	Immunoassays (such as enzyme-linked immunosorbent assay (ELISA))	(16-20)
Tumor antigens (e.g., carcinoembryonic antigen (CEA) and prostate-specific antigen (PSA))	Immunoassays (fluorescence immunoassay (FIA) for PSA, immunoenzymometric assay for CEA)	(18, 21-23)

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Table S2. List of reviews about nipple aspirate fluid or where nipple aspirate fluid is mentioned amongst other approaches in the manuscript. Abbreviations: R, review; SR, systematic review; W, workshop.

Type	Title	Year	Author(s)	References
R/W	Physiologic, biochemical, and cytologic aspects of nipple aspirate fluid.	1986	Petrakis	(1)
SR	Factors associated with obtaining nipple aspirate fluid: analysis of 1428 women and literature review.	1990	Wrensch et al.	(2)
R	Epidemiology and prevention of breast cancer.	1996	Kelsey and Bernstein	(3)
R	Nipple aspirate fluid in relation to breast cancer.	1999	Phillips et al.	(4)
R	Lavage and nipple aspiration of breast ductal fluids: a source of biomarkers for environmental mutagenesis	2002	Klein and Lawrence	(5)
R	Breast cancer chemoprevention: current challenges and a look toward the future.	2002	Fabian and Kimler	(6)
R	Ductal lavage, nipple aspiration, and ductoscopy for breast cancer diagnosis	2003	Dooley WC	(7)
R	The role of ductal lavage in the management of women at high risk for breast carcinoma	2004	Khan SA	(8)
R	Ductal lavage in the screening of high-risk women	2004	Kenney PJ, Ellison MC	(9)
R	The local hormonal environment and related biomarkers in the normal breast	2005	Khan SA et al.	(10)
R	The Fourth International Symposium on the Intraductal Approach to Breast Cancer, Santa Barbara, California, 10–13 March 2005	2005	King BL, Love SM et al.	(11)
R	Improved peak detection and quantification of mass spectrometry data acquired from surface-enhanced laser desorption and ionization by denoising spectra with the undecimated discrete wavelet transform.	2005	Coombes et al.	(12)
R	Breast-tissue sampling for risk assessment and prevention.	2005	Fabian, C J et al.	(13)
R	The intraductal approach to breast cancer biomarker discovery	2006	Dua et al.	(14)
R	The clinical applications of mammary ductoscopy	2006	Escobar PF et al.	(15)
R	Human body fluid proteome analysis	2006	Hu, Shen et al.	(16)
R	Breast ductal secretions: clinical features, potential uses, and possible applications	2007	Lang JE, Kuerer HM.	(17)
R	Proteomics of nipple aspirate fluid, breast cyst fluid, milk, and colostrum.	2007	Ruhlen and Sauter	(18)

Type	Title	Year	Author(s)	References
R	Proteomic analysis of breast tissue and nipple aspirate fluid for breast cancer detection.	2007	Ruhlen and Sauter	(19)
R	Proteomic approaches for serum biomarker discovery in cancer.	2007	Maurya et al.	(20)
R	Human breast biomonitoring and environmental chemicals: use of breast tissues and fluids in breast cancer etiologic research.	2007	LaKind et al.	(21)
R	Molecular analysis of nipple fluid for breast cancer screening	2008	Suijkerbuijk et al.	(22)
R	Analysis of the intraductal microenvironment for the early diagnosis of breast cancer: identification of biomarkers in nipple-aspirate fluids.	2008	Mannello	(23)
R	Nutrients and nipple aspirate fluid composition: the breast microenvironment regulates protein expression and cancer aetiology.	2008	Mannello, et al.	(24)
R	Increased shedding of soluble fragments of P-cadherin in nipple aspirate fluids from women with breast cancer.	2008	Mannello et al.	(25)
R	Intracrinology of breast microenvironment: hormonal status in nipple aspirate fluid and its relationship to breast cancer.	2009	Mannello et al.	(26)
R	Protein profile analysis of the breast microenvironment to differentiate healthy women from breast cancer patients.	2009	Mannello et al.	(27)
R	Non-invasive proteomics-thinking about personalized breast cancer screening and treatment	2010	Debald M et al.	(28)
R	Breast cancer risk assessment, prevention, and the future.	2013	Green, Victoria L	(29)
R	Aluminium and breast cancer: Sources of exposure, tissue measurements and mechanisms of toxicological actions on breast biology.	2013	Darbre et al.	(30)
R	Resolving breast cancer heterogeneity by searching reliable protein cancer biomarkers in the breast fluid secretome.	2013	Mannello and Ligi	(31)
R	The human mammary gland as a target for isoflavones: how does the relation vary in individuals with different ethnicity?	2013	Maskarinec, Gertraud	(32)
R	The cancer secretome, current status and opportunities in the lung, breast and colorectal cancer context.	2013	Schaaij-Visser et al.	(33)
R	Development of a novel approach for breast cancer prediction and early detection using minimally invasive procedures and molecular analysis: how cytomorphology became a breast cancer risk predictor.	2015	Masood, Shahla	(34)

Type	Title	Year	Author(s)	References
SR	Proliferative epithelial disease identified in nipple aspirate fluid and risk of developing breast cancer: a systematic review.	2015	Hornberger et al.	(35)
R	The In's and Out's of Ductography: A Comprehensive Review.	2016	Sheiman et al.	(36)
R	Evaluation of nipple aspirate fluid as a diagnostic tool for early detection of breast cancer.	2018	Shaheed et al.	(37)
R	The power of small changes: Comprehensive analyses of microbial dysbiosis in breast cancer.	2019	Parida, Sheetal and Sharma, Dipali	(38)
SR	Diagnostic Accuracy of Nipple Aspirate Fluid Cytology in Asymptomatic Patients: A Meta-analysis and Systematic Review of the Literature	2020	Jiwa N et al.	(39)
R	Lessons Learned from Setting Up a Prospective, Longitudinal, Multicenter Study with Women at High Risk for Breast Cancer	2020/ 2021	Patuleia SIS, Hagenaars SC et al.	(40)
R	Non-Invasive Biomarkers for Early Detection of Breast Cancer	2020	Li J et al.	(41)

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Figure S1

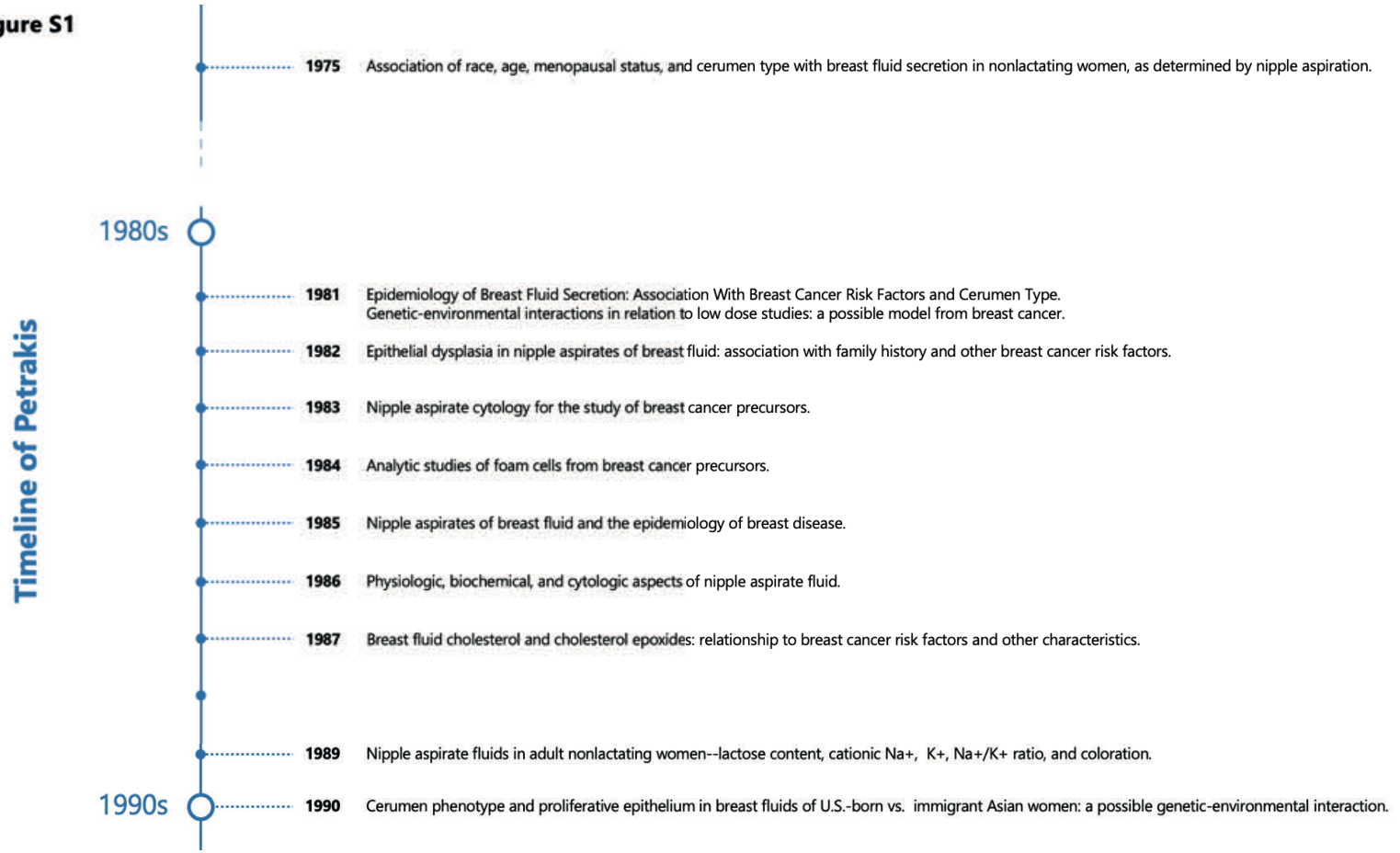

Timeline of Petrakis
1975
Association of race, age, menopausal status, and cerumen type with breast fluid secretion in nonlactating women, as determined by nipple aspiration.
1980s
1981
Epidemiology of Breast Fluid Secretion: Association With Breast Cancer Risk Factors and Cerumen Type.
Genetic-environmental interactions in relation to low dose studies: a possible model from breast cancer.
1982
Epithelial dysplasia in nipple aspirates of breast fluid: association with family history and other breast cancer risk factors.
1983
Nipple aspirate cytology for the study of breast cancer precursors.
1984
Analytic studies of foam cells from breast cancer precursors.
1985
Nipple aspirates of breast fluid and the epidemiology of breast disease.
1986
Physiologic, biochemical, and cytologic aspects of nipple aspirate fluid.
1987
Breast fluid cholesterol and cholesterol epoxides: relationship to breast cancer risk factors and other characteristics.
1989
Nipple aspirate fluids in adult nonlactating women--lactose content, cationic Na+, K+, Na+/K+ ratio, and coloration.
1990s
1990
Cerumen phenotype and proliferative epithelium in breast fluids of U.S.-born vs. immigrant Asian women: a possible genetic-environmental interaction.

Figure S1

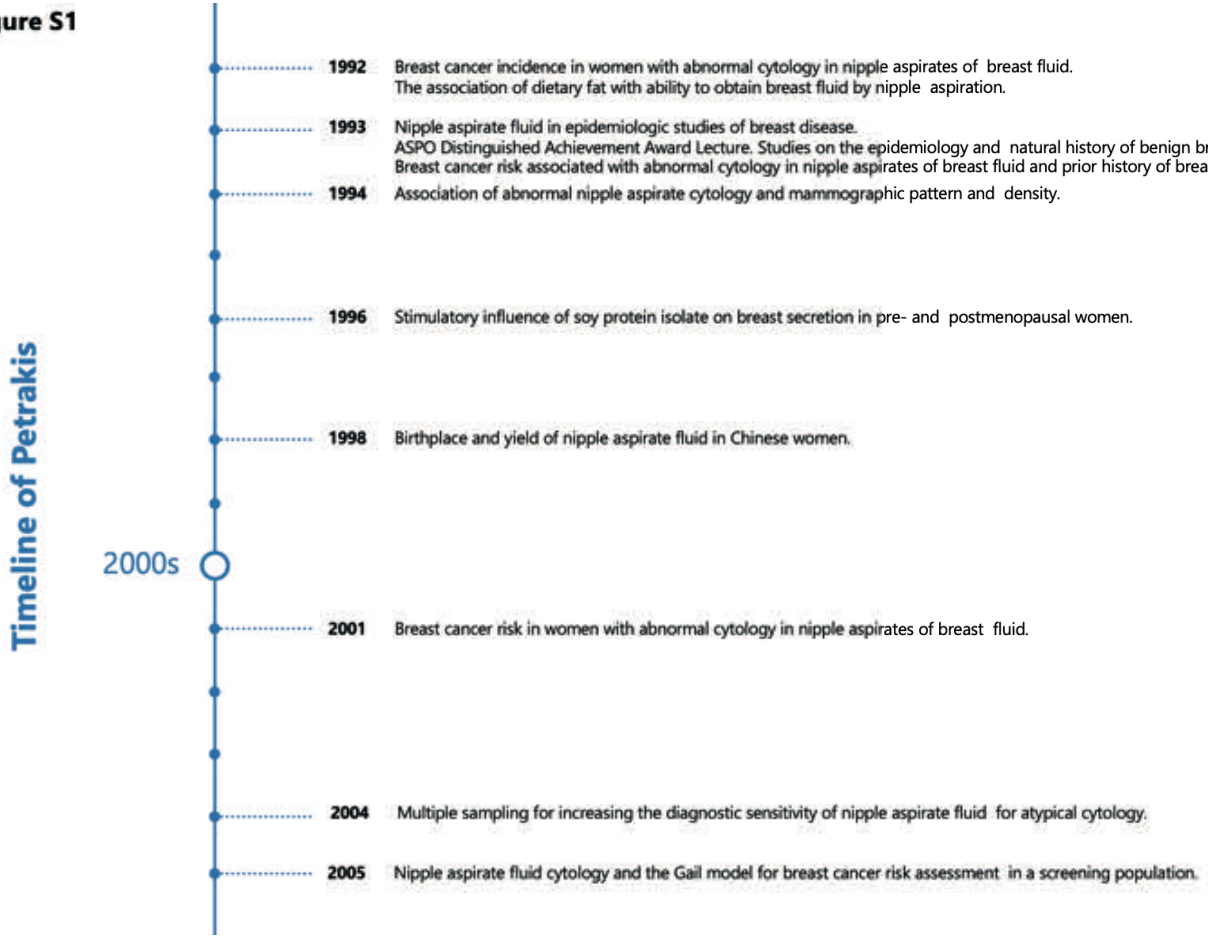

Timeline of Petrakis
1992
Breast cancer incidence in women with abnormal cytology in nipple aspirates of breast fluid.
The association of dietary fat with ability to obtain breast fluid by nipple aspiration.
1993
Nipple aspirate fluid in epidemiologic studies of breast disease.
ASPO Distinguished Achievement Award Lecture. Studies on the epidemiology and natural history of benign breast disease and breast cancer using nipple aspirate fluid.
Breast cancer risk associated with abnormal cytology in nipple aspirates of breast fluid and prior history of breast biopsy
1994
Association of abnormal nipple aspirate cytology and mammographic pattern and density.
1996
Stimulatory influence of soy protein isolate on breast secretion in pre- and postmenopausal women.
1998
Birthplace and yield of nipple aspirate fluid in Chinese women.
2000s
2001
Breast cancer risk in women with abnormal cytology in nipple aspirates of breast fluid.
2004
Multiple sampling for increasing the diagnostic sensitivity of nipple aspirate fluid for atypical cytology.
2005
Nipple aspirate fluid cytology and the Gail model for breast cancer risk assessment in a screening population.

Figure S2

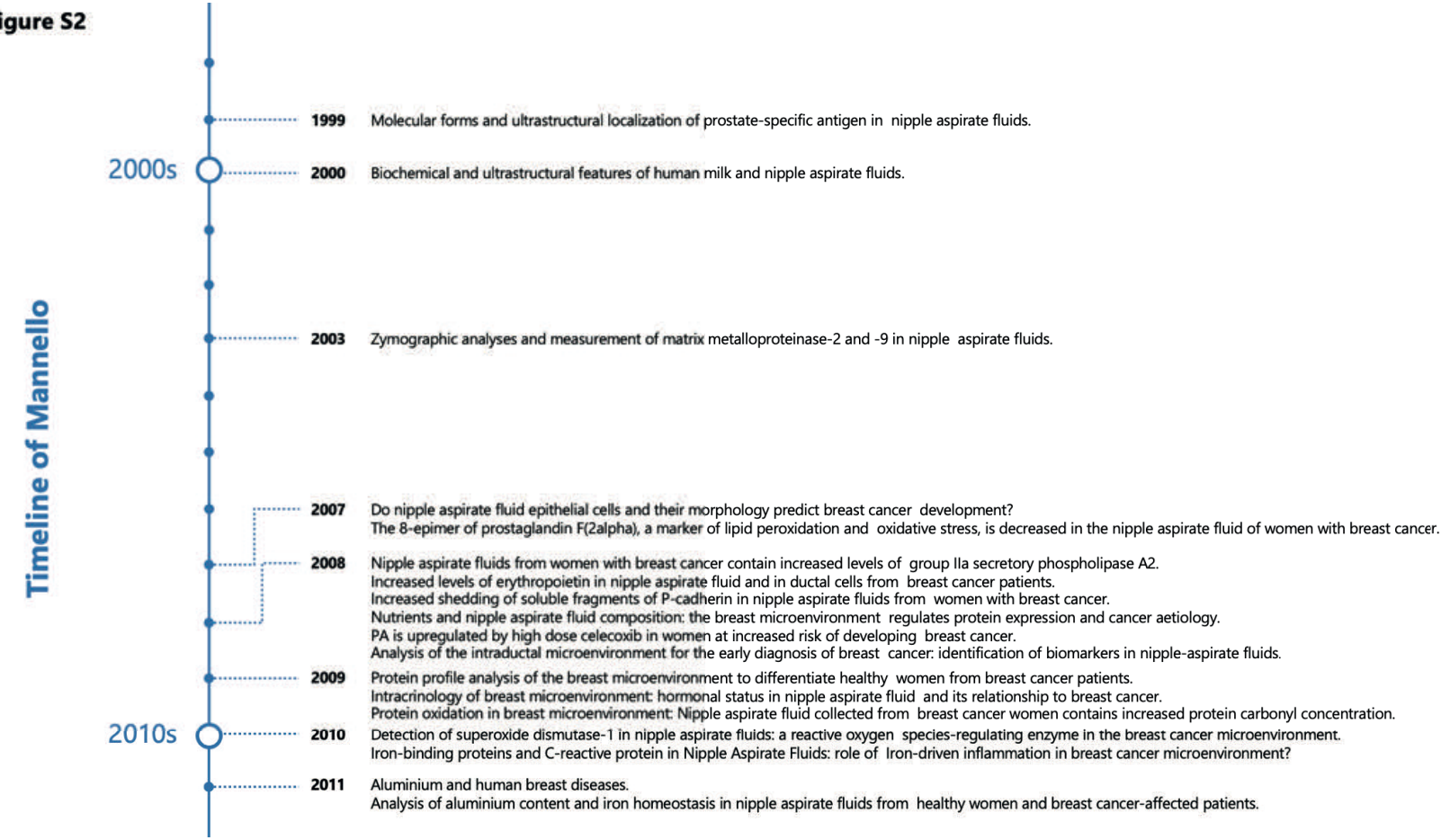

Timeline of Mannello
2000s
2010s
1999
Molecular forms and ultrastructural localization of prostate-specific antigen in nipple aspirate fluids.
2000
Biochemical and ultrastructural features of human milk and nipple aspirate fluids.
2003
Zymographic analyses and measurement of matrix metalloproteinase-2 and -9 in nipple aspirate fluids.
2007
Do nipple aspirate fluid epithelial cells and their morphology predict breast cancer development?
The 8-epimer of prostaglandin F(2alpha), a marker of lipid peroxidation and oxidative stress, is decreased in the nipple aspirate fluid of women with breast cancer.
2008
Nipple aspirate fluids from women with breast cancer contain increased levels of group IIa secretory phospholipase A2.
Increased levels of erythropoietin in nipple aspirate fluid and in ductal cells from breast cancer patients.
Increased shedding of soluble fragments of P-cadherin in nipple aspirate fluids from women with breast cancer.
Nutrients and nipple aspirate fluid composition: the breast microenvironment regulates protein expression and cancer aetiology.
PA is upregulated by high dose celecoxib in women at increased risk of developing breast cancer.
Analysis of the intraductal microenvironment for the early diagnosis of breast cancer: identification of biomarkers in nipple-aspirate fluids.
2009
Protein profile analysis of the breast microenvironment to differentiate healthy women from breast cancer patients.
Intracrinology of breast microenvironment: hormonal status in nipple aspirate fluid and its relationship to breast cancer.
Protein oxidation in breast microenvironment: Nipple aspirate fluid collected from breast cancer women contains increased protein carbonyl concentration.
2010
Detection of superoxide dismutase-1 in nipple aspirate fluids: a reactive oxygen species-regulating enzyme in the breast cancer microenvironment.
Iron-binding proteins and C-reactive protein in Nipple Aspirate Fluids: role of Iron-driven inflammation in breast cancer microenvironment?
2011
Aluminium and human breast diseases.
Analysis of aluminium content and iron homeostasis in nipple aspirate fluids from healthy women and breast cancer-affected patients.

Figure S2

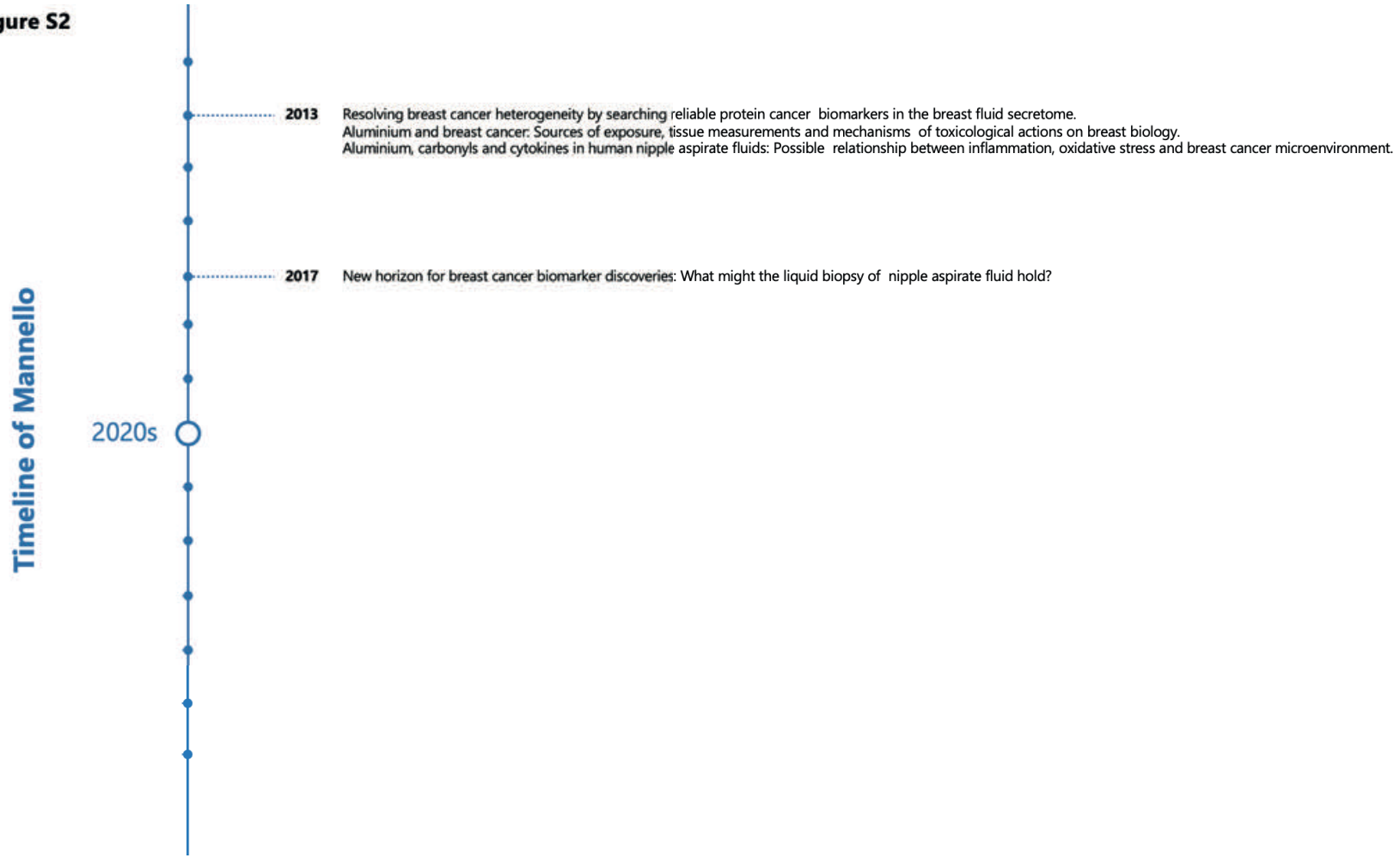

Timeline of Mannello
2020s
2013
Resolving breast cancer heterogeneity by searching reliable protein cancer biomarkers in the breast fluid secretome.
Aluminium and breast cancer: Sources of exposure, tissue measurements and mechanisms of toxicological actions on breast biology.
Aluminium, carbonyls and cytokines in human nipple aspirate fluids: Possible relationship between inflammation, oxidative stress and breast cancer microenvironment.
2017
New horizon for breast cancer biomarker discoveries: What might the liquid biopsy of nipple aspirate fluid hold?

Figure S3


Timeline of Sauter
2000s
1996
Prostate-specific antigen levels in nipple aspirate fluid correlate with breast cancer risk.
1997
Nipple aspirate fluid: a promising non-invasive method to identify cellular markers of breast cancer risk.
1998
Prostate-specific antigen production in the female breast: association with progesterone.
1999
Biological markers of risk in nipple aspirate fluid are associated with residual cancer and tumour size.
2000
Expression of a prostate-associated protein, human glandular kallikrein (hK2), in breast tumours and in normal breast secretions.
2001
Prostate-specific antigen levels in nipple aspirate fluid.
Nipple aspirate cytology and pathologic parameters predict residual cancer and nodal involvement after excisional breast biopsy.
Cellular characteristics of nipple aspiration fluid during the menstrual cycle in healthy premenopausal women.
2002
Circulating testosterone and prostate-specific antigen in nipple aspirate fluid and tissue are associated with breast cancer.
Proteomic analysis of nipple aspirate fluid to detect biologic markers of breast cancer.
Prostate-specific antigen and insulin-like growth factor binding protein-3 in nipple aspirate fluid are associated with breast cancer.
High basic fibroblast growth factor levels in nipple aspirate fluid are correlated with breast cancer.
Ethnic variation in kallikrein expression in nipple aspirate fluid.
Prostate-specific antigen in nipple aspiration fluid: menstrual cycle variability and correlation with serum prostate-specific antigen
2003
Microsatellite changes in nipple aspirate fluid and breast tissue from women with breast carcinoma or its precursors.
Association of uPA, PAT-1, and uPAR in nipple aspirate fluid (NAF) with breast cancer.
Nipple aspirate fluid expression of urokinase-type plasminogen activator, plasminogen activator inhibitor-1, and urokinase-type plasminogen activator receptor predicts breast cancer diagnosis and advanced disease.
2004
Association of kallikrein expression in nipple aspirate fluid with breast cancer risk.
Detection of breast cancer in nipple aspirate fluid by CpG island hypermethylation.
Prostate-specific antigen expression in nipple aspirate fluid is associated with advanced breast cancer.
Leptin expression in breast nipple aspirate fluid (NAF) and serum is influenced by body mass index (BMI) but not by the presence of breast cancer.
Lack of effect of celecoxib on prostaglandin E2 concentrations in nipple aspirate fluid from women at increased risk of breast cancer.
Proteomic analysis to identify breast cancer biomarkers in nipple aspirate fluid.
2005
Mitochondrial DNA mutations in breast cancer tissue and in matched nipple aspirate fluid.
Proteomic analysis of nipple aspirate fluid using SELDI-TOF-MS.
Thomsen-Friedenreich and Tn antigens in nipple fluid: carbohydrate biomarkers for breast cancer detection.
Analysis of nipple aspirate fluid for diagnosis of breast cancer: an alternative to invasive biopsy.
2006
Nipple aspirate fluid color is associated with breast cancer.
Celecoxib decreases prostaglandin E2 concentrations in nipple aspirate fluid from high-risk postmenopausal women and women with breast cancer.

Figure S3

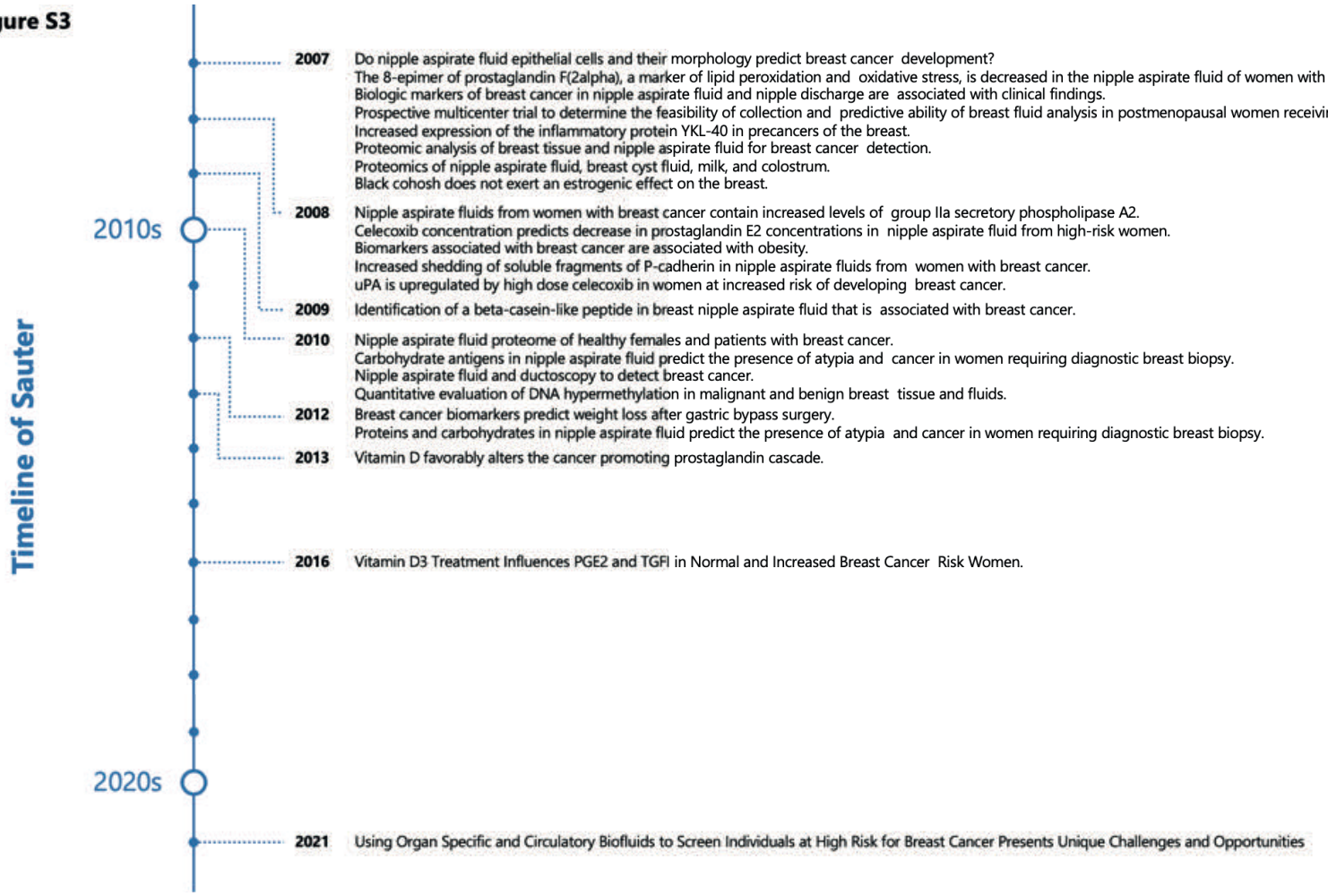
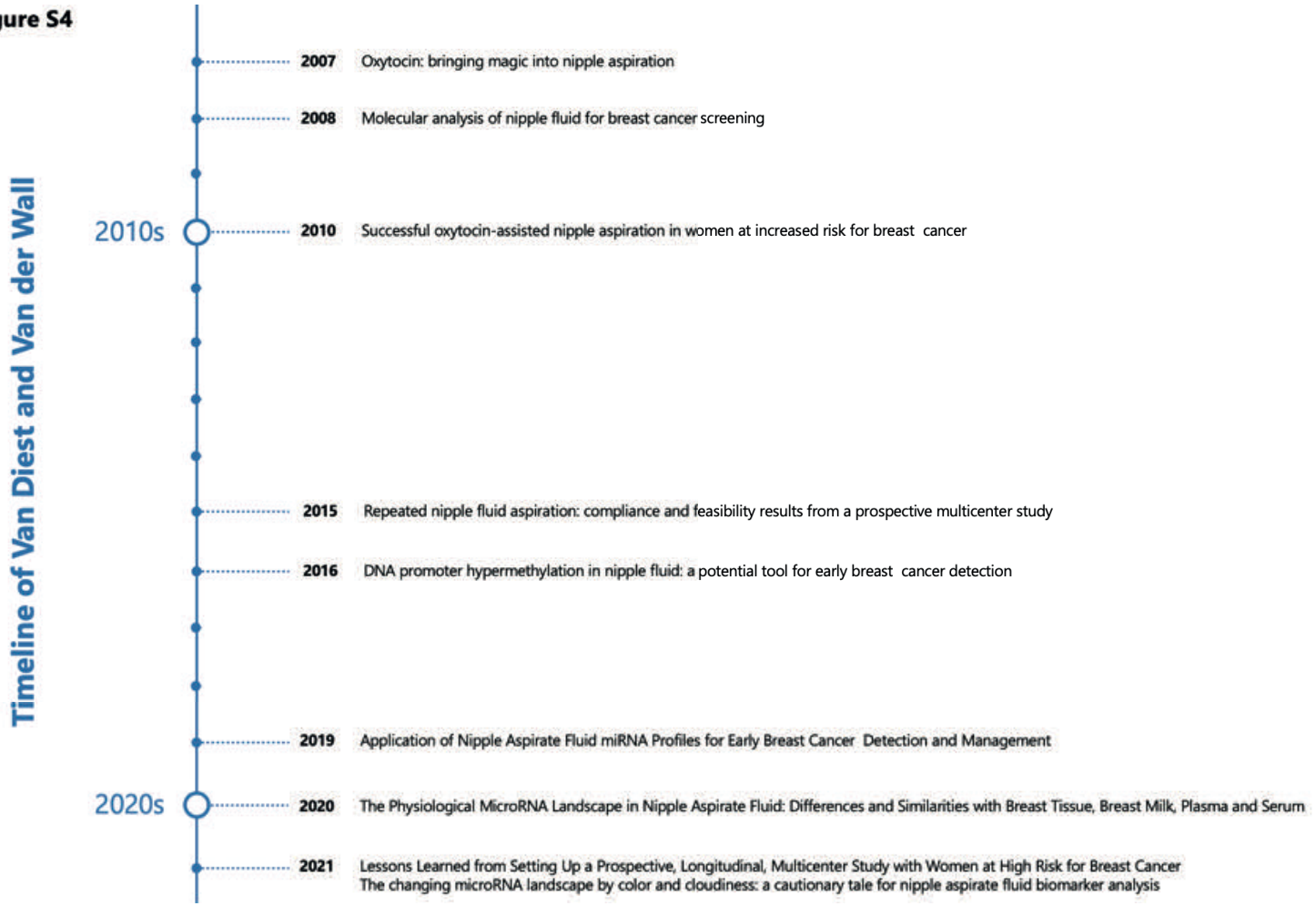

Timeline of Sauter
2010s
2020s
2007
Do nipple aspirate fluid epithelial cells and their morphology predict breast cancer development?
The 8-epimer of prostaglandin F(2alpha), a marker of lipid peroxidation and oxidative stress, is decreased in the nipple aspirate fluid of women with breast cancer.
Biologic markers of breast cancer in nipple aspirate fluid and nipple discharge are associated with clinical findings.
Prospective multicenter trial to determine the feasibility of collection and predictive ability of breast fluid analysis in postmenopausal women receiving SERMs.
Increased expression of the inflammatory protein YKL-40 in precancers of the breast.
Proteomic analysis of breast tissue and nipple aspirate fluid for breast cancer detection.
Proteomics of nipple aspirate fluid, breast cyst fluid, milk, and colostrum.
Black cohosh does not exert an estrogenic effect on the breast.
2008
Nipple aspirate fluids from women with breast cancer contain increased levels of group IIa secretory phospholipase A2.
Celecoxib concentration predicts decrease in prostaglandin E2 concentrations in nipple aspirate fluid from high-risk women.
Biomarkers associated with breast cancer are associated with obesity.
Increased shedding of soluble fragments of P-cadherin in nipple aspirate fluids from women with breast cancer.
uPA is upregulated by high dose celecoxib in women at increased risk of developing breast cancer.
2009
Identification of a beta-casein-like peptide in breast nipple aspirate fluid that is associated with breast cancer.
2010
Nipple aspirate fluid proteome of healthy females and patients with breast cancer.
Carbohydrate antigens in nipple aspirate fluid predict the presence of atypia and cancer in women requiring diagnostic breast biopsy.
Nipple aspirate fluid and ductoscopy to detect breast cancer.
Quantitative evaluation of DNA hypermethylation in malignant and benign breast tissue and fluids.
2012
Breast cancer biomarkers predict weight loss after gastric bypass surgery.
Proteins and carbohydrates in nipple aspirate fluid predict the presence of atypia and cancer in women requiring diagnostic breast biopsy.
2013
Vitamin D favorably alters the cancer promoting prostaglandin cascade.
2016
Vitamin D3 Treatment Influences PGE2 and TGFI in Normal and Increased Breast Cancer Risk Women.
2021
Using Organ Specific and Circulatory Biofluids to Screen Individuals at High Risk for Breast Cancer Presents Unique Challenges and Opportunities

Figure S4


Timeline of Van Diest and Van der Wall
2010s
2020s
2007 Oxytocin: bringing magic into nipple aspiration
2008 Molecular analysis of nipple fluid for breast cancer screening
2010 Successful oxytocin-assisted nipple aspiration in women at increased risk for breast cancer
2015 Repeated nipple fluid aspiration: compliance and feasibility results from a prospective multicenter study
2016 DNA promoter hypermethylation in nipple fluid: a potential tool for early breast cancer detection
2019 Application of Nipple Aspirate Fluid miRNA Profiles for Early Breast Cancer Detection and Management
2020 The Physiological MicroRNA Landscape in Nipple Aspirate Fluid: Differences and Similarities with Breast Tissue, Breast Milk, Plasma and Serum
2021 Lessons Learned from Setting Up a Prospective, Longitudinal, Multicenter Study with Women at High Risk for Breast Cancer
The changing microRNA landscape by color and cloudiness: a cautionary tale for nipple aspirate fluid biomarker analysis

CHAPTER 3

Lessons learned from setting up a prospective, longitudinal, multicenter study with women at high risk of breast cancer

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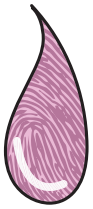
and Elsken van der Wall

* shared first authorship

Abstract

Women identified with an increased risk of breast cancer due to mutations in cancer susceptibility genes or a family history of breast cancer undergo tailored screening with the goal of detecting tumors earlier, when potential curative interventions are still possible. Ideally, screening would identify signs of carcinogenesis even before a tumor is detectable by imaging. This could be achieved by timely signaling altered biomarker levels for pre-cancerous processes in liquid biopsies.

Currently, the Nipple Aspirate Fluid (NAF) and the Trial Early Serum Test BREAST cancer (TESTBREAST), both ongoing, prospective, multicenter studies, are investigating biomarkers in liquid biopsies to improve breast cancer screening in high-risk women. The NAF study focuses on changes over time in microRNA expression levels in both blood and NAF samples, whereas the TESTBREAST study analyzes changes in protein levels in blood samples at sequential interval time points. These within-subject changes are studied in relation to later breast cancer occurrence using a nested case-control design.



These longitudinal studies face their own challenges in their execution, such as logistic and sample processing hindrances that were difficult to anticipate. This paper offers insight into these challenges and concurrently aims to provide useful strategies for the setup of similar studies.

Introduction

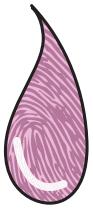
Screening of women at high risk of breast cancer

Women identified with mutations in breast cancer susceptibility genes or with a family history of breast cancer have a moderate or strong increased lifetime risk (LTR) of developing breast cancer. The LTR can reach 72% (1) and therefore requires adequate screening programs to spare these women the physical and psychosocial sequelae of breast cancer. Current screening practices in high-risk women, especially in carriers of genetic mutations, comprise adapted, more intensive programs than the regular nationwide screening for women at a population risk. The tailored screening starts at an earlier age, is more frequent, is mostly combined with a clinical breast examination and often includes breast MRI depending on the risk group and age (2-4). Advantages of breast MRI over the widely used mammography are its higher sensitivity (5) and absence of radiation, and hence the possible radiation-induced side effects (6, 7). However, whereas the improvement of applied imaging techniques has led to a better detection of smaller and *in situ* tumors, the fact remains that imaging detects cancer when it has already developed. Another issue is the timing of the screening. Although the screening is performed at regular intervals (biannually or annually), around 3-17% of the detected breast tumors are diagnosed between these scheduled screening moments (i.e. interval cancers) (8-11). Finally, another drawback is the postponement of screening by means of mammography and MRI during pregnancy and lactation. These imaging techniques are not indicated during these periods, due to the potentially harmful effects of mammography and the decreased specificity and sensitivity of both (12). Although breast ultrasound can represent an alternative for these women according to some guidelines (13), it is still not ideal given its low positive predictive value and high false positive rates (12). Taken all of this together, it is clear that there remains an urgent need to improve current screening protocols.

As scientific knowledge on the biology of cancer is still accumulating, extending the screening with the analysis of biological tumor markers in so-called liquid biopsies becomes an increasingly realistic approach. In these biofluids, tumor-derived material shed by cancerous cells, such as DNA, RNA, and proteins, can be found. Serial monitoring of these markers bear the potential to reveal early carcinogenesis by a non-invasive approach, as has already been shown for lung cancer in the MILD trial (14, 15) and for *BRCA2*-associated prostate cancer in the IMPACT trial (16), among others (17-21). The IMPACT trial has even led to the implementation of biomarker monitoring in current Dutch screening practice (22, 23). Such biomarkers could also be used as an indicator to anticipate the next screening moment and hence decrease the occurrence of interval cancers. These advantages would also apply to a non-high-risk population, for instance,

for women with dense breast tissue, for whom mammography is less sensitive (24) and the additional value of supplemental MRI is being studied in the DENSE study (25). Furthermore, as there is currently no international consensus on the best age for carriers of *BRCA1* and *BRCA2* mutations to undergo prophylactic mastectomy (26), biomarker monitoring could play an additional role in personalizing this delicate decision.

The NAF and TESTBREAST studies



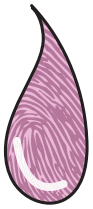
The exploratory work to identify a panel of predictive biomarkers in liquid biopsies as an additional screening tool requires an appropriate study design. The prospective, longitudinal follow-up of an ample cohort of the targeted population at risk with repeated liquid sampling before cancer onset and at the moment of cancer discovery is the optimal design. Two studies, both of which have received strong support from patient advocates, have been designed accordingly. These concern the Dutch Nipple Aspirate Fluid (NAF) study (Dutch trial register number NL8661 (27)), initiated by the University Medical Center Utrecht, and the Trial Early Serum Test BREAST cancer (TESTBREAST) study (Dutch trial register number NL8724 (28)), initiated by the Leiden University Medical Center. The setup of these studies allows the identification of prediagnostic changes in biomarker levels between serial samples from the same study subject. A certain proportion of study subjects will develop breast cancer after being recruited (cases). Serial comparison of samples facilitates investigation of whether biomarker levels were already altered before tumor was diagnosed and, if so, how long before these changes could already be measured (Figure 1). These changes over time in the cases could then be compared with those determined in study subjects who have not developed breast cancer (controls).

Both are long-term, ongoing studies: the NAF study started in 2008 and the TESTBREAST study was initiated in 2011. In the TESTBREAST study, blood samples from high-risk women are prospectively collected at the time of regular screening appointments. The last sample is collected if and when an event occurs, i.e., invasive breast cancer or carcinoma *in situ* (ductal or lobular). The NAF study uses the same setup, but in addition to blood, it also collects NAF samples from these high-risk women. The additional hypothesis tested in the NAF study is whether NAF samples mirror the breast microenvironment best, as this “liquid biopsy” is directly derived from the ducts and/or lobules including those that harbor the cancer cells. The biomarkers investigated in both studies differ: while the NAF study focuses on microRNA expression levels, the TESTBREAST study analyzes protein changes over time. The overarching objective of both research groups is similar, namely, to identify a combination of biomarkers for early detection of breast cancer.

For this paper, both research groups convened to reflect on the challenges that go hand in hand with conducting such a prolonged, longitudinal, multicenter biomarker study. Our goal was to provide an overview of these challenges and their possible solutions to support future researchers who intend to design similar cohort studies.

Lesson 1. Study phases and cohort size

The first and predominant challenge is that, in order to obtain a longitudinal series of prediagnostic samples from a sufficient number of breast cancer cases, a very large, long-running biobank needs to be constructed. Even in cohorts of women at high risk for breast cancer, only about 1-6% of the study participants develop an event over a period of 4-8 years (9-11). Therefore, much effort is needed to set up the biobank and to acquire and store the large number of samples, while final analysis will only be performed in a nested case-control design on a limited number of cases and controls to answer the research question. From the beginning, the focus of the study is on establishing a large longitudinal cohort of healthy high-risk women. The required time to reach a large number of inclusions and a sufficient follow-up period makes this "inclusion and biobanking phase" the most time-consuming part of the study phases. When enough cases have developed to provide sufficient statistical power to test the predictive value of changes in biomarker levels in relation to cancer occurrence, the next phase can be initiated. This "sample and data analysis phase" includes selecting matching controls based on the characteristics of the cases, retrieving samples of the selected study subjects from the biobank, analyzing biological samples in the laboratory and performing subsequent statistical analyses. In the meantime, the "inclusion and biobanking phase" becomes a "follow-up and biobanking phase", allowing for more women to develop events for the subsequent validation studies.



A few potential pitfalls should be taken into account. The first pitfall is to only engage experts in specific phases during the study. Early commitment and participation of several experts helps the study run smoothly from the start and prevents making incorrect assumptions or decisions. We recommend involving all necessary experts who should be involved from the start of the study. A list of experts suggested for the presented study design and cohort is presented in Table 1.

Another pitfall is to mainly focus on the number of inclusions in the cohort, while it is also relevant to regularly monitor more aspects of the cohort, such as study dropouts. Specifically for biomarker studies, issues such as the number of successfully serially acquired samples for analysis should be closely monitored. Some samples may be absent due to missed visits, lost by unexpected flaws, processing errors, and/or laboratory technical failures. These issues and recommendations on how to avoid them will be further elucidated in Lessons 3, 4, 7 and 9. Lastly, predicting the time frame of the study is challenging. From our experience, more than five years are needed for the initial "inclusion and biobanking phase". This depends on the extent of the abovementioned hurdles, which cannot be anticipated beforehand. We recommend monitoring the participation rate and being prepared for a prolonged time frame of the study.

Table 1. List of suggested team members.

Core team	Consulting experts	Additional essential members
· PIs · PhD student · Project coordinator · Research nurses · Biomedical expert · Datamanager · Laboratory technician	· Clinical geneticist(s) · Epidemiologist and methodologist · Statistician · Biobank coordinator	· Screening doctors and nurses · Local PIs, research nurses, and lab staff in side centers · CRA

PIs: principal investigators; CRA: clinical research associate

Lesson 2. Funding

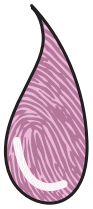
The lack of fast results in prolonged longitudinal studies makes funding less attractive, both to governmental and private sponsors. Preliminary data and the achievement of milestones are usually the basis for applying for the next grant. However, given the long "inclusion and biobanking phase," which was described in Lesson 1, limited data will be generated for a long period of time. This is a relevant and delicate challenge, since the progress of the study is highly dependent on the continuous and long-term funding for one project. The discontinuation of funding is detrimental to any phase of the project. Essential budgetary costs also refer to the salaries of the team members involved in running the study (Table 1); intermittently downsizing the team due to financial shortcomings endangers, amongst others, adequate data acquisition and, in the end, its interpretation.

To ensure continuous funding, principal investigators (PIs) should apply for several grants throughout the course of the study. Each grant application should focus on one of the study aims. Infrastructural grants are ideal to achieve biobanking aims in the "inclusion and biobanking phase", whereas research grants, including high-risk pilot grants and proof of concept grants, are more suited for aims focused on acquiring results as part of the "sample and data analysis phase". Private sponsors that share the vision that a prolonged study is required to obtain translational results may be instrumental.

Lesson 3. The inclusion and exclusion criteria are dynamic

Inclusion criteria

The underlying causes for having an increased risk of developing breast cancer are quite diverse, as these include, among others, pathogenic variants of one of the breast cancer genes (e.g. *BRCA1*, *BRCA2*, *CHEK2*, *PALB2,* and *ATM*), a personal and/or a family history of breast cancer, and a medical history of radiotherapy in the thoracic field. Inclusion criteria can be defined based on a threshold LTR percentage or based on a specific high-risk group for the cohort. The pitfall of applying LTR as the main inclusion criterion lies in the inclusion of a wide range of high-risk subgroups. Note that if the predictive value of the biomarker panel varies per subgroup, but it is analyzed as the total group, there will be a diluted effect, and biomarkers that are relevant for one subgroup but not for others may be missed. If there are strong initial indicators that relevant biomarkers may differ per high-risk subgroup, it is advisable to calculate the necessary sample size per subgroup.



One can also choose to only include mutation carriers of a certain age to make the study financially efficient in terms of costs for sample collection, processing, and storage. However, this would mean that the biomarker panel could only be tested for this group and would not give the opportunity to explore whether it is also usable for other high-risk women, who also need such an early detection screening tool. Therefore, including several high-risk categories has the advantage that one large high-risk breast cancer biobank can be established that allows biomarker testing in a wider scope. The increased LTR for developing breast cancer is well defined for most subgroups, e.g. women with an established genetic mutation. Yet, the risk may be dynamic due to increasing age, a new breast cancer diagnosis in the family, new scientific developments that lead to the discovery of additional gene mutations, expansion of the tested gene mutation panel (29) and new algorithms for risk assessment (30) that result in renewed LTR estimates and screening guidelines. For women with a 50% chance of having a *BRCA1* or *BRCA2* gene mutation, their LTR lowers if genetic testing by the study subject and/or a family member is negative for mutation(s). For these cases, the study protocol should state whether they should be excluded. If so, the research team should decide beforehand whether the already acquired samples will then be used for other research purposes and describe this possibility in the informed consent form. Arguably, follow-up of these women could still be continued, as a lower risk does not rule out the possibility of developing breast cancer. The study team should be aware of the possibility of this decreased LTR and keep this particular screening information of study subjects up-to-date, which can be facilitated by keeping close collaboration with the clinical geneticists involved.

Exclusion criteria

A criterion to consider for exclusion in a biomarker study is a personal history of breast cancer. Although these women have an increased risk of developing breast cancer in the contralateral breast (31), the past treatment may by itself influence the biomarker pattern of choice. For the same reason, a medical history of other malignancies could be considered an exclusion criterion, as adopted by the TESTBREAST study team (Table 2). Given the novelty component that comes with the NAF samples and, therefore, the lack of literature-based arguments for the implementation of these criteria, these were not adopted by the NAF study team. Nevertheless, this information will be considered in the statistical analysis.

Commonly used study exclusion criteria are pregnancy and lactation. One of the reasons why these criteria were implemented in the NAF study was to avoid milky sample collection; as such, a study visit can only be planned more than 3 months after completing breastfeeding. Still, samples collected between 3 and 24 months after the end of lactation may contain milk components. These components may influence biomarker analyses when these samples are being compared to NAF samples collected more than 24 months after the end of breastfeeding, as are most of the samples. Other reasons were the potential rare side effects of the oxytocin nose spray, such as uterus contractions (32). Although oxytocin is used in every study visit in a very low dosage to increase the success rate of bilateral NAF harvesting (which currently is 65.8% for the entire study period and was previously reported to be 62-73% (33-35)), a total absence of effect on uterine muscle and biomarker patterns cannot be guaranteed. As these are long-term studies and young participating women could become pregnant during the study period, these criteria should be defined as 'temporary exclusion criteria' to allow the continuation of the study participation later. From another perspective, since there are currently no alternatives for screening during that period, measurement of blood-based biomarkers in pregnant high-risk women may be even more valuable. This could, however, be limited by the fact that pregnant women do not undergo routine screening during this period, so they would have to visit the hospital only to donate blood samples for research purposes, which could be an extra burden for some. Lastly, in countries where chemoprevention is advised (3, 36), this should be considered as an exclusion criterion due to the woman's lowered LTR and the possible influence of chemoprevention on the biomarker panel.

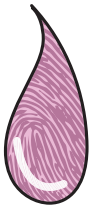
To sum up, the study protocol should identify which measures should be taken when the LTR of a woman decreases, and this should be clearly stated in the study information form. In this form, the subsequent consequences regarding participation in the study and handling of already acquired data and biobank material should be included. Finally, PIs should define criteria that may influence the biomarker of choice and add them to the list of exclusion criteria in the study protocol.

Table 2. Inclusion and exclusion criteria for the NAF and TESTBREAST studies.

	NAF study	TESTBREAST study
Inclusion criteria	· Female ≥ 18 years of all ethnic backgrounds · A > 20% LTR of developing breast cancer, including germline *BRCA1* or *BRCA2* mutations and previous DCIS/invasive breast cancer	· Female between 25-75 years of age · Screening indication due to a familiar or genetically increased risk of developing breast cancer or LTR > 15%
Exclusion criteria	· Bilateral ablative breast surgery · Bilateral breast reduction with nipple graft · Pregnancy or lactation · Active breast infection · Disseminated breast cancer	· Previous invasive breast cancer · Other malignancies < 10 years (other than basal cell carcinoma)

LTR: lifetime risks; *BRCA*: breast cancer gene; DCIS: ductal carcinoma *in situ*

Lesson 4. Delayed inclusion moment and discontinuation of participation



Both studies aimed to place as little extra burden on study participants with regard to hospital visits as possible. This means, in theory, that the first opportunity to inform potential candidates about the study is during their regular scheduled screening appointment. By Dutch law, it is required that individuals who are asked to participate in a scientific study should have a reasonable amount of time to consider this request, in principle prohibiting start of the study on the day that information about the study is provided. Consequently, by holding on to having sample acquisition coincide with a regular screening appointment, the actual moment of study inclusion could be delayed for six to twelve months (i.e. when the next screening appointment is scheduled). This observation led to an adapted protocol in the TESTBREAST study, where research nurses now attempt to inform potential participants about the study in advance by post or email, so that inclusion in the study can start at the time of the already scheduled screening visit.

Given the long time period until publication of study results, discontinuation of participation among enrolled women is prone to occur. Therefore, efficient strategies to maintain compliance should be applied. An essential starting point is having dedicated research nurses who clearly describe the aim of the study to participants and the importance of collecting serial samples. In addition, a movie depicting a study visit and/or explaining the setup of the study provides further clarification to participating women. To keep study subjects informed about the course of the study, one should consider sending regular study newsletters, frequently updating the study website, and organizing annual meetings where the study team is present. The latter provides study participants ample opportunity to ask questions, which in our studies has proven to be very well appreciated.

A combination of the strategies mentioned above leads to a clear realization of the importance of their participation in the study. This is relevant not only for the participating women but also for team members in participating hospitals.

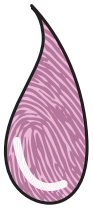
Despite these various strategies, dropouts are almost an implicit component of long-term clinical studies. The main reasons for women to stop participating in the NAF study, in rank order (from highest to lowest) were: 1) reaching the end of the study according to protocol (established as 10 years in the NAF study) (18%), 2) repeated unsuccessful aspiration (17%), 3) preventive bilateral mastectomy (8.6%), 4) loss to follow-up (7.4%), 5) no screening appointments at the hospital (6.7%), 6) development of breast cancer (6%), amongst other reasons. Planned prophylactic mastectomies, specifically for carriers of the *BRCA1* or *BRCA2* mutations, are unavoidable dropouts that can be expected. For the NAF study, a specific drop-out reason was repeated unsuccessful NAF acquisition (i.e. 0 µL), which can be related to parameters such as menopausal status, spontaneous nipple discharge, breast size, bilateral oophorectomy and previous use of hormone replacement therapy or anti-hormonal treatment (34). Other reasons are logistic issues, such as relocation of participants to another area and hospital, no longer having time to come to the outpatient clinic or the end of hospital screening (i.e. return to regular out-of-hospital nationwide screening). These problems could theoretically be surpassed if blood draws could be taken at any location (e.g. at a blood draw clinic or at the general practitioner's office). However, in practice, women may overlook or postpone having their blood drawn. To avoid long waiting times as a potential barrier, the TESTBREAST study team established a priority rule for blood sampling in the lab. This resulted in an increased willingness of up to 86% of the study population to have their blood samples taken. All of these factors should be considered when estimating the number of required inclusions.

To summarize, to safeguard the inclusion rate, the study team should ideally inform potential candidates about the study before the next scheduled clinical screening visit. For subsequent study visit invitations, the research team should create and maintain a recall system to invite study subjects in a timely manner, and sample acquisition should be facilitated by implementing a fast track for obtaining blood samples. On top of that, the core study team should keep the study subjects and other participants in the team informed about the course of the study. These strategies reduce the number of unnecessary dropouts and missed study visits.

Lesson 5. Logistics

Given the high number of participants and an even higher number of study appointments in this trial design, it can be challenging to maintain continuous registration of the incoming data. For example, in the NAF study, a two-center study in which every study visit takes about one hour, up to 12 study visits are

planned every week. The study visit registration aspects include the digital processing of a questionnaire and the collection, processing, registration and biobanking of NAF and blood samples that are aliquoted into 22 vials (three vials per breast for NAF, ten vials for serum and six vials for plasma). Questionnaire data comprise lifestyle and hormonal factors. Sample registration data comprise NAF sample color, including bloody appearance, consistency, and volume per aliquot. NAF color can range between study subjects, between breasts of the same woman, and even within a breast. NAF color registration provides insight into the color variation and permits exploration of its association with breast cancer risk. NAF samples should be labeled as bloody or non-bloody before analysis, as bloody NAF samples could lead to alterations in biomarker analyses compared to non-bloody NAF. The TESTBREAST study visits comprise a questionnaire and a blood draw, which, unlike the NAF study, does not need to be performed by trained study team members, but can be performed by regularly trained hospital personnel. Since TESTBREAST is a nine-center study with one to four visits per participant per year (as established by hospital-specific screening guidelines and commitment of study participants), the number of questionnaires and blood samples incoming to be processed and stored is high. Therefore, it is key to set up a well-established administrative process. To guarantee a good cohesive administration that is adequately maintained according to data monitoring rules, annual monitoring by an independent clinical research associate (CRA) is advisable throughout the course of the study. Additionally, both a data manager and a(n) (online) data management system (e.g. ProMISe for the TESTBREAST study) are essential to keep the incoming information up to date and facilitate the traceability of samples (37). Recent developments in online databases also allow the integration of online questionnaires. This ensures completeness of the questionnaires and automated integration of the information directly into the database.



A turnover of study team members is expected during a long-term study. This type of turnover can occur in team members who are daily and routinely involved in the study, such as research nurses and the clinical study coordinator. In the Netherlands, coordination of a clinical study is usually performed by an MD-PhD student. As they should finish their research project within four years, several PhD students are usually involved in such a project before it reaches its end. A way to pertain consistent working procedures is to establish and frequently update standardized operating procedures (SOPs), which are supervised by a CRA. Also, contact information of team members should be updated and departing team members should train new incoming members about the SOPs. In multicenter studies, a fixed contact person on site is responsible for the correct local execution of the study. A site training of a specific study technique (such as nipple fluid aspiration) might be necessary in some studies. Structured meetings with local study coordinators are recommended to ensure proper data collection.

Lesson 6. Sample processing

A difficulty of long-term prospective studies is to guarantee the optimal quality of biological material for multiple years until the time of analysis. For example, prolonged freezer storage time is accompanied by the risk of loss of sample quality and loss of sample by evaporation (38). Therefore, correct handling of the sample during collection and storage is essential. Sample collection and processing aspects to consider include consistent use of the same type of collection tubes and buffers, a pre-defined time until sample processing and freezing, and a defined monitored temperature at which samples are stored. Additionally, aliquoting samples is a good strategy to later avoid freeze-thaw cycles, which possibly influences sample quality. Lastly, pilot sample analysis should not be postponed until the end of the study, as it allows timely sample quality monitoring and assessment of natural temporal biomarker fluctuations (Figure 1). In this context, pilot tests should be performed to ensure that collected samples are suitable for biomarker analyses. As an example, in the NAF study, pilot testing has shown that, regardless of the RNA starting concentration, microRNA qPCR can always be measured given the high sensitivity of this technique (39).

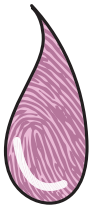
Working with a number of hospitals over a long period of time comes with the potential pitfall that different types of collection tubes and a variety of buffers are used across centers or even within centers, which could hamper a comparable sample analysis. It is important to maintain consistency among and within participating hospitals. Recommended approaches include providing sets of collection tubes, labels, lab forms, and questionnaires to all centers, as has been part of the standard procedure in the NAF and TESTBREAST studies.

Finally, prolonged processing time of samples can lead to alteration of biomarker characteristics and cause the final results to deflect (40). Therefore, the continuous presence of a team member in the laboratory is required to process new samples within the defined time limit. The standards used for sample processing in the NAF and TESTBREAST studies are depicted in Table 3.

Table 3. Sample processing in the NAF and TESTBREAST studies.

	NAF study	TESTBREAST study
Sample processing time	< 60 minutes	< 4 hours
Centrifuging speed	15 minutes 300g (1200 rpm) for serum Mini-centrifugation for NAF	10 minutes 1000g
Storage temperature	-80 °C	-80 °C
Sample volume	10x 600 µL for serum 6x 600 µL for plasma 6x 0-30 µL for NAF	550 µL and 4x 500 µL for serum
Type of collection tubes	BD Vacutainer SST II Advance for serum BD Vacutainer K2E (EDTA) for plasma Brooks FluidX 0.7 mL external threat tube for NAF	BD Vacutainer SST II Advance for serum
Analysis	RT-qPCR	Mass-spectrometry methods

NAF: Nipple Aspirate Fluid; TESTBREAST: Trial Early Serum BREAST cancer; RT-qPCR: reverse transcription quantitative real-time PCR



Lesson 7. Sampling at events

For both the longitudinal cohorts of the NAF and the TESTBREAST studies, multiple samples from study subjects are prospectively acquired; the last sample is collected if and when an event occurs. As the primary objective of studies is to compare data from liquid biopsies obtained at the time of an event with those acquired before diagnosis, sampling at the time of an event is valuable. This sample is relevant in the investigational setting, as biomarker levels at the time of the event best reflect the carcinogenetic signature and, as such, function as reference levels in the paired analysis (Figure 1).

As participating women follow an intensive regular screening program, an event can be detected during such an appointment, which is usually combined with a study visit. In those cases, sampling at the time of an event is guaranteed. However, for interval cancers, chances are that liquid biopsy collection is missed, since neither the treating physician nor the study participant is sufficiently aware of the importance of informing the study team: the physician because he/she might not be informed about study participation and the participant may neglect to inform the study team about her diagnosis because of her overwhelming present situation. An active, regular search by the study team to rule out or confirm new breast-related pathology is inefficient, labor-intensive, and has a high chance of still leading to discovering the events too late (i.e. after they started treatment). A practical and automated solution is to generate a 'study alert notification' on electronic platforms such as electronic health records (EHR) or in the nationwide online registry of pathology reports (in the Netherlands, this

has the acronym PALGA (41)) so that new events will be reported to the study team before the start of treatment. This interconnection between the cohort and tissue repositories and cancer registries is also essential to ensure complete follow-up, provided that this interconnection is included in the informed consent form. Probably the most practical approach is to instruct study participants to inform the research team every time they have additional hospital visits for breast-related problems. Therefore, participants must be well-informed, well-instructed, and perhaps regularly reminded that when they develop an event they should contact the study team for final sampling. Still, it is understandable that this is a delicate time for a woman to consider an additional hospital visit, especially in the context of a clinical study.

Lastly, there is a chance that women develop an event after having completed the study period. According to the Medical Research Ethics Committee guidelines, the protocol of a clinical study is required to have a specified delimited study period. The absence of such a limit could lead to endless clinical studies with the threat of losing aim and perspective. However, we recommend adding a prolonged time margin to the study protocol, which would allow women to participate for a longer period and to have an additional sample taken at the time of an event, provided that this sample is acquired within a reasonable amount of time after the end of the study period.

By combining the above-mentioned approaches, chances of acquiring a sample at the time of an event are increased, leading to a more complete intra-subject sample series in the biobank and, thereby, achieving the main aim of the study.

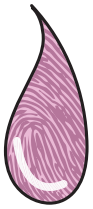
Lesson 8. Biobanking

As a consequence of the high number of samples in these specific studies, storage room might become an issue. As explained in Lesson 5, around 1 to 22 aliquots per study visit per study subject were acquired throughout the TESTBREAST and the NAF studies, respectively. To give an impression, in the NAF study, over 15000 vials were stored in 11 years' time for 555 study subjects. In the TESTBREAST study, more than 3000 samples were acquired between 2011 and 2019 for more than 930 participants (some blood samples for the same women exceed the 20 visits). Therefore, it is essential to ensure substantial biobank storage in a timely manner, which will allow the samples to be kept safely for many years. In parallel, a well-defined biobanking system that allows for continuous sample registration in a database and consensual labeling throughout the study should be operational. Labels should be cold and moisture resistant, printed, and include standardized information, ideally the name of the team member who acquired and handled the sample, study subject number, visit number, sample type, volume, aliquot

number, sample date, and a QR code. Specifically, for the NAF study, the breast side of the NAF sample should be added to the label.

Lesson 9. Sample handling at the time of analysis

As highlighted in Lesson 1, only a relatively small part of the cohort is expected to develop an event. Depending on the ratio of sample volume attained and technical volume required, samples could perhaps only be used once. For example, in the NAF study, given the small volume acquired (10-50 µL), the majority or the complete volume may be needed for a single experiment. Thus, the application of a trustworthy, familiar technique that has already been optimized to analyze the presence of biomarkers provides reinsurance.



The exploration of improved, cheaper, new, faster, and potentially better techniques developed during the course of the study should be tested. This is inherent in technological developments and cannot be anticipated. Therefore, a sufficient sample volume has to be acquired to allow for such technical exploration and comparisons. We recommend including technical testing in the list of secondary aims of the study protocol. Obtaining more volume is understandably less of a limitation for blood samples than for low-volume NAF samples. As highlighted in Lesson 8, a downside of obtaining higher volumes in general is that even more samples will have to be stored in the biobank, which demands more storage.

Lesson 10. Adjustment of the biomarker of choice

The biomarker of choice to investigate in the high-risk cohort often derives from case-control studies. Specifically, for the TESTBREAST study, the focus of research is based on previous promising results in proteomic expression profiling, showing very high sensitivity and specificity for the detection of breast cancer (42-44). However, preliminary case-control studies may also lead to alteration of the initially chosen biomarker due to insufficient diagnostic accuracy in interim analyses or in other studies. For instance, in the NAF study, gene methylation was the subject of investigation at first, but when interim analyses did not reveal a sufficient area under the curve to justify further translation into a clinical test (45), the biomarker of choice changed into microRNA. Furthermore, alterations may occur as a result of the discovery of new classes of biomarkers. Therefore, it is important to create the ideal conditions to be able to switch to another biomarker during the study, among others, by obtaining broad informed consent. Nevertheless, it is difficult to define the ideal conditions for switching to new biomarkers, as this anticipates future developments. The use of buffers that allow different types of isolations of e.g. DNA, RNA, and proteins is advisable. Storing sufficient amounts of biofluids in different aliquots allows even more types of

analyses than initially anticipated for the project. Moreover, storing different blood (half)products, such as whole blood, plasma and serum is recommended but, as a downside, leads to increased pre-processing costs. When switching to another biomarker or technical platform, one has to be aware that new markers and platforms will have to be technically validated, and the validity of the "old" data has to be established and might even have to be discarded.

Lesson 11. Nested case-control analysis

There are a few issues that hamper nested case-control analysis. First, the envisioned time frames of sampling (Figure 1) are seldom exactly reached. The main reasons include delayed hospital screening appointments due to hospital logistics, a woman's preference, pregnancy (a contraindication to imaging) or lactation (leads to reduced imaging sensitivity) (12). Since study visits are preferentially combined with hospital screening visits, a change in the latter affects the study time points. Consequently, in practice, there will be variation in the sample acquisition intervals within the same subject and between study subjects. Secondly, another naturally occurring issue is incompleteness of sample series. This is due to missed study visits or unsuccessful sample collection. The statistical approach that allows data analysis with individual repeated measurements in case of missing data points and flexible time schedules includes the use of linear mixed models (46). A solution could be to cluster data sampled at several time points into categorized subgroups (e.g. <6, 6-12 and >12 months before the event, as clustered by Weber et al. (47)). Still, to optimally allow for paired comparison analysis, it is of importance to continuously keep a real-time overview of the number of events and also to keep the number of successfully acquired samples and timing of sample acquisition up-to-date. If incomplete series are noticed in a timely manner, adjusting the sample sizes accordingly is still possible.

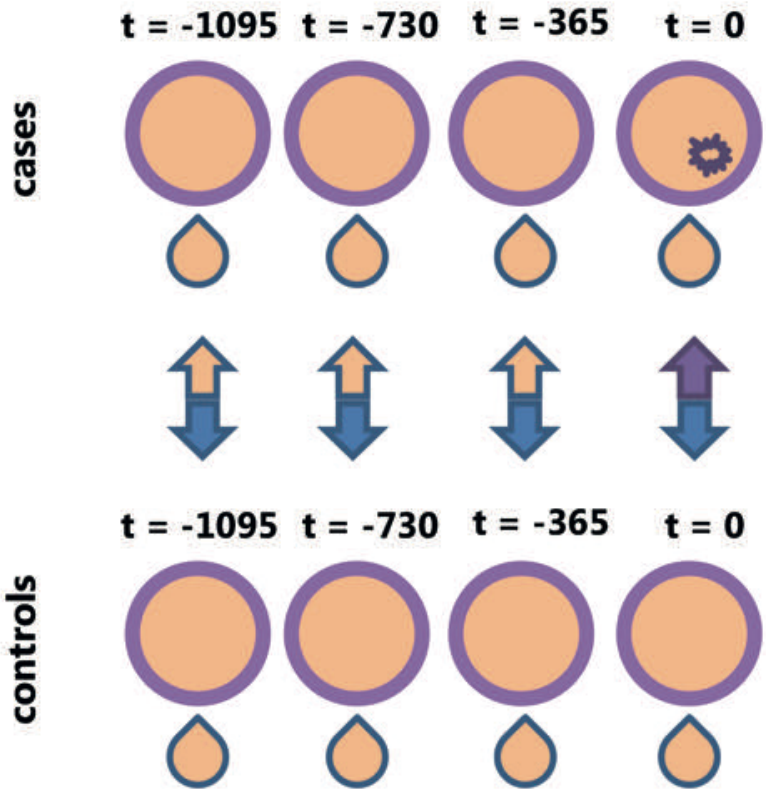


Figure 1. Nested case-control analysis. In the described prospective, longitudinal cohort, healthy high-risk women were included and serial samples are collected over time. The subjects who developed breast cancer after they were recruited in the cohort become the "cases". "Controls" are all the cohort members who did not develop breast cancer. In this figure, the circles represent the study visits over time, and the droplets underneath represent sample acquisition. Any "t" (in days) stands for the number of days before the event. In the case groups, t=0 represents the time of the event; in the control group, the time point t=0 represents the moment at which the most recent sample was acquired. Arrows represent the comparisons between cases and controls that allow for a nested case-control analysis.

Discussion

Biological markers in liquid biopsies reflecting carcinogenesis could be of great additional value to further improve the breast cancer screening program, specifically in high-risk women. To find a suitable panel of biomarkers for early breast cancer detection, a prospective longitudinal cohort should be established to allow serial collection of samples from high-risk women until an event occurs. This allows serial analysis of changes in biomarker levels over time and especially how long biomarkers can signal the onset of breast cancer beforehand. However, this study design is also accompanied by pitfalls that were encountered by two independent research teams who set up similar long-lasting studies. Some of the pitfalls can be circumvented by defining crucial elements in the protocol before the start of the study. In addition, proper SOPs should be written to contribute to uniformity in the execution of the study, as a change of personnel is unavoidable in view of the long study period. Furthermore, substantial biobank storage needs to be established promptly and study subject withdrawal and missing data should be closely monitored. However, one of the greatest challenges is to have a sustained flow of funding by, for instance, research foundations or generous private donors. On a general note, advances in knowledge and technology, such as database management, online questionnaires, QR codes, and laboratory technologies, cannot all be anticipated at the time of the study set-up, but should be actively tailored throughout the course of the study. Table 4 provides a summary of these different pitfalls, lessons learned by us, and recommendations that may be of use for other research groups setting up similar long-lasting cohort studies.

Additional file

The published version of this article was commented by the expert Dr. Edward Sauter. With his consent, this comment is included into this thesis in pages 85-91.

Acknowledgments

We are greatly indebted to all women who participated and are participating in the NAF and TESTBREAST studies.

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Table 4. Summary of the challenges, lessons learned, and recommendations for prospective, longitudinal, multicenter studies of women with a high risk of developing breast cancer.

Challenges	Lessons learned and recommendations
1. Study phases and cohort size	· Engage all necessary experts from the start of the study. · Consider in the sample size calculation of the complete cohort, matters like study withdrawals, lowered LTRs, and incomplete sample series. Customizing the sample size during the study and extending the study time frame may be necessary.
2. Funding	· Divide the study objectives into parts to apply for several infrastructure and research funding grants throughout the years.
3. The inclusion criteria and exclusion criteria are dynamic	· A woman's LTR may lower: describe which measures to take in the study protocol and in the study information form. · Investigate which factors may influence the biomarker of choice and add these to the list of exclusion criteria in the study protocol.
4. Delayed inclusion moment and discontinuation of participation	· Inform potential candidates about the study before the scheduled screening appointment. This allows the first study visit to take place together with the upcoming scheduled screening moment, avoiding study inclusion delay. · Provide regular study updates to study subjects and team members.
5. Logistics	· Ensure a data management system that allows data overview and sample traceability. · Monitor the administration of the study annually. · Generate SOPs and use them to train new members of the study team.
6. Sample processing	· Keep consistency by using the same collection tubes and buffers. · Assure sample quality during multiple years by choosing the best buffer, aliquoting samples, defining the maximum time until sample processing, time until freezing, and storage temperature. · Periodically test sample quality. · Compare serial samples to assess natural temporal biomarker fluctuation.
7. Sampling at events	· Study subject participation and dedicated research nurses are relevant strategies to increase sampling at events. · Add a time margin in the protocol for sampling at events. · Awareness among study subjects about the relevance of notifying the study team in case of an event is crucial. · Interconnection of the cohort with tissue repositories and cancer registries is essential to ensure complete follow-up, provided that such an interconnection is included in the informed consent form.
8. Biobanking	· Ensure the availability of substantial biobanking storage. · Establish a well-defined biobanking system with consensual labeling.
9. Sample handling at the time of analysis	· Use a trustworthy, optimized, familiar technique. · Compare promising new, cheap, and evolved techniques to perform the primary analysis with the most optimal one. Include such an analysis in the secondary aims of the study.
10. Adjustment of the biomarker of choice	· Get informed consent as broad as possible to allow analysis of various and emerging biomarker classes.
11. Nested case-control analysis	· Keep a real-time overview of the number of events, including the timing and number of successfully acquired samples. Envisioned time frames for sampling and completeness of sample series are seldom reached. This can be overcome in statistical analysis by applying mixed linear models.

LTRs: lifetime risks; SOPs: standardized operating procedures

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3

ADDITIONAL FILE:

editorial comment in the journal Cancer Epidemiology, Biomarkers & Prevention by Dr. Edward Sauter about the original article in chapter 3.

Cancer Epidemiology, Biomarkers & Prevention,
March 2021;30(3):429–31

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Edward R. Sauter

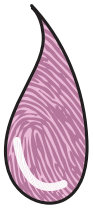
Using Organ Specific and Circulatory Biofluids to Screen Individuals at High Risk for Breast Cancer Presents Unique Challenges and Opportunities

By Edward R. Sauter

Published in Cancer Epidemiology, Biomarkers & Prevention, March 2021;30(3): 429–31

Abstract

Intraductal assessment of the breast holds the potential to provide useful information regarding breast cancer risk assessment, early diagnosis, and/or response to therapy. Intraductal assessment can be through imaging (ductography), direct visualization (mammary ductoscopy), or evaluation of the intraductal fluid collected. The most common nonradiologic approaches to intraductal assessment that provide intraductal fluid for evaluation include breast nipple aspiration fluid (NAF), spontaneous nipple discharge (SND), mammary ductoscopy, and ductal lavage. The first two approaches are entirely noninvasive while the latter are considered minimally invasive. Nipple aspiration is performed both on women with and without evidence of possible disease in the breast. On the other hand, unilateral SND suggests the presence of a lesion in the incident breast, while bilateral SND is most often physiologic. The focus of the report by Patuleia and colleagues is on challenges, lessons learned, and recommended solutions in the identification of women with increased breast cancer risk who are more likely to develop *in situ* or invasive breast cancer based on sequential collection and subsequent analysis of biofluids (NAF and serum). The lessons learned that are discussed can also be applied to other types of biofluid studies for cancer early detection and response to treatment.



Introduction

The authors provide insights on 11 challenges that they encountered in the establishment of risk assessment biofluid studies at two centers in the Netherlands using NAF (https://www.trialregister.nl/trial/8661) and serum (https://www.trialregister.nl/trial/8661) in women at elevated risk for breast cancer (1). At sequential time points up to and including the development of a breast event, defined as *in situ* or invasive breast cancer, the plan is to compare miRNA expression levels in both NAF and blood, and protein levels in blood.

Topics that the report addresses well

The authors accomplish the stated goal of the report to "...provide an overview of these challenges and its possible solutions to support future researchers that intend to design similar cohort studies."

Sequential biofluid collection

An important reason to consider sequential biofluid collection and analysis in their cohort as a means to assess risk, as discussed by the authors, is the postponement of screening by means of mammography and MRI during pregnancy and lactation, as well as the low positive predictive value and high false positive rates of breast ultrasound. Whether to include high risk women who are pregnant or lactating in the cohort is an important topic for many reasons, not the least of which is the potential for delayed diagnosis, less accurate breast imaging and lower survival after treatment compared with nonpregnant/lactating women of similar age and risk characteristics (2).

Establishment of a biobank

The authors rightly outline the importance of a establishing a large biobank, expecting enrollment time to last more than five years before sample evaluation with attendant startup and maintenance costs (and therefore needed funding) involved, and close tabs needs to be kept on dropouts as well as samples not collected or not evaluable for other reasons to determine when sample evaluation can begin. They point out that keeping participants regularly informed of study progress is an important lesson learned to minimize dropouts, and for participants to inform the study team if a new breast related event develops, so that breast cancers and precancers, especially those diagnosed at a time other than at a planned screening visit, are not missed.

Which cohorts to include in a high-risk study

Deciding on which high risk groups to include in the cohort are discussed, balancing the benefits of broad inclusion with the potential dilutional effects of evaluating diverse groups. They rightly discuss the potential downside of including subjects with a history of breast or another cancer whose future risk, as well as risk assessment biomarkers, may be altered by the treatment of incident disease.

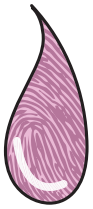
What information and samples to collect when designing a study in a high-risk cohort

Determining what information to collect at study start is also very important. While deciding once the study is ongoing that additional information should be collected is common, minimizing these additions is important because the added information will not be available from subjects already enrolled, and baseline comparisons not possible. As is pointed out, having standard operating procedures, including providing to all enrollment sites sample collection containers, diluents, and consistent diluent volume into which a sample may be placed, as well as hiring a data manager who has access to an online data

management system before or soon after study start are also keys to success. The report does not provide detail on biomarker analysis, including detailed results of biomarkers analyzed. The authors do, however suggest that investigators conduct biomarker analysis on a biofluid sample set before picking the biomarker(s) for further study in NAF and/or blood.

What factors to consider when choosing a biomarker

The authors discuss important factors to consider when choosing a biomarker for biofluid analysis, especially when the sample is limited. For instance, NAF samples are often 50 mL or less. As such, most or all of the entire sample may be needed for a single experiment. It is therefore important to use "a trustworthy, familiar technique that has already been optimized." It is also useful to determine the minimum sample volume required to reliably obtain a signal for a given biomarker. In addition to reliable detection, the biomarkers chosen for analysis should, whenever possible, be based on promising prior findings in the same or another biofluid. It is nonetheless possible that once the biomarker is chosen, new findings that are less promising may lead investigators to alter their biomarker choice(s).



Additional considerations

NAF color

NAF can range in color from clear to milky to yellow to green to brown to red. Clear and red NAF may indicate a higher risk of breast cancer (3). The authors suggest to label NAF as bloody or nonbloody, but I believe that more detail regarding color is desirable. Red NAF may be come from at least two sources, inside the breast ducts or on the nipple surface. Blood contamination from the nipple surface is not associated with risk, whereas intraductal NAF may indicate an intraductal papilloma, *in situ*, or invasive breast cancer (3).

NAF producers versus nonproducers

Despite its importance, this topic is not well addressed. One of the primary criticisms of using NAF to evaluate risk, especially when successful collection of sequential samples is required to compare changes in a given breast, is collection success, both at baseline and at each subsequent visit. Indeed, the authors use oxytocin nasal spray in an attempt to optimize NAF collection in women who participated in their study to increase collection success. Reports of collection success vary widely, ranging from <50 to 90+% (4, 5). The definition of NAF collection success is often not clearly defined, but usually implies a visible NAF sample from one or both breasts. Real success for long term cohort studies, as well as studies that use biofluids for clinical trials to monitor medication or other intervention effects, implies successful collection of a NAF sample from each

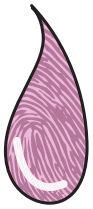
breast at each visit which is sufficient to detect the biomarkers of interest (should they be present) and that the NAF sample is not diluted with milk or blood. Some investigators, including those in this report, have used oxytocin nasal spray to increase NAF collection success. As such, more detail on NAF collection success should be provided, and the choice of NAF biomarkers should include those that are most likely to be reliably detected in all NAF samples collected, should the biomarker be present in the sample. This implies that the biomarker is generally present in high concentration in NAF, since NAF volume is limited. The authors indicate that their success rate of bilateral NAF harvesting is 65.8%. It is not clear if this means that bilateral NAF was collected at every visit for 65.8% of participants, whether each sample was adequate for biomarker analysis, or that bilateral NAF was collected at least once in this percent of participants. The more time points at which samples are available, the greater the ability to identify useful biomarkers. We assessed our success in women from whom sequential NAF samples were collected and considered two factors regarding NAF yield. The first was the ability to obtain a sample. Of 113 aspiration visits made, on only one occasion was fluid not obtained (6). The ability to obtain a NAF samples did not decrease over time. The second factor considered was whether NAF volume decreased over time. There was not a significant decline in fluid volume over time in either pre- or postmenopausal participants, considering all aspirations, the first versus the second aspiration in each subject, the first versus the third aspiration, the first versus the last aspiration, the second versus the third aspiration, or the second versus the last aspiration. Age did not significantly influence NAF volume.

What "events" should prompt evaluation of biomarkers from biofluid samples collected

The authors define events as the development of *in situ* or invasive breast cancer, at which time the last biomarker samples are collected. While it is clear that the development of breast cancer is the most important event in a cohort of individuals at increased breast cancer risk, there are other events which should be considered. The diagnosis of atypical hyperplasia (ductal or lobular) on needle biopsy frequently prompts an excisional biopsy, which may or may not identify more advanced disease. Either way, the surgical intervention alters the locoregional NAF milieu, ductal scarring occurs, and future NAF collection from the incident breast may have biomarker changes independent of disease considerations within the breast ducts and lobules. Indeed, any event prompting surgery, such as the identification of a mass leading to surgical intervention, even if the lesion which prompted surgery is found to be benign, should raise the question of whether future NAF collection provides a valid comparison with prior NAF samples, at least in the short term. The effects of needle biopsy are also likely to influence the locoregional NAF milieu at least in the short term,

although perhaps not as significantly due to the less invasive nature of the intervention. Another important event is breast irradiation. While this is most often due to the treatment of breast cancer, it has also occurred in women being treated for Hodgkin lymphoma. It has been our experience that breast irradiation significantly decreases the investigator's ability to collect NAF, if successful the NAF volume is rarely more than 10 mL, and the NAF sample generally lacks epithelial cells.

Collection of NAF during pregnancy and lactation



The collection of NAF during pregnancy and lactation raises at least potential concerns. The authors explain that NAF is highly diluted with milk in lactating women, which can alter biomarker results. Their strategy was to collect NAF starting three months after lactation ceased. As they note, milk components have been reported to exist in NAF up to 24 months after breastfeeding ends (7), so samples collected sequentially starting less than 24 months after breastfeeding ceases compared with later samples may have biomarker changes due to the influence of milk rather than risk increasing changes in the breast epithelium. There is also the low but potential risk of inducing uterine contractions among investigators who administer oxytocin nasal spray to women of childbearing potential to increase NAF collection success (8). In summary, this report provides a useful discussion of challenges, lessons learned, and possible solutions among investigators wishing to conduct studies with biofluids such as NAF and serum. While information provided focuses on subjects at increased risk of breast cancer who are followed from enrollment until a breast event, the challenges regarding study design and funding, biomarker choice, sample collection, storage, and analysis are also largely applicable to the development of biofluid studies for cancer early detection and response to treatment studies. Biofluids have untapped potential for translational evaluation and clinical care. The value of biofluid analysis will continue to increase as our ability to collect evaluable samples is optimized and technology allows us to identify useful biomarkers with increasingly small concentrations of these biomarkers.

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CHAPTER 4

Patient-centered research: how do women tolerate the nipple fluid aspiration technique as a potential screening tool for breast cancer?

Submitted

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Abstract

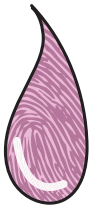
Background

Nipple fluid aspiration (NFA) is a technique to acquire nipple aspirate fluid (NAF), which is considered a rich source of breast-specific biomarkers. Originating directly from the mammary ducts, this liquid biopsy can offer insight into the process of carcinogenesis at its earliest stage and therefore could be of added value to the current imaging-based breast cancer screening tools. With that in mind, it is necessary to know how well NFA is tolerated.

Aim

To evaluate the participants' tolerability of NFA compared to breast imaging screening methods and blood draws.

Materials and methods



Three cohorts of women underwent NFA: healthy women (n=190), women diagnosed with breast cancer (n=137) and women at high risk of developing breast cancer (n=48). A 0-10 discomfort score of NFA, mammography, breast MRI and blood draws, was filled in at the study visits, which took place once or annually.

Results

The median discomfort rate of NFA was 1, which was significantly lower than the median discomfort of mammography and breast MRI (5 and 3, respectively, $p<0.001$), but significantly higher than median discomfort for blood draws (0, $p<0.001$). The great majority of women would undergo the procedure again (98%) and recommend it to others (97%).

Conclusion

This study shows that NFA was well tolerated by healthy women, women diagnosed with breast cancer and high-risk women. This makes NFA a feasible method to pursue as a potential future breast cancer early detection tool, based on resident biomarkers. Finding biomarkers that prove its clinical utility is the next step.

Introduction

Breast cancer is the most common cancer amongst women (1) and detection at an early stage is key for better treatment and survival outcomes (2-4). Well-established imaging-based population and high-risk screening programs (5-11) have contributed to improved early breast cancer detection. Still, a significant proportion (12-47%) of the diagnosed invasive breast tumors and ductal carcinoma *in situ* tumors are not diagnosed at scheduled screening visits (12, 13). Therefore, efforts focus on the development of tools beyond current imaging-based screening. In that context, liquid-based biomarkers for early detection of breast cancer are being investigated as these have the potential to be implemented as an add-on or triage tool in the early detection workup (14). Liquid-biopsy based screening holds promise as it allows repeated sampling by non-invasive means with potential high accuracy, simple and fast interpretation at low costs (15, 16). This could especially be of value for women with a higher chance of false negative results, such as women with dense breasts (17). Moreover, mammography is generally perceived as uncomfortable and painful, which can lead to reluctance to comply to screening guidelines (18, 19). Liquid biopsy-based screening could potentially be more tolerable than imaging-based screening and, as such, reduce the threshold for women to attend screening.

A specific liquid biopsy of the breast is nipple aspirate fluid (NAF), a biofluid that accumulates at small amounts in the breast ducts of non-lactating women (14). As such, it can provide information about the breast microenvironment and its subtle changes. The possibility of synchronous acquirement of matched pairs of bilateral NAF samples makes this liquid biopsy, from a research point of view, even more interesting as it provides an intra-patient control for unilateral disease. The collection technique is called nipple fluid aspiration (NFA), a non-invasive technique that was first described by George Papanicolaou (20, 21), the developer of the Pap smear test which is widely used in early detection of cervical cancer. The NFA procedure uses a manual vacuum breast pump to obtain breast fluid from the duct openings of the nipple after oxytocin nasal spray stimulation (22, 23). While results from ongoing studies investigating the potential role of biomarkers found in NAF await (24-26), it is vital to investigate the tolerability of NFA by the most relevant stakeholders: women.

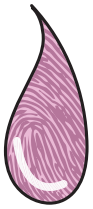
We previously published the feasibility results of (repeated) NFA in separate cohorts of healthy women (22) and high-risk women (23, 27), including adherence to study visits and discomfort associated with the procedure. Here, we provide an update of the discomfort scores of NFA, now also compared to blood draw, in three cohorts at different stages of the breast cancer care pathway: healthy women at population risk undergoing population screening, women diagnosed with breast cancer and high-risk women undergoing intense surveillance.

Materials and Methods

Study cohorts, setting and ethics

Three cohorts of women were included in the present analysis: healthy women, women with breast cancer and women at high-risk of developing breast cancer. These cohorts are all part of the Dutch Nipple Aspirate Fluid project (24-26, 28). Healthy women and women with breast cancer underwent one study visit, whereas high-risk women underwent sequential visits with a preferential regularity of 1 year in between, according to the Dutch national screening guidelines.

Inclusion of the healthy cohort (26) was started in August 2017 and closed in February 2021. Only women who were 45 years or older, did not have breast cancer and were not at increased risk for developing breast cancer according to personal and familial history were eligible. Women were recruited by word of mouth, radio announcement and flyers at general practitioner offices, the University Medical Center Utrecht (UMCU) hospital, breast cancer screening units and blood banks, amongst others. A total of 190 women were included.



In the second cohort, breast cancer patients (25) were included from January 2017 until March 2021. Patients were identified at multidisciplinary expert team meetings of four hospitals: UMC Utrecht, Alexander Monro hospital, Diakonessenhuis hospital and Alrijne hospital. Patients having untreated primary invasive breast cancer were eligible to participate. A total of 137 women were included.

Women at high risk of developing breast cancer and undergoing routine surveillance were included in the high-risk cohort (24) between May 2017 and February 2020; yearly study visits were performed until March 2021 at the UMC Utrecht. This subcohort (n=48) of high-risk women was selected based on overlapping study participation period with the healthy and breast cancer cohorts and comprises consecutive inclusions of high-risk women, except for two women with a history of invasive breast cancer, who were excluded from the analyses. Participants were informed about the study at the outpatient clinics of the genetics department and familial cancer clinic in the UMC Utrecht. Inclusion criteria comprised having a cumulative lifetime risk (LTR) higher than 20% estimated at the genetics department at the time of inclusion (29) as described in Table S1.

Exclusion criteria for all cohorts comprised active breast infection, pregnancy and lactation. See File S1 for specified inclusion and exclusion criteria per cohort. Written informed consent was obtained from all participants and the ethical review boards within the participating hospitals approved the studies (NL41845.041.12, NL57343.041.16 and NL11690.041.06).

Nipple fluid aspiration and blood collection

Study visits were carried out by trained research nurses, and included NFA, phlebotomy and questionnaires. Performing NFA requires a short practical training and can be easily learned. Participants were asked to apply an anesthetic cream onto the nipple covered with an occlusive plaster prior to the study visits (for at least one hour), to minimize discomfort due to the NFA. At the study visit, participants were seated in an upright position. The trained research nurse cleansed the breast, smeared a scrub gel to remove any occluding keratin plugs and disinfected the nipple with alcohol. This was followed by inhalation of one spray of oxytocin nasal spray in both nostrils, in a dose of 4 IU per spray, to stimulate NAF flow towards the nipple. Subsequently, a suction cup (also known as modified Sartorius cup (30)) was placed over the nipple attached to a plastic tube and a 50 cc syringe (Figure S1a). Repeated gentle plunger withdrawal of the syringe created a vacuum around the breast and led to the flow of NAF to the nipple surface. When a droplet appeared, the cup was detached and the fluid droplets were collected from the nipple surface using glass capillaries (Figure S1b). The procedure was defined as successful when droplets were visible on the nipple surface and could be collected. A total of three attempts were performed within a maximum of 20 minutes per breast. Vacuum suction was then repeated on the other breast. The obtained nipple fluid was transferred from the capillary into barcoded biobanking tubes, after which RLT plus buffer (Qiagen, Venlo, The Netherlands; supplemented with 1:100 v/v beta-mercaptoethanol) was added. Samples were immediately stored at -80°C. NAF characteristics like volume, color and viscosity (31), together with duration of NFA, who performed the NFA and the breast side order of NFA procedure were registered.

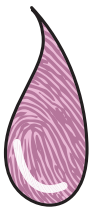
Blood was collected by phlebotomy in the median cubital vein. After collection, serum (in BD Vacutainer SST II Advance tubes) and plasma (in BD Vacutainer K2E (EDTA) tubes) were processed within 1 hour by centrifugation at 1500x g for 20 min. Aliquots of serum (n=10) and plasma (n=6) were immediately biobanked at -80°C. Blood collection was initially not part of the high-risk cohort study protocol, and hence, not included in the discomfort assessment of our previous studies (22, 23, 27).

Questionnaire

After blood and bilateral NAF collection, participants completed a discomfort questionnaire that we adapted from a questionnaire first described by Klein *et al* (22, 23, 27, 32). Discomfort regarding NFA, blood collection, breast surveillance techniques (mammography and breast MRI), breast physical exam and breastfeeding was scored on a scale from zero (no discomfort) to ten (worst discomfort imaginable); see File S2. Breastfeeding and breast physical exam were

included as a means of comparison; the first because it is comparable to the NFA procedure and as such might cause a similar discomfort, the latter because the breast exposure by itself can cause discomfort. It was chosen to evaluate discomfort rather than pain to facilitate comparison with our previous studies, but also due to its applicability for all these techniques and circumstances as the word 'discomfort' engulfs more (implicit) aspects such as e.g. embarrassment and duration of a technique. For instance, discomfort associated to a breast physical exam is possibly associated to breast exposure and physical contact; pain would not be an applicable measure to assess this variable. Additional questions comprised whether participants would undergo the NFA procedure again and recommend it to others. In 2019, the question whether participants would accept NFA as a screening tool was added to the questionnaires.

Statistical analyses



Statistical analyses were performed using IBM SPSS Statistics for Windows version 25.0.0.2 (IBM Corp., Orchard Road Armonk, New York, US) and GraphPad Prism for Windows version 8.0 (GraphPad Software, La Jolla, California, US). A two-tailed p-value <0.05 was considered statistically significant. Data are presented as median with interquartile range (IQR) or mean with standard deviation (SD) when appropriate for continuous data, and counts with percentages for categorical data. Normality of data distribution was evaluated by Kolmogorov-Smirnov test.

The chi-square test was used to compare the binary variables breastfeeding, mammography, breast MRI, blood collection, breast MRI, breast physical exam (yes vs. no) between cohorts. The Mann Whitney test was used to compare age between cohorts. The Mann-Whitney test was used to compare median discomfort of NFA with the median discomforts of breastfeeding, breast physical exam, mammography, breast MRI and blood collection. Violin plots were made in GraphPad to display experienced discomfort distribution and medians. In the high-risk cohort, discomfort scores of the first study visit were included in the analyses. The Hodges-Lehmann Estimate (HLE) (33) was used to compare the median discomfort of NFA with the discomfort of mammography, breast MRI, blood collection, breastfeeding and breast physical exam and significance was calculated with Wilcoxon signed rank test. Discomfort scores between study visits in the high-risk participants were compared by Wilcoxon signed ranks tests. Of note, the variable now used to report discomfort of NFA was defined as the discomfort experienced by the vacuum created by the modified breast pump, while in our previous studies we reported mean discomfort of several aspects of the study visit, including discomfort of waiting, filling in a questionnaire, NFA and the nose spray (27).

The Spearman's test was used to investigate correlation between discomfort scores with age, parity, breastfeeding, duration of breastfeeding, history of spontaneous nipple fluid discharge, breast size, use of contraception, age at menarche, menopausal status, NAF sample volume, duration of the NFA and successful NFA (at least one droplet). A significance value below <0.05 was deemed necessary to indicate a correlation and a minimum of 0.7 was considered a lower bound value to indicate a strong correlation between variables. For logistic regression analyses, the enter method was used and NFA discomfort was dichotomized into 'no discomfort' (scores 0-3) and 'discomfort' (scores 4-10). A significance value below <0.05 was deemed necessary for a variable to indicate a relevant effect on NFA discomfort.

Results

Cohort characteristics

A total of 375 women of three cohorts were included in the study (Table 1). Median age was 54 years old (IQR 47-62). A total of 250 women had experienced a breast physical exam, 254 had breastfed, 319 had undergone a mammography, 114 a breast MRI and 358 underwent a blood draw in the context of the study visit (Table 1).

Women in the healthy volunteer and breast cancer cohorts were significantly older than women in the high-risk cohort (both p<0.001; Table 1). In the three cohorts, the majority of women had undergone a mammography (Table 1). Only 2% of the women in the healthy volunteer cohort had experienced a breast MRI, whereas 56% of the breast cancer cohort and 72% of the high-risk cohorts had undergone a breast MRI. Blood was obtained in 95.5% of all participants.

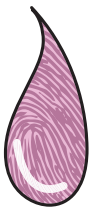


Table 1. Cohort characteristics.

Cohort	All	Healthy volunteers cohort	Breast cancer cohort	High-risk cohort	P value HC vs. BC	P value BC vs. HR	P value HC vs. HR
n	375	190	137	48			
Age, median (Q25-Q75)	54 (47-62)	54 (49-60)	56 (49.5-67.5)	36 (27-48)	0.093	<0.001	<0.001
Mammography, n	319	154 (81%)	137 (100%)	31 (62%)	<0.001	<0.001	0.002
Breast MRI, n	114	3 (2%)	76 (56%)	36 (72%)	<0.001	0.034	<0.001
Blood collection, n	358	184 (96.8%)	130 (94.9%)	44 (91.7%)	0.950	0.082	0.064
Breast physical examination, n	250	92 (48%)	112 (82%)	43 (86%)	<0.001	0.563	<0.001
Ever breastfed, n	254	139 (73%)	91 (66%)	27 (56%)	0.245	0.001	<0.001

In the high-risk cohort, only data from the first visit was considered for this baseline table. Abbreviations: Q25-Q75: inter-quartile range between quartile 25 and quartile 75; MRI: magnetic resonance imaging; HC: healthy volunteers cohort; BC, breast cancer cohort; HR, high-risk cohort.

In the three cohorts, the majority of women were parous (83%, 82% and 62% of women in the healthy, breast cancer and high-risk cohorts, respectively) (Table S2). From the parous women, 89%, 81% and 87% had breastfed in the healthy, breast cancer and high-risk cohorts, respectively. Most of the women in the breast cancer cohort (82%) and high-risk cohort (86%) had experienced

a breast physical exam, in contrast to the healthy volunteers, of whom 48% had undergone a breast physical exam.

Within the high-risk cohort, participants underwent a NFA between 1 to 4 times (median study visits=1, IQR= 1-2) with a median time of one year in between visits (median 384 days; IQR 363-499).

Discomfort of nipple fluid aspiration compared to mammography, breast MRI, blood collection, breast physical exam and breastfeeding

Overall, NFA discomfort scored a median of 1 on a scale from 0-10. NFA discomfort scores were significantly lower compared to breastfeeding (median=2; p=0.001), breast MRI (median=3, p<0.001) and mammography (median=5; p<0.001); NFA scores were nevertheless significantly higher compared to discomfort of blood collection (median=0; p<0.001; see Figure 1 and Table S3). There was no significant difference between the discomfort experienced by NFA and a breast physical exam (median=2; p=0.057). These results were confirmed with a Hodges-Lehmann Estimate analysis (HLE, Table S4). To put these data in perspective, 72% and 41% of the women gave a discomfort score of 4 or higher to mammography and breast MRI, respectively, whereas this was only the case for 21% of the women regarding NFA (Table S5).

NFA was assigned a significantly lower discomfort score in the healthy volunteers cohort (median=1) and the breast cancer cohort (median=1), compared to the median NFA score of 3 in the high-risk cohort (p=0.005) (Table S3). To understand whether this could be related to other variables, correlation analyses were performed, but there were no clinically relevant correlations between baseline factors and NFA discomfort (all rho/coefficients<0.3, Table S6). As the high-risk cohort had a significant younger age compared to the other cohorts, discomfort scores between cohorts were then compared, correcting for age, with a hierarchical logistic regression analysis. With this correction, there was no significant difference between cohorts regarding NFA discomfort scores (Table S7). A stratification analysis by age groups further supported this as it showed that NFA discomfort medians significantly diminish with older age (p<0.001, Table S8). As such, the higher discomfort scores of NFA in high-risk women could at least partly be explained by their younger age.

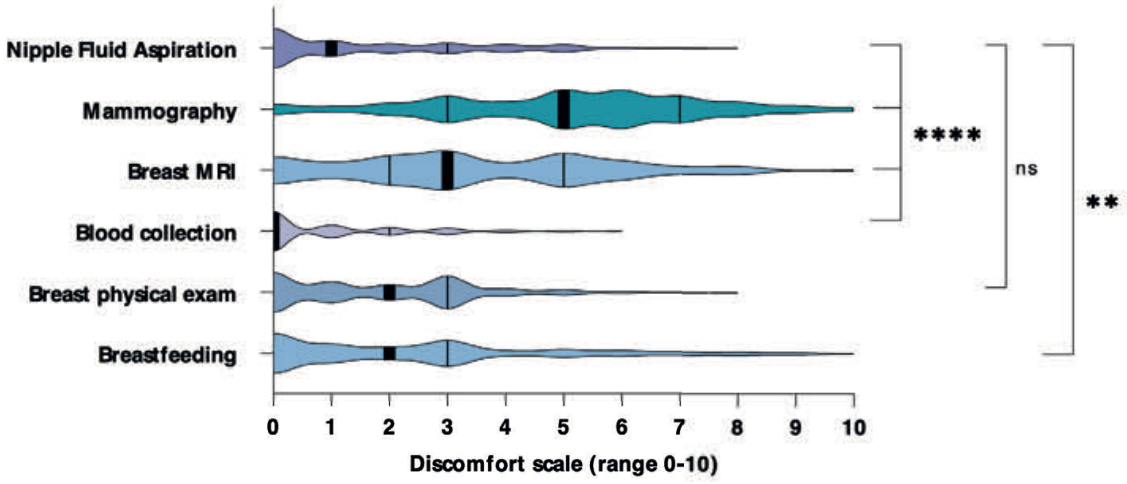


Figure 1. Discomfort of nipple fluid aspiration (NFA) compared to mammography, breast MRI, blood collection, breast physical exam and breastfeeding, in all cohorts. The thickened line in each violin plot represents the median. Abbreviations and symbols: MRI: magnetic resonance imaging; ns: not significant; ****: p-value < 0.0001; **: p-value < 0.01 statistically significant. Significances were calculated with the Mann Whitney U test.

Within cohorts, discomfort of NFA was consistently lower than in the case of mammography ($p<0.0001$ in the three cohorts) and breast MRI ($p<0.008$ and $p<0.0001$ in the high-risk and breast cancer cohort, respectively). NFA discomfort was consistently higher than blood collection ($p<0.002$) within cohorts. Discomfort regarding breastfeeding and breast physical exam was not significantly different compared to the discomfort rates of NFA within the breast cancer cohort and the high-risk cohort (Table S3).

Repeated NFAs in high-risk participants did not significantly affect the NFA discomfort score (Table S9). Almost all participating women would opt for repeated NFA (98%, n=311/318) and would recommend NFA to others (97%, n=308/317). Additionally, we asked whether women would endorse NFA in the context of screening, and most responses were positive (95%, n=36/38), if proven effective (Table S10). In the high-risk group, 13 women dropped out (27%) due to breast cancer diagnosis, bilateral prophylactic mastectomy or because they found NFA unpleasant or were disappointed by the volume obtained, amongst other reasons (Table S11). Reported adverse events in all cohorts were rare (2% of all the NFA procedures) and self-limiting. These comprised fainting after blood collection or NFA, hematoma, headache and abdominal cramps (Table S12).

Discussion

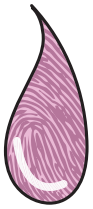
Early involvement of women in research related to the development of new breast cancer screening tools is essential. One upcoming line of investigation involves the integration of liquid biopsies as a minimally invasive, safe and sensitive additive early detection tool (16, 17, 34). Even though there is a great number of articles on the subject of biomarkers in liquid biopsies for early detection of breast cancer, there are almost no studies reporting the women's perspectives about the different aspects that come with the scenario of introducing liquid biopsies in screening. Here, we assessed how women from three different cohorts (healthy women undergoing or about to undergo population screening, women diagnosed with breast cancer and women at high risk of breast cancer undergoing intensive surveillance) experienced liquid biopsy acquisition (NAF and blood) in comparison to breast imaging.

Discomfort experienced during the NFA technique was significantly lower (median 1) than the discomfort experienced from the conventional screening/diagnostic tools mammography and breast MRI. The discomfort scores presented here are comparable to our previous reports in other cohorts of healthy women (22) and high-risk women (23, 27) (mean discomfort of 1.3, 0.6 and 0.71, respectively). Another study from Klein *et al.* (32) reported a higher score (median=2; range=1-7) for the discomfort associated to NFA compared to the cohorts in the present study, when taken together. Those higher scores were acquired from a cohort with fewer and younger women (25 healthy women with a mean age of 38 years old) compared to our three cohorts altogether. Since we show that younger age is associated with higher NFA discomfort, this might explain the observed difference in discomfort. Within all cohorts, NFA had consistently significantly lower discomfort scores than mammography and breast MRI which are now widely used screening/diagnostic techniques. Given the overall low discomfort scores, NFA, if proven useful, could be widely applicable for the general female population.

As a means of comparison between liquid biopsies, discomfort of blood collection, the most regular applied source of liquid biopsy, was taken along in our analyses. Blood collection is a fast, widely performed and accepted procedure that only requires exposure of the arm. As such, its discomfort scored unsurprisingly the lowest. Still, the advantages of NAF as a valuable information source for biomarkers about alterations in intraductal health, together with information about breast side and intra-patient control, have the potential to surpass the advantages of blood-based biomarkers. Such studies are under way (24-26).

The high (intended) willingness of the women in our cohorts to participate again is consistent with our previous studies (22, 23, 27). Interestingly, a recently published

questionnaire-based study reported data of 3178 women about awareness, information and preferences about breast cancer screening, including questions regarding knowledge about nipple aspirate fluid (35). When asked about their willingness to produce NAF samples themselves at home, over 70% of the respondents would be willing to do so. The great majority of the respondents in this study were under the age of 50 and had never underwent a NFA themselves and 88% had never heard or were not aware of nipple fluid aspiration. One other study showed that there is a great willingness of women to provide liquid biopsies (blood and saliva) for biobanking at the time of breast cancer screening (36). Altogether, these studies show that the use of nipple aspirate samples, including the NFA approach, would be very well accepted by women and is complementary to our data from women who underwent NFA.



The presented results might be influenced by self-selection bias. That is, by participating in the study, women are already willing to collect NAF and blood. Similarly, women who previously experienced a high discomfort during mammography and/or breast MRI, could be more motivated to participate in our study. Still, our discomfort scores for mammography are in line with the reported means for pain in other studies, which vary between 4 and 7 (scale 0-10) (37-39). While discomfort associated to mammography is mostly associated to pain provoked by the plates that compress the breasts, discomfort associated to breast MRI is a result of intravenous contrast injection and lying on an MRI table while being subject to intermittent loud noises for about one hour (40-44). And while these breast imaging disadvantages have been reported by women who have undergone these imaging techniques, it could be a reason for women to not comply to screening. As such, low discomfort associated to liquid biopsy acquisition could possibly represent an opportunity to offer a surveillance alternative at a low threshold for women who experience the imaging techniques as a burden.

Conclusions

In summary, NFA and blood collection are simple, minimally invasive, repeatable methods that are very well accepted and endured by women. The great majority of participating women would undergo NFA again in the context of research and screening. These data indicate that a liquid biopsy biomarker-based (repeated) surveillance testing tool could be well received by women, provided that the biomarkers found in NAF and blood hold promise.

Supplementary Materials

File S1. Eligibility criteria per cohort.
File S2. Discomfort questionnaire.
Figure S1a. Picture of syringe attached to the plastic tube on one end and to the plastic cup on the other end.
Figure S1b. Picture of a glass capillary used to collect nipple fluid droplets.
Table S1. Inclusion reason in the high-risk cohort
Table S2. Cohort characteristics in breast cancer, healthy volunteers and high-risk cohorts.
Table S3. Discomfort of nipple fluid aspiration (NFA) compared to breast physical exam, breastfeeding, mammography, breast MRI and blood collection in the breast cancer cohort, healthy volunteers cohort and
high-risk cohort.
Table S4. Hodges-Lehmann Estimate (HLE) of discomfort scores for nipple fluid aspiration (NFA), mammography, breast MRI, breast physical exam, breastfeeding and blood collection.
Table S5. Percentage of women of all cohorts who reported a discomfort score of 5 or above for NFA, mammography, breast MRI, breast physical exam, breastfeeding and blood collection.
Table S6. Correlation tests comparing NFA discomfort with other variables and significance values from the linear regression analyses.
Table S7. Logistic regression showing that, when corrected for age, NFA discomfort scores are not significantly different between cohorts.
Table S8. Nipple fluid aspiration discomfort scores by age in all cohorts.
Table S9. Association between discomfort NFA and repeated NFA in the high-risk cohort.
Table S10. Response of women in all cohorts and specifically in the breast cancer, healthy volunteers and high-risk cohorts.
Table S11. Drop-out reasons in high-risk cohort.
Table S12. Adverse events in all cohorts and specifically in the breast cancer, healthy volunteers and high-risk cohorts.

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Supplementary Data

File S1. Eligibility criteria per cohort.

Eligibility criteria for the breast cancer cohort

Inclusion Criteria	Exclusion Criteria
Patients must meet ***all*** of the following criteria ***to be eligible*** for enrollment as study participants:	Patients who meet ***any*** of these criteria are ***not eligible*** for enrollment as study participants:
· Female, age ≥18 years · Proven invasive breast carcinoma	· Bilateral breast reduction with nipple graft · Bilateral ablative breast surgery · Pregnancy or lactation · Active breast infection · Disseminated breast cancer

Eligibility criteria for healthy volunteers cohort

Inclusion Criteria	Exclusion Criteria
Patients must meet ***all*** of the following criteria ***to be eligible*** for enrollment as study participants:	Patients who meet ***any*** of these criteria are ***not eligible*** for enrollment as study participants:
· Female, age ≥45 years	· Bilateral breast reduction with nipple graft · Breast cancer or history of breast cancer · Pregnancy or lactation · Active breast infection · Lifetime risk ≥ 20% for breast cancer

Eligibility criteria for high-risk cohort

Inclusion Criteria	Exclusion Criteria
Patients must meet all of the following criteria ***to be eligible*** for enrollment as study participants:	Patients who meet ***any*** of these criteria are ***not eligible*** for enrollment as study participants:
· Female, age ≥18 years · Lifetime risk ≥ 20% for breast cancer (due to family history or high-risk breast cancer susceptibility genes)	· Bilateral breast reduction with nipple graft · Bilateral ablative breast surgery · Pregnancy or lactation (temporary exclusion criteria) · Active breast infection · Disseminated breast cancer

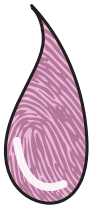
File S2. Discomfort questionnaire. This is a selection of questions regarding discomfort included in the questionnaire. Other questions regarding anthropomorphic characteristics were removed for this supplementary file to keep the overview.

1. Have you ever breastfed?
   ☐ Yes
   ☐ No

2. Can you indicate the degree of discomfort of breastfeeding on a scale of 0 (no discomfort at all) tot 10 (the worst I can imagine)?

0	1	2	3	4	5	6	7	8	9	10	N.A.

3. How long ago have you breastfed for the last time?[1] _____



4. Have you ever had spontaneous nipple discharge (apart from periods of breastfeeding)?
   ☐ Yes
   ☐ No

5. Have you ever had a mammogram?
   ☐ Yes
   ☐ No

6. Can you indicate the degree of discomfort of the mammogram on a scale from 0 (no discomfort at all) to 10 (the worst you can imagine)?

0	1	2	3	4	5	6	7	8	9	10	N.A.

7. How long ago have you had a mammogram for the last time?[1] _____

8. Have you ever had a breast MRI?
   ☐ Yes
   ☐ No

9. How long ago have you had a breast MRI for the last time?[1] _____

10. Can you indicate the degree of discomfort of the MRI on a scale from 0 (no discomfort at all) to 10 (the worst you can imagine)?

0	1	2	3	4	5	6	7	8	9	10	N.A.

11 Have you ever had a breast physical exam in a hospital or at the general practitioner's office?

☐ Yes
☐ No

12. Can you indicate the degree of discomfort of the physical examination on a scale from 0 (no discomfort at all) to 10 (the worst you can imagine)?

0	1	2	3	4	5	6	7	8	9	10	N.A.

13. How long ago have you had a breast physical exam for the last time? [1]_____

14. If there was anything less pleasant or discomfortable during the study visit, what was it?

    14a. If yes, Is there a way we might prevent this, or make it less discomfortable?

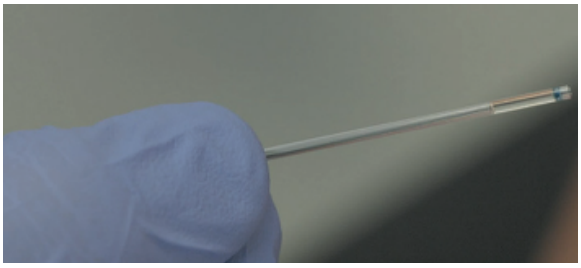
15. Would you undergo the procedure again:
    b. In the context of study trial?[1]
    c. If this would be a standard screening method?1

16. Would you recommend the procedure to someone else, if this would be a standard screening method?
    a. In the context of study trial?[1]
    b. If this would be a standard screening method?[1]

1 This question was added in the end of 2019.

Please circle in every row the number that best fits the discomfort you experienced during the procedure.

Prior to the procedure												
Waiting	0	1	2	3	4	5	6	7	8	9	10	
Insecure about what will happen	0	1	2	3	4	5	6	7	8	9	10	N.A.
Questionnaire	0	1	2	3	4	5	6	7	8	9	10	
During the procedure												
Application of the anaesthetic cream, cleansing of the nipple	0	1	2	3	4	5	6	7	8	9	10	N.A.
Nasal spray	0	1	2	3	4	5	6	7	8	9	10	N.A.
Vacuum aspiration	0	1	2	3	4	5	6	7	8	9	10	
Blood draw	0	1	2	3	4	5	6	7	8	9	10	N.A.
Overall during the procedure												
Duration	0	1	2	3	4	5	6	7	8	9	10	
Pain	0	1	2	3	4	5	6	7	8	9	10	
Shame	0	1	2	3	4	5	6	7	8	9	10	
After the procedure												
Pain	0	1	2	3	4	5	6	7	8	9	10	
Overall discomfort of the study visit												
Discomfort	0	1	2	3	4	5	6	7	8	9	10	



S1a. **S1b.**

Figure S1. (S1a) Picture of syringe attached to the plastic tube on one end and to the plastic cup on the other end, (S1b) Picture of a glass capillary used to collect nipple fluid droplets.

Supplementary Tables

Table S1. Inclusion reason in the high-risk cohort. PV: pathogenic variant.

Inclusion reason in high-risk cohort	n
BRCA1 PV	20
BRCA2 PV	13
Familial history of breast cancer	6
Chek2 PV	4
E-cadherin PV	1
NF1 PV	1
PALB2 PV	2
PTEN PV	1

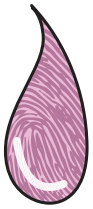


Table S2. Cohort characteristics in breast cancer, healthy volunteers and high-risk cohorts.

Cohort	Breast cancer cohort	Healthy volunteers cohort	High-risk cohort
n	137	190	48
Age, median (IQR)	56 (18)	54 (11)	36 (21)
BMI, median (IQR)	25.5 (0.7)	24.2 (5.5)	24.07 (5.55)
Use of contraception, n	18 (13%)	44 (23%)	17 (34%)**
Postmenopausal, n	81 (64%)*	120 (63%)**	14 (28%)
History of spontaneous nipple discharge, n	17 (12.5%)	22 (11.6%)	5 (10.4%)
Parous, n	112 (82%)	156 (83%)**	31 (62%)
_Breastfeeding, n	91 (81%)	139 (89%)	27 (87%) ***
Mammography, n	137 (100%)	154 (81%)	29 (60.4%)
Breast MRI, n	76 (56%)	3 (2%)	35 (72.9%)
Breast physical examination, n	112 (82%)	92 (48%)	41 (85.4%)
Blood collection, n ****	130 (94.9%)	184 (96.8%)	44 (91.7%)
Number of study visits, minimum and maximum	1	1	1 - 4
Missed study visits, n	N.A.	N.A.	2
Drop-outs, n	N.A.	N.A.	13

* 5 missings; ** 1 missing; *** 3 missings; **** Almost all women consented on taking a blood draw, except for four in the breast cancer cohort and one in the high-risk cohort. From the women who consented, blood draw was not successful in 7 women. One blood draw was not performed in one woman because the research nurse did not have the qualification to do it yet. Blood draw was not tried in the first four inclusions in the breast cancer cohort because it was not part of the protocol at the beginning.

Table S3. Discomfort of nipple fluid aspiration (NFA) compared to breast physical exam, breastfeeding, mammography, breast MRI and blood collection in the breast cancer cohort, healthy volunteers cohort and high-risk cohort. Abbreviations and symbols: a: Mann Whitney test; comparison with NFA; IQR: inter-quartile range; MRI: magnetic resonance imaging; HC: healthy volunteers cohort; BC, breast cancer cohort; HR, high-risk cohort. N.A.: not applicable.

	All	P value[a]	Breast cancer cohort	P value[a] BC	Healthy volunteers cohort	P value[a] HC	High-risk cohort	P value[a] HR
n								
NFA, median (IQR)	1	N.A.	1 (3)	N.A.	1 (3)	N.A.	3 (4)	N.A.
Mammography, median (IQR)	5	<0.0001 ****	5 (3)	<0,0001 ****	5 (4)	<0.0001 ****	6 (2)	<0.0001 ****
Breast MRI, median (IQR)	3	<0.0001 ****	3 (3)	<0,0001 ****	2 (N.A.)	N.A.	4 (4)	0.0079 **
Blood, median (IQR)	0	<0.0001 ****	0 (1)	<0,0001 ****	0 (1)	0.0125 *	1 (3)	0,0176 *
Breast physical exam, median (IQR)	2	0.0569 (ns)	1 (3)	0,5647 (ns)	2 (3)	0.0016 **	1.5 (3)	0.1054 (ns)
Breastfeeding, median (IQR)	2	0.0012 **	1 (3)	0,3171 (ns)	2 (4)	<0.0001 ****	3 (3)	0.6278 (ns)

a: Mann Whitney test; comparison with NFA; ns: not significant
P value summary: **** p-value < 0.0001; *** p-value < 0.001; ** p-value < 0.01; * p-value < 0.05
Abbreviations: NS, non-significant; IQR: inter-quartile range; MRI: magnetic resonance imaging; HC: healthy volunteers cohort; BC, breast cancer cohort; HR, high-risk cohort. N.A.: not applicable.

Table S4. Hodges-Lehmann Estimate (HLE) of discomfort scores for nipple fluid aspiration (NFA), mammography, breast MRI, breast physical exam, breastfeeding and blood collection. Significance calculated with Wilcoxon signed rank test.

Median NFA discomfort score minus:	HLE	95% C.I. HLE		p value
		Lower	Upper	
median mammography	4,0	-4,0	-3,5	<0,001
median breast MRI	2,0	-2,5	-1,0	<0,001
median breast physical exam	0,0	-0,5	0,5	0,645
median breastfeeding	1,0	-1,5	-0,5	<0,001
median blood collection	1,5	1,5	1	<0,001

Table S5. Percentage of women of all cohorts who reported a discomfort score of 5, 4 or 3 or above for NFA, mammography, breast MRI, breast physical exam, breastfeeding and blood collection.

	Discomfort ≥3	**Pearson Chi-square between cohorts for NFA**
NFA, %	31,7%	p=0.002
Mammography, %	85,9%	
Breast MRI, %	63,2%	
Breast physical exam, %	40,0%	
Breastfeeding, %	44,5%	
Blood collection, %	15,7%	

	Discomfort ≥4	**Pearson Chi-square between cohorts for NFA**
NFA, %	20,6%	p=0.007
Mammography, %	72,1%	
Breast MRI, %	42,1%	
Breast physical exam, %	14,0%	
Breastfeeding, %	23,2%	
Blood collection, %	7,0%	

	Discomfort ≥5	**Pearson Chi-square between cohorts for NFA**
NFA, %	12,2%	p=0.181
Mammography, %	65,2%	
Breast MRI, %	36,8%	
Breast physical exam, %	8,0%	
Breastfeeding, %	19,3%	
Blood collection, %	3,4%	

Table S6. Correlation tests comparing NFA discomfort with other variables.

NFA discomfort and:	Spearman's test	P value	
Age	- 0.224	<0.0001	**
Parity	0.019	0.716	
Breastfeeding	- 0.083	0.140	
Duration breastfeeding	- 0.103	0.088	
Spontaneous NFA	0.011	0.840	
Breast size	- 0.075	0.201	
Use of contraception	0.114	0.028	*
Age menarche	- 0.039	0.453	
Menopausal status	- 0.166	0.022	**
Volume NAF sample	- 0.010	0.855	
Duration of NFA	0.043	0.431	
Successful NFA	0.088	0.091	

* Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed).
[a] p value from linear regression analyses with variables that significantly correlated with NFA discomfort.

Table S7. Logistic regression showing that, when corrected for age, NFA discomfort scores are not significantly different between cohorts. In model 1, the variable 'cohort' was introduced. In model 2, the variables age, menopause and contraception were added. Healthy controls were used as a reference for the variable cohort. Discomfort was dichotomized based on the scores of 0-3 and 4 or higher.

Model 1				**Odds ratio 95% confidence interval**	
	B	**Significance**	**Odds Ratio**	**Lower Bound**	**Upper Bound**
Breast cancer cohort	0,349515	0,227	1,418	0,805	2,499
High-risk cohort	1,120966	0,002	3,068	1,501	6,270

Model 2				**Odds ratio 95% confidence interval**	
	B	**Significance**	**Odds Ratio**	**Lower Bound**	**Upper Bound**
Breast cancer cohort	0,398	0,175	1,489	0,837	2,649
High-risk cohort	0,25	0,583	1,284	0,526	3,132
Age	-0,048	0,001	0,953	0,928	0,98
Menopause	0,087	0,827	1,091	0,5	2,381
Contraception	-0,051	0,888	0,951	0,47	1,922

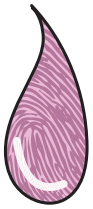


Table S8. Nipple fluid aspiration discomfort scores by age in all cohorts. Within the high-risk cohort, solely the first visit was considered.

Age category	n	Median NFA discomfort	IQR	95% Confidence Interval		Kruskall Wallis test
				Lower Bound	Upper Bound	
30 or younger	20	3,5	5	1,57	3,73	<0.001
31-40	21	3	4	1,8	3,73	
41-50	106	1	3	1,62	2,38	
51-60	120	1	3	1,18	1,82	
61 or older	102	0	2	0,88	1,59	

Table S9. Association between discomfort NFA and repeated NFA in the high-risk cohort. Significance was calculated with Wilcoxon signed rank test.

	Visit 1	Visit 2	Visit 3	p value visit 1 vs. visit 2	p value visit 1 vs. visit 3
n	46	32	6	0.132	0.180
Median discomfort NFA	3	3	3		

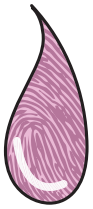


Table S10. Response of women in all cohorts and specifically in the breast cancer, healthy volunteers and high-risk cohorts.

		All	Breast cancer cohort	Healthy cohort	High-risk cohort[a]
Repeat	n	318	97	174	47
	Yes, n (%)	311 (97,7%)	96 (99%)	168 (96.6%)	47 (100%)
	Missing, n		0	10	1
Recommend	n	317	96	173**	48
	Yes, n (%)	308 (97.2%)	95 (99%)	165 (95.4%)	48 (100%)
	Missing, n		0	11	0
Endorse NFA in the context of screening	n	38	12	24	2
	Yes, n (%)	36 (94.7%)	11 (91.7%)	23 (95.8%)	2 (100%)
	Missing, n		0	160	46

a: based on first visit

Table S11. Drop-out reasons in high-risk cohort.

Drop-outs, n	**13**
Reasons	
Developed breast cancer	2
Bilateral prophylactic mastectomy	5
Unsuccessful NFA	1
Unpleasant NFA	1
Disappointed by the volume obtained	1
Lack of time	2
Corona pandemic	1

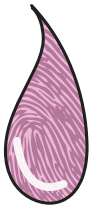


Table S12. Adverse events in all cohorts and specifically in the breast cancer, healthy volunteers and high-risk cohorts.

	All	Breast cancer cohort	Healthy cohort	High-risk cohort
Adverse events, n	8 (2.1% of the 375 women; 1.9% of the 417 NFAs)	1 (1%)	4 (2.1%)	3 (6% of the 48 women, 3% of the 90 NFAs)
Description				
Fainted after blood draw, n	1	1	0	0
Fainted after NFA, n	2	0	0	2
Alergic dermatitis, n	1	0	1	0
Dizziness, n	1	0	1	0
Abdominal cramps, n	1	0	1	0
Headache, n	1	0	0	1
Hematoma, n	1	0	1	0

PART 2

CHAPTER 5

The physiological microRNA landscape in nipple aspirate fluid: differences and similarities with breast tissue, breast milk, plasma, and serum

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Abstract

Background

MicroRNAs (miRNAs) target 60% of human messenger RNAs and can be detected in tissues and biofluids without loss of stability during sample processing, making them highly appraised upcoming biomarkers for evaluation of disease. However, the reporting of abundantly expressed miRNAs in healthy samples is often surpassed. Here, we characterized for the first time the physiological miRNA landscape in a biofluid of the healthy breast - nipple aspirate fluid (NAF) - and compared NAF miRNA expression patterns with publicly available miRNA expression profiles of healthy breast tissue, breast milk, plasma, and serum.

Methods

MiRNA RT-qPCR profiling of NAF (n = 41) and serum (n = 23) samples from two healthy female cohorts was performed using the TaqMan OpenArray Human Advanced MicroRNA 754-Panel. MiRNA quantification data based on non-targeted or multi-targeted profiling techniques for breast tissue, breast milk, plasma, and serum were retrieved from the literature by means of a systematic search. The miRNAs of each individual study were ranked in order between 1 and 50, combined into an overall ranking per sample type, and compared.

Results

NAF expressed 11 unique miRNAs and shared 21/50 miRNAs with breast tissue. Seven miRNAs were shared between the five sample types. The overlap between the sample types ranged between 42 and 62%. Highly ranked NAF miRNAs have established roles in breast carcinogenesis.

Conclusions

This is the first study to characterize and compare the unique physiological NAF-derived miRNA landscape with the physiological expression pattern in breast tissue, breast milk, plasma, and serum. Breast-specific sources did not mutually overlap more than with systemic sources. Given their established role in carcinogenesis, NAF miRNA assessment could be a valuable tool in breast tumor diagnostics.

Introduction

MicroRNAs (miRNAs) are small, non-coding RNAs of ~22 nucleotides that regulate around 60% of messenger RNAs in humans (1). MiRNAs are released into many biofluids as a result of cellular apoptosis or necrotic cell death, or by active secretion from their cells of origin, engulfed in extracellular vesicles such as exosomes, or by forming complexes with RNA-binding proteins (2-4). The fact that they are easily detectable in biofluids and remain stable during sample processing makes miRNAs suitable as candidate biomarkers for diseases (4-9). It has been established that miRNAs are involved in many signaling pathways and are associated with numerous disorders in the oncological, cardiovascular, and neurological fields (10-13). Yet, such findings are usually based on studies that focus solely on differentially expressed miRNAs between two or more groups and fail to define the physiological miRNA landscape of controls. It is, however, of utmost importance to establish and make available the baseline values of these biomarkers, especially given the potential clinical use of miRNAs. Such reference data can be established by investigating the most abundantly expressed miRNAs in healthy samples.

The establishment of such a baseline requires the use of large-scale profiling methodology for miRNA detection and quantification, such as RT-qPCR profiling, microarray, and next-generation sequencing techniques (14). These techniques facilitate simultaneous assessment of hundreds of miRNAs or even theoretically allow detection of all known 2656 mature miRNAs in Homo sapiens (15). When establishing a baseline ranking of miRNAs, the sample source must be taken into account. Different body sources may have a different set of physiologically expressed miRNAs (16) and, in theory, liquid biopsies closest to the tissue of origin may best reflect its miRNA pattern. As such, we hypothesized that nipple aspirate fluid (NAF) is a potential useful source of miRNAs derived from the breast. NAF is secreted by the breast ducts of adult non-lactating women and is readily accessible by oxytocin-supported non-invasive vacuum aspiration, yielding sufficient material for molecular analysis (17-21). NAF aspiration is associated with significantly less discomfort compared to mammography and MRI and may therefore represent a promising tool for breast cancer screening (20). To date, characterization of the miRNAs present in NAF has never been reported. Such a characterization, together with a systematic comparison between healthy NAF and other breast-specific and systemic healthy samples provides insight into the source specificity of miRNAs prior to breast cancer case-control studies.

This study focuses on defining the naturally occurring most abundantly expressed miRNAs in NAF compared to other (non-)liquid sample types, namely breast tissue, breast milk, plasma, and serum, as measured by multi-targeted

profiling techniques in our cohorts and in other studies. The data was extracted chiefly from the literature and combined with data from our own discovery experiments with NAF and serum (Figure 1). Breast tissue was included since this sample has a well-established diagnostic role in breast disease and is a specific source of breast-derived biomarkers. NAF and breast milk were included because these are likely liquid representatives of the breast microenvironment. The systemic readily available biofluids plasma and serum, which are the most commonly used liquid biopsies in research and the clinic, allowed comparison with site-specific biosamples.

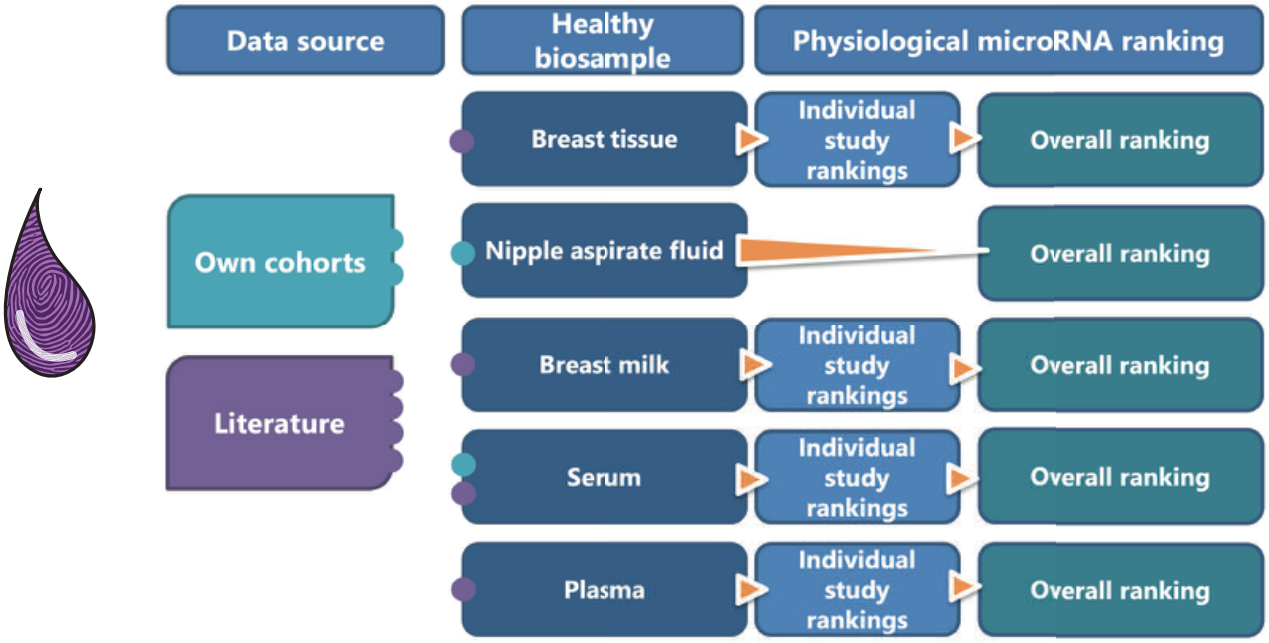


Figure 1. Schematic overview of the microRNA ranking. MicroRNA data was extracted from the literature for breast tissue, breast milk, serum, and plasma samples (purple circles) and combined with data from our own discovery experiments with NAF and serum samples (blue circles). With this data, we were able to make an overall physiological microRNA ranking per sample type.

Materials and Methods

Nipple aspirate fluid and serum collection and processing

NAF had already been collected from 41 women in the context of the Dutch nationwide multicenter Dense tissue and Early breast Neoplasm ScrEening (DENSE) trial (NCT01315015 (22, 23)). Samples were derived from women with extremely high (D, n = 21) or very low (A, n = 20) mammographic density (MD), which are the two extreme categories within the four-category MD spectrum (A—D (24)). Serum was obtained from 23 women included in the healthy cohort of another NAF study (NL41845.041.12 (25)). Hereafter, these cohorts will be referred to as the "NAF cohort" and the "serum cohort", respectively. These studies were approved by the Institutional Review Boards of the UMC Utrecht and other participating hospitals (study number 12-495 approved on January 29 2013) and the UMC Utrecht Biobank Research Ethics Committee (TCBio; biobank study number 14-467 approved on June 17 2015). Informed consent was obtained from all participating women.

NAF samples were collected between June 2015 and March 2016, and serum samples were collected between August 2017 and November 2019. At the time of sample collection, all participants were healthy and had a minimum age of 50 and 45 years old according to inclusion criteria for the NAF and serum cohorts, respectively. For the NAF cohort, healthy was defined as not having breast abnormalities on recent mammography and/or magnetic resonance imaging. For the serum cohort, this was defined as not having breast cancer and not having an increased risk for developing breast cancer according to personal and familial history. The NAF samples were acquired after nasal oxytocin administration as described previously [19,20]. Bilaterally acquired samples were conserved in a buffer solution (50 mM Tris pH 8.0, 150 mM NaCl, 2mM EDTA). Serum samples were acquired by phlebotomy in the median cubital vein. After collection, serum was processed by centrifugation at 300 g for 15 min. Aliquots were immediately stored at –80 °C until use.

Extraction and quantification of RNA from nipple aspirate fluid and serum

Total RNA extraction from pooled NAF samples (intra-individual samples from left and right breast combined) and serum was performed according to the manufacturer's protocol using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) and the miRNeasy serum/plasma kit (Qiagen), respectively. All extractions were performed starting with 20 µL of pooled NAF and 200 µL of serum. For the NAF samples, non-human ath-mir-159 (with a 5' phosphate) was spiked in as quality control at 300 pg by pre-mixing with RLT plus lysis buffer. For the serum samples, 50 pg non-human ath-mir-159 (with a 5' phosphate) and 1 µg MS2 carrier RNA (Roche) were spiked in as quality control

after Qiazol incubation. RNA was eluted in 30 µL or 17 µL of RNAse-free water for the NAF and serum samples, respectively. The concentration of the extracted RNA was measured by Qubit 3.0 (ThermoFisher Scientific, MA, USA) fluorometric quantification. All RNA samples were then stored at −80 °C until further analysis.

Reverse transcription, preamplification, and Taqman OpenArray profiling analysis of nipple aspirate fluid and serum

The expression levels of 754 human mature miRNAs (Supplementary Methods) that have been functionally validated with miRNA artificial templates were profiled using the fixed-content TaqMan OpenArray Human Advanced MicroRNA Panel on a QuantStudio 12 K Flex system (ThermoFisher Scientific, MA, USA). According to the manufacturer's instructions, total RNA (8 ng from NAF and 2 µL from serum) was first poly-A tailed, and after adaptor ligation and reverse transcription, pre-amplified for 19 cycles. The pre-amplification product was subsequently diluted 20× in 0.1× TE buffer pH 8.0. The samples were loaded from a 384-well plate onto the TaqMan OpenArray Human Advanced MicroRNA Panel array slide using the OpenArray AccuFill system. Relative threshold values (CRT (26)), proven to be more robust than baseline threshold values for analyzing data generated using nanoliter fluidics-based OpenArray plates, were automatically calculated using the ThermoFisher Cloud system (27). Analysis settings included the following restrictions: a minimum CRT of 10, a minimum AMPSCORE (low signal in linear phase) of 1, a minimum calculated confidence in the quantification cycle (CQCONF) (Cq) value of 0.6, a maximum CRT of 28 with inclusion of maximum CRT in calculations. Additionally, all miRNA amplification plots were visually inspected on curve shape and signal timing.

A technical validation (i.e., quality control of NAF profiling data using the same samples) was performed using individual Taqman advanced miRNA assays hsa-miR-29a-3p, hsa-miR-324-5p and hsa-miR-181a-5p according to the manufacturer's instructions. Pearson correlation was calculated and graphically visualized using GraphPad 8.0 for Windows (San Diego, CA, USA).

Literature search: search strategy, study selection and data extraction

Four systematic searches were performed on PubMed for studies published up until May 2020 for samples of normal breast tissue or cells, breast milk, plasma and serum. Each search comprised words and synonyms of the biosample and of "microRNA", "profiling techniques" and "healthy women". Only those papers in which extensive miRNA profiling was performed either by deep sequencing, microarray or RT-qPCR profiling on either of these samples were selected for further ranking and comparison.

Full syntaxes and detailed criteria for inclusion and exclusion are presented in Supplementary Methods. Two reviewers (S.I.S.P. and A.M.O.C.) independently screened the titles, abstracts and full texts of the studies retrieved from the searches. Any disagreement was solved through discussion to reach a consensus. In total, 17 studies and our own serum and NAF data were selected to establish rankings of the most highly expressed miRNAs per biosample. The process of article selection followed adapted Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (28) and is presented in Figure 2 and detailed in Supplementary Methods. Quality assessment is one of the steps of the PRISMA guidelines, but was not performed as the studies were not diagnostic, therapeutic nor prognostic and hence did not fit to any of the existing quality assessment tools (29). Extracted data of the selected articles included normalized read counts, units of fluorescence intensity, and C(R)T values of individual miRNAs measured in the healthy population, anthropomorphic characteristics of the healthy cohort (such as age, body mass index and breast density), details concerning sample fraction and sample processing, and techniques of choice used for (mi)RNA isolation, miRNA detection and quantification.

MiRNA ranking

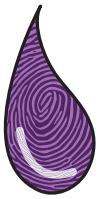
Extracted data of the selected articles included normalized read counts (reads per kilobase million, transcripts per kilobase million, counts per million and others), units of fluorescence intensity, relative abundance and C(R)T values of individual miRNAs measured in the healthy population. This heterogeneity of measures used across studies, including studies within one sample type, led us to apply the strategy of developing a top 50 rank. Given that 2656 microRNAs are known (15), highlighting the top 50 miRNAs was considered reasonable to highlight the most abundant miRNAs in each sample type.

To perform a miRNA ranking of the top 50 most highly expressed miRNAs in NAF samples, miRNAs detected in ≥80% of the NAF samples were selected. Secondly, miRNAs were orderly ranked based on their mean CRT values. The same strategy was applied to the miRNA profiling expression data from serum samples. This serum ranking was then combined with the serum rankings acquired from the literature into an overall serum ranking.

MiRNA data was retrieved from selected articles to perform a ranking of the top 50 most abundantly expressed miRNAs in breast tissue, breast milk, plasma and serum. As a first step, the miRNAs were ranked from highest to lowest expression (Supplementary Table S1). Listed miRNAs that were retracted from miRBase due to misclassification were excluded from the rankings, if present. Then, the rankings of the individual studies were combined into overall rankings per biosample. The

formulas applied for the overall ranking weighted in the number of samples used in each study and the top 50 rank number (the consistency) across studies per miRNA (Supplementary Table S2). This ranking formula is similar to the ranking method applied for meta-analysis of miRNA case-control studies developed by Griffith et al. (30) and Chan et al. (31), and used by many for miRNA profiling studies (32-36), which focuses on consistency in direction of expression change, frequency of reporting a particular miRNA, total sample size for consistently reported miRNAs and average fold change. Afterwards, miRNAs in the overall rankings for breast tissue, breast milk, plasma and serum that were not present in the 754-panel used for the NAF profiling from own data were excluded for comparative analysis. This was solely the case for one miRNA in the plasma ranking (hsa-miR-135a-3p). Consequently, the plasma top 50 was supplemented with the 51st most highly expressed miRNA, allowing a fair ranking comparison.

Ranking comparison



MiRNA nomenclature was adapted for consistency across sample type rankings into the most recent nomenclature according to miRBase (v22) (37, 38). The number of overlapping miRNAs across sample types was then displayed in Venn diagrams using the web tool VENN (39). GraphPad Prism 8.0 for Windows (San Diego, CA, USA) was used for graphical visualization of the ranking ranges of the top 20 miRNAs in breast tissue in the top 50 of the other four biosamples.

Physiological roles, pathway and target analysis of miRNAs detected in nipple aspirate fluid

Physiological roles and pathway involvement of NAF-derived miRNAs were retrieved from miRPathDB v2.0 (40) using the KEGG, WikiPathway and GO databases. Stringent criteria for miRPathDB query included selection of data based on strong experimental evidence and with at least 10 significant miRNAs per pathway. Involvement of these miRNAs in the immune processes was cross-checked with the Pathway Central database (SABiosciences, MD, USA) and Abcam's Multiplex Circulating miRNA Immunology Fixed Panel (ab204064). Experimentally validated common targets of the NAF top 20 miRNAs were obtained from miRTarBase v7 (41). Again, strict criteria were applied, namely that common mRNAs needed to be targeted by at least five miRNAs based on strong experimental evidence, which was defined as data derived from luciferase reporter assays or Western blot experiments.

Results

Study selection for ranking of breast tissue, breast milk, plasma and serum

In total, 341 records were screened, out of which 17 studies were included for miRNA ranking per biosample (four for breast tissue/cells (42-45), ten for breast milk (46-55), two for plasma (47, 56) and two for serum (57, 58)). One article reported data for breast milk as well as plasma ranking (47). Additionally, data from our own serum cohort were included in the ranking. This led to a total of 18 included studies for the ranking of these four biosamples. The flow of the search strategy is shown in Figure 2 and presented in detail in Supplementary Methods.

MiRNA expression data were derived from a total of 331 women. In the included literature studies, women were on average around 25 years younger in the breast milk and plasma studies (mean 30.3 and 32.93, respectively, based on available data from merely 28/173 women) compared to the women in the serum and NAF studies (mean 58.69 and 55, respectively, based on data from 158/158 women). The limited anthropomorphic characteristics of the healthy women that could be extracted from the included studies are shown in Supplementary Table S3. Most of the studies applied RNA sequencing techniques, only one study used a microarray platform (43) and two studies and our cohort were based on miRNA profiling with a wide panel of miRNAs (667-754 miRNA targets) (55, 56). A wide variety of (mi)RNA isolation kits were used. Detailed technical information is shown in Supplementary Table S3.

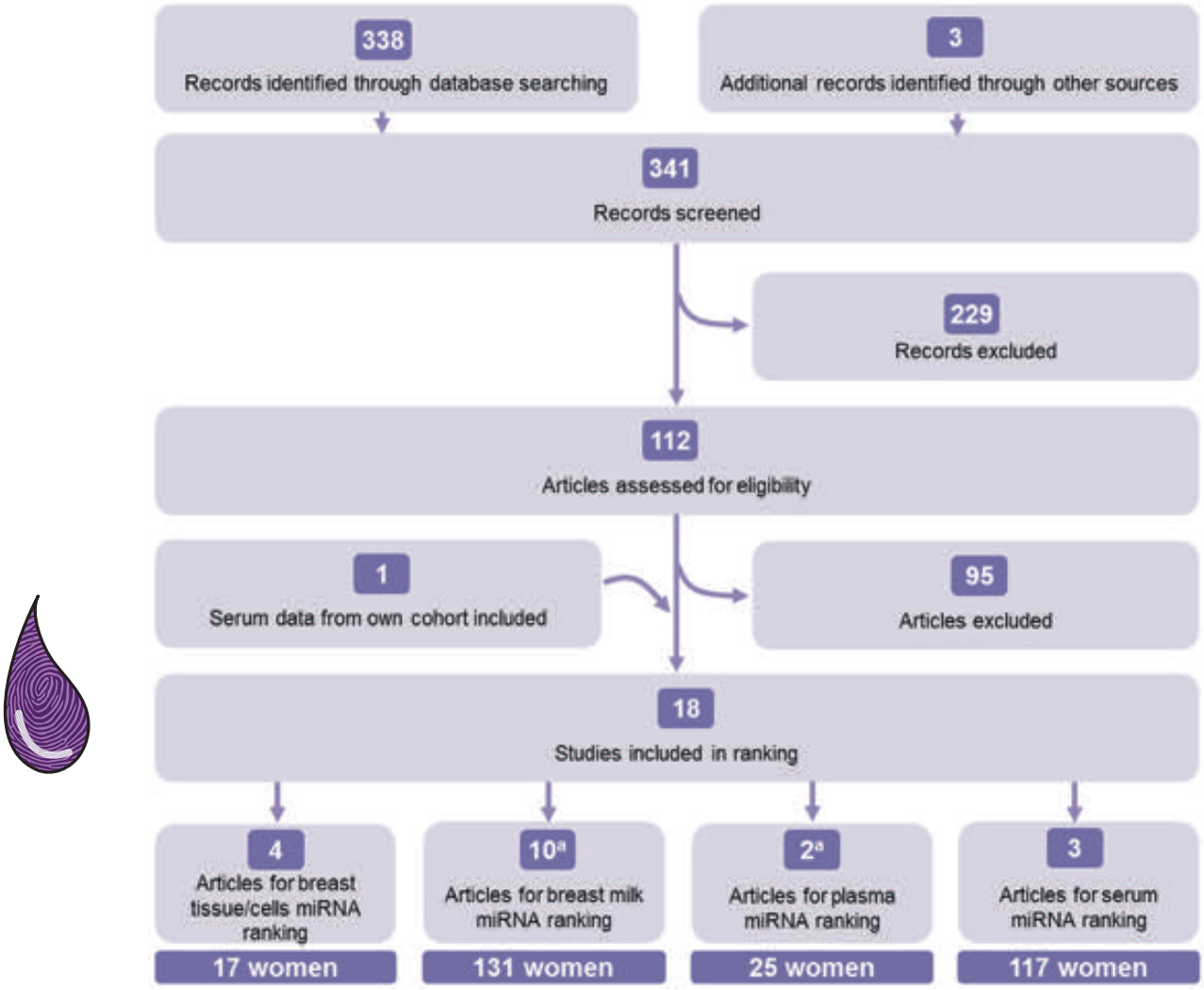


Figure 2. Flowchart of the study selection strategy used for miRNA ranking in four biosamples: breast tissue, breast milk, plasma and serum. Adapted from Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (28). Detailed flowcharts are shown in Supplementary Methods. The total number of women included in the selected studies per sample type was added underneath. a. One article provided data for the breast milk miRNA ranking and for the plasma miRNA ranking.

MiRNA expression profiling in nipple aspirate fluid and serum and ranking

The concentration of total RNA extracted from NAF varied between 3 and 134 ng/µL, with a mean of 42.2 ng/µL (SD 35.2). Nanoliter fluidics-based miRNA profiling was successful in all samples, with 296 out of the 754 miRNAs in the profiling panel (39%) determined with mean 10 ≤ CRT ≤ 29. To add perspective, 29/754 (4%) miRNAs were detected in all samples (n = 41), 85/754 (11%) miRNAs were detected in 90% of the samples (n ≥ 37) and 186/754 miRNAs (25%) were detectable in 50% of the samples (n ≥ 20). Mean and median expression for each miRNA present in at least 10/41 NAF samples is available in Supplementary Table S1. NAF ranking was performed with miRNAs detected in at least 80% of the samples (n ≥ 33), which was a total of 118/754 miRNAs (16%). The 50 most highly expressed microRNAs discovered in NAF are presented in Table 1. Correlation between profiling and individual qPCR technical validation for three miRNAs in the top 50 was highly significant ($p < 0.0001$; R squared between 0.6344 and 0.8644; Supplementary Figure S1).

In serum samples, total RNA concentration varied between 52 and 73 ng/µL, with a mean of 61.9 ng/µL (SD 6.1). Using the same 754-miRNA profiling panel as for the NAF samples, 89/754 (12%) miRNAs were detected in at least 90% of the samples (n ≥ 20), 110/754 miRNAs (15%) were detected in 80% of the samples (n ≥ 18) and 153/754 miRNAs (20%) were detected in 50% of the samples (n ≥ 11). Similarly, to the NAF samples, an 80% detection cut-off was applied to select the miRNA data used for serum ranking.

Table 1. Ranking of the 50 most highly expressed miRNAs in five healthy sample types. Individual rankings per study are presented in Supplementary Table S1. MiRNAs in bold are abundant in all interrogated sample types. In Supplementary Table S7, the unique physiological roles and pathway involvement for the 7 shared miRNAs (bold) are shown.

Ranking	Breast tissue (42-45)	Nipple Aspirate Fluid (this study)	Breast milk (46-55)	Plasma (47, 56)	Serum ((57, 58) and this study)
1	**hsa-miR-24-3p**	hsa-miR-205-5p	**hsa-miR-148a-3p**	hsa-miR-451a	hsa-miR-451a
2	**hsa-miR-21-5p**	hsa-miR-203a-3p	**hsa-miR-22-3p**	hsa-miR-92a-3p	hsa-miR-92a-3p
3	hsa-miR-23a-3p	hsa-miR-26b-5p	hsa-miR-146b-5p	**hsa-miR-21-5p**	**hsa-miR-26a-5p**
4	hsa-let-7f-5p	hsa-miR-221-3p	hsa-miR-30d-5p	hsa-miR-126-3p	**hsa-miR-22-3p**
5	**hsa-let-7b-5p**	**hsa-let-7b-5p**	hsa-miR-30a-5p	hsa-miR-320a-3p	hsa-miR-25-3p
6	**hsa-miR-22-3p**	hsa-miR-27a-3p	**hsa-miR-26a-5p**	hsa-miR-122-5p	hsa-miR-423-5p
7	hsa-let-7a-5p	hsa-miR-451a	hsa-miR-375-3p	**hsa-let-7b-5p**	**hsa-miR-21-5p**
8	hsa-miR-30a-5p	hsa-miR-92a-3p	hsa-let-7a-5p	hsa-miR-191-5p	hsa-miR-16-5p
9	hsa-miR-125b-5p	hsa-miR-16-5p	hsa-miR-200a-3p	hsa-miR-223-3p	**hsa-miR-148a-3p**
10	**hsa-let-7g-5p**	**hsa-let-7g-5p**	hsa-miR-141-3p	**hsa-miR-24-3p**	hsa-let-7a-5p
11	hsa-miR-143-3p	**hsa-miR-24-3p**	**hsa-miR-21-5p**	hsa-miR-16-5p	hsa-let-7f-5p
12	hsa-miR-451a	hsa-miR-200c-3p	hsa-let-7f-5p	hsa-miR-19b-3p	hsa-miR-27a-3p
13	hsa-miR-99a-5p	hsa-miR-200b-3p	hsa-miR-181a-5p	hsa-miR-486-5p	hsa-miR-122-5p
14	hsa-miR-320a-3p	hsa-miR-99a-5p	hsa-miR-30b-5p	hsa-miR-150-5p	**hsa-let-7b-5p**
15	hsa-miR-145-5p	**hsa-miR-21-5p**	**hsa-let-7b-5p**	hsa-miR-17-5p	hsa-miR-142-5p
16	hsa-miR-205-5p	hsa-miR-92b-3p	**hsa-let-7g-5p**	hsa-miR-484	hsa-miR-30d-5p
17	hsa-miR-126-3p	hsa-miR-15a-5p	hsa-miR-92a-3p	hsa-miR-106a-5p	hsa-miR-181a-5p
18	**hsa-miR-26a-5p**	hsa-miR-193b-3p	hsa-miR-191-5p	hsa-miR-20a-5p	hsa-miR-126-3p
19	hsa-miR-19b-3p	hsa-miR-30b-5p	hsa-miR-320a-3p	hsa-miR-146a-5p	hsa-miR-191-5p
20	hsa-let-7i-5p	hsa-miR-29a-3p	hsa-miR-182-5p	**hsa-miR-26a-5p**	hsa-let-7i-5p
21	hsa-let-7c-5p	hsa-miR-34a-5p	hsa-miR-423-5p	hsa-miR-25-3p	hsa-miR-101-3p
22	**hsa-miR-148a-3p**	**hsa-miR-26a-5p**	hsa-miR-99b-5p	hsa-miR-222-3p	**hsa-miR-24-3p**
23	hsa-miR-199a-3p	hsa-miR-125b-5p	hsa-miR-200c-3p	hsa-miR-342-3p	**hsa-let-7g-5p**
24	hsa-miR-101-3p	**hsa-miR-22-3p**	hsa-let-7i-5p	hsa-miR-30a-5p	hsa-miR-26b-5p
25	hsa-miR-100-5p	hsa-miR-365a-3p	hsa-miR-200b-3p	hsa-miR-126-5p	hsa-miR-146a-5p
26	hsa-miR-29a-3p	**hsa-miR-148a-3p**	hsa-miR-101-3p	hsa-miR-30c-5p	hsa-miR-486-5p
27	hsa-miR-103a-3p	hsa-miR-17-5p	**hsa-miR-24-3p**	hsa-miR-142-3p	hsa-miR-223-3p
28	hsa-let-7d-5p	hsa-miR-20a-5p	hsa-miR-27b-3p	hsa-miR-30d-5p	hsa-miR-144-3p
29	hsa-miR-125a-5p	hsa-miR-652-3p	hsa-miR-29a-3p	hsa-miR-30e-5p	hsa-miR-143-3p

Ranking	Breast tissue (42-45)	Nipple Aspirate Fluid (this study)	Breast milk (46-55)	Plasma (47, 56)	Serum ((57, 58) and this study)
30	hsa-miR-141-3p	hsa-miR-144-3p	hsa-miR-99a-5p	hsa-miR-30b-5p	hsa-miR-30e-5p
31	hsa-miR-199b-3p	hsa-miR-125a-5p	hsa-miR-429	hsa-miR-192-5p	hsa-miR-320a-3p
32	hsa-miR-199a-5p	hsa-miR-210-3p	hsa-miR-16-5p	hsa-miR-425-5p	hsa-miR-103a-3p
33	hsa-miR-181a-5p	hsa-miR-25-3p	hsa-miR-378a-3p	hsa-miR-423-5p	hsa-miR-10b-5p
34	hsa-miR-30b-5p	hsa-miR-361-5p	hsa-miR-103a-3p	hsa-let-7e-5p	hsa-miR-423-3p
35	hsa-miR-497-5p	hsa-miR-29b-3p	hsa-miR-186-5p	hsa-miR-106b-5p	hsa-miR-126-5p
36	hsa-miR-30e-5p	hsa-miR-195-5p	hsa-miR-151a-3p	**hsa-miR-148a-3p**	hsa-miR-27b-3p
37	hsa-miR-140-3p	hsa-miR-181b-5p	hsa-miR-10a-5p	hsa-miR-19a-3p	hsa-miR-16-2-3p
38	hsa-miR-27a-3p	hsa-miR-30c-5p	hsa-miR-25-3p	hsa-let-7i-5p	hsa-miR-150-5p
39	hsa-miR-130a-3p	hsa-miR-342-3p	hsa-miR-30e-5p	hsa-miR-378a-3p	hsa-miR-92b-3p
40	hsa-miR-191-5p	hsa-miR-181a-5p	hsa-miR-181b-5p	**hsa-miR-22-3p**	hsa-miR-484
41	hsa-miR-195-5p	hsa-miR-223-3p	hsa-miR-125a-5p	hsa-miR-146b-5p	hsa-let-7d-5p
42	hsa-miR-939-5p	hsa-miR-146a-5p	hsa-miR-30c-5p	hsa-miR-151a-3p	hsa-miR-151a-3p
43	hsa-let-7e-5p	hsa-miR-324-5p	hsa-miR-193b-3p	hsa-miR-195-5p	hsa-miR-185-5p
44	hsa-miR-221-3p	hsa-miR-150-5p	hsa-miR-335-5p	hsa-miR-185-5p	hsa-miR-23a-3p
45	hsa-miR-26b-5p	hsa-miR-145-5p	hsa-miR-27a-3p	hsa-miR-143-3p	hsa-miR-107
46	hsa-miR-10b-5p	hsa-miR-103a-2-5p	hsa-miR-19b-3p	hsa-miR-186-5p	hsa-miR-199a-3p
47	hsa-miR-143-5p	hsa-miR-193a-3p	hsa-miR-148a-5p	hsa-miR-93-5p	hsa-miR-148b-3p
48	hsa-miR-628-3p	hsa-miR-484	hsa-miR-146a-5p	hsa-miR-140-3p	hsa-miR-140-3p
49	hsa-miR-423-5p	hsa-miR-130a-3p	hsa-miR-29c-3p	hsa-miR-375-3p	hsa-miR-17-5p
50	hsa-miR-519d-3p	hsa-miR-19a-3p	hsa-let-7e-5p	**hsa-let-7g-5p**	hsa-miR-19b-3p

Roughly half of the highly expressed miRNAs in breast tissue and nipple aspirate fluid are also present in other samples

The lists of the 50 most abundant miRNAs per healthy sample type (breast tissue, NAF, breast milk, plasma and serum) are shown in Table 1 and the rankings per individual study are available in Supplementary Table S1. A total of 110 unique miRNAs were found when comparing the top 50 across all overall rankings. Out of these, only seven miRNAs were highly ranked in all five sample types, namely hsa-miR-148a-3p, hsa-let-7b-5p, hsa-miR-26a-5p, hsa-miR-24-3p, hsa-let-7g-5p, hsa-miR-21-5p and hsa-miR-22-3p.

The number of overlapping miRNAs between breast tissue and each biofluid was investigated. With the top 50 of breast tissue as a reference, the highest number of overlapping miRNAs was found in serum (n = 28; 56%), followed by breast milk (n = 26) and subsequently NAF (n = 21) and plasma (n = 21) (Figure 3a). A total of 17 miRNAs was found overlapping between breast tissue and the two systemic biofluids serum and plasma (Figure 3c). A somewhat lower number of miRNAs (n = 13) overlapped between breast tissue and the two breast-derived biofluids (NAF and breast milk) (Figure 3d).

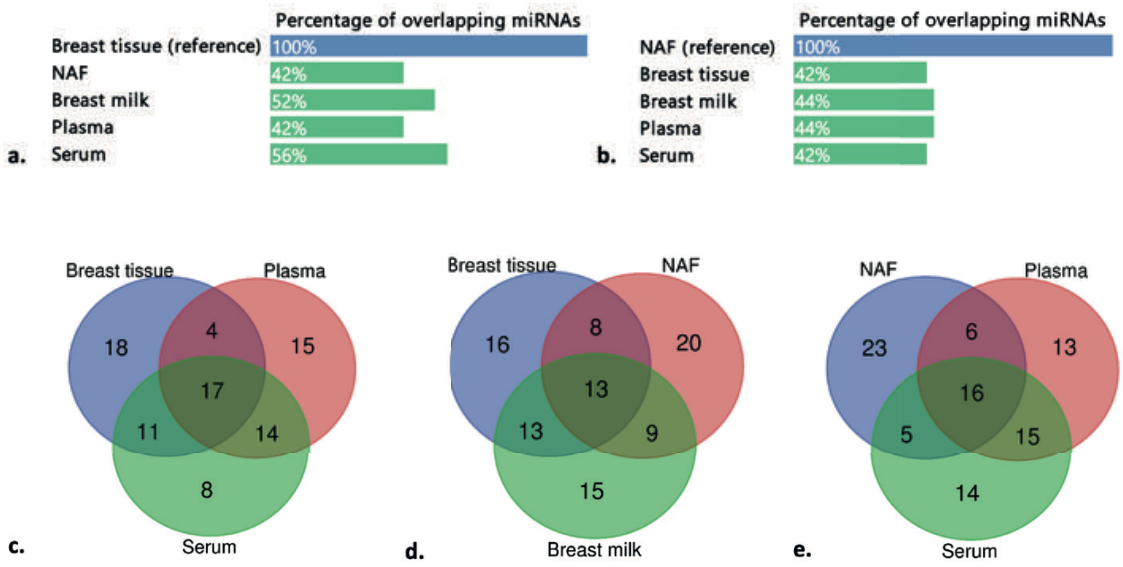


Figure 3. Overlapping miRNAs between sample types. (a,b): percentage of overlapping miRNAs between the top 50 of breast tissue (reference, a) and nipple aspirate fluid (NAF; reference, b) and the top 50 of the other four sample types. (c,d): number of overlapping miRNAs between the top 50 of breast tissue and the top 50 of two systemic biofluids (plasma and serum, c) and two breast-derived biofluids (NAF and breast milk, d). (e): number of overlapping miRNAs between de top 50 of NAF and the top 50 of two systemic biofluids: plasma and serum. The list of shared miRNAs is shown in Supplementary Table S4 and Supplementary Figure S2. A 5-way Venn diagram is shown in Supplementary Figure S4.

When the top 50 of NAF was used as a reference compared to each of the other sample types, a comparable number of overlapping miRNAs was found, namely 21–22 miRNAs (42–44%) (Figure 2b). Comparing the top 50 of NAF and both systemic biofluids, a total of 16 miRNAs overlapped (Figure 2e). Five miRNAs were exclusively shared between NAF and breast tissue, namely hsa-miR-221-3p, hsa-miR-205-5p, hsa-miR-125b-5p, hsa-miR-145-5p and hsa-miR-130a-3p (Supplementary Table S4). Four miRNAs were exclusively shared between NAF and breast milk, namely hsa-miR-181b-5p hsa-miR-193b-3p hsa-miR-200c-3p and hsa-miR-200b-3p. In Supplementary Table S4 and Supplementary Figure S2, these and additional overlapping comparisons are presented. In general, the overlap between the top 50 ranking of individual sample types varied marginally between 42–62%. The greatest overlap was observed between serum and plasma (62%). The overlap amongst breast-specific sample types varied between 42–52% whereas the overlap between breast-specific samples and systemic sample types was 42–56%.

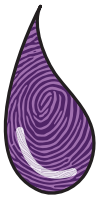
Non-overlapping miRNAs were also observed. A list of these potentially unique biosample-enriched miRNAs is presented in Table 2. NAF and breast tissue contained the highest number of unique miRNAs (n = 11 and n = 9, respectively), while serum had the lowest number of unique miRNAs (n = 5).

Table 2. List of unique miRNAs in the top 50 of each of the five healthy sample types. For each miRNA, the individual rank is provided between brackets. In Supplementary Table S7, the unique physiological roles and pathway involvement to each set of specimen-specific miRNAs are shown. NAF: nipple aspirate fluid.

Samples	Number of miRNAs	miRNAs
Breast tissue	9	hsa-let-7c-5p (21) hsa-miR-100-5p (25) hsa-miR-199b-3p (31) hsa-miR-199a-5p (32) hsa-miR-497-5p (35) hsa-miR-939-5p (42) hsa-miR-143-5p (47) hsa-miR-628-3p (48) hsa-miR-519d-3p (50)
NAF	11	hsa-miR-203a-3p (2) hsa-miR-15a-5p (17) hsa-miR-34a-5p (21) hsa-miR-365a-3p (25) hsa-miR-652-3p (29) hsa-miR-210-3p (32) hsa-miR-361-5p (34) hsa-miR-29b-3p (35) hsa-miR-324-5p (43) hsa-miR-103a-2-5p (46) hsa-miR-193a-3p (47)
Breast milk	8	hsa-miR-200a-3p (9) hsa-miR-182-5p (20) hsa-miR-99b-5p (22) hsa-miR-429 (31) hsa-miR-10a-5p (37) hsa-miR-335-5p (44) hsa-miR-148a-5p (47) hsa-miR-29c-3p (49)
Plasma	7	hsa-miR-106a-5p (17) hsa-miR-222-3p (22) hsa-miR-142-3p (27) hsa-miR-192-5p (31) hsa-miR-425-5p (32) hsa-miR-106b-5p (35) hsa-miR-93-5p (47)
Serum	5	hsa-miR-142-5p (15) hsa-miR-423-3p (34) hsa-miR-16-2-3p (37) hsa-miR-107 (45) hsa-miR-148b-3p (47)

The majority of abundant miRNAs in nipple aspirate fluid are immune-related and established tumor suppressors

To obtain insight into the biological processes in which the most highly expressed NAF miRNAs are involved, miRPathDB v2.0 (59, 60) was used. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that the top 20 of the most highly expressed miRNAs in NAF are annotated for, amongst others, "MicroRNAs in cancer", "Pathways in cancer", "Focal adhesion" and "PI3K/AKT signaling pathway". Interestingly, WikiPathway showed that most of the miRNAs in NAF have been categorized as being involved in the "breast cancer pathway". Moreover, these miRNAs were annotated for "regulation of cell population proliferation", "response to organic substance", "cell death" and "(cellular) protein metabolic process" according to Gene Ontology (GO) biological processes enrichment analysis. The three pathway analyses are shown in Supplementary Figure S3. In Supplementary Table S7, the unique physiological roles and pathway involvement to each set of specimen-specific miRNAs (Table 2) and for the seven shared miRNAs (Table 1, bold) are shown. Interestingly, "integrated breast cancer pathways" are related to the majority of unique NAF-miRNAs and "breast cancer pathways" are related to unique breast milk miRNAs and unique NAF-miRNAs.



Additionally, of the 50 most abundant miRNAs in NAF, 39 (78%) have been annotated as immune-related miRNAs, i.e., related to immunity pathways and/or targets reported to be involved in immunity (Supplementary Table S5). All of the 10 most highly expressed miRNAs in NAF have been listed as immune-related miRNAs.

Lastly, a target analysis was performed for the top 20 NAF miRNAs. Common established targets were BCL2 (a suppressor of apoptosis, targeted by 9/20 miRNAs), PTEN (a tumor suppressor that antagonizes the PI3K-AKT signaling pathway, targeted by 8/20 miRNAs), VEGFA (a proto-oncogene involved in angiogenesis, targeted by 8/20 miRNAs), BMI1 (a proto-oncogene involved in stem cell renewal and DNA repair, targeted by 7/20 miRNAs), CCND1 (an oncogene involved in cell cycle progression, targeted by 7/20 miRNAs), TP53 (a tumor suppressor that induces growth arrest and induces apoptosis, targeted by 6/20 miRNAs) and SP1 (a proto-oncogenic transcription factor, targeted by 5/20 miRNAs), amongst others (IGF1R, KRAS, ZEB2, AKT2, CDK6, MYC and RECK). Depending on their targets, miRNAs can be classified into tumor suppressor miRNAs or oncogenic miRNAs. Notably, 15 (75%) of the 20 most highly expressed miRNAs in NAF have an established tumor suppressor role in breast cancer. Supplementary Table S6 provides a summary of the most relevant established targets and cellular processes in which each of the top 20 NAF miRNAs has been shown to be involved in.

Discussion

MiRNAs have the potential to serve as novel clinical biomarkers as they can reflect subtle changes that occur in easily accessible biofluids, better known as liquid biopsies. Thousands of research papers related to miRNAs have been published in relation to disease, usually focusing on the significance and fold change of differentially expressed miRNAs between cases and controls, sometimes correlating with endpoints such as overall survival and prognosis (61-63). However, reporting of baseline miRNA levels in healthy controls is often overpassed and not systematically reported (64). As a consequence, data on normally expected miRNAs in the healthy state are very limited. Here, using literature data supplemented with our own data, we present the 50 most highly expressed miRNAs in five healthy sample types, namely breast tissue, and the liquid biosamples NAF, breast milk, plasma, and serum. Furthermore, this is the first study to define the physiological miRNA landscape in NAF and its overlap with other biosample types.

To establish a baseline ranking of highly expressed miRNAs, a systematic literature search was applied to ensure that all the relevant articles were found. As quantitative measurements of miRNAs across studies could not be compared due to the application of different techniques and platforms, a ranking from 1 to 50 was established. The overall overlap analysis of the five top 50 miRNA rankings showed that merely seven out of the 110 unique miRNAs were shared between the interrogated sample types, confirming the importance of biosample or biofluid choice for biomarker analysis. These seven shared microRNAs across all sample types overlapped for a great number of unique pathways (Supplementary Table S7), which could imply that these microRNAs are abundant in the whole body and involved in many, diverse, and (hence) aspecific pathways. Furthermore, the overlap analyses unexpectedly showed that highly expressed miRNAs in breast-specific sources did not mutually overlap more than with systemic sources. For example, the greatest miRNA overlap between breast tissue and another sample type was observed with serum (28 miRNAs). This either suggests that most miRNAs derived from breast tissue are released into this blood fraction, or that other non-investigated tissues might express overlapping miRNAs that are released into the bloodstream. An additional comparison with a miRNA ranking from minimally one other healthy tissue would provide insight into the latter. A publicly available “healthy genome atlas” for tissue and liquid biopsy data would facilitate such miRNA specificity analyses.

Given that this is the first study to present data on miRNA profiling in healthy NAF, detailed analyses from the perspective of NAF were performed. Here, we show that miRNA profiling using a wide panel of 754 miRNA targets was successful in all

NAF samples. In addition, the technical validation of three miRNAs demonstrated reproducibility. An overlap analysis using highly expressed miRNAs in NAF as a reference compared to the other samples showed that a minority of NAF miRNAs are also abundant in the four other sample types (21-22/50 miRNAs), regardless of whether it is a liquid or solid sample, a local or a peripheral/systemic sample.

Exploring the relevance of the most highly expressed miRNAs in NAF revealed that the majority of NAF miRNAs are tumor suppressors involved in breast carcinogenesis pathways. The highest-ranked miRNA in NAF was hsa-miRNA-205-5p, also positioned in the top 50 of breast tissue. This tumor suppressor miRNA, known to be exclusively expressed in myoepithelial cells, has been associated with the regulation of adherens junctions and focal adhesion. As such, downregulation of hsa-miRNA-205-5p leads to tumor invasion (65-67). Two shared miRNAs between NAF and milk were hsa-miRNA-200b-3p and hsa-miRNA-200c-3p, belonging to the tumor suppressor miRNA-200 family (68, 69). Hsa-miRNAs 200b/c-3p directly target E-cadherin transcriptional repressors ZEB1/ZEB2, thereby inhibiting epithelial–mesenchymal transition (EMT) (70-74). Downregulation of these miRNAs has been associated with breast cancer metastasis (75).

A total of 11 NAF miRNAs were not expressed in the top 50 of the other sample types and are therefore likely NAF-enriched. The most highly ranked among them, hsa-miR-203a-3p (second ranked) is an established tumor suppressor that has been shown to compromise cellular migration and invasive capacity in vitro, and tumor initiation and metastasis in vivo (76). Downregulation of hsa-miR-203 has been associated with increased extracellular matrix stiffness (77). Increased breast stiffness is visible in radiological findings (“dense breasts”), and is a known relevant risk factor for breast cancer development, biologically still far from understood (78, 79). Other highly ranked miRNAs unique for the top 50 of NAF were hsa-miR-15a-5p and hsa-miR-193b-3p. Likewise, their downregulation has been related to breast cancer by influencing migration (80), cell cycle progression, and apoptosis (81, 82). Not only these miRNAs, but the majority of highly ranked miRNAs in NAF are involved in cellular proliferation, differentiation, apoptosis and/or target commonly known mRNAs involved in carcinogenesis, such as PTEN, BCL2, and VEGFA. In addition, all the top 10 miRNAs in NAF play a prominent role in immunity, which is of increasing relevance in the context of breast cancer immunotherapy treatment (83-85). Altogether, this shows that miRNAs detected in NAF have established roles in breast carcinogenesis. Given their source specificity and tumor suppressor role, they may be overlooked or detected with lower sensitivity in case-control studies that use other sample sources.

From the above, it can be concluded that having insight into the miRNA patterns of normal tissues and biological fluids is key to be able to correctly interpret miRNA patterns as biomarkers of disease. Nonetheless, this study has some limitations. First, with a range of 1 to 10 studies per sample type for the overall ranking, a relatively low number of studies was included. This was the result of strict inclusion and exclusion criteria for article selection. For example, it was chosen to select studies with miRNA data for women, as female gender was the only possible miRNA confounding variable (86) that could be filtered a priori. The miRNA data from The Cancer Genome Atlas (TCGA (87, 88)) was queried but not used because miRNA data from normal breast tissue adjacent to the tumor is believed to have a different profile compared to normal healthy breast tissue (89). Furthermore, many studies were excluded because individual quantitative data per miRNA in the healthy cohort were not provided (62 studies out of the 92 excluded based on the full text, Supplementary Methods), which strengthens the reason and need for the present study. Still, the data for the rankings are derived from an ample number of women, namely 331 women.

Second, the presented ranking positions per miRNA may not be entirely accurate as these positions are dependent on the ranking strategy, isolation method (90), sample pooling, anthropomorphic characteristics of the cohort, and/or cut-off values for miRNA selection. To mention a few possible confounders, for the overall ranking of breast milk in this study, a combination of individual rankings per milk fraction (lipid fraction, skim milk, cells or exosomes) was applied, and for the ranking of breast tissue, two in vitro studies using mammary epithelial cells were integrated into the ranking. Regarding anthropomorphic characteristics, based on available data from a minority of women, the breast milk and plasma cohorts in this study were collected from younger women compared to the cohorts for the other samples (Supplementary Table S3), which may have altered miRNA expression (86, 91). Another baseline characteristic relevant for women in the context of breast cancer is mammographic density, which was only known within our own NAF cohort. NAF samples were derived from women with extremely high or very low MD, together with a 20% prevalence in the female population (92). Whether this influences the data presented is unclear as the influence of breast density on miRNA expression has not been studied yet. Regarding cut-off values, in the NAF and serum cohorts that produced our own data, miRNAs had to be expressed in at least 80% of the interrogated samples to qualify for ranking selection; this was probably not applied by others, or at least not reported. Adaptation of any of these variables may shuffle individual miRNAs to another ranking position. A similar study comparing these (and more) sample types from the same subjects would be ideal, but challenging, with the unique combination of samples chosen for this study.

In conclusion, this is the first study to establish and compare the physiological miRNA expression pattern in three breast-specific and two systemic healthy sample types. A publicly available genomic data atlas repository for healthy samples currently lacks and should be created to allow further comparisons and evaluations, such as the investigation of sample type (a)specificity of potential biomarker miRNAs. Such data needs to be accompanied with a clear definition of what is healthy, with anthropomorphic characteristics and sample fraction specification and should be based on uniform sample processing, techniques, and platforms. Most importantly, this is the first study that characterizes the unique physiological NAF-derived miRNA landscape. Surprisingly, in terms of abundant physiological miRNAs, healthy NAF does not appear to be a better representative of the breast microenvironment than commonly used biofluids such as serum or plasma. Nevertheless, healthy NAF contains measurable amounts of miRNAs with established roles in breast carcinogenesis, some of them likely enriched compared to serum and plasma. As such, there is a possible clinical applicability of NAF-derived miRNAs that should be further explored.

Supplementary Materials

Supplementary Methods S1*. List of Advanced assay names present in the 754-miRNA panel used for NAF sample profiling.
Supplementary Methods S2. Search syntaxes in PubMED used per biosample for the systematic literature search.
Supplementary Methods S3. Inclusion and exclusion criteria used for article selection.
Supplementary Methods S4. Flow Diagram: selection of articles for the physiological microRNA ranking based on breast tissue samples (A), breast milk samples (B), plasma samples (C) and serum samples (D).
Supplementary Figure S1. Pearson correlation analysis between RT-qPCR profiling results and technical individual RT-qPCR assay validation (quality control) for three selected miRNAs showing high concordance.
Supplementary Figure S2*. Ranking range of the 20 most highly expressed miRNAs in breast tissue compared to the top 50 miRNAs from each study of each biosample: nipple aspirate fluid (NAF), breast milk, plasma and serum.
Supplementary Figure S3. Physiological roles and pathway involvement of the top 20 NAF-derived miRNAs as retrieved from miRPathDB v2.0 using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (A), WikiPathway (B) and Gene Ontology (GO) biological processes (C) databases.
Supplementary Figure S4. Five-way Venn diagram showing the number of overlapping top 50 microRNAs across five sample types: breast tissue, NAF, breast milk, plasma and serum.
Supplementary Table S1*. Individual expression levels, read counts or units of fluorescence units of the top 55–60 miRNAs per study that used breast tissue samples or breast tissue cell lines, breast milk, plasma, serum and NAF.
Supplementary Table S2*. Overall ranking of the top 50 miRNAs in studies that used breast tissue samples or breast tissue cell lines, breast milk, plasma and serum.
Supplementary Table S3*. Study subjects' and sample characteristics of the studies included in the ranking.
Supplementary Table S4*. Overlap from the perspective of each sample as a reference and overall overlap.
Supplementary Table S5*. Proportion of immune-related miRNAs of the 50 most abundant miRNAs in NAF, breast milk, plasma and serum.
Supplementary Table S6. Overview of the most relevant established mRNA targets and breast cancer related cellular processes of each top 20 NAF miRNA shown in Table 1.
Supplementary Table S7*. Unique physiological roles and pathway involvement to each set of specimen-specific miRNAs (Table 2) and for the 7 shared miRNAs (Table 1, bold).

* Not shown in this thesis due to the extent of the data, but available online at: https://www.mdpi.com/1422-0067/21/22/8466#supplementary

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Supplementary Methods S2. Search syntaxes in PubMED used per biosample for systematic literature search

Breast milk

("microRNAs" [MeSH Terms] OR microrna*[Title/Abstract] OR micro rna [Title/Abstract] OR micro rnas [Title/Abstract] OR mirna* [Title/Abstract]) AND ("milk, human"[MeSH Terms] OR breast milk [Title/Abstract] OR breastmilk [Title/Abstract] OR human milk[Title/Abstract]) AND ("microarray analysis"[MeSH Terms] OR microarray* [Title/Abstract] OR array* [Title/Abstract] OR profil* [Title/Abstract] OR sequenc* [Title/Abstract])

Tissue

("microRNAs" [MeSH Terms] OR microrna* [Title/Abstract] OR micro rna [Title/Abstract] OR micro rnas [Title/Abstract] OR mirna* [Title/Abstract]) AND ("mammary glands, human" [MeSH Terms] OR mammary gland* [Title/Abstract] OR breast tissue* [Title/Abstract] OR breast connective tissue*[Title/Abstract] OR mammary tissue[Title/Abstract]) AND ("healthy volunteers"[MeSH Terms] OR healthy volunteer*[Title/Abstract] OR healthy participant*[Title/Abstract] OR healthy subject*[Title/Abstract] OR human volunteer*[Title/Abstract] OR normal volunteer*[Title/Abstract] OR control*[Title/Abstract] OR healthy women[Title/Abstract]) AND ("microarray analysis"[MeSH Terms] OR microarray*[Title/Abstract] OR array*[Title/Abstract] OR profil* [Title/Abstract] OR sequenc*[Title/Abstract])

Plasma[a]

("microRNAs" [MeSH Terms] OR microrna* [Title/Abstract] OR mirna* [Title/Abstract]) AND ("plasma"[MeSH Terms] OR plasma[Title/Abstract]) AND (women[Title/Abstract]) AND ("microarray analysis"[MeSH Terms] OR microarray*[Title/Abstract] OR array*[Title/Abstract] OR profil*[Title/Abstract] OR sequenc*)

Serum[a]

("microRNAs" [MeSH Terms] OR microrna* [Title/Abstract] OR mirna* [Title/Abstract]) AND ("serum"[MeSH Terms] OR serum[Title/Abstract]) AND (women[Title/Abstract]) AND ("microarray analysis"[MeSH Terms] OR microarray*[Title/Abstract] OR array*[Title/Abstract] OR profil*[Title/Abstract] OR sequenc*)

a. For plasma and serum, we decided to only search for articles that performed miRNA non-targeted analysis, to ensure that a ranking based on data from women was obtained.

Supplementary Methods S3. Inclusion and exclusion criteria used for article selection

Inclusion criteria

Studies were included if they met the following inclusion criteria:

(1) samples were collected from healthy women,
(2) microRNA expression analysis was performed in breast tissue, breast milk, plasma or serum,
(3) microRNA relative quantitative measure presentation based on non-targeted techniques such as profiling, sequencing and microarray,
(4) -3p and -5p arms of microRNAs mentioned (if applicable).

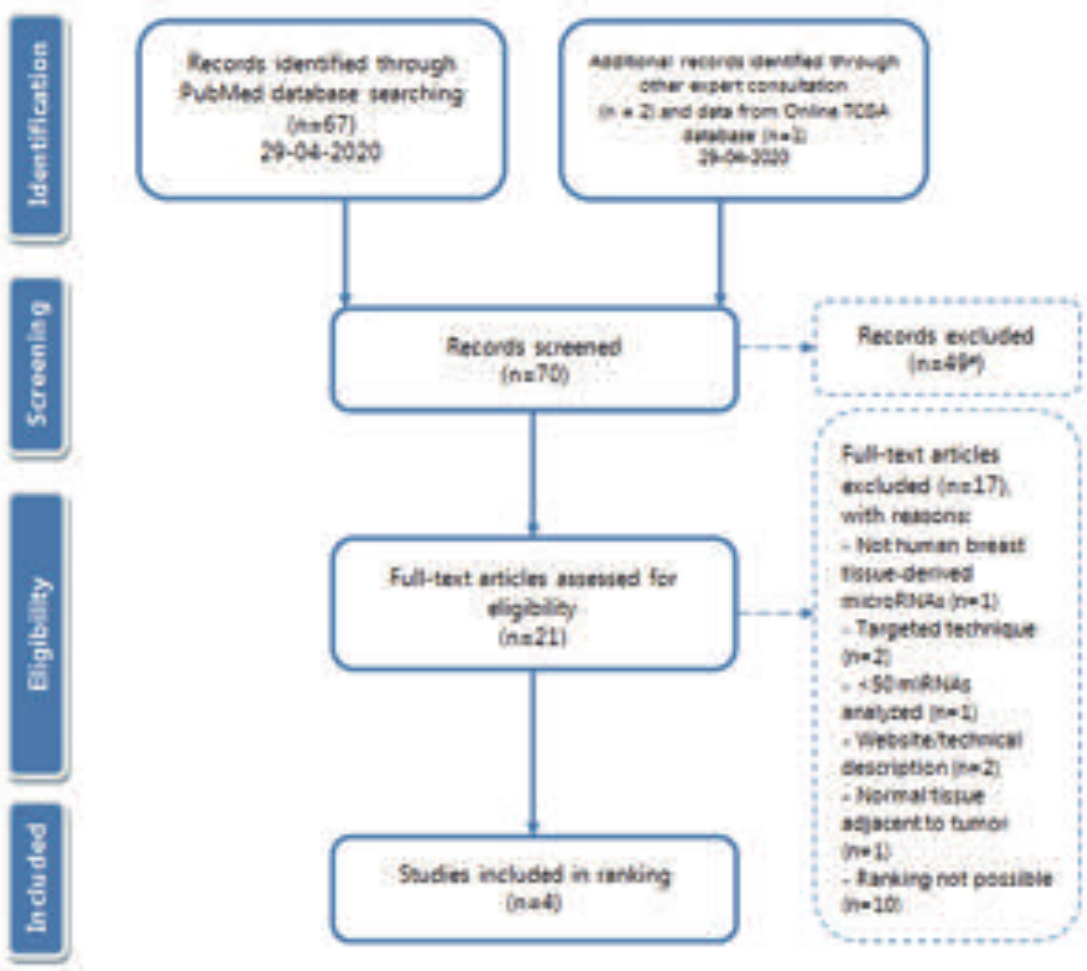
Exclusion criteria:

Studies were excluded if:

(1) the format was a review, conference abstract, comment, perspective or book chapter,
(2) not written in English,
(3) full text was not available,
(4) samples were from animals,
(5) miRNA analysis was performed in cell lines[a]
(6) samples from women with benign disease were defined as the control samples,
(7) tissue samples originated from healthy adjacent tumor area,
(8) samples were from pregnant women
(9) samples were from women with an explicit condition or complaint,
(10) blood samples, without explicit differentiation between serum or plasma fraction and hence separated data,
(11) data from samples from men that could not be separated from that of women,
(12) individual quantitative measures (Ct values or read counts) for the analyzed miRNAs were absent either in the article, supplementary data or data repositories,
(13) less than 50 miRNAs were analyzed.

a. Given the low number of articles that allowed generating a ranking for breast tissue, an exception was made for studies using breast cell lines that resemble epithelial cells.

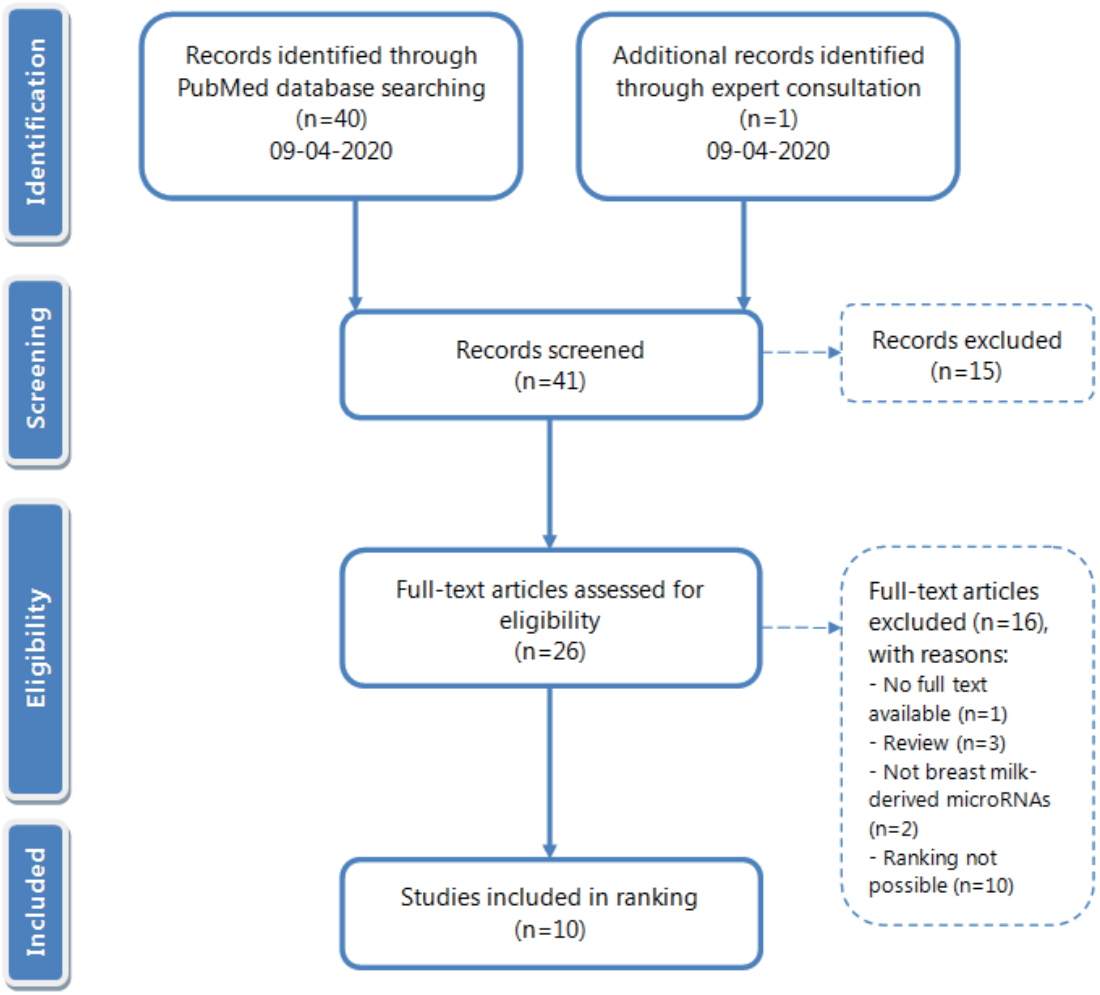
Supplementary Methods S4A. Flow Diagram: selection of articles for the physiological microRNA ranking based on breast tissue samples



a. This includes exclusion of the online The Cancer Genome Atlas (TCGA) database given that 'normal breast tissue' samples referred to samples adjacent to tumor tissue.

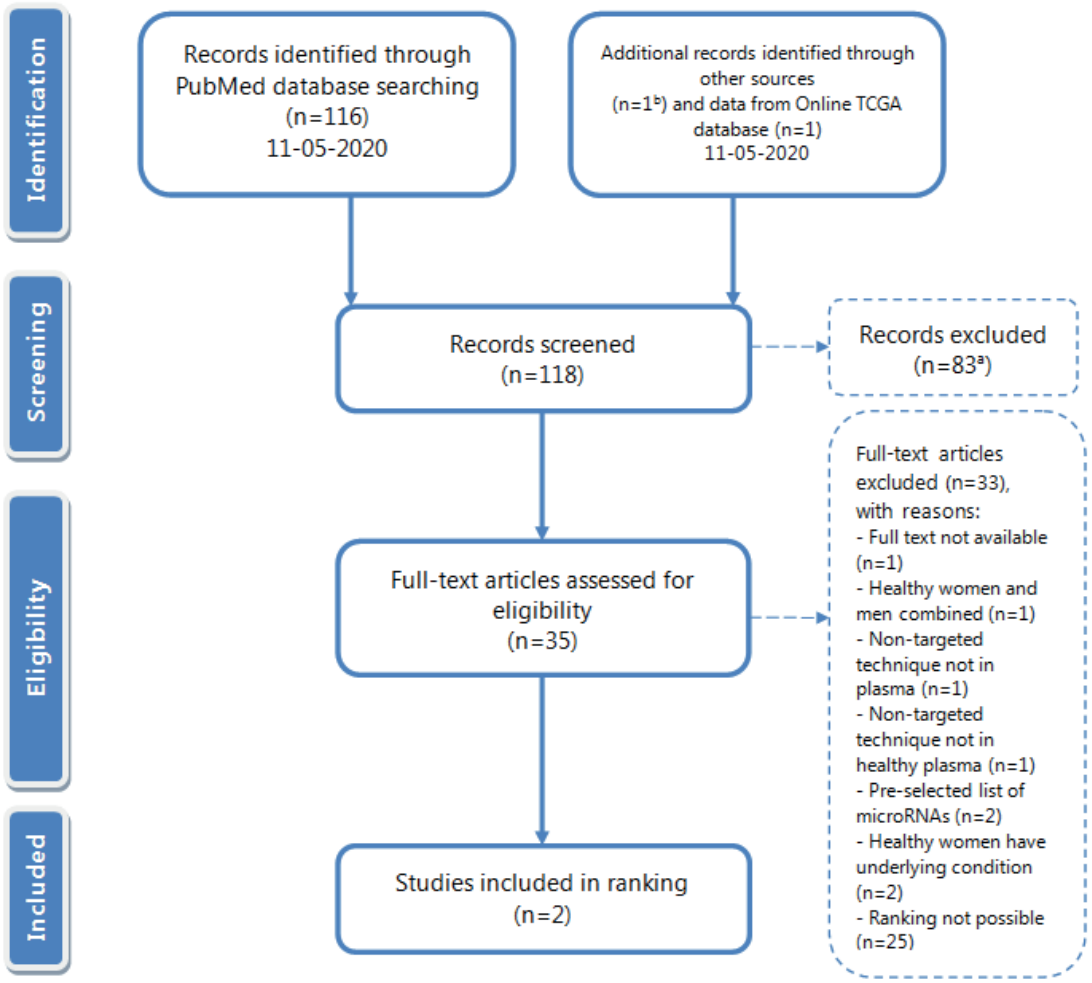
Based on: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097
For more information, visit www.prisma-statement.org.

Supplementary Methods S4B. Flow Diagram: selection of articles for the physiological microRNA ranking based on breast milk samples



Based on: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097
For more information, visit www.prisma-statement.org.

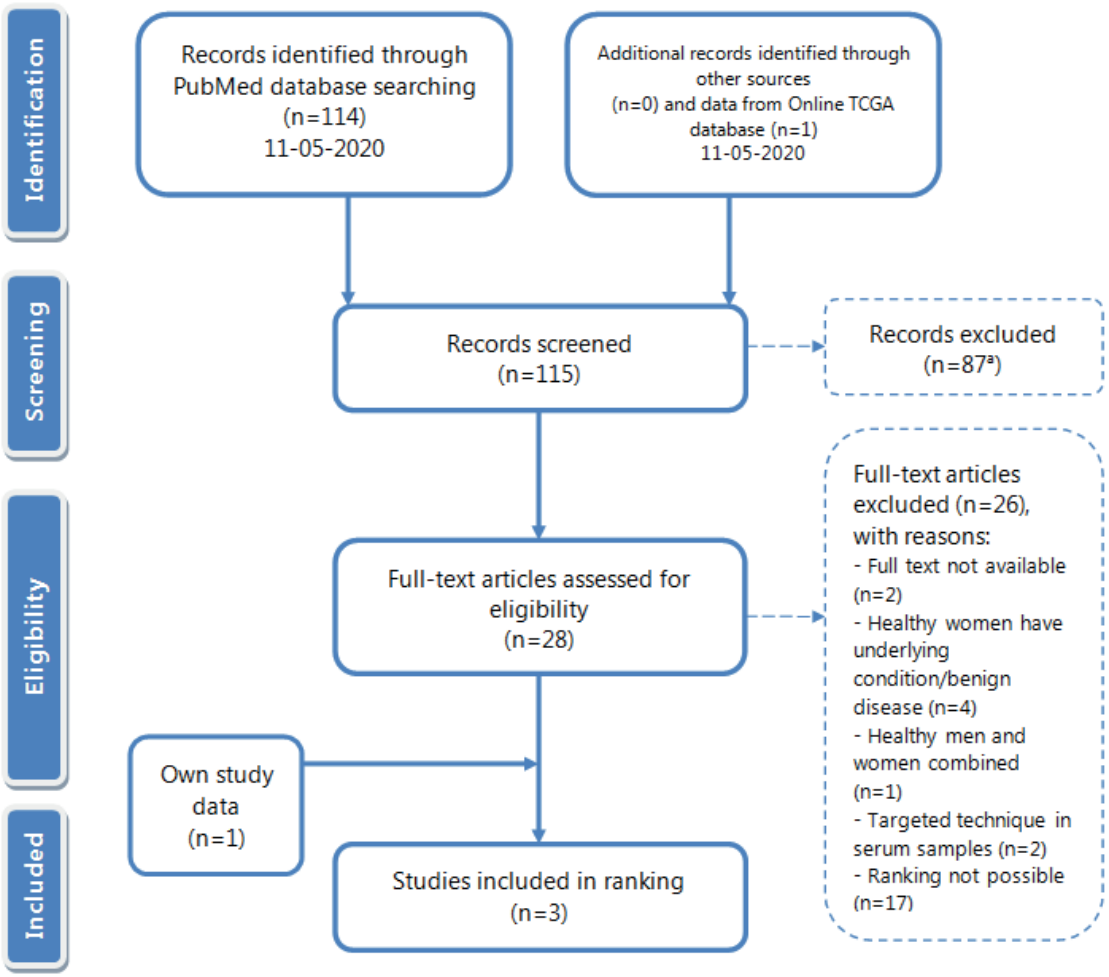
Supplementary Methods S4C. Flow Diagram: selection of articles for the physiological microRNA ranking based on plasma samples



a. This includes exclusion of the online The Cancer Genome Atlas (TCGA) database given that 'plasma' was not specified for microRNA data derived from blood samples.
Article added from "breast milk" search

Based on: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097
For more information, visit www.prisma-statement.org.

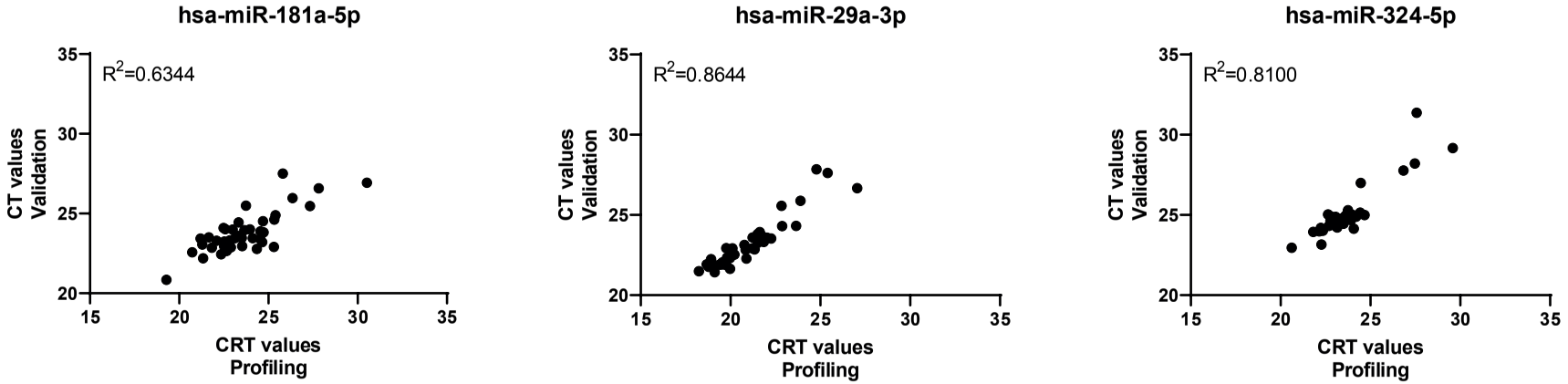
Supplementary Methods S4D. Flow Diagram: selection of articles for the physiological microRNA ranking based on serum samples



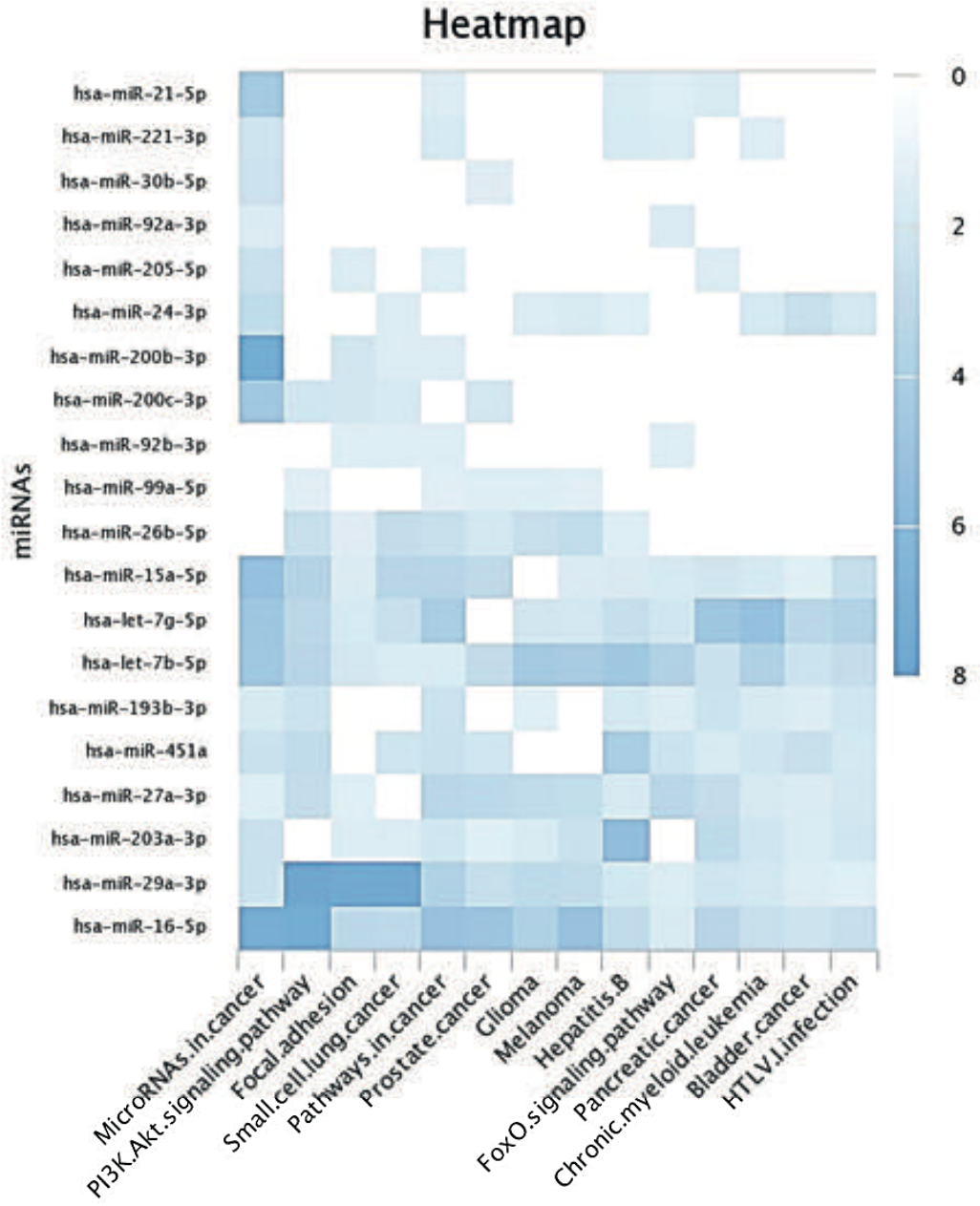
a. This includes exclusion of the online The Cancer Genome Atlas (TCGA) database given that 'serum' was not specified for microRNA data derived from blood samples.

Based on: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097
For more information, visit www.prisma-statement.org.

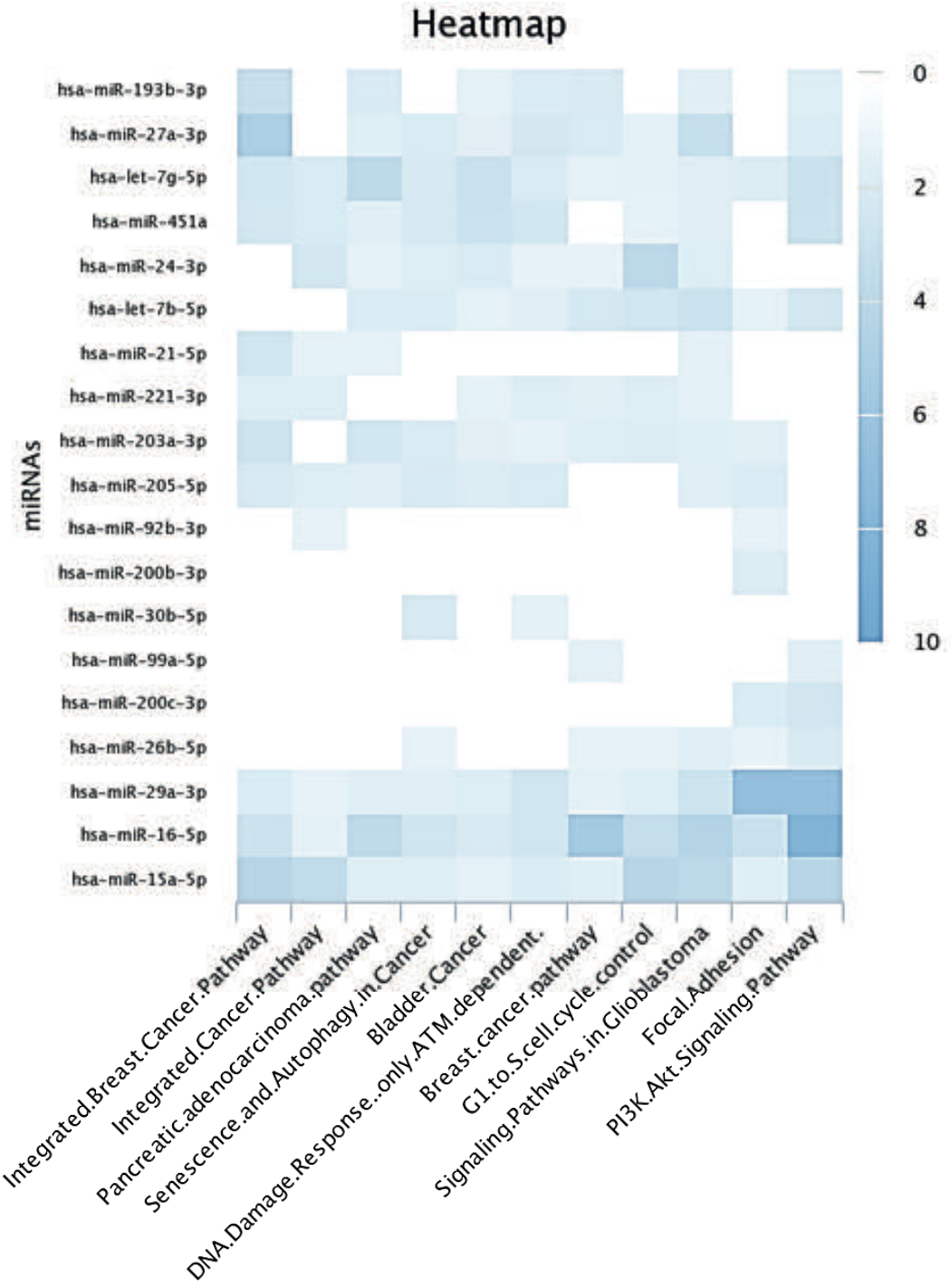
Supplementary Figure S1. Pearson correlation analysis between RT-qPCR profiling results and technical individual RT-qPCR assay validation (quality control) for three selected miRNAs showing high concordance. Each dot represents a measurement of a NAF sample (n=41 NAF samples for hsa-miR-181a and hsa-miR-29a and n=40 NAF samples for hsa-miR-324).



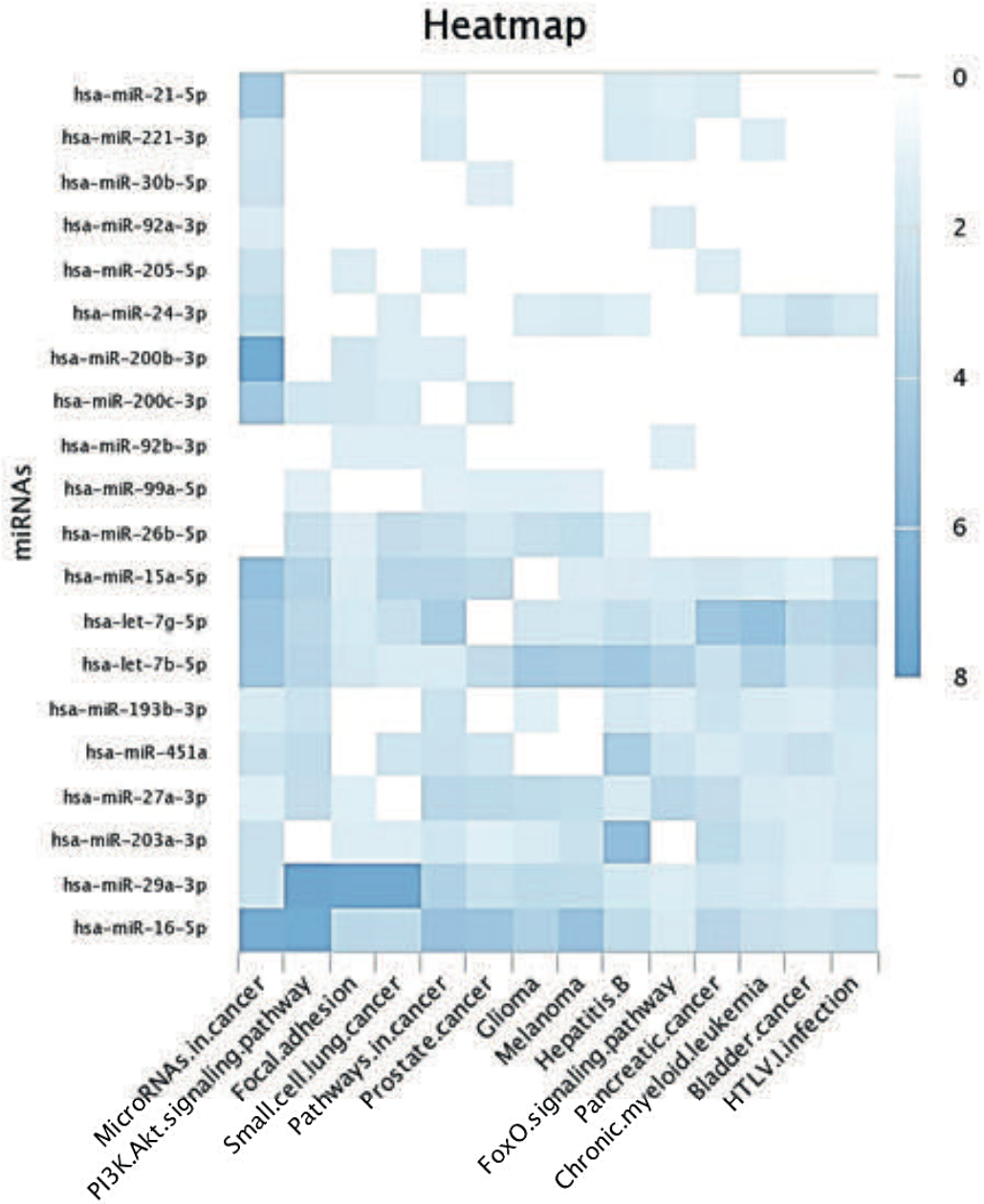
Supplementary Figure S3. Physiological roles and pathway involvement of the top 20 NAF-derived miRNAs as retrieved from miRPathDB v2.0 (1) using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (A), WikiPathway (B) and Gene Ontology (GO) biological processes (C) databases. Stringent criteria for miRPathDB query included selection of data based on strong experimental evidence and with at least 10 significant miRNAs per pathway.



A.

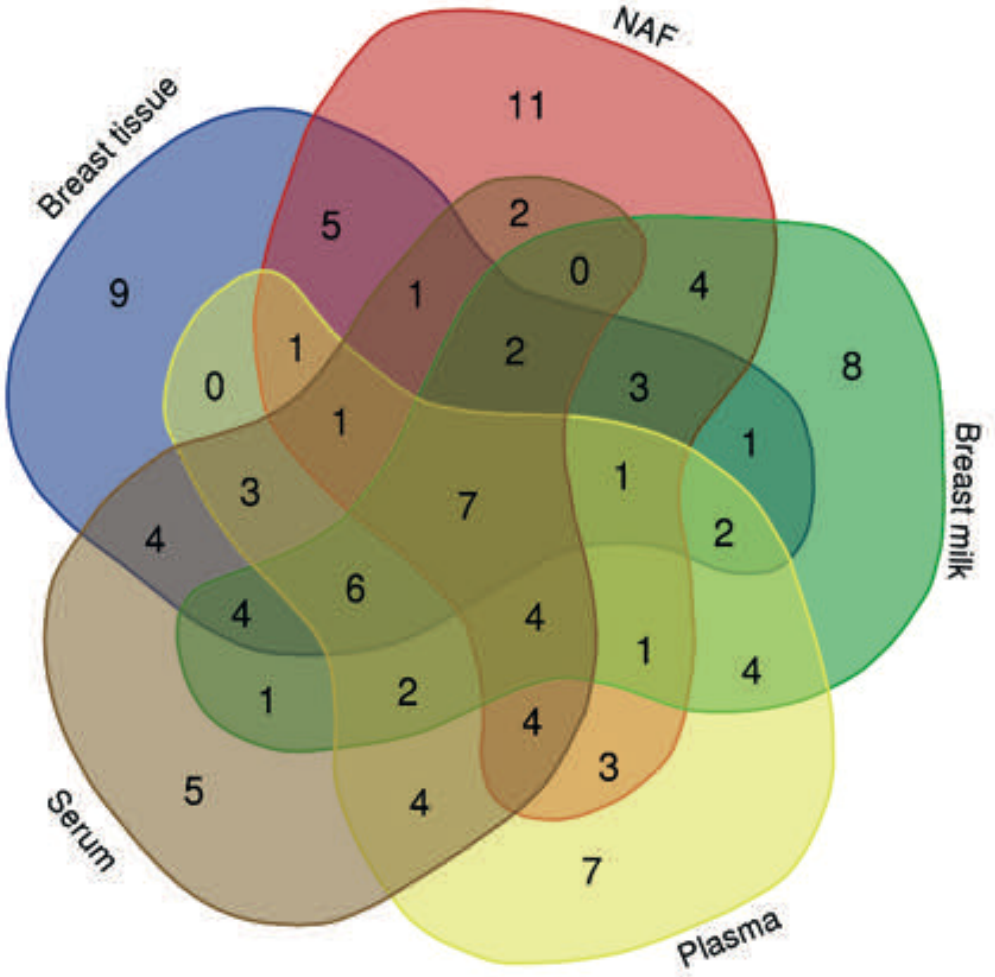

Heatmap
miRNAs
hsa-miR-193b-3p
hsa-miR-27a-3p
hsa-let-7g-5p
hsa-miR-451a
hsa-miR-24-3p
hsa-let-7b-5p
hsa-miR-21-5p
hsa-miR-221-3p
hsa-miR-203a-3p
hsa-miR-205-5p
hsa-miR-92b-3p
hsa-miR-200b-3p
hsa-miR-30b-5p
hsa-miR-99a-5p
hsa-miR-200c-3p
hsa-miR-26b-5p
hsa-miR-29a-3p
hsa-miR-16-5p
hsa-miR-15a-5p
0
2
4
6
8
10
Integrated.Breast.Cancer.Pathway
Integrated.Cancer.Pathway
Pancreatic.adenocarcinoma.pathway
Senescence.and.Autophagy.in.Cancer
Bladder.Cancer
DNA.Damage.Response..only.ATM.dependent.
Breast.cancer.pathway
G1.to.S.cell.cycle.control
Signaling.Pathways.in.Glioblastoma
Focal.Adhesion
PI3K.Akt.Signaling.Pathway

B.


Heatmap
miRNAs
hsa-miR-21-5p
hsa-miR-221-3p
hsa-miR-30b-5p
hsa-miR-92a-3p
hsa-miR-205-5p
hsa-miR-24-3p
hsa-miR-200b-3p
hsa-miR-200c-3p
hsa-miR-92b-3p
hsa-miR-99a-5p
hsa-miR-26b-5p
hsa-miR-15a-5p
hsa-let-7g-5p
hsa-let-7b-5p
hsa-miR-193b-3p
hsa-miR-451a
hsa-miR-27a-3p
hsa-miR-203a-3p
hsa-miR-29a-3p
hsa-miR-16-5p
MicroRNAs.in.cancer
PI3K.Akt.signaling.pathway
Focal.adhesion
Small.cell.lung.cancer
Pathways.in.cancer
Prostate.cancer
Glioma
Melanoma
Hepatitis.B
FoxO.signaling.pathway
Pancreatic.cancer
Chronic.myeloid.leukemia
Bladder.cancer
HTLV.I.infection
0
2
4
6
8

C.

Supplementary Figure S4. Five-way Venn diagram showing the number of overlapping top 50 microRNAs across five sample types: breast tissue, NAF, breast milk, plasma and serum. See Supplementary Table A4F for the lists of microRNAs.



References

1. Backes C, Kehl T, Stockel D, Fehlmann T, Schneider L, Meese E, Lenhof HP, Keller A. miRPathDB: a new dictionary on microRNAs and target pathways. Nucleic Acids Res. 2017;45(D1):D90-D6.

Supplementary Table S6. Overview of the most relevant established mRNA targets and breast cancer related cellular processes of each top 20 NAF miRNA shown in Table 1. Messenger RNAs targeted by six or more top 20 miRNAs are indicated in bold. TAM: tumor associated macrophage; CSC: cancer stem cell; EMT: epithelial to mesenchymal transition. *same cluster: miR 27a~24-2; #same cluster: miR 15a~16-1.

	Tumor suppressor or oncogenic role	Established targets	Processes	References
hsa-miR-205-5p	Tumor suppressor	ERBB2/3, ZEB1/2, VEGFA, E2F1, HMGB1/3, PTEN, MED1, SMAD1/2/4, BCL2/6	EMT; proliferation; stemness; expansion of the progenitor-cell population	(1-8)
hsa-miR-203a-3p	Tumor suppressor	ZEB2, TP63, BMI1, SNAI1/2, SOCS3, SRC, BIRC5, CDK6, SMAD4, VEGFA, MMP10, ABL1	EMT; cell shape; matrix adhesion; migration/ invasion; stemness	(9-13)
hsa-miR-26b-5p	Tumor suppressor	PTGS2, GATA4, EZH2, COL1A2, LARP1, CDK6, CCNE1, TAB1, EPHA2, KPNA2, PGR, PTEN	apoptosis; proliferation	(14-17)
hsa-miR-221-3p	Oncogenic	CDKN1B/c, BCL2L11, DDIT4, KIT, ETS1, ESR1, MDM2, ZEB2, PIK3R1, PTEN, FOXO3, BNIP3, PARP	apoptosis; proliferation	(18, 19)
hsa-let-7b-5p	Tumor suppressor	HMGA1/2, CCND1/2, CDC34, IGF1R, AGO1, PRDM1, CDK6, CDC25A, IGF2BP1/2	migration/invasion	(20, 21)
hsa-miR-27a-3p*	Oncogenic	PHB, FOXO1, SPRY2, ZBTB10, SMAD2/4/5, CDC27, SFRP1, TP53, FBXW7, SP1, EGFR, MET, CCND1	proliferation; apoptosis; EMT; migration/invasion; angiogenesis; TAM polarization	(22-29)
hsa-miR-451a	Tumor suppressor	MIF, ABCB1, CAB39, STAT3, IL6/R, MYC, AKT1, BCL2, MAP3K1, TSC1	proliferation; migration/ invasion; apoptosis; autophagy	(30-33)
hsa-miR-92a-3p	Tumor suppressor	BMPR2, HIPK1, KLF2, ITGA5, TP63, STAT3, PTEN, FBXW7, MAP2K4	migration/invasion	(14, 34)
hsa-miR-16-5p#	Tumor supressor	CCNE1, FGF2, ARL2, BCL2, HMGA1, CDK6, CCND1, VEGFA, RECK, PRDM4, PPM1D, WEE1, CHEK1, BMI1	proliferation; apoptosis; stress response; CSC growth	(35-39)
hsa-let-7g-5p	Tumor suppressor	HMGA2, IGF2BP1, COL1A2, MYC, CDKN2A, BCL2L1, AGO1, THBS1, AKT2, FN1, SMAD2, TGFBR1, FOXC2, BMI1	proliferation; migration/ invasion	(21, 40-42)

	Tumor suppressor or oncogenic role	Established targets	Processes	References
hsa-miR-24-3p*	Oncogenic	CDKN1B, FEN1, CDK4, CCNA2, AURKB, MYC, E2F2, DHFR, HNF4A, SP1, BRCA1, POLD1,H2AFX, CCND1, CDK1	proliferation; apoptosis	(43-46)
hsa-miR-200c-3p	Tumor suppressor	ZEB1/2, TUBB3, BMI1, FN1, RNF2, DUSP1, ZNF217, KRAS, CDK2, SP1, XIAP, BCL2, PTEN, VEGFA	EMT; migration/invasion; stemness	(5, 47-56)
hsa-miR-200b-3p	Tumor suppressor	ZEB1/2, FN1, BMI1, RNF2, ETS1, GATA4, WNT1, CDKN1B, VEGFA, XIAP, BCL2, CREB1, MYB, SP1	proliferation; migration/ invasion; stemness	(5, 56-61)
hsa-miR-99a-5p	Tumor suppressor	MTOR, SMARCA5, IGF1R, FGFR3, AGO2, CAPNS1, HOXA1, CDC25A	EMT; migration/invasion; proliferation; apoptosis	(62-67)
hsa-miR-21-5p	Oncogenic	RASGRP1, CDC25A, BTG2, PDCD4, SERPINB5, BCL2, RPS7, TIMP3, SOX5, SP1, RECK, TGFBR2/3, PTEN, VEGFA	proliferation; apoptosis, EMT; migration/invasion	(68-73)
hsa-miR-92b-3p	Tumor suppressor/ Oncogenic	SLC15A1, DAB2IP, CDKN1C, PTEN, SMAD3/7, PRMT5, RECK, ITGA6, NLK, DKK3, EZH2	proliferation; migration/ invasion; autophagy	(74, 75)
hsa-miR-15a-5p#	Tumor suppressor	BCL2, CCNE1, VEGFA, BMI1, MYB, CCND1, CRKL, MN1, CLCN3, FOXO1, WEE1, CHEK1, YAP1, RECK	proliferation; apoptosis; migration/invasion; EMT	(76-80)
hsa-miR-193b-3p	Tumor suppressor	CCND1, PLAU, MCL1, KRAS, RAD51, ETS1, SMAD3, MAX, NF1, ESR1, PRAP1, MYB	proliferation; migration/ invasion	(81-85)
hsa-mir-30b-5p	Oncogenic	TP53BP2, CCNE2, PDGFRB, TP53, BCL2	proliferation; migration/ invasion; apoptosis	(86, 87)
hsa-mir-29a-3p	Tumor suppressor/ Oncogenic	CDK6, CDC42, SPARC, DNMT3A/B, MCL1, TET1, SUV420H2, BCL2, PTEN, VEGFA, CCND1, AKT2	EMT; proliferation; metastasis	(88-90)

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CHAPTER 6

The changing microRNA landscape by color and cloudiness: a cautionary tale for nipple aspirate fluid biomarker analysis

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Abstract

Purpose

Investigation of nipple aspirate fluid (NAF)-based microRNAs (miRNAs) as a potential screening tool for women at increased risk of developing breast cancer is the scope of our research. While aiming to identify discriminating NAF-miRNAs between women with different mammographic densities, we were confronted with an unexpected confounder: NAF sample appearance. Here, we report and alert for the impact of NAF color and cloudiness on miRNA assessment.

Methods

Seven classes of NAF colors coupled with cloudiness appearance were established. Using 173 NAF samples from 154 healthy women (19 samples were bilaterally collected), the expression of 14 target and 2 candidate endogenous control (EC) miRNAs was investigated using Taqman Advanced miRNA assays to identify significant differential expression patterns between color-cloudiness classes. Inter- and intra-individual variation of miRNA expression was analyzed using the coefficient of variation (CV).

Results

We found that between the seven NAF classes, fold change miRNA expression differences ranged between 2.4 and 19.6 depending on the interrogated miRNA. Clear NAF samples exhibited higher miRNA expression levels compared to cloudy NAF samples with fold change differences ranging between 1.1 and 6.2. Inter-individual and intra-individual miRNA expression was fairly stable (CV < 15%), but nevertheless impacted by NAF sample appearance. Within NAF classes, inter-individual variation was highest for green samples (CV 6-15%) and lowest for bloody samples (CV 2-6%).

Conclusions

Our data indicate that NAF color and cloudiness influence miRNA expression and therefore should be systematically registered using an objective color classification system. Given that sample appearance is an inherent feature of NAF, these variables should be statistically controlled for in multivariate data analyses. This cautionary note and recommendations could be of value beyond the field of NAF-miRNAs, given that variability in sample color and cloudiness is likewise observed in liquid biopsies such as urine, cerebrospinal fluid, and sputum, and could therefore influence the levels of miRNAs and other biomarkers.

Introduction

MicroRNAs (miRNAs) are key regulators in many cellular biological processes. They represent a class of short (~22 nucleotides long) non-coding RNAs that modulate gene expression at the post-transcriptional level (1). The miRNA repository miRBase (release 22.1) currently lists 2,654 mature miRNAs in Homo sapiens (2), and an estimated 30–80% of human genes are influenced by at least one of these miRNAs (3, 4). Cellular miRNAs can be released into biofluids as a result of apoptotic or necrotic cell death or active secretion (5). These circulating, cell-free miRNAs hold great promise as a new class of cancer biomarkers due to their surprisingly high stability in biofluids, correlation with carcinogenesis and disease state, and ease of measurement (5-13). Among the various body biofluids in which miRNAs can be measured, nipple aspirate fluid (NAF) has attracted attention for the early detection and management of breast cancer because it can reflect pathological changes in the breast microenvironment (14-16). NAF is secreted in small amounts by the breast ducts of adult non-lactating women and can be collected by non-invasive vacuum aspiration preceded by oxytocin nasal spray administration (17-19). The use of oxytocin promotes the release of already existing fluid in the milk ducts, thereby yielding sufficient material (on average 10-20 µl) for molecular analysis in the majority of healthy volunteers and patients (14, 15, 17, 20-23). NAF collection causes less discomfort compared to other breast cancer screening modalities (14, 20), and the synchronous acquisition of matched pairs of bilateral NAF samples provides intra-patient control samples for unilateral disease.

The evaluation of NAF-based miRNAs in the context of early detection of breast cancer in women at increased risk of developing breast cancer, such as those with high mammographic density, is the focus of our ongoing investigations. While searching for discriminatory miRNAs between two groups of healthy women differing in mammographic density, we identified NAF sample color and cloudiness as relevant confounders. Although these sample characteristics have previously been described as being associated with lifestyle factors and nutrient composition (24-26), they have never been described as having a notable influence on biomarker discovery. Moreover, interpretation of NAF sample appearance is prone to subjectivity, and hence its reporting lacks consistency. The following color designations were extracted from the literature: pale-yellow, dark yellow, brown, brown-black, brown-cloudy, green-brown, light-green, greenish-clear, olive color, green, green-milky, white, white cloudy, milky, colored, colorless, gray-cloudy, black, clear, cloudy, and opaque (21, 24-29). This highlights the need for a standardized system to allow future comparisons between studies.

To elucidate the influence of the color and cloudiness on miRNA biomarker assessment, we established seven classes of NAF sample appearance and studied their association with miRNA expression levels.

Materials and Methods

Ethics, sample collection and processing

To evaluate the effect of NAF color and cloudiness on miRNA expression levels, 173 NAF samples from 154 women were included (19 samples were bilaterally acquired from the same women) from the DENSE-on biobank (biobank numbers 14-467 and 15-356). 92 of these NAF samples were collected from 82 women at screening age (50-74 years old) with extremely dense breasts according to Volpara imaging software, version 1.5 (Volpara Health Technologies) (30), i.e. with a Volpara density 4 or 'd'; this is the highest of a 4-point radiological classification, which is comparable to a 'd' in the breast density categories of the Breast Imaging, Reporting and Data System (BI-RADS) of the American College of Radiology (31). 81 samples were collected from 72 women ≤ 60 years old undergoing screening with a breast density at the other end of the spectrum, i.e., Volpara density 1 or 'a', which is a result of an almost entirely fatty breast tissue composition. The DENSE-on biobank was set up to gain a better biological understanding of breast density, e.g. why some women have extremely dense breasts and if there are biomarkers for early breast cancer detection in these women. The biobank is an extension of the Dutch nationwide multicenter Dense tissue and Early breast Neoplasm ScrEening (DENSE) trial (NCT01315015 (32-34)), which aims to investigate the additional value of MRI in screening for women with extremely dense breasts.

The studies were approved by the Institutional Review Boards of the participating hospitals, and the UMC Utrecht Biobank Research Ethics Committee. Written informed consent was provided by all participants. Samples were collected between June 2015 and March 2016. The median age of the participants was 55 years (interquartile range (IQR) = 6). Other anthropomorphic characteristics are listed in Supplementary Table S1. All participants were healthy at the time of NAF collection, without abnormalities on recent mammography and/or MRI. NAF samples were collected after nasal oxytocin administration and application of vacuum around the breast by a modified breast pump, as described previously (20, 21). The collected fluid was conserved in a buffer solution (50 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA) and, without centrifugation, immediately snap-frozen at -80°C until analysis.

NAF color and cloudiness categorization into NAF classes

Upon NAF collection, the research nurses assessed and registered NAF appearance on color and cloudiness. Given that 22 different NAF appearances were reported during study visits (Supplementary Table S2), a lumped classification was made based on the most prevalent NAF appearances registered to reduce the number of NAF classes for subsequent statistical analysis. The 173 NAF samples were subdivided into 7 color classes (Supplementary Table S2) coupling colors with cloudiness appearance: 53 clear-colorless samples (31%), 36 bloody samples (including red, orange and pink; 21%), 30 cloudy-white samples (17%), 20 clear-yellow samples (11%), 19 cloudy-yellow samples (11%), 8 green (5%) and 7 brown samples (4%).

RNA extraction and quantification

For all experiments, an AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) was used to extract total RNA according to the manufacturer's protocol. Ten µl NAF was used to evaluate the effect of NAF color and cloudiness on miRNA expression levels. Non-human ath-mir-159 (with a 5' phosphate) was spiked-in at 300 pg by pre-mixing with RLT plus lysis buffer. RNA was eluted in 30 µl RNAse-free water. The concentration of the extracted RNA was measured by Qubit 3.0 (ThermoFisher Scientific, MA, USA) fluorometric high sensitivity quantification. Next, all RNA samples were stored at -80°C until further analysis.

Reverse transcription, pre-amplification and Taqman Advanced miRNA analysis
To study associations between NAF color and cloudiness and miRNA expression levels, the expression of 16 human mature miRNAs was evaluated using individual Taqman Advanced miRNA assays (ThermoFisher Scientific, Catalog number A25576): hsa-miR-19a-3p, hsa-miR-25-3p, hsa-miR-29a-3p, hsa-miR-29b-3p, hsa-miR-125a-5p, hsa-miR-99b-5p, hsa-miR-155-5p, hsa-miR-181a-5p, hsa-miR-186-5p, hsa-miR-187-3p, hsa-miR-222-3p, hsa-miR-324-5p, hsa-miR-339-5p, hsa-miR-361-5p, hsa-miR-425-5p and hsa-miR-660-5p (see Supplementary Materials for assay identification numbers (IDs)). These microRNAs were evaluated because they initially demonstrated potential discriminatory power between women with Volpara 1 and Volpara 4 mammographic densities when no correction for NAF appearance had been performed.

Hsa-miR-99b-5p and hsa-miR-25-3p were used as suitable endogenous control (EC) miRNAs for NAF as they demonstrated the lowest geNorm M-values in previous miRNA profiling experiments using the same platform (low M-values indicate high expression stability) (35). Hsa-miR-99b-5p, with the lowest M-value of the two candidate EC miRNAs, was selected as EC for differential expression analysis of target miRNAs in this study.

According to the manufacturer's instructions, 5 ng total RNA was first poly-A tailed, and after adaptor ligation and reverse transcription, pre-amplified for 14 cycles. The pre-amplification product was subsequently diluted 10x in 0.1x TE buffer pH 8.0. qPCR was performed in duplicate in a 20 µl final volume using a Taqman Fast Advanced Mastermix (ThermoFisher Scientific) on a ViiA7 realtime PCR device. All miRNA amplification plots were visually inspected on curve shape and signal timing. Threshold cycles (CTs), i.e., the cycle at which the fluorescence level reaches a certain value (the threshold), above the threshold of 35 were omitted, as were samples with aberrant spike-in values. Next, CT values were used for calculation of the delta CT (DCT = CT(target miRNA)–CT(endogenous control miRNA)). Subsequently, the commonly used delta delta CT (DDCT) was calculated (2–DDCT, where DDCT = DCT(target sample)-DCT(reference sample)) (36, 37) resulting in relative fold changes in target miRNA expression between color classes.

Statistical analyses

Statistical analyses were performed using IBM SPSS Statistics for Windows version 25.0.0.2 (IBM Corp., Orchard Road Armonk, New York, USA). Variables analyzed in relation to NAF appearance were age (continuous), body mass index (BMI, continuous), breast density (dichotomous) and miRNA expression levels (continuous). Normality of data distribution was evaluated by Kolmogorov-Smirnov test. Data are presented as median with IQR or mean with standard deviation. A p value < 0.05 was considered statistically significant.

MiRNA median CT differences (median CT (target miRNA class 1)-median CT (target miRNA class 2)) and subsequent fold changes (FCs) between NAF classes (clear-colorless, bloody, cloudy-white, clear-yellow, cloudy-yellow, green and brown) were calculated. Kruskall-Wallis test was used to identify miRNAs with significant differential expression (based on DDCT) between NAF classes. Mann-Whitney U test was used to identify miRNAs with significant differential expression between clear and cloudy NAF samples, and between any two NAF classes. The coefficient of variation (CV) was calculated as the ratio of the standard deviation to the mean miRNA expression (CT value) within and across all color classes, as well as intra-individually between left and right breasts. A criterion for intra-individual CV analysis was a minimum detection of 6 out of the 16 miRNAs per sample. Consequently, three NAF pairs were omitted from final intra-individual analysis. CVs below 15% were considered to have acceptable technical reliability (38). Unsupervised hierarchical clustering of NAF samples based on their miRNA expression pattern was performed using the web tool ClustVis (39). Missing values were automatically imputed by ClustVis. GraphPad Prism 8.3 for Windows (San Diego, California USA) was used for graphical visualization of the results.

Results

NAF appearance classes are associated with age

We found that NAF appearance classes were significantly associated with age (p = 0.001 between 7 NAF classes). Bloody NAF samples were more frequently observed in older women, especially compared to green NAF (p = 0.002) and cloudy-white NAF (p = 0.014). Overall, the order of most commonly observed NAF classes, from older to younger age (ranging between 50 and 74 years old), was as follows: bloody, clear-colorless, clear-yellow, cloudy-yellow, cloudy-white, brown and green samples (Figure 1). No significant association between NAF appearance and BMI or breast density was noted.

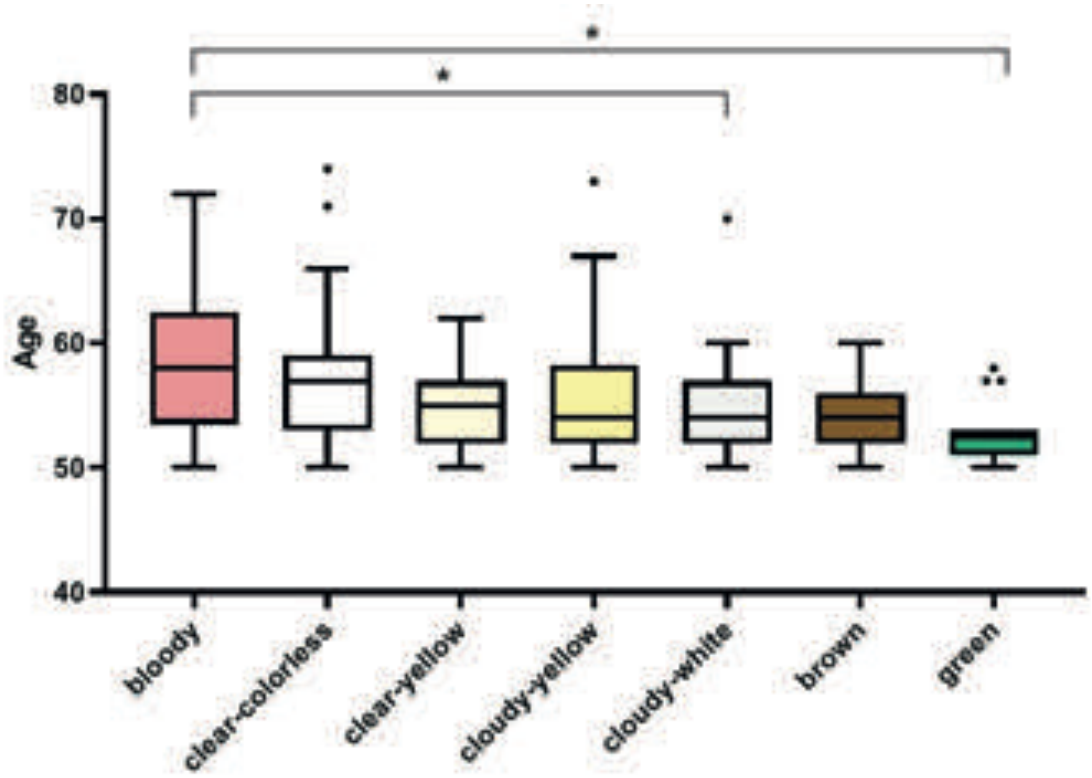

Figure 1. Association between nipple aspirate fluid classes and age. The most commonly occurring NAF appearance classes from older to younger age were bloody, clear-colorless, clear-yellow, cloudy-yellow, cloudy-white, brown and green. Boxes extend from the 25th to 75th percentiles (IQR). The horizontal line in the box is plotted at the median. Tukey method was used to indicate outliers (25/75th percentile ± 1.5* interquartile range). Significant differences are indicated with *.

RNA concentration varies significantly with NAF color and cloudiness

Next, we found that the total RNA concentration significantly varied per NAF appearance class (p < 0.0001), with highest concentrations observed in bloody (n = 48; 19.9 ng/µl, IQR = 43.29) and brown (n = 14; 29.5 ng/µl, IQR = 56.79) NAF

samples. This was followed by clear-yellow NAF (n = 19; 9.97 ng/µl, IQR = 19.69), clear-colorless NAF (n = 48; 6.35 ng/µl, IQR = 8.71), green NAF (n = 12; 4.02 ng/µl, IQR = 12.44), cloudy-white (n = 15; 3.01 ng/µl, IQR = 5.70) and cloudy-yellow (n = 17; 3 ng/µl, IQR = 2.99) NAF (Supplementary Figure S1). The RNA concentration was significantly higher when it was extracted from clear compared to cloudy NAF samples ($p < 0.0001$). Median concentrations were 12 ng/µl (IQR = 26.9) and 3.85 ng/µl (IQR = 5.79) in clear NAF (n = 111) and cloudy NAF (n = 57), respectively.

MiRNA expression varies significantly with NAF color and cloudiness

Of the 15 interrogated target miRNAs, all but one (hsa-miR-187-3p) were significantly differentially expressed between NAF colors (all miRNAs $p < 0.0001$, except hsa-miR-125-5p with $p = 0.008$) and NAF cloudiness (all miRNAs $p < 0.0001$ except hsa-miR-125-5p with $p = 0.036$). In general, clear NAF samples showed higher miRNA expression levels compared to cloudy NAF samples with median CT (expression) differences between cloudy and clear samples ranging from 0.16 for hsa-miR-125a-5p (FC = 1.12) to 2.63 for hsa-miR-222-3p (FC = 6.19) (Supplementary Table S3A). Interestingly, we found that hsa-miR-155-5p was the only miRNA with a higher expression in cloudy versus clear NAF (FC = 3.81). Between the seven NAF classes, median CT differences ranged from 1.26 for hsa-miR-187-3p (FC = 2.39) to 4.29 for hsa-miR-222-3p (FC = 19.64) despite identical RNA input based on Qubit total RNA measurements. Combinatorial analyses among the seven NAF classes were also performed, leading to a total of 21 class comparisons. In general, cloudy-white, cloudy-yellow and green NAF showed lower miRNA expression levels compared to red, brown and clear-colorless NAF. In contrast, hsa-miR-155-5p showed a higher expression in green samples compared to clear-colorless and clear-yellow samples (Supplementary Table S3B). In almost every NAF class comparison, at least one miRNA showed a significantly different pattern. Exceptions were green, cloudy-white and cloudy-yellow NAF, exhibiting no significantly different miRNAs between NAF classes. Brown and clear-yellow NAF were also highly similar for all interrogated miRNAs. Greatest differences were observed between bloody and green NAF, between bloody and cloudy-yellow or cloudy-white NAF and between clear-colorless and cloudy-yellow or cloudy-white NAF, with at least 13/15 interrogated miRNAs showing significantly different expression levels. Accordingly, unsupervised hierarchical clustering of NAF samples based on their 15-miRNA expression pattern resulted in two major clusters, one containing mainly cloudy NAF samples (white, yellow, green or brown), and the other containing mainly clear NAF samples (Figure 2). Within the latter cluster, clear-colorless and bloody samples were separated from clear-yellow and brown samples.

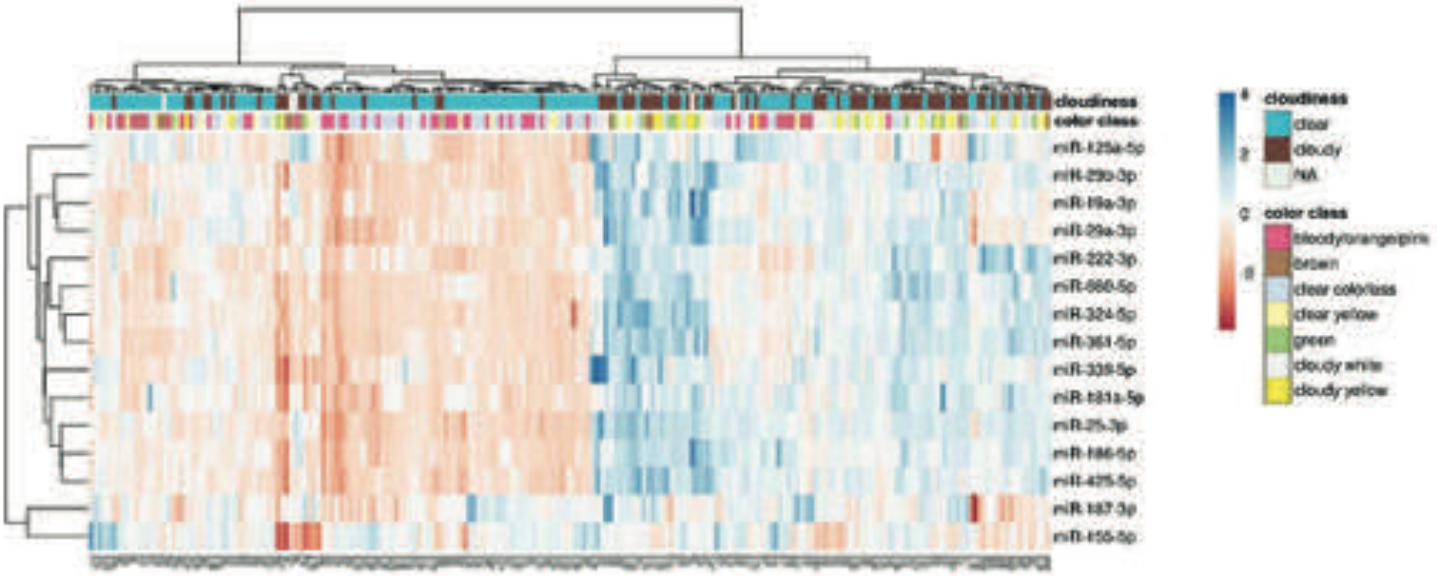

Figure 2. Unsupervised hierarchical clustering of 211 nipple aspirate fluid samples based on the expression pattern (delta delta CT) of 15 miRNAs. Rows are centered and unit variance scaling is applied to rows. Imputation was used for missing value estimation. Both rows and columns are clustered using Euclidean distance and Ward linkage.

Candidate endogenous control miRNA choice significantly impacts variability between NAF color and cloudiness classes

Since miRNA expression is influenced significantly by NAF color, choosing the right endogenous miRNA may prove to be difficult. Figure 3 illustrates the effect of candidate endogenous control choice on between-sample variability in the context of color. Overall, variability between bloody and clear-colorless samples, and between cloudy-white and cloudy-yellow samples was acceptable, regardless of the chosen endogenous control. Variability was, however, much larger between other NAF classes (e.g., bloody or clear-colorless versus cloudy-white). For hsa-miR-324-5p, for instance, a miRNA with significantly lower CT values (higher non-normalized expression) in bloody and clear-colorless versus cloudy-white and cloudy-yellow NAF, after normalization with hsa-miR-99b-5p (DDCT) showed median FCs between 11.6 and 7.0 depending on the comparison (red and colorless vs. cloudy-white and cloudy-yellow). However, when normalized against hsa-miR-25-3p, showing an effect of similar magnitude as observed for hsa-miR-324-5p, median FCs varied between 1.27 and 2.0. As hsa-miR-155-5p seems to show an opposite effect compared to the other interrogated miRNAs (higher CT values and thus lower expression in bloody and colorless samples), a suitable endogenous control miRNA for proper normalization is yet to be determined.

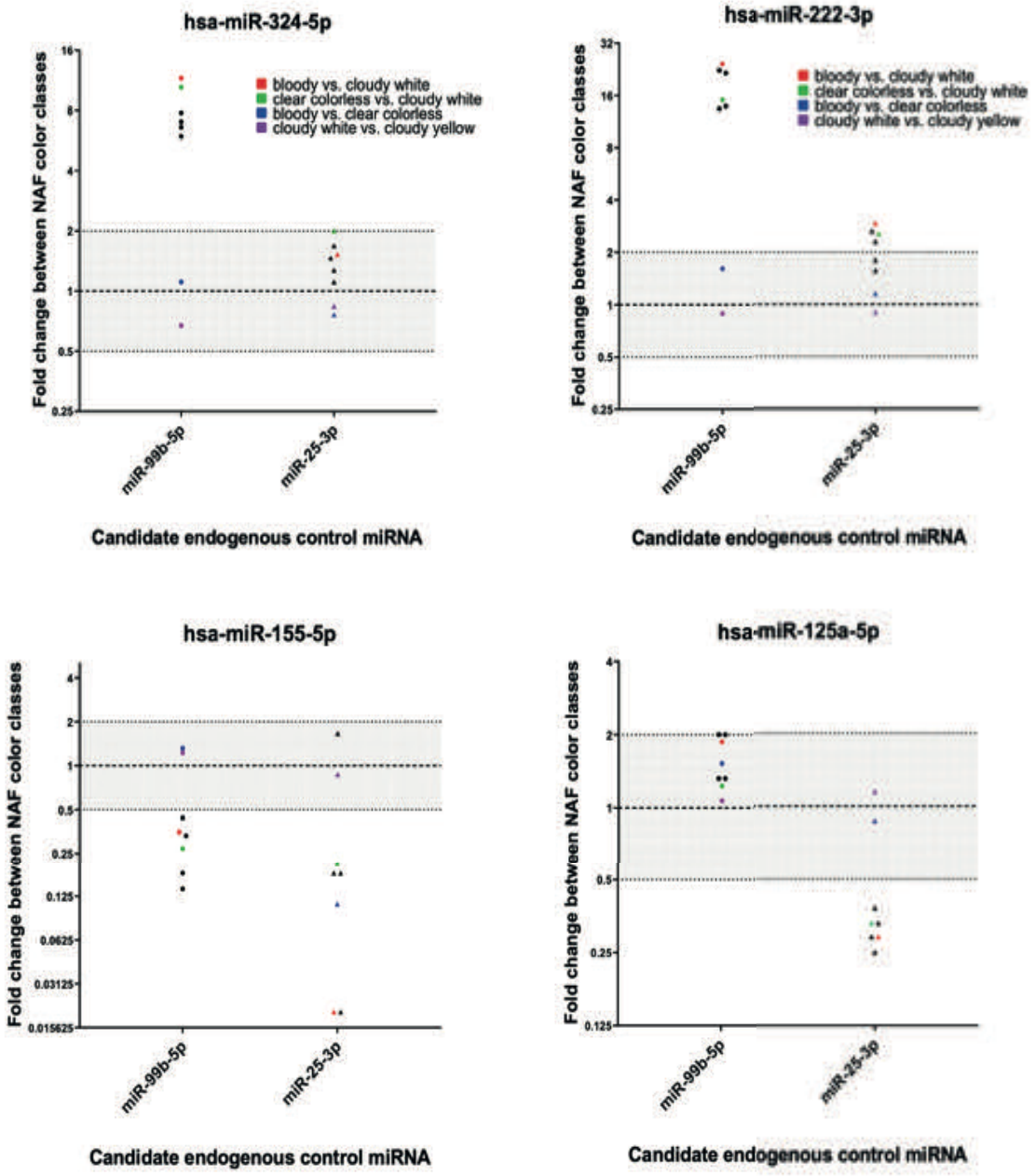

Figure 3. The effect of endogenous control miRNA choice on variability between nipple aspirate fluid (NAF) color-cloudiness classes. Fold changes between NAF color classes (derived from median DDCT) are plotted for 4 target miRNAs (hsa-miR-324-5p, hsa-miR-222-3p, hsa-miR-155-5p and hsa-miR-125a-5p) based on two endogenous controls (hsa-miR-99b-5p and hsa-miR-25-3p). Fold changes between 0.5 and 2 are shaded grey. The upper two target miRNAs show less variability between color classes when using hsa-miR-25-3p as endogenous control, the lower two miRNAs show less variability when using hsa-miR-99b-5p as endogenous control. Overall, variability between bloody and clear-colorless samples (blue), and between cloudy-white and cloudy-yellow samples (purple) was acceptable, regardless of the chosen endogenous control. Variability was larger between other color classes (e.g., colors red = bloody versus cloudy-white, and green = clear-colorless versus cloudy-white).

Inter-individual and intra-individual microRNA expression variation in the context of NAF color

Inter-individual coefficients of variation (CV) across all NAF classes ranged between 5% for hsa-miR-187-5p and hsa-miR-99b-5p, and 11% for hsa-miR-222-3p, hsa-miR-324-5p and hsa-miR-29b-3p. Within NAF classes, inter-individual variation was largest for green samples (CV 6-15%) and smallest for bloody samples (CV 2-6%). Intra-individual differences between left and right breast, based on 16 pairs of samples, were of similar magnitude, with smallest CV for hsa-miR-187-5p and hsa-miR-19a-3p (both 1-5%), and largest CV for hsa-miR-222-3p (0.3-19%). Of the 16 pairs of samples, 7 pairs had the same NAF appearance while 9 pairs had different NAF appearances. Inter-breast differences were generally larger with differing NAF color. For example, hsa-miR-361-5p and hsa-miR-425-5p tended to show notable inter-breast differences with differing NAF color ($p = 0.053$ and $p = 0.085$, respectively) while no significant difference was observed when the NAF color class was identical between breasts ($p = 0.341$ and $p = 0.384$, respectively). Figure 4 depicts a heatmap of CV percentages for NAF miRNA expression levels within and between women. In general, using hsa-miR-99b-5p as endogenous control, the maximal absolute DDCT difference between left and right breasts varied between 2.1 (hsa-miR-339-5p) and 6.1 (hsa-miR-222-3p). Maximal absolute DDCT differences between individual women varied between 5.5 (hsa-miR-125a-5p) and 14.3 (hsa-miR-155-5p). Using hsa-miR-25-3p as endogenous control, the maximal absolute DDCT difference between left and right breasts varied between 1.3 (hsa-miR-324-5p) and 5.0 (hsa-miR-222-3p). Maximal absolute inter-individual DDCT differences varied between 4.6 (hsa-miR-186-5p) and 12.5 (hsa-miR-155-5p).

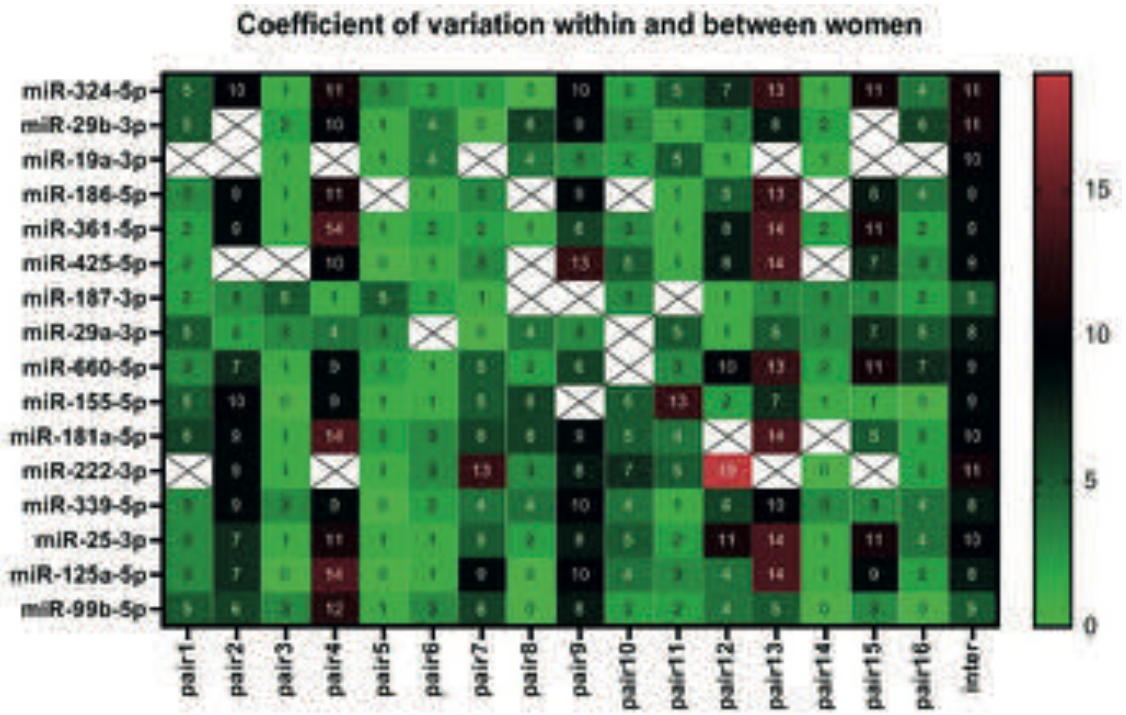

Figure 4. Heatmap of coefficient of variation (CV) for nipple aspirate fluid miRNA expression within and between women. CV was based on mean CT values and standard deviations determined for 16 miRNAs in 135 women (inter-individual variation; 1 breast) and an additional 16 women (intra-individual variation; left and right breast pairs). Samples with reliable measurements for at least 6 of the 16 interrogated miRNAs (15 miRNAs and EC hsa-mir-99b-5p) were selected for this figure. Crossed boxes are missing values for the miRNAs.

Discussion

MicroRNAs are considered promising disease biomarkers given their renowned stability in liquid biopsies and their expression levels reflecting subtle changes in pathological state (5-11). Before they can make the step from bench to bedside, their longitudinal, intra-subject and inter-subject stability should be explored in independent cohorts. Alongside, factors that may cause intrinsic noise in miRNA expression, such as study subject characteristics, pre-analytic procedures, or sample characteristics must be identified and controlled for. Many of such factors have been identified in blood samples (40-42), but have not yet been explored in NAF. NAF can present itself with a variety of colors in combination with a cloudy or clear appearance. Even though liquid sample appearance is quite relevant for clinical assessment (e.g., urine, sputum, drain fluids, or cerebrospinal fluid), such a variable is scarcely reported in the evaluation of new biomarkers in liquid biopsies (43-52) and has seldom been investigated with the purpose of evaluating its intrinsic influence on (mi)RNA levels. Here, we present the association of NAF sample appearance with individual characteristics and evaluate its effect on intra-individual and inter-individual miRNA expression variability and normalization.

We found that NAF color-cloudiness is associated with woman's age. Overall, red, clear-colorless and clear-yellow NAF were found to be more common in older women. The only other study that compared NAF color-cloudiness and age showed that older age increased the chance of lighter NAF colors (26) which is in line with our findings. However, that study used other color classes and a broader age range (20-70 years compared to 50-74 years in our study). Furthermore, we show that NAF color and cloudiness significantly influence total RNA concentrations and miRNA expression levels. A more specific analysis stratifying NAF for color and cloudiness showed that the effect of the former seems to be somewhat secondary to the effect of the latter. Unsupervised cluster analysis clearly separated cloudy from clear NAF samples based on miRNA expression patterns, but subsequent sub-clustering into NAF colors was less distinct, except for the highly similar clear-colorless and bloody NAF specimens.

Intra-individual and inter-individual miRNA expression variability were overall below the CV threshold of 15%, thereby showing acceptable miRNA stability in NAF samples within and among study subjects. As expected, variability of miRNA expression levels was greater inter-individually than intra-individually. Specifically for intra-individual variability, a sub-analysis showed that this was somewhat greater between samples from different color categories compared to those of the same color category. Sample appearance not only influenced inter-individual and intra-individual miRNA expression variation, but also posed a problem for normalization, as candidate endogenous control miRNAs might

not vary with cloudiness and color to the same extent as target miRNAs. We therefore recommend using a combination of endogenous control miRNAs and to correct for sample characteristics in multivariable analysis.

Identifying the underlying cause of these different NAF colors in healthy women may explain our findings. We hypothesize that several potential factors may contribute to NAF color, such as diverse cellular (53) or bacterial (54) compositions in NAF samples, nutrients, and proteins (55-57), medications (58-61), or food intake (61-67). However, studies addressing the association between these possible factors and color classes are limited. Only two studies by Petrakis et al. more than three decades ago reported an association between NAF colors and nutrients (24, 25). In those studies, NAF samples of darker colors (dark yellow, brown, green, and black) were shown to have a higher concentration of lipids, cholesterol, estrogens, NA+ and K+, and a lower lactose concentration compared to NAF samples of lighter colors. A comparison between our data and those of Petrakis et al. is hampered by the different color classes used and the fact that we did not obtain the nutrient information studied by Petrakis et al. for our cohorts.

A more recent and meticulous study on NAF composition per sample color is lacking. We attempted to identify NAF cell types per NAF class using fluorescence-activated cell sorting (FACS). However, this was hampered by sample viscosity, sparse volume, and the presence or very limited numbers of cells in the NAF samples, a finding also reported by others (62, 68-74). Single cell sequencing, a technique that allows analyzing samples with a small number of cells, may help decompose the cellular composition of NAF per color class, but this technique still needs to be tested in this context.

Cellular composition is important, as several studies have shown that blood cells, especially red blood cells, white blood cells, and platelets, are major contributors to cell-free miRNAs (75-79). This has resulted in the introduction of a routine quality control step for serum and plasma miRNA analysis (80, 81), consisting of calculating the ratio of hsa-miR-451a (enriched in red blood cells) to hsa-miR-23a-3p (not affected by hemolysis). Since bloody was one of our NAF classes, we hypothesized that those samples would contain more red blood cells, in line with the fact that these samples have the highest RNA concentration and a high miRNA expression. However, we found that the suggested ratio for serum and plasma could not be extrapolated to NAF due to a different physiological expression of both miRNAs in this particular biofluid. NAF samples that were visually red, orange or pink did not show CT differences > 5 (indicating hemolysis according to the above-mentioned ratio) but rather < 0, with no clear differences compared to non-red samples (data not shown). Another method for the identification of red blood cell contamination is measuring oxy-hemoglobin

absorbance by spectrophotometry (76, 82). Again, the use of this technique was hampered by the viscosity of the NAF sample and the cloudiness.

The analysis of a restricted number of miRNAs, namely 15 or 5 miRNAs, can be considered a limitation of the present study. An investigation of a broader range of miRNAs using a non-targeted or multi-targeted approach, such as profiling or sequencing, may allow the identification of miRNAs most susceptible to NAF appearance. Another limitation of the present study is that color and cloudiness are subjective variables. Future research with a standardized, objectified color scale, such as the international RAL ("ReichsAusschuss für Lieferbedingungen" in German) (83) or Pantone (84) systems for color categories, should be performed to allow uniformity of data reporting and, hence, data comparison between future studies. As an alternative to wallpaper matching of NAF colors with RAL colors, image analysis techniques or, even better, artificial intelligence techniques could be employed. However, the latter approach requires large sample sizes to properly train convolutional neural networks. As a consequence of the accidental findings reported here, we have already started implementing the RAL system in our NAF studies (Supplementary Figure S2) and a study investigating discriminatory miRNAs between density classes, correcting for NAF color classes.

Conclusions

In conclusion, we show that NAF sample color and cloudiness may serve as relevant variables that should be systematically registered by adopting an objective color classification system such as RAL, and that may be taken into consideration in biomarker analyses. We recommend using a combination of endogenous control miRNAs and correcting for sample characteristics in statistical multivariate analyzes. Our cautionary note and recommendations could be of value beyond the field of NAF-miRNAs, given that sample color variability is also seen in other liquid biopsies such as urine, cerebrospinal fluid, and sputum, and could influence miRNA and other biomarker levels, thereby hampering biomarker discovery and validation.

Supplementary Materials

Supplementary Figure S1. Association between nipple aspirate fluid appearance classes and total RNA concentration.
Supplementary Figure S2. Selection of RAL colors to register upon nipple aspirate fluid collection.
Supplementary Materials*. Assay identification numbers (IDs) of the human mature microRNA used.
Supplementary Table S1. Anthropomorphic characteristics of the study subjects from whom nipple aspirate fluid (NAF) samples were used to evaluate the effect of NAF color and cloudiness on miRNA expression levels, including intra-individual and inter-individual variation.
Supplementary Table S2. *Frequencies of registered NAF appearances in the samples from the DENSE-on cohorts.*
Supplementary Table S3*. *A: Descriptives per cloudiness, B: Descriptives per color class.*

**Not shown in this thesis due to the extent of the data, but available online at: https://link.springer.com/article/10.1007/s13402-021-00641-w#additional-information*

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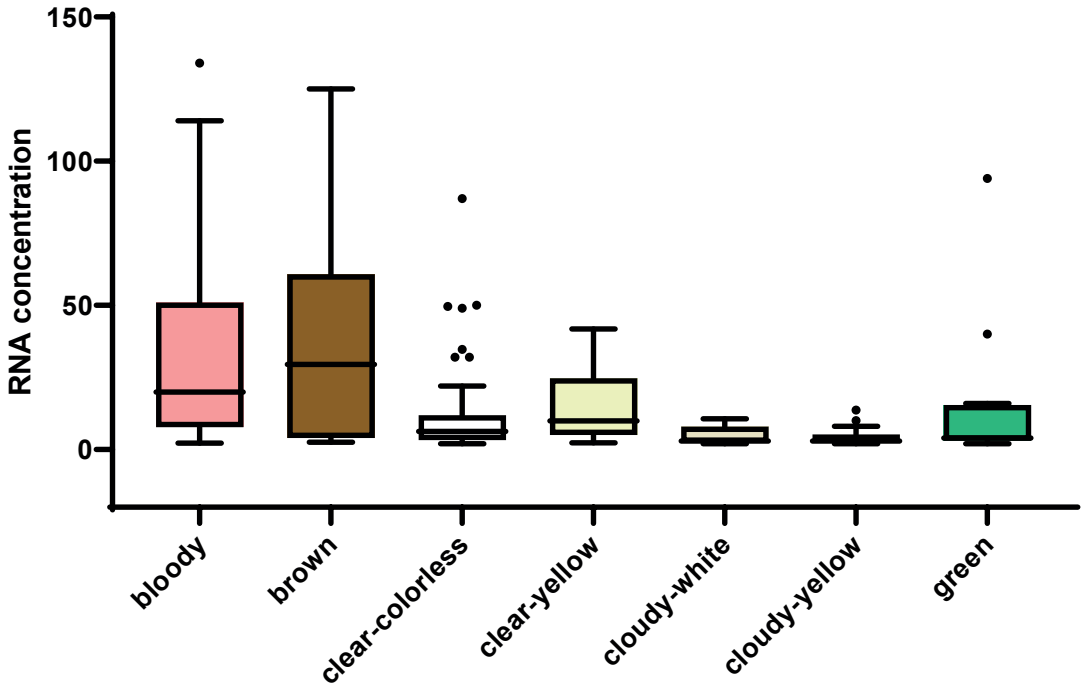
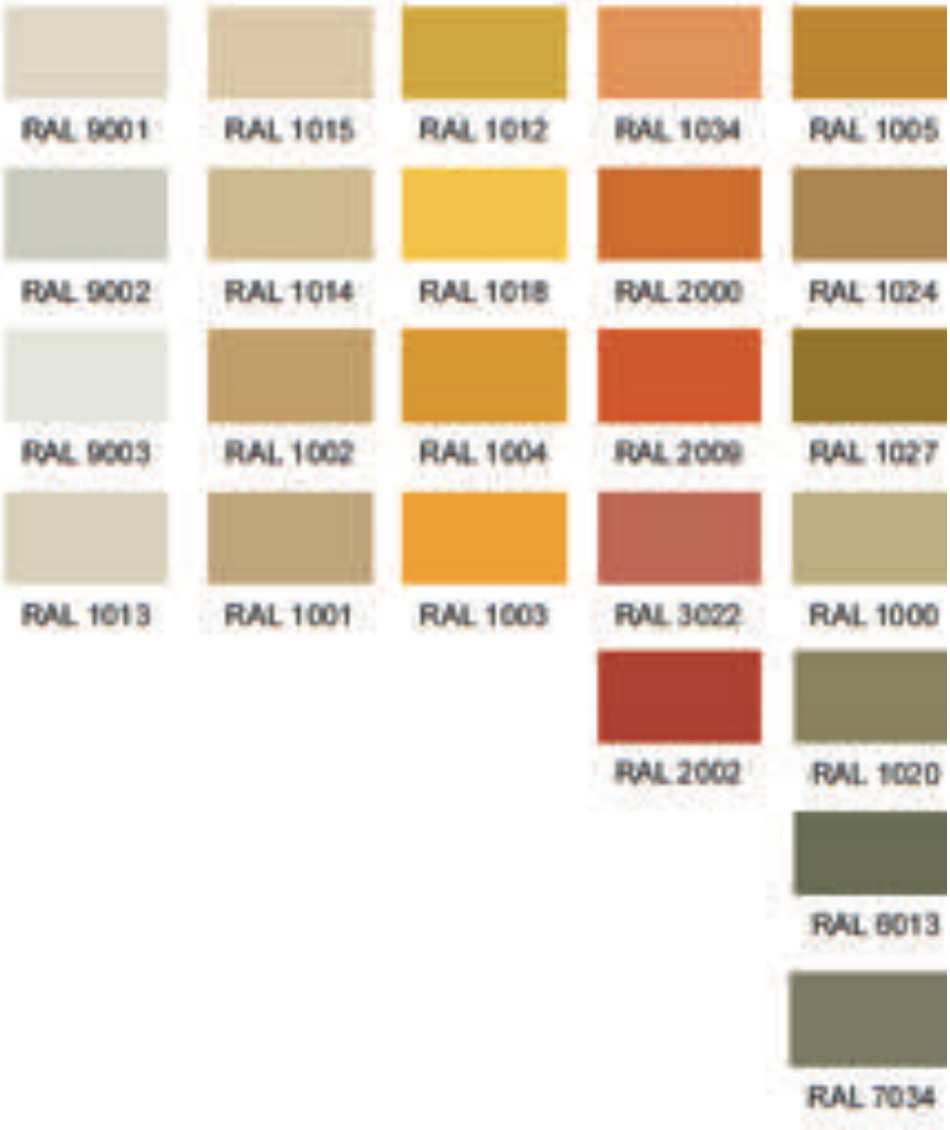
Supplementary Figure S1. Association between nipple aspirate fluid appearance classes and total RNA concentration.



Supplementary Figure S2. Selection of RAL colors to register upon nipple aspirate fluid collection.



Supplementary Table S1. Anthropomorphic characteristics of the study subjects from whom nipple aspirate fluid (NAF) samples were used to evaluate the effect of NAF color and cloudiness on miRNA expression levels, including intra-individual and inter-individual variation.

Characteristics	**n (%) or median (quartile 1 – quartile 3 (Q1-Q3))**
DENSE-on cohort	154
Age	55 (52-58)
BMI	24.7 (21.6-29.1)[a]
Mammographic density	
Volpara 1 or 'a'	73 (46.8%)
Volpara 4 or 'd'	83 (53.2%)
Age at menarche	13 (12-14)[b]
Parous	129 (87.2%)[c]
Age at first live birth	
<25	29 (22.5%)
25-29	57 (44.2%)
≥ 30	43 (33.3%)
Postmenopausal	59 (39.6%)[d]
First degree relative with breast cancer	24 (19.9%)[e]

a: 10 missings, b: 8 missings, c: 6 missings, d: 5 missings, e: 33 missings. NA: not applicable, LTR: lifetime risk. Valid percentages are shown i.e. without missings and not applicable data.

Supplementary Table S2. Frequencies of registered NAF appearances in the samples from the DENSE-on cohorts.

NAF color classes	Registered NAF color and cloudiness	Frequencies (n)
Clear-colorless	clear colorless	53
Bloody	clear orange	14
	clear pink	11
	clear bloody	2
	clear white/orange	1
	clear yellow/orange	1
	cloudy pink	4
	cloudy white/pink	1
	cloudy bloody	1
	cloudy orange	1
Cloudy-white	cloudy white	30
Clear-yellow	clear yellow	20
Cloudy-yellow	cloudy white/yellow	4
	cloudy yellow	15
Green	clear green	1
	clear green/blue	1
	cloudy green	4
	cloudy green/blue	1
	cloudy yellow/green	1
Brown	cloudy brown	4
	cloudy brown/yellow	1
	cloudy brown/white	2

SUMMARY AND DISCUSSION

CHAPTER 7

Summary of main research questions and conclusions

Summarizing discussion

Future projects

Perspectives on the future of liquid biopsy-based biomarkers for early detection of breast cancer

Summary of main research questions and conclusions

Chapter	Main study questions and conclusions
2	**What is known about NAF?** NAF is an intraductal mammary fluid, which can be collected non-invasively, safely, and repeatedly in the great majority of women for the purpose of investigating biomarkers for breast cancer detection or risk. **How could NAF (or other liquid biopsies) be used in the breast cancer diagnostic process?** The possible applications of a liquid biopsy test in the breast cancer detection pathway include a triage, add-on, or replacement test.
3	**What are the lessons learned from performing the NAF study, with focus on the high-risk cohort?** In this chapter we report our first-hand experience of conducting a prolonged, longitudinal, multicenter biomarker study, organized in 11 “lessons learned”. These lessons incorporate the following topics: awareness of the study phases and the need of a large cohort, funding challenges, participant inclusion details, logistics, sample processing, timing, storage and handling, the flexibility of adjusting the biomarker of choice and finally the particulars of the nested case–control analysis.
4	**How do women tolerate nipple fluid aspiration and blood draws?** Discomfort for collection of NAF and blood samples is significantly lower than for current imaging methods for detection and diagnosis of breast cancer, namely mammography, and breast MRI.
5	**Is it feasible to detect microRNAs in NAF?** For the first time, we showed that it is possible to detect microRNAs in NAF using RT-qPCR. **Which microRNAs can be expected in healthy controls?** A list of the 50 most highly expressed microRNAs detected in healthy samples of NAF, breast tissue, breast milk, plasma and serum is presented in this chapter. **Do microRNAs in healthy breast-specific samples overlap more than in circulating samples?** Breast-specific sources (breast tissue, breast milk and NAF) did not mutually overlap more than with systemic sources (plasma and serum). **Do the microRNAs found in NAF play a role in breast cancer?** Many of the microRNAs found in NAF were annotated in breast cancer pathways. As such, NAF microRNA assessment could be a valuable tool in breast tumor diagnostics.
6	**Does NAF sample color and cloudiness influence microRNA expression?** We found that NAF sample color and cloudiness can influence microRNA expression and, as such, should be systematically registered and taken into consideration in biomarker analyses and choice of endogenous control.

Summarizing discussion

Detection of disease in its early stage is imperative to achieve the best outcome for those affected. Since breast cancer lacks effective non-mutilating preventive measures, being able to intervene at the first signs of carcinogenesis will lead to improved outcomes, including a better quality of life. This need for early detection requires the most sensitive and specific screening methods that are easily applicable in routine care. Of all breast cancers diagnosed in the Netherlands today, only around 40% are discovered by image-based population screening, despite a high adherence rate of women to the screening, illustrating the need for additional or improved screening methods. One such method could be the detection of tumor-associated biomarkers in liquid biopsies. In recent years, the value of biomarker-based liquid biopsy screening has been evaluated in various malignancies. The ability to use biomarkers as an early warning sign for breast cancer development would represent an opportunity to minimize or even avoid the disease burden of this highly prevalent malignancy.

With this in mind, we set out to analyze the potential of tumor-associated biomarkers in nipple aspiration fluid (NAF). To do so, we established three cohorts that together form the basis of our NAF research. A cohort of healthy volunteers was established to provide us with data on physiological NAF and a second cohort of patients diagnosed with primary invasive breast cancer as a source of NAF material. This second cohort not only provides us data on NAF of the breast with malignancy but also allows for a comparison between the affected and contralateral breast. Combining data from both cohorts will help identify a biomarker pattern that signals tumor development. The third cohort is our target cohort, consisting of women at high risk of developing breast cancer, including *BRCA1/2* germline mutation carriers, of whom we have annual NAF samples for a period of up to 10 years. NAF of women in this cohort who developed breast cancer will be analyzed for biomarker patterns years before image-based tumor detection to hopefully provide us with a biomarker-signature of early carcinogenesis. Blood was drawn in parallel with NAF and will also be available for analysis and comparison.

In the current thesis, the initial work is presented for the detection of breast cancer at its earliest development using NAF for the discovery of biomarkers (Figure 1).

The first part of this thesis (**Chapters 2, 3, and 4**) provides background information on NAF research, summarizes the practical aspects of creating the biobank, and describes the NAF collection process from the perspective of participants.

In **Chapter 2**, an introduction to NAF research is provided in the form of a narrative review. It describes NAF as a physiological fluid in the ductal tree of the breast that, because of its proximity to the tissue in which breast cancer originates, is an interesting potential source for biomarkers of breast disease. In addition to common notions in the field of NAF and the hurdles of NAF research, we address possible future roles of NAF samples and their molecular material in the context of breast cancer detection in clinical practice. These could comprise a tool for the detection of breast carcinogenesis in its earliest stage (before a tumor mass can be seen by imaging), or as a supporting diagnostic tool for imaging, such as when imaging is less reliable (to rule out false positives from imaging) or when imaging is not advisable (such as during pregnancy and breastfeeding). The eventual application of a NAF test will depend on the accuracy of the biomarkers for disease detection and the characteristics of the cohorts used in clinical studies to test the NAF samples. In addition, participation of medical decision-making experts and patient advocate groups should be involved to discuss translational applications.

More than 200 articles have been published on NAF, ranging from studies investigating biomarkers for breast cancer or evaluating the sensitivity of NAF cytology for breast cancer diagnosis, to interventional studies trying to find the effect of food components or topical agents on the composition of the NAF yield. Like other NAF researchers, we were interested in these samples because of their proximity to the tissue in which breast cancer originates. Due to the upsurge in research on microRNAs (microRNAs) as promising signatures for disease in other biofluids, together with our own pilot data in NAF samples, we set out to investigate whether we could find a microRNA signature in NAF for breast cancer.

Conducting a clinical study in which the three cohorts are included brought to light some day-to-day pitfalls, but also the experience on how to manage these. Specifically for the high-risk cohort, these hurdles are discussed in **Chapter 3** together with recommendations, broken down into eleven “lessons learned.” These cover a wide range of practical aspects, ranging from the setup of the research team, the challenges in acquiring funding, the importance of communication with team members and study subjects, and the initial aspects to consider in the laboratory and data analysis. The strength of this chapter is that the pitfalls described were encountered by two independent research groups that conducted similar trials, namely long-term studies with cohorts of women at high risk of breast cancer.

Many of the lessons learned are also applicable to our two other cohorts that run for a shorter period of time (the healthy cohort and the breast cancer cohort), particularly the study phases, logistics, sample processing, biobanking, and sample handling at time of the analysis. The greatest challenge in these

two cohorts proved to be obtaining the intended total number of inclusions. For healthy volunteers, collaboration between the study team and general practitioners, blood bank and population screening managers in the region proved to be essential, leading to a maximum inclusion rate of 120 women in the year 2019. For comparison, in the breast cancer cohort, it took 4.5 years (2017-2021) to reach a total of 140 inclusions in the study, with a maximum of 45 inclusions in the year 2019. Essential for increased inclusion rate in the breast cancer cohort were adding non-academic hospitals as collaborating centers, being present at multi-disciplinary meetings, sending timely reminders to the medical professionals, and keeping good contact with all team members in the side centers. Having more side centers and more personnel able to perform the study procedures would have increased the number of included women at a faster pace. By adapting such a strategy, the initial costs may seem higher, but a faster completion of the study would probably make it more cost-effective. However, the inclusion of every side center necessitates a new assessment by the local Internal Review Boards, which can be a bureaucratic and slow process (1-3). Finally, the timing of the NAF procedure in the breast cancer cohort, that is, shortly after diagnosis and before primary treatment, makes inclusion challenging as women hardly have had the time to process the diagnosis, let alone consider participation in the study. Women who participated in the study were enthusiastic about its aims. Some of these women even gave the healthy control recruitment flyer to friends to encourage them to participate in the healthy cohort as well.

While in **Chapter 3** we provide the researchers' perspective on conducting the study, in **Chapter 4** we describe how participating women experienced the nipple fluid aspiration (NFA) procedure. Our data showed that women experienced significantly less discomfort during the NFA than during mammography and breast MRI. These data corroborate earlier data in previous NAF cohorts of healthy and high-risk women (4-6) and suggest that women would participate in and adhere to a NAF-based screening/testing tool.

In the second part of this thesis (**Chapters 5 and 6**), we focused on the microRNA in NAF samples. To assess the value of NAF for breast cancer diagnosis and risk prediction, investigations into epigenetic changes (hypermethylation of tumor suppressor genes) and cytological changes upon carcinogenesis were carried out. A study in our group showed that significant differences in methylation levels were found between NAF from cancerous breasts and NAF from healthy controls, but these were not large enough to warrant further studies to work toward a clinically useful test (7, 8). The diagnostic accuracy of nipple smear cytology was recently evaluated in a systematic meta-analysis of 19 studies and showed that cytological atypia in NAF has a poor (pooled) sensitivity (9). As such, epigenetic

changes and cytological changes have not been pursued further. MicroRNAs are recent, upcoming biomarkers for oncological disease, as microRNAs can influence the output of many oncogenes and tumor suppressor genes. These biomarkers have been implicated as critical regulators of carcinogenesis (10) and have been shown to be easily measured in body fluids (11), but have not been tested in NAF yet. As such, our objective was to investigate microRNAs in NAF.

In **Chapter 5**, we present a list of microRNAs that physiologically have the highest expression in NAF samples of healthy women. This was achieved by using an RT-qPCR profiling technique, demonstrating its feasibility for the first time. The assembled microRNA list can be seen as a physiological reference point that can be used for comparison with a breast cancer setting. This chapter originated from the notion that reporting on the normal expression pattern of microRNAs in healthy samples is often omitted. This became clear when performing the literature search to put together lists of the most abundantly expressed microRNAs using high-throughput-based techniques in healthy breast tissue, breast milk, plasma, and serum: only 18 articles had healthy microRNA data available, even though thousands of case-control studies have been published. Furthermore, it was also clear that many studies did not register the anthropomorphic characteristics of healthy study subjects. In particular, breast density, the second highest risk factor for developing breast cancer (12), is one of the systematically unreported variables, which also holds for our healthy cohort from which we used serum samples for this chapter. This can be explained by the fact that, being a radiological finding, women must have had a mammogram, but density assessment is still not commonly reported in the mammographic screening report. Ideally, if informed consent would allow it, mammograms obtained at the time of inclusion in the study could be retrieved and the breast density could be evaluated by a breast radiologist.

Quantitatively, we unexpectedly found that the overlap of microRNAs between tissue and serum was higher compared to the overlap between breast tissue, breast milk, and NAF. However, this is an overlap in the number of highly expressed microRNAs from different cohorts, using different techniques and normalization strategies, and consequently cannot lead to solid conclusions. Only a study comparing samples from the same subject and using the same sample processing procedures would provide the right conditions for comparison, but such is unlikely with the unique combination of samples chosen for this study (i.e., acquiring breast milk, breast tissue, and NAF from the same woman is challenging). A combination of fewer sample types would be one alternative. Since women who provided NAF samples for the DENSE-on biobank (a biobank with samples from healthy women undergoing screening with extremely high and very low breast density), also provided serum samples, a study using paired samples could still be performed for overlapping analysis and correlation with

the serum microRNA list presented in the literature. A comparison between the microRNAs that have proven to be biomarkers for breast cancer across sample types would be more relevant to elucidate whether NAF represents a specific breast-microRNA source compared to systemic liquid biopsies. Qualitatively, microRNA annotation in pathways was performed to identify microRNAs in the healthy state that, in the diseased state, could have a different expression. Interestingly, annotation analysis showed that NAF-microRNAs involved in breast cancer pathways are also involved in pathways for other types of cancer, such as melanoma, pancreatic cancer, small cell lung cancer, and colorectal cancer. This could mean that the microRNAs in NAF are not specific for the breast microenvironment but can also be explained by the fact that each microRNA can influence hundreds of targets.

NAF samples can be in a variety of colors in combination with a cloudy or clear appearance. Although this cannot go unnoticed when handling these samples, this is a neglected aspect in data analysis in most NAF studies. In **Chapter 6**, we report for the first time that there is an association between the appearance of the NAF sample and microRNA expression. This was an accidental finding that we encountered when investigating discriminating NAF-microRNAs between women with different breast densities. Since NAF appearance (color and viscosity), reflecting biomarker content and cellular (e.g., blood) composition, proved to be a relevant confounder in data interpretation, we first prepared this dedicated report in the form of a 'cautionary note' prior to carrying on with the NAF-DENSE project (which has as aim to identify microRNAs in NAF that are discriminating between women with extremely high and very low breast density). It would be interesting if other researchers in the NAF field or doing research with liquid biopsies such as urine, cerebrospinal fluid, and sputum, would test whether color influences the expression levels of microRNAs or of other biomarkers. If microRNA investigation is performed for translational purposes, factors that may cause intrinsic noise must be identified and controlled. MicroRNA levels have been shown to vary with age, gender, and smoking habits (13-15). As such, our finding about the influence of NAF appearance on microRNA expression does not dissuade the potential of (NAF-) microRNAs nor should it discourage further investigation, but rather makes us aware of another factor to consider. It is relevant to maintain an overview of the known confounding factors per microRNA so that researchers are aware of these. A web-based application that shows which microRNA varies with gender and age already exists (microRNACon (16)) and could be further expanded by including more interfering factors per microRNA and per sample type. This chapter would be more complete with analyses of the causes of different colorations and how to deal with different colorations to minimize confounding, but addressing these proved to be unfeasible because of the volume and viscosity of NAF. However, the objective of the chapter was to raise awareness that the appearance of the NAF sample is

a confounder of microRNA expression, which we believe was achieved with this cautionary tale.

Conclusions of this thesis

Performing biomarker research with NAF samples due to their potential to mirror the breast microenvironment has been the focus of several research groups over the last decades. The search for biomarkers for early breast cancer detection requires a well-designed protocol and several cohorts. From the perspective of the researchers, carrying out such a study takes many years during which there are challenges to be overcome, some of which can be avoided. From the perspective of the women who participated in the study, liquid biopsy acquisition was considered less uncomfortable than other breast imaging techniques, and the women were overall positive about the concept of using liquid biopsies in the screening. For the first time, we have investigated microRNA levels in NAF, and RT-qPCR profiling in these very small sample volumes has proven to be feasible. There is a lack of integration of anthropomorphic characteristics, including risk factors for breast cancer, in microRNA studies. Translationally-minded researchers should integrate these data into their articles and analyses to be able to transfer their data into actionable findings with clinical utility. Finally, NAF sample appearance is a confounder in NAF-microRNA analyses, an effect that could also be relevant for other biomarker-based liquid biopsy studies. This finding shows that being susceptible to subtle changes could be one of the greatest advantages of microRNAs, but also their greatest danger to the unwary researcher.

Future projects

The work presented in this thesis sets the foundation for future projects. These projects focus on microRNAs in the context of breast density and microRNAs for early breast cancer detection. An overview of follow-up, feasible and necessary projects is highlighted below, together with their potential translational applications (see Figure 1 for a schematic overview).

1. MicroRNAs in the context of breast density

Using microRNAs to help understand the biology of high mammographic breast density

Chapters 5 and 6 combine NAF microRNA data from healthy women with extremely high and very low breast density. These data are currently being used to identify microRNAs that discriminate between these two groups of women. Getting to know which microRNAs are aberrantly expressed among women with high breast density opens the way to biologically understand why this characteristic is associated with an increased risk of breast cancer. Follow-up target and pathway analyses could lead to the development of targeted therapies to lower breast cancer risk.

2. MicroRNAs for the detection of breast cancer

MicroRNAs for the diagnosis of breast cancer and microRNAs for the early detection of breast cancer

Case-control analysis

As explained above, during the 2017-2021 period, NAF, serum and plasma samples from 137 women with breast cancer and 190 healthy women were collected for a case-control study to compare microRNA profiles. Analyses with NAF samples from this case-control study will be performed to find a microRNA signature for breast cancer. Biobanked samples from a third group of women with benign breast disease, who were identified due to pathological symptoms of nipple discharge, will be included in the analyses to test the specificity of the panel. The microRNA signature will be selected based on multiple combinatorial analyses, to indicate which combination of microRNAs provides the highest performance, based on an area under the curve analysis. This will allow calculating measures of accuracy, such as sensitivity, specificity, negative predictive value, and positive predictive value. Analyses will correct for sample color, as presented in Chapter 6. Variables like tumor size, tumor subtype, and risk factors for breast cancer such as age and breast density are available and will be included in the analyses. An independent set of NAF samples from a breast cancer cohort that was put together in Poland (Gdansk) will allow an independent external validation.

Nested case-control analysis

The microRNA signature with the best performance will then be tested for its utility for early detection of breast cancer in high-risk women who developed breast cancer, from whom serial samples were collected before the onset of breast cancer by comparing them with serially acquired samples from high-risk women who did not develop breast cancer (nested case-control analyses). Samples will be matched by age, high-risk subgroup, and date of sampling. Moreover, since a heterogeneous group of women at high risk was included (carriers of *BRCA1/2* but also other germline mutations and women with a family history or personal history of breast cancer), stratified analyses will be performed for specific high-risk groups. There are a few possible outcomes: it could be that one panel is found for the high-risk group or that several panels are found per high-risk subgroup. Moreover, it could be that the panel in the high-risk group overlaps with a panel for women at population risk.

Technique of choice for the first NAF-microRNA analyses

In order to acquire the best microRNA signature, usage of a non-targeted platform, such as bulk single-cell small RNA sequencing, could be of additive value. This not only allows investigating all the microRNAs (and other small RNAs) without making a pre-selection, it would also allow investigating the best microRNA signatures per subgroup or patient characteristic using bioinformatics. Experiments to test the applicability of such a technique in NAF samples are ongoing. Of course, a technical validation with a low-cost laboratory test with correlation analysis such as RT-qPCR should follow in order to make results future-proof and translational. A great advantage of qPCR is that diagnostic lab technicians are already trained to use it, as it is already widely used to investigate other markers for disease.

Parallel, additional, in-depth experiments

An additional experiment to perform would be to test whether the microRNA signature found in NAF also shows the same pattern in breast tumor tissue. Such a correlation analysis of microRNA expression between NAF and tumor tissue may provide valuable information on the biological role of microRNAs. Nevertheless, such analyses may prove inconclusive because of a selective release of microRNAs from the tissue to the liquid biopsies as shown by some blood-based studies (17). A weak correlation between NAF and breast tissue would be consistent with our own findings in Chapter 6 and would have no actionable consequences for our NAF panel. Moreover, it is relevant to be aware that experiments with breast tissue are at risk to be of less clinical relevance in the screening setting, given that repeated breast tissue sampling is undesirable and tumor tissue microRNA expression may be heterogeneous. Finally, it is more demanding to

find control breast tissue samples; ways to tackle this include acquiring samples from breast reductions or using tissue surrounding the tumor. In addition to exploring the correlation between NAF and tumor tissue microRNA expression, further biological understanding of the functions of discriminatory microRNAs and their role in tumor formation could be confirmed with cell line or organoid experiments.

Other key elements to investigate, especially within the samples from the high-risk cohort, include the effect of various factors that can fluctuate over time and thereby potentially exert different effects on the microRNA expression levels in the serially obtained NAF samples. These factors vary from effects of storage time to changed characteristics of participants such as body mass index, parity, and menopause.

Investigations in the long run

After finding a panel (or panels) with high accuracy for early detection of breast cancer, this should be validated. Validation could be performed using a set of remaining samples from our own cohort. If validation proves to be successful, the next step comprises careful implementation of the microRNA-panel in screening practice by prospectively analyzing the biomarker levels in samples of high-risk women undergoing screening. Parallel to this, a self-test could be developed for NAF samples. If developed into a test of straightforward sample collection and result interpretation, specialized trained personnel would not be necessary nor sample processing and transportation, this way reducing time and costs. This would be in line with the needs of the Health Council of the Netherlands, who expressed interest in more efficient screening methods (18). These projects comprise a few of those that should be performed in the long run to evaluate whether there is a place for a biomarker in the context of early breast cancer detection.

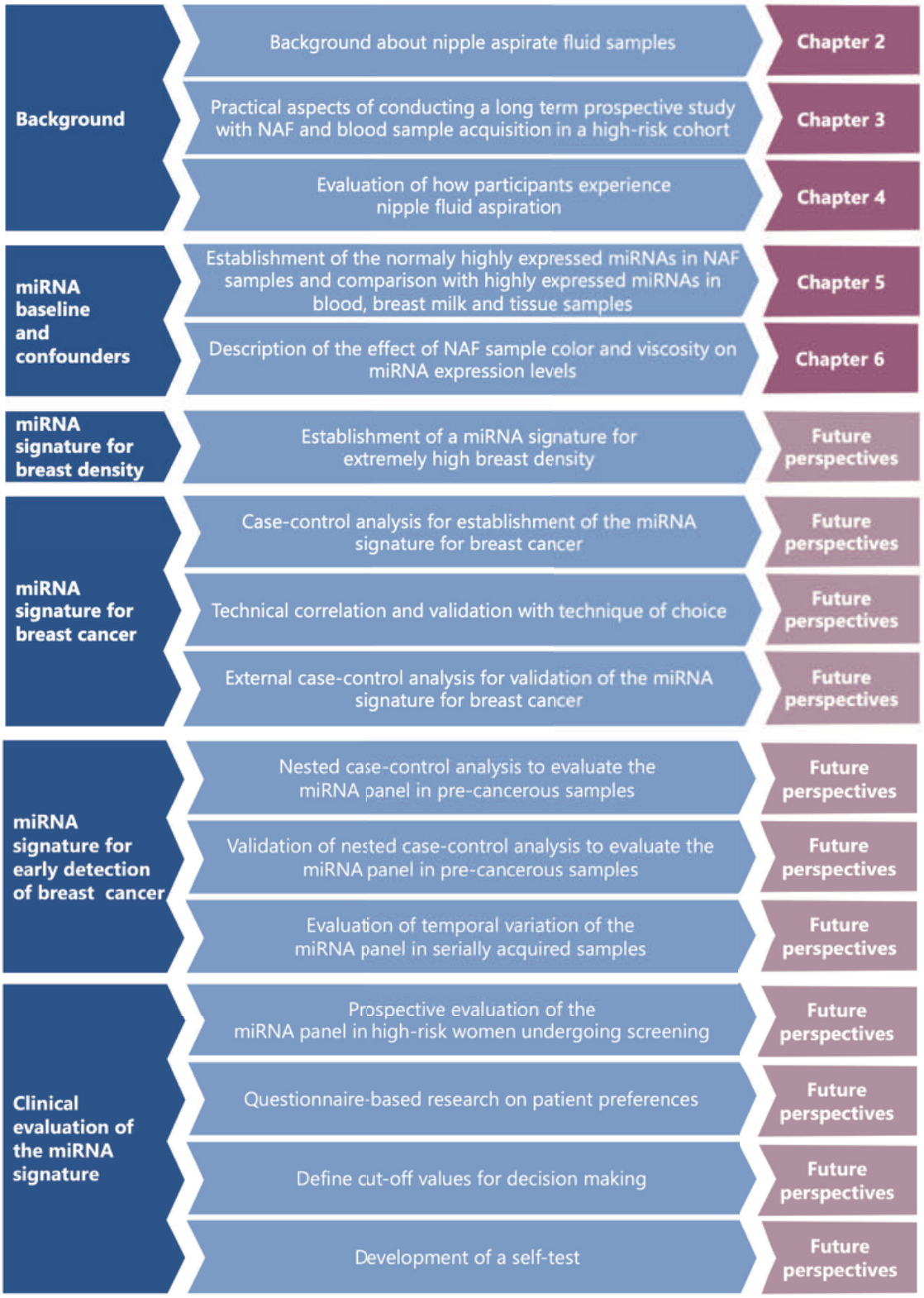


Figure 1. Schematic overview of this thesis with future perspectives.

Perspectives on the future of liquid biopsy-based biomarkers for early detection of breast cancer

The microRNA road to the clinic: a bumpy ride

From our pilot experiments, we have indications that there are microRNAs in NAF that are associated with breast density and breast cancer. This confirms that NAF-based microRNAs are representative biomarkers for biological processes and disease. While searching for microRNAs related to breast density has not been done before, many studies have already proposed very promising microRNA signatures (based on 3-9 microRNAs) for breast cancer detection in blood, with sensitivities ranging between 78% and 99%, and specificities ranging between 88%-100% (19-26). This includes microRNAs that can identify hormonal subtypes, such as triple negative breast cancer (27, 28) and different stages of breast cancer (22). However, these studies seem to identify microRNA signatures with little-to-no overlap with the signature of other publications. This could be due to the use of different techniques to process and analyze the samples or to differences between cohort sizes and cohort characteristics, which are seen not only between studies but also within (case-control) studies, in which samples of the cases are collected at diagnosis, and controls come from a very different setting and, as such, are not comparable. In addition, the microRNAs found in blood originate from many organs and cells, including blood cells. There are also some studies that used urine samples (easy to approach and non-invasive sources) to evaluate the accuracy of microRNAs for the diagnosis of breast cancer. However, also in these three studies, there is a lack of overlap between microRNA panels (29-31).

In addition to this, there is often a missing link with the potential clinical application, as there is a lack of follow-up validation studies of the identified biomarker(s), and most studies often lack a baseline anthropomorphic and clinicopathological table and adequate sample sizes (24, 32). These are relevant elements because if characteristics of the investigated population are unknown, possible future extrapolation of the data is hampered. The translational application for early breast cancer detection also misses in prospective nested case-control studies, as the microRNA signature found can only say something about being able to detect disease at the same moment that conventional imaging methods are able to, but not before. A recent article did present a microRNA signature for early breast cancer detection, up to ten years before diagnosis; however, the sample size was low (n=10) (33). As such, overall, there is a need for standardization of techniques, publications with large sample sizes, nested case-control analyses, attention for confounding factors, and completeness of data from biobanks, including documentation of sample processing. These and other suggestions are summarized and explained further in Table 1, which is in line and complementary to the suggestions described in Chapter 3.

Table 1: Suggestion of elements to consider and type of information that should be acquired and reported in any biomarker discovery study for early breast cancer detection.

Element to consider	Description of the suggestion
Cohorts	Include a cohort of healthy volunteers (control group), a cohort of patients with benign disease, and a cohort of patients with invasive disease to compare biomarker performance. In addition, consider including a group of patients with another oncological disease that is not breast cancer. In this way, you can test whether the biomarker (source) is specific for the disease of interest. Moreover, include and prioritize cohorts of women in the subgroups who have the biggest disadvantages or needs in current screening: women at high risk for breast cancer, pregnant women, lactating women, and women with high breast density. Discuss with a statistician how large the cohorts need to be in order to be able to draw solid conclusions from the data.
Medical history	Register a medical history from the participating women (including the healthy controls) that can influence results. In this way, it can be tested whether the biomarker is influenced by other underlying diseases.
Anthropomorphic information	Collect anthropomorphic information, including information related to risk factors for the disease. This allows testing whether the biomarker adds up to the risk factors and, consequently, can have a predictive role in disease development.
Pathological information	Collect pathological information of the tumor: side, breast quadrant, size, receptor status, lymph node status, grade, molecular subtype.
Radiological information	Collect radiological information about the tumor, namely BIRADS classification for breast lesions and breast density.
Timing of sampling	Aim to acquire the sample for biomarker analysis prior to biopsy or systemic treatment, to avoid these to influence the biomarker levels, if the protocol allows. In addition, acquire samples before and after tumor resection to evaluate the biomarker load. In this way, it can be tested whether there is a change in biomarker levels once the tumor has been removed. After sample collection, process and store these on time to avoid loss of quality, and report these processing details in the sample management system.
Reproducible endogenous control and normalization strategy	Establish a reproducible endogenous control and normalization strategy for data analysis. This enables validation of the results.
Technical correlation and validation	Perform a technical correlation and validation analysis with a technique that is less expensive. In this way, a validation step is performed with translational potential.

Other biomarkers in liquid biopsies under investigation for breast cancer

Potential liquid biopsy-based biomarkers currently being investigated include, in addition to microRNAs, tumor-educated platelets (TEPs), circulating tumor DNA (ctDNA), tumor-associated auto-antibodies (TAAbs), and protein alterations (34-36).

One example is Videssa breast, a blood-based diagnostic biomarker test based on serum protein biomarkers (SPBs) and TAAbs that has been shown to be capable of detecting established breast cancer in conjunction with imaging BIRADS 3 or 4 in women under 50 years of age (37). This was later also validated in women over the age of 50 years (38). Methodologically, this included the use of samples from several cohorts from different medical institutes, starting with the comparison of healthy vs. breast cancer and followed by comparisons between benign breast disease and invasive breast cancer. The investigations included selection of a number of biomarkers, (blind) validation, and addition of biomarkers from the literature to the set (39). Comparison of the published articles does however show that the number of SPBs and TAAbs seems to vary between 8-11 and 10-33 in different publications. The purpose of Videssa breast is to be used as an additional tool for patients with abnormal or difficult-to-interpret mammograms. However, a recent congress abstract showed that this test was not good enough to defer tissue biopsy in patients with indeterminate calcifications on imaging, nor to help predict results in high-risk solid lesions in clinical practice (40).

Another example is the work of Opstal-van Winden et al. who investigated the combination of breast cancer serum biomarkers together with cancer antigens (e.g., cancer antigen 15-3, carcinoembryonic antigen, cancer antigen 125, cancer antigen 19-9, and prolactin). They showed that, using a nested case-control study that matches cases and controls on age and sample storage time, serum protein profiles were already altered up to three years before breast cancer detection (41). However, this was not confirmed in a follow-up study (42).

In addition, there seems to be a surge in interest in liquid biopsy-based ctDNA detection of mutations related to oncological diseases, including breast cancer. An example is CancerSeek, which is a blood-based marker for five types of cancer based on frequently prevalent mutations detected in cell-free tumor DNA. However, accuracy was lowest for breast cancer, and the sensitivity decreased with lower stages of breast cancer, limiting its applicability for early detection of breast cancer (34, 43). This is probably due to the low residual ctDNA that can be found in blood samples (the fraction of tumor-derived DNA in the blood is typically much lower than 5% and fractions as low as 0.1% have been observed) (44-46). These low concentrations make detection at early stages of tumor development challenging (47).

The studies mentioned above all made use of blood samples. There are also studies that have analyzed biomarkers for breast cancer detection in sweat, urine, and tear samples, and breath (48). Several biomarkers have also been evaluated in NAF, such as the Thomsen-Freidenreich (TF) antigen and its biosynthetic precursor Tn antigen, a combination of TF, uPA and PAI-1, deglycase DJ-1 protein, dehydroepiandrosterone concentration, and proteomic profiles. However, the accuracy measures sensitivity and specificity are commonly not reported, making evaluation and comparison of diagnostic performance difficult. Currently, to our knowledge, there is only one group in the United Kingdom actively investigating protein level alterations in NAF samples, namely Natasha Jiwa and colleagues at Imperial College London, United Kingdom.

In the end, the ideal biomarker for early breast cancer detection could be a panel of microRNAs, one of the mentioned biomarker classes, or maybe even a combination of biomarker classes.

When is a biomarker for early breast cancer detection good enough?

The introduction of a new tool in clinical practice is demanding, especially if another tool already exists for many years, as is the case of mammography and breast MRI for breast cancer detection and diagnosis. As such, the stakes are high for biomarkers to be considered for implementation. A list of aspects that should be taken into consideration when investigating the translational potential of a biomarker is presented below in three sections.

Depending on the acquired data on the biomarker's performance, its role in the detection pathway can be established. As such, a biomarker tool should:

- have a **high sensitivity** in the screening situation if to be used as a **triage biomarker**
- have a **high positive predictive value** if used as a biomarker to contribute to a decrease in the **false positive rate of MRIs**
- have a **high specificity** in the diagnostic setting if to be used as an **add-on tool**

Furthermore, the interpretation of biomarker levels should be tested for various conditions that can be of influence. There is a chance that biomarker levels could be susceptible to certain conditions and variables, but investigators should be able to control for the following:

- **Variability over time.** This is relevant if the biomarker test is to be used as a repeated monitoring biomarker. If the levels of the biomarker vary over time, one strategy would be to first establish individual baseline biomarker levels and to interpret the levels as divergent when they change significantly in follow-up measurements.
- **Sample characteristics.** In the case that biomarker levels vary for sample characteristics, a strategy to take is to be able to correct for that characteristic in the analysis.
- **Women's characteristics** (e.g., menopausal status, parity, and ethnicity). The test should be able to correct for a woman's (changing) characteristic. If this cannot be achieved, it should be considered to implement the biomarker only in subpopulations, instead of the entire population.

Finally, the following should be considered:

- **Cost-effectiveness studies** should be performed to investigate the health costs that result from the introduction of a biomarker tool.
- Especially in the case of biomarkers for early detection of breast cancer, it is relevant to **include radiologists** in the research team to discuss current imaging challenges and the opportunities for biomarkers to overcome them.
- It should be investigated whether such a panel will lead to **overdiagnosis**, i.e., the detection of disease that would not develop into an invasive stage.
- If the levels vary by **technique used for analysis**, only one technique should be used and implemented.

Are there interventional/therapeutic consequences of a positive NAF biomarker result for early breast cancer detection?

One of the criteria defined for screening developed by Wilson and Jungner is that the detected asymptomatic disease should have treatment at that stage to cure or control disease (49, 50). A positive result in the screening is followed by further evaluation to establish the disease. If the diagnosis is established, early intervention should change the course of the disease, resulting, among other things, in decreased mortality and less intense treatments. The problem with an early detection tool in practice is what to do if detection is so early that one cannot see the tumor. This would make a diagnostic process and treatment as we know it difficult, as this is now based on tumor location, tumor size, tumor subtype, and affected lymph nodes. If a tumor is not visible, pathological confirmation cannot

be obtained, which precludes treatment based on the molecular characteristics of the tumor. The standard of care for confirmed non-metastasized breast cancer is usually upfront breast surgery and (neo-)adjuvant systemic treatment. Having invisible disease hampers local and systemic intervention: a surgeon will not perform surgery to remove a tumor that she/he will not be able to see, especially because this makes a lumpectomy impossible. Neoadjuvant chemotherapy is also an impactful treatment with significant side effects and is difficult to opt for if the molecular characteristics are unknown.

So what could be the action when the result of a marker indicates that carcinogenesis has started? A possibility would be to give endopreventive treatment either systemically, as already exists and is already given for women at high risk or women with DCIS, or topically or even intra-ductally (both would still have to be developed). Or, a new type of preventive systemic or local treatment could be developed based on anti-biomarker mechanisms (e.g., anti-microRNA); another idea would be to give a single dose of radiotherapy on the whole breast (this would have to be investigated first). The European Collaborative on Personalized Early Detection and Prevention of Breast Cancer (ENVISION) statement, published in 2020, endorses the development of novel non-surgical preventive measures to reduce the incidence of breast cancer of poor prognosis (51). Another role of such a biomarker panel would be to determine whether it is necessary to intensify the next screening moment(s) and screening techniques. In that case, it would be used as a monitoring tool for triage; after a positive result, an imaging-based screening moment could be planned earlier until the tumor can be seen. However, this comes with an immense amount of stress for women with a positive biomarker result. This is why such an early panel is, in its early phases, specifically indicated for women at high risk who are already considering undergoing a prophylactic mastectomy. However, one could say that performing a prophylactic mastectomy when carcinogenesis is already ongoing is still lagging behind events. In conclusion, detection of breast carcinogenesis before visibility is only of additional value if it results in adequate preventive measures that improve patient outcome and quality of life. Ideally, these measures would be restricted to local interventions that already are the subject of intensive research but have not yet resulted in soon to be expected clinical applications. Most of all, further research is needed to produce a solid biomarker tool to guide clinical decision-making where its implementation should be discussed together with patient advocate groups and medical decision-making experts.

The future of early breast cancer detection

Breast cancer screening programs for women at population risk and women at high risk are susceptible to change in light of epidemiological insights and technological innovations. These programs have already been adapted through the years, for example, with the implementation of digital mammograms and adaptation of screening for *BRCA1/2* germline mutation carriers with high breast density. Possible changes to be expected in the future include invitation of women with extremely high breast density to undergo a supplemental breast MRI (52) and the application of artificial intelligence methods in breast MRI scans to reduce false positives (53). There are currently studies investigating the implementation of a personalized breast cancer screening, such as the Dutch Prisma trial (54), and the international My Personal Breast Cancer Screening (MyPeBS) (55) and Tomosynthesis Mammographic Imaging Screening Trial (TMIST) (56), which are in line with one of the main goals established in the ENVISION statement (51). This could mean that women with a higher population risk would undergo more intense surveillance compared to women with a lower population risk (within the ranges of population risk); the latter could, however, lead to anxiety for women (57, 58) and healthcare professionals (59). In view of the surge of liquid biopsy research, it is not unlikely that a combinatorial approach of imaging together with the measurement of (organ-specific or circulating liquid biopsy) biomarkers will be used in the near future. However, this requires a large longitudinal collection of samples and data from studies that are set to have reproducible and actionable findings.

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7

APPENDICES

Background information

Ia. Early breast cancer detection and prognosis

Table 1. Percentage of detected breast tumors per (invasive) stage. Since the introduction of breast cancer screening, there is a relative higher percentage of stage 1 breast tumors at diagnosis compared to stage 2 breast tumors.

Invasiveness	Stage at diagnosis	Percentage of breast tumors per stage	
		1989 (before breast cancer screening in the Netherlands)	2019 (breast cancer screening fully implemented for decades)
DCIS		4%	14%
Invasive breast cancer	**Stage 1**	28%	40%
	Stage 2	51%	33%
	Stage 3	10%	10%
	Stage 4	5%	5%

DCIS: ductal carcinoma *in situ*, which is considered a pre-invasive breast cancer stadium.

Table 2. Current survival of breast tumors per (invasive) stage. Survival is strongly influenced by stage at diagnosis.

Invasiveness	Stage at diagnosis	Current 10-year survival
DCIS		99%
Invasive breast cancer	**Stage 1**	95%
	Stage 2	86%
	Stage 3	64%
	Stage 4	12%

DCIS: ductal carcinoma *in situ*, which is considered a pre-invasive breast cancer stadium.

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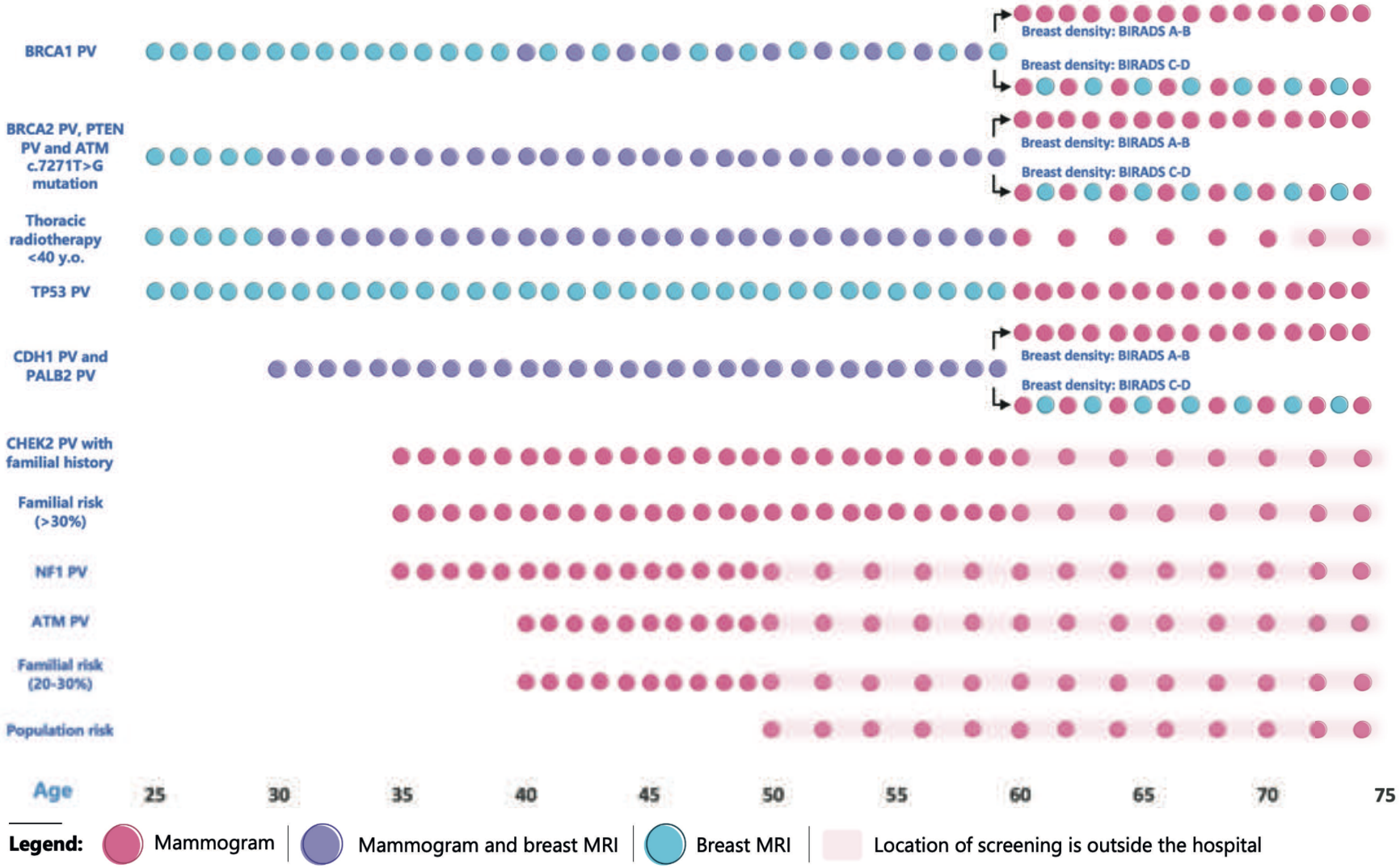
Ib. Breast cancer screening schemes by age and imaging technique of women at population risk and with elevated risk[1].



A

[1] Breast cancer screening for women at population risk is currently temporarily triennial after having been biannual until 2020. It is represented here as a biannual screening because expectations are that soon this will be biannual again after the logistic hindrances are solved. PV: pathogenic variant. Source: https://richtlijnendatabase.nl/richtlijn/borstkanker/screening/screening_buiten_het_bob/screening_buiten_het_bevolkingsonderzoek.html

Ic. BI-RADS classification of breast masses and breast density.

BI-RADS (Breast Imaging-Reporting and Data System) is a standardized risk assessment and quality assurance tool developed by American College of Radiology that provides a widely accepted lexicon and reporting schema for imaging of the breast (mammogram, ultrasound, and breast MRI) (1). Table 1 shows the BI-RADS classification of *breast masses* in seven categories and Table 2 shows the BI-RADS classification of *breast density* in four categories.

Table 1. BI-RADS classification of breast masses.

BI-RADS category	Explanation	Management	Likelihood of breast cancer
0	Need additional imaging or prior examinations	Recall for additional imaging and/or await prior examinations	Not applicable
1	Negative	Routine screening	0%
2	Benign	Routine screening	0%
3	Probably benign	Short interval follow-up (6 months) or continued	0-2%
4	Suspicious	Tissue diagnosis	4A: low suspicion for malignancy (2-9%) 4B: moderate suspicion for malignancy (10-49%) 4C: high suspicion for malignancy (50-94%)
5	Highly suggestive of malignancy	Tissue diagnosis	>95% probability of malignancy
6	Known biopsy-proven malignancy	Surgical excision when clinical appropriate	Not applicable

Table 2. BI-RADS classification of breast density.

Classification	Description
A or 1	The breasts are almost entirely fatty
B or 2	There are scattered areas of fibroglandular density
C or 3	The breasts are heterogeneously dense, which may obscure small masses
D or 4	The breasts are extremely dense, which lowers the sensitivity of mammography

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Id. Accuracy measures in the context of breast cancer.

Table 1. Accuracy measures in the context of breast cancer. Percentage of breast cancers detected in women at age of screening is 61% (a total of 6919 breast tumors were found in nationwide screening, of the 10933 breast tumors found in women aged 50-74 years old; data from 2019). The percentage of breast cancers in women detected thanks to screening compared to all breast cancers detected in women is 40% (a total of 6919 breast tumors were found in nationwide screening, of the 17114 breast tumors detected in 2019). Of note that these numbers do not include the tumors detected in high-risk women at the hospital and that breast tumors were considered invasive breast tumors and ductal carcinoma *in situ*.

Definitions in the context of breast cancer screening					
Term	**Definition**	**Current percentages of mammogram screening, according to the Dutch nationwide registration**	**Percentages, specifically for BRCA1/2 pathogenic variant carriers and/or women with a familial history of breast cancer, according to Dutch cohort studies**		
			Mammogram	**Breast MRI**	**Mammogram and breast MRI**
Screen-detected breast cancer (sensitivity)	Breast cancers diagnosed after a recalled screening examination (positieve mammogram or breast MRI). High sensitivity is associated with fewer false negatives. The number of screen-detected tumors is divided by the sum of screen-detected tumors and interval tumors.	75% This percentage varies between 61% and 86%, depending on breast density.	26-58%	59-96%	79-98%
True negative (specificity)	Screening examinations that did not lead to recall (negative mammogram) and no breast cancer was diagnosed within 24* months after the examination, or before the next scheduled screening examination. High specificity is associated with fewer false positives. The number of true negatives is divided by the sum of true negatives and false positives.	98%	88-97%	79-90%	73-86%

Definitions in the context of breast cancer screening

Term	Definition	Current percentages of mammogram screening, according to the Dutch nationwide registration	Percentages, specifically for BRCA1/2 pathogenic variant carriers and/or women with a familial history of breast cancer, according to Dutch cohort studies	
			Mammogram	Breast MRI
Interval breast cancer (false negatives)	Breast cancers diagnosed within 24* months after a screening examination that did not lead to recall (negative mammogram or breast MRI), and before the next scheduled screening examination. False-negative mammograms or breast MRIs can give women a *false sense of security*, thinking that they don't have breast cancer when in fact they do. Next to false reassurance, this leads to delayed presentation/diagnosis when symptoms appear.	21%	13%	3-28%
False positives	Screening examinations that led to a recall (positive mammogram or breast MRI), but not to a breast cancer diagnosis within 24* months after the examination, or before the next scheduled screening examination. It is the probability that a *false alarm* will be raised: that a positive result will be given when the true value is negative. Furthermore, these women will undergo further 'unnecessary' diagnostic procedures.	1.7% of the screened women. This comprises 73% of the referrals. This percentage varies between BIRADS 0 (86%-96%), BIRADS 4 (51%-75%) or BIRADS 5 (3-12%) and varies between first screeners and regular screeners (higher percentage in first-time screeners).	22-57%	3-61%

MRI: magnetic resonance imaging. BIRADS: Breast Imaging Reporting and Data System.

* Sometimes, a time period of 30 months is considered.

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Ie. Toolkit for interpretation of microRNAs

Nomenclature

A total of 2656 microRNAs (miRNAs) are known so far and these can be found in the central repository for miRNA nomenclature and nucleotide sequence data, miRBase (1, 2). The nomenclature of each miRNA includes a prefix indicative of the species; 'hsa' stands for a miRNA in humans. MiRNAs are only around 18-22 nucleotides long (hence the designation "micro") and if miRNA sequences differ at only one or a few nucleotide positions, each miRNA is given lettered suffices (e.g. -a or -b) (3, 4). Mature miRNAs that have an identical sequence but arise from different genes are given additional numerical suffices (e.g. -1 or -2). A deviation from this nomenclature are the "let" microRNAs (e.g. hsa-let-7f-5p) (5, 6). A schematic explanation of the miRNA nomenclature is shown in Figure 1.

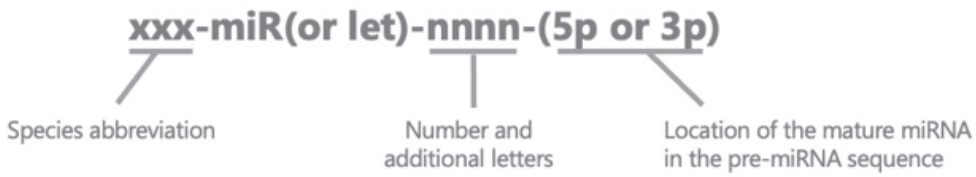

Figure 1. Nomenclature of miRNAs. Example: hsa-miR-29c-5p, which stands for human microRNA-29c-5p. Adapted from miRNAtools.eu(7).

MicroRNA expression: interpretation of ups and downs

Interpretation of microRNAs comprises analyzing their levels of expression. By comparing the miRNA levels of a cohort with cases to the miRNA levels of a cohort with healthy controls, one can conclude which miRNAs are significantly aberrantly expressed based on their fold change between those groups and their associated p-values.

A technique that allows miRNA expression interpretation and that was used in this thesis is reverse transcription quantitative real-time PCR (RT-qPCR). In short, this technique starts with a reverse transcription, i.e. complementary DNA (cDNA) is made from miRNAs, which is followed by real-time PCR amplification. During this amplification, fluorescence of a miRNA-specific probe binding to the cDNA is detected by a sensor and quantified. See Figure 2 for a schematic overview of the technical steps of RT-qPCR. RT-qPCR can be applied as a targeted technique when singular miRNAs are investigated per sample or as a multi-targeted technique (which is designated RT-qPCR profiling) when a wide range of miRNAs can be analyzed per sample, which is less labor intensive but more expensive (8).

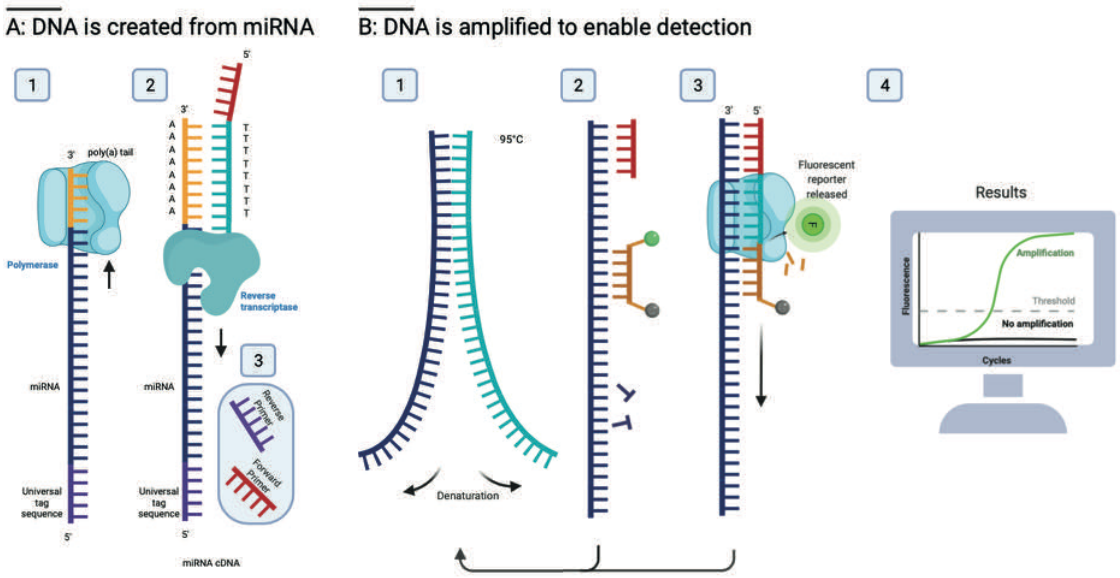

Figure 2. RT-qPCR. A1. Polyadenylation step and adapter ligation step: poly-A tail is added (a strand containing only adenine nucleotides), which is afterwards recognized by universal poly-T primers. A2. Reverse transcription step: reverse transcriptase generates a complementary DNA (cDNA) strand from miRNAs. A3. miR-amplification step: universal forward and reverse primers are added to increase the number of cDNA molecules (sometimes called 'miRNA cDNA' strands). B1. Heat is applied to break the (miRNA c)DNA strands apart. B2. Primers and probes (both short stretches of nucleic acids), attach to matching parts of the DNA. The probes have a fluorescence reporter (green circle) and a quencher (grey circle). The primers allow the polymerase to elongate the strands. B3. Upon cleavage of the probe by the polymerase, the fluorophore sends a fluorescent signal which is detected by a sensor and quantified. B4. Steps B1-B3 are repeated several times (cycles), which leads to generation of PCR curves (exponential increase of fluorescent signal); these curves are later further analyzed. This figure was generated using BioRender.com.

The role of miRNAs in cancer can be deduced from their relative change in expression in the cancer versus healthy state and/or by knowledge of their targets and pathways. Oncogenic miRNAs act by targeting tumor suppressor genes and are hence upregulated in the cancer state (9, 10) . Tumor suppressor miRNAs act by repressing the expression of oncogenes and are frequently downregulated in cancer (Figure 3). These roles in cancer can then be further explored and confirmed by investigating the targets and pathways of the differentially expressed miRNAs. miRTarBase is an example of a website that allows identification of relevant established or predicted mRNA targets and pathways (https://miRTarBase.cuhk.edu.cn/).

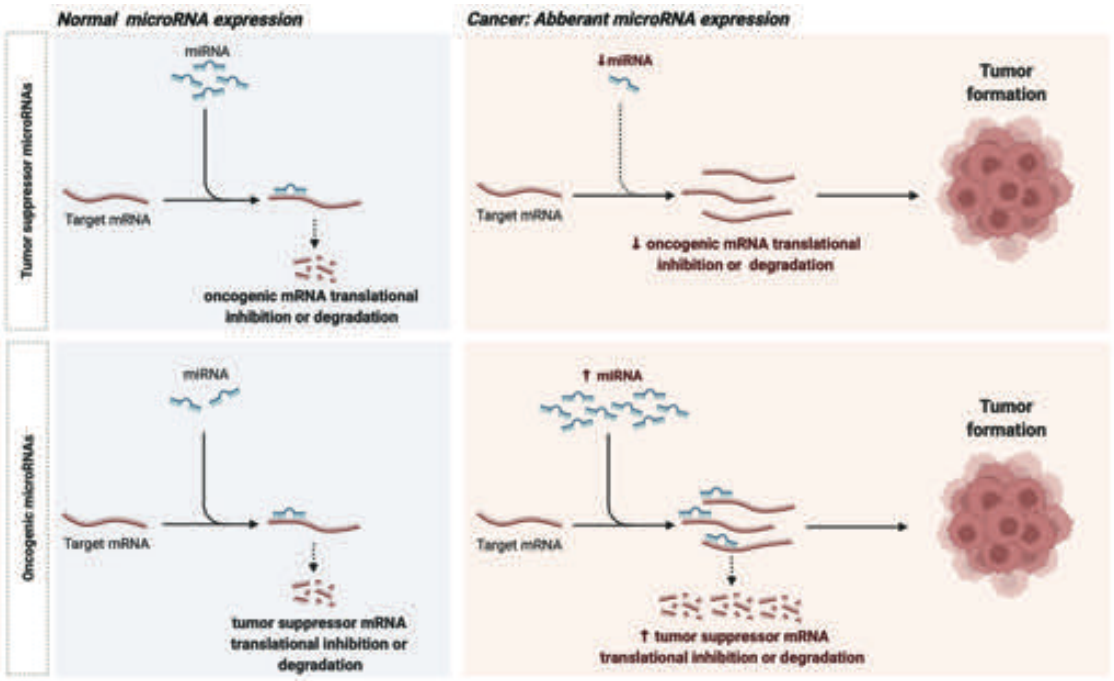

Figure 3. The effect of microRNA expression changes on tumor behavior depends on the function of the messenger RNA (mRNA) target gene. This figure was generated using BioRender.com.

Biomarker studies: the two phases and signature building

The search for biomarkers of disease ideally includes a stepwise approach: the discovery phase followed by a validation phase.

Discovery phase

Within the discovery phase, a category of biomarkers, in this case microRNAs, are investigated in samples from cases (with disease) compared to controls (no disease). The acquired profiles are then compared to identify discriminating miRNAs. These profiles show the miRNAs' relative expression when using qPCR profiling platforms or read counts when using sequencing platforms (11). MiRNA sequencing platforms are sequence agnostic, and as such, have the advantage of being able to identify and quantitate novel miRNAs (that is, sequences that are likely to be miRNAs but are not yet annotated in miRBase). Using the discovery approach, a set of (usually) hundreds of miRNAs are measured.

Validation phase

The follow-up validation phase is a candidate-based method. In this phase, the biomarkers that were selected in the discovery phase, are compared in a new set of samples from cases and controls. After these steps, analyses are performed to find the combination of microRNAs with the best performance.

Alternative or complimentary approaches

An alternative or complimentary approach to these two phases includes testing a miRNA signature or setting up a panel of miRNAs based on previously published studies. However, this is not often performed as it comes with the risk of a lower performance due to possible differences in used samples, sample processing steps, platforms of choice and normalization methods.

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If. Eligibility criteria for the three cohorts.

Participant eligibility criteria and recruitment

The NAF study consists of three cohorts of women: a breast cancer (n=137), a healthy (n=190) and a high-risk cohort (n=555). Within all three cohorts, serum, plasma and NAF samples were collected. Study participants of the three cohorts were selected according to the criteria presented in Table 1. The rationale for the eligibility criteria and recruitment is described below.

Table 1 – Eligibility criteria for the three cohorts.

	Healthy controls cohort	Breast cancer cohort	High-risk cohort
Inclusion criteria	• Women ≥ 45 years old • Lifetime risk < 20%	• Women with primary invasive breast cancer • Before start of treatment	• Women ≥ 18 years with a lifetime risk > 20% for developing breast cancer • Previous DCIS/invasive breast cancer
Exclusion criteria	• Pregnancy or lactation • Active breast infection • History of breast reduction with nipple replacement	• Pregnancy or lactation • Active unilateral or bilateral breast infection • Post-neoadjuvant chemotherapy • Metastasized breast cancer • History of breast reduction with nipple replacement	• Pregnancy or lactation at the time of a study visit* • Active breast infection • Disseminated breast cancer • Bilateral mastectomy • History of breast reduction with nipple replacement
Participant recruitment	Flyers in general practices, hospitals, blood banks, mammography screening posts and word of mouth	Multidisciplinary meetings of four hospitals: 1 teaching hospital and three non-teaching hospitals	Outpatient clinics of hereditary breast cancer and clinical genetics of two teaching hospitals
Number of study visits	One	One	Annual study visits within 10 years or until an event occurred

* Given the longitudinal character of the study, this is a temporary exclusion criteria for planning a study visit rather than participation in the study.

A

Healthy controls were self-referred by acquaintance with the flyers. The pre-requisites of being healthy were not described in the flyer because our belief and experience is that the word already leads to a selection of self-considered healthy women. Age at the time of participation deemed to be minimally 45 years so that the cohort could match the age of the breast cancer cohort, which was expected to have a mean age of 63 years old according to the national data of breast cancer incidence age (1). Healthy controls were considered to be eligible if they had a life time risk (LTR) < 20%, which was controlled for by a questionnaire that included questions about a history of breast cancer, a family history of breast, ovarian or prostate cancer and any previous referral to or advice from a clinical geneticist according to the criteria to refer to a clinical geneticist (2). In case of doubt, the questionnaire was controlled by a clinical geneticist and/or additional questions were asked to the candidate (2, 3).

Women in the breast cancer cohort could participate in the study if there was a pathologically confirmed invasive breast cancer. This way, women with breast cancer diagnosed at different stages were included, which enables investigating whether the panel has a better performance for early stage compared to late-stage non-metastasized breast cancer. We excluded women with metastasized breast cancer and women with recurrent breast cancer given that the aim of our study is to find a biomarker for early detection of breast cancer. Moreover, to avoid possible influence of previous treatments on the biomarker levels, women with a history of breast cancer were excluded. Eligible women had to participate before the start of any treatment for their current disease as systemic treatment could influence the biomarker levels and breast surgery would not only remove the tumor, leading to a possible lower tumor load of miRNAs in blood samples (4), but would also hamper NFA.

For the high-risk cohort, women that are regularly checked because of an increased risk of developing breast cancer were asked to participate. Women were included in this cohort if their risk was defined as high according to the criteria adopted by the clinical geneticist (2, 3). If during follow-up or after participation it became clear that a high-risk participant did not have a high risk for breast cancer development, registration of such was done in the samples and study participants' study data.

For the three cohorts, breast infection was considered an exclusion criterion because this might influence the composition of NAF and hence influence the biomarker pattern. A history of breast reduction with nipple replacement was considered an exclusion criterion because it might influence the connection of ducts to the nipple. If a woman had a retracted nipple or a history of spontaneous bloody nipple discharge, these were advised to go to the general practitioner for eventual referral to our outpatient clinic. Women with pathological nipple discharge were not included in the cohorts as this may be a sign of breast disease (5). For safety reasons and to avoid a milky composition of NAF, pregnancy was considered an exclusion criteria.

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II

List of abbreviations

AUC	Area under the curve
CEA	Carcioembryonic antigen
CI	Confidence Interval
CT	Threshold cycles
CV	Coefficient of variation
DCIS	Ductal carcinoma *in situ*
DCT	Delta CT
DDCT	Delta delta CT
DENSE trial	Dutch nationwide multicenter Dense tissue and Early breast Neoplasm ScrEening trial
EC	Endogenous control
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FC	Fold change(s)
FIA	Fluorescence immunoassay
GLC	Gas liquid chromatography
GO	Gene Ontology
IQR	Interquartile range
KEGG	Kyoto Encyclopedia of Genes and Genomes
LTR	Lifetime risk
MALDI-TOF	Matrix-assisted laser desorption ionization – time of flight
MCv2	Mitochondrial resenquencing array 2.0
MD	Mammographic Density
miRNA	MicroRNA
MREC	Medical Research Ethics Committee
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
MSP	Methylation-specific PCR
NAF	Nipple aspirate fluid
NFA	Nipple fluid aspiration
ORNAMENT	A multicenter, crOss sectional, study to assess microRNA expression in Nipple Aspirated Fluid, blood and tuMor material in women with primary brEast caNcer compared with healthy conTrols
PCR	Polymerase chain reaction
PND	Pathological nipple discharge
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
PSA	Prostate-specific antigen
RAL	ReichsAusschuss für Lieferbedingungen
RIE	Rocket immunoelectrophoresis
RT-qPCR	Reverse transcription quantitative real-time PCR

SD	Standard deviation
SELDI-TOF	Surface-enhanced laser desorption/ionization – time of flight
SOP	Standard operating procedure
TCBio	UMC Utrecht Biobank Research Ethics Committee
TCGA	The Cancer Genome Atlas
TESTBREAST	Trial Early Serum Test BREAST cancer trial

A

III

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IV

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V

Summary in Dutch
Nederlandse samenvatting

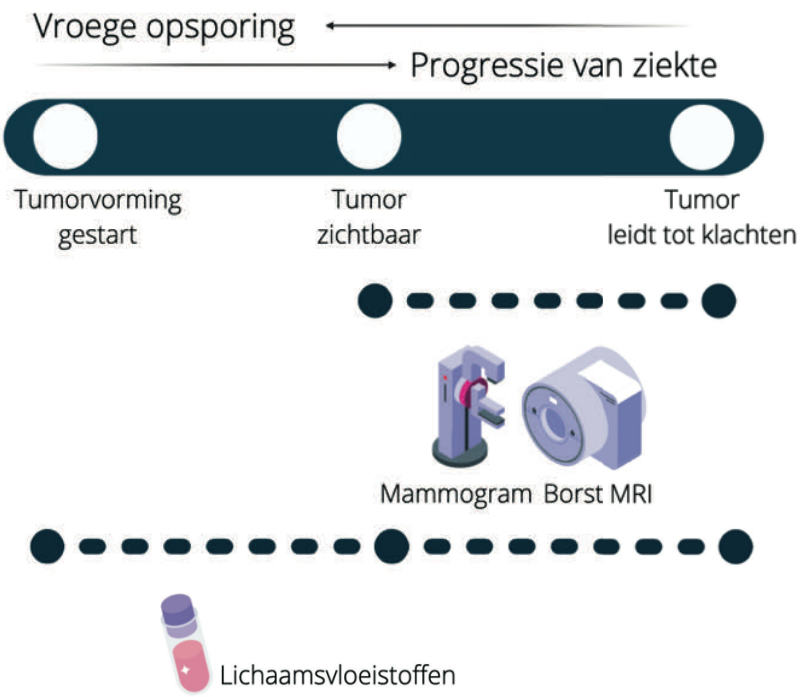
Vroege opsporing van borstkanker

Borstkanker is de meest voorkomende vorm van kanker bij vrouwen, zowel wereldwijd als in Nederland. In 2019 hebben in Nederland in totaal 17.114 vrouwen de diagnose primair ductaal carcinoma *in situ* (DCIS, wat wordt beschouwd als een voorstadium van borstkanker) of invasieve borstkanker gekregen. Borstkanker komt ook bij mannen voor en werd in Nederland in 2019 bij 139 mannen vastgesteld. De behandeling van deze ziekte betreft in de meeste gevallen een operatie al dan niet gecombineerd met radiotherapie, en, afhankelijk het type borsttumor, anti-hormonale therapie, chemotherapie en/of immunotherapie. Om de intensiteit van deze behandelingen en de nadelige gevolgen daarvan te beperken wordt er al jaren geïnvesteerd in vroege opsporing van borstkanker. Zo werd in 1989 in Nederland het 2-jaarlijkse landelijke bevolkingsonderzoek borstkanker geïmplementeerd, gebruik makend van een mammografie. Een aantal jaar later werd een jaarlijkse screening met mammografie of borst-MRI gestart voor vrouwen met een verhoogd risico op borstkanker, zoals voor hen die draagster zijn van een erfelijke afwijking in de *BRCA1* of *BRCA2* genen. Het feit dat vroege opsporing nu afhankelijk is van medische beeldvorming (mammografie of borst-MRI) maakt dat een borsttumor pas vastgesteld kan worden als het een omvang heeft die zichtbaar is bij beeldvorming. Het is bekend dat de prognose van de patiënt met borstkanker verbetert als de tumor kleiner is; in dat licht zou het nog beter zijn als de ontwikkeling van kanker ontdekt zou kunnen worden nog voordat deze zich tot een zichtbare tumor heeft ontwikkeld. De laatste jaren richt het onderzoek naar de vroege opsporing van kanker zich vooral op de rol van zogenaamde biomarkers, 'signaalstoffen' die, wanneer zij in het lichaam gevonden worden, o.a. kunnen duiden op een vroege ontwikkeling van kanker. Voorbeelden van biomarkers zijn cel-vrij DNA, eiwitten en microRNA's. Het aantonen van deze biomarkers kan dan een teken zijn dat een behandeling nodig is om de verdere groei tot een zichtbare tumor tegen te houden. Daarmee zou het mogelijk worden om de ziektelast van borstkanker te minimaliseren of zelfs te voorkomen.

Biomarkers in lichaamsvloeistoffen voor vroege opsporing van borstkanker

Met dit in gedachten hebben we in ons onderzoek onze focus gericht op het analyseren van biomarkers in tepelvocht (NAF, in het Engels 'nipple aspirate fluid') en in bloed. Samples van NAF en bloed hebben we afgenomen bij drie verschillende groepen vrouwen: 1) gezonde vrijwilligers (controlegroep), 2) vrouwen met de diagnose invasieve borstkanker, en 3) vrouwen met een hoog risico op het ontwikkelen van borstkanker, zoals de *BRCA1/2*-genmutatiedraagsters.

In het onderzoek hebben we in eerste instantie de samples van de groep gezonde vrijwilligers vergeleken met die van de groep vrouwen met de diagnose invasieve borstkanker. Door vergelijking van de samples van vrouwen met bewezen borstkanker met de samples van gezonde vrouwen, kan vastgesteld worden welke biomarkers meer of minder aanwezig zijn als zich borstkanker in het lichaam ontwikkeld heeft. Dit geeft dus de mogelijkheid om een panel van biomarkers specifiek voor borstkanker samen te stellen. Dit biomarker-panel wordt vervolgens ingezet bij vrouwen uit het derde cohort. Bij vrouwen die deelnamen in dit cohort zijn gedurende een periode van maximaal 10 jaar, jaarlijks NAF en bloed afgenomen. Bij een aantal van deze vrouwen is in de loop van die 10 jaar helaas borstkanker ontstaan. Bij deze vrouwen worden de samples, die jaren voorafgaand aan de diagnose borstkanker afgenomen zijn, onderzocht op de aanwezigheid van het biomarker-panel. Op deze manier kan onderzocht worden of deze biomarkers ook de ontwikkeling van borstkanker kunnen aantonen, jaren *voordat* het tot een tumor van een zichtbare afmeting heeft geleid (Figuur 1).



Figuur 1. Schematische weergave van het uiteindelijke doel van het onderzoek: een samenstelling van biomarkers identificeren in lichaamsvloeistoffen die toegepast kunnen worden voor vroege opsporing van borstkanker. Hierdoor zou opsporing mogelijk zijn, bijvoorbeeld, nog vóór dat een borsttumor middels medische beeldvorming zichtbaar is.

Bevindingen beschreven in dit proefschrift

Om de vraag te beantwoorden of NAF een toegevoegde waarde heeft in de vroege opsporing van borstkanker hebben wij gekozen voor een meervoudig cohort-biobank onderzoek zoals eerder beschreven. De aspecten die voor een goede uitvoering van een dergelijk onderzoek noodzakelijk zijn, worden in dit proefschrift beschreven.

In deel 1 geven we een inleiding over wat tepelvocht (NAF) is, hoe het verkregen wordt en waarop gelet moet worden als NAF gebruikt wordt voor biomarker analyse. Tevens worden in dit deel de praktische aspecten bij het uitvoeren van een dergelijk langlopend cohortonderzoek beschreven en wordt aangegeven hoe de deelnemende vrouwen de tepelvochtafname (NFA, in het Engels 'nipple fluid aspiration') hebben ervaren. In deel 2 van dit proefschrift beschrijven we analyses van de door ons onderzochte biomarkers, microRNA's, in NAF en bloedsamples.

Deel 1

Hoofdstuk 2: Inleiding over tepelvocht, NAF

In hoofdstuk 2 geven we een inleiding over NAF. NAF is een lichaamsvloeistof die zich bevindt in de kanalen (melkgangen) van de borst die uitmonden in de tepel. Borstkanker ontstaat in deze melkgangen en daardoor is NAF mogelijk een waardevolle vloeistof om te onderzoeken op biomarkers voor borstkanker. NAF kan via de tepel gemakkelijk, veilig en regelmatig worden verkregen door gebruik te maken van de neusspray oxytocine en een aangepast kolfsysteem. Bij gebruik van NAF dient met een aantal aspecten rekening gehouden te worden, zoals de kleine volumes, de viscositeit van NAF en de verschillende kleuren. Mondiaal hebben meerdere onderzoeksgroepen zich beziggehouden met het onderzoeken van NAF samples. Wij zijn de eersten die hebben gewezen op de impact van de kleuren van NAF op biomarker analyses (zoals later in hoofdstuk 6 beschreven). Tot slot beschrijven wij de theoretische (diagnostische) toepasbaarheid van biomarkers in NAF (of bloed) binnen de zorgketen.

Hoofdstuk 3: Ervaringen van onderzoekers over het uitvoeren van het onderzoek

Bij het uitvoeren van een lang lopend cohortonderzoek zoals dit, waaraan vrouwen met een verhoogd risico op borstkanker gedurende maximaal 10 jaar jaarlijks hebben deelgenomen, worden diverse ervaringen opgedaan. Deze ervaringen hebben wij in dit hoofdstuk door middel van een aantal aanbevelingen beschreven. Deze aanbevelingen omvatten een scala aan praktische aspecten, variërend van het opzetten van het onderzoeksteam,

uitdagingen bij het verkrijgen van financiering, het belang van communicatie met teamleden en proefpersonen, alsmede initiële aspecten waarmee rekening moet worden gehouden in het laboratorium. De beschreven ervaringen zijn niet alleen gedeeld door ons, maar ook door de onderzoeksgroep van de zogenaamde TESTBREAST studie (trialregister nummer NL8724), oftewel, door twee onafhankelijke onderzoeksgroepen die een vergelijkbare onderzoeksopzet hebben, namelijk lange termijn studies met cohorten van vrouwen met een verhoogd risico op borstkanker.

Hoofdstuk 4: Ervaringen van de deelnemende vrouwen over de tepelvochtafname

Terwijl in hoofdstuk 3 het perspectief van de onderzoekers op het uitvoeren van het onderzoek wordt weergegeven, beschrijft hoofdstuk 4 hoe de deelnemende vrouwen uit alle drie de cohorten de NFA hebben ervaren. Onze gegevens toonden aan dat vrouwen duidelijk minder ongemak ervoeren tijdens de NFA (ongemak score van 1 op een schaal van 0-10) in vergelijking met de mammografie en borst-MRI (ongemak score van respectievelijk 5 en 3). Deze gegevens bevestigen eerder gepubliceerde resultaten over ongemakscores bij NFA bij gezonde vrouwen en vrouwen met een verhoogd risico op borstkanker. De bloedafname werd als het minst belastend ervaren. Dit was een te verwachten uitkomst, aangezien een bloedafname een snel en algeheel geaccepteerde procedure is. Het is echter voor het eerst op deze manier vergeleken en vastgelegd. Voorts blijkt uit onze vragenlijsten dat vrijwel geen van de vrouwen bezwaar zou hebben tegen een tepelvochtafname in het kader van toekomstige wetenschappelijke studies dan wel in de klinische praktijk voor opsporing van borstkanker.

Deel 2

Hoofdstuk 5: Het normale patroon van de biomarkers “microRNA’s” in NAF, borstweefsel, borstmelk, plasma en serum

MicroRNA’s zijn een van de vele soorten biomarkers. MicroRNA’s zijn stoffen die invloed kunnen hebben op de aanmaak van eiwitten. Door de aanmaak van bepaalde eiwitten te verstoren, kunnen tumoren zich gaan ontwikkelen – daarom dat microRNA’s een belangrijke rol spelen in tumorvorming. Op dit moment zijn er meer dan 2000 microRNA’s bekend. Aangezien microRNA’s geassocieerd worden met het ontstaan van kanker, zijn wij deze gaan onderzoeken in NAF. In dit kader hebben wij in dit hoofdstuk voor het eerst vastgelegd dat het mogelijk is om microRNA’s te detecteren in NAF. Wij hebben beschreven welke microRNA’s aanwezig zijn in NAF samples van gezonde vrouwen. Het viel ons op dat in de onderzoeken die gepubliceerd waren de meest voorkomende microRNA’s in gezonde samples vaak niet worden gerapporteerd. Daarom hebben wij deze

lijsten samengesteld voor NAF, borstweefsel, borstmelk, plasma en serum. Onze veronderstelling was dat de microRNA's in NAF het meest zouden overlappen met de lijst microRNA's in borstmelk en borstweefsel, omdat dit immers borst-specifieke samples zijn. Verrassend genoeg blijken de meest voorkomende microRNA's in NAF niet een betere afspiegeling van de borst dan bloedsamples. Wel komt naar voren dat de meeste microRNA's in NAF een belangrijke rol kunnen spelen in de biologische processen die leiden tot tumorvorming in de borst.

Hoofdstuk 6: De invloed van verschillende kleuren NAF op het normale microRNA patroon

NAF samples hebben verschillende kleuren en helderheid. In dit hoofdstuk hebben wij onderzocht of de verschillende kleuren en helderheid van de NAF samples invloed zouden hebben op het microRNA patroon. Hoewel dit voor de hand zou liggen, zijn deze kenmerken niet eerder meegenomen in (andere) biomarker analyses van NAF samples. Uit het onderzoek beschreven in dit hoofdstuk, blijkt dat er wel degelijk een verband bestaat tussen het uiterlijke kenmerk van NAF samples en de hoeveelheid microRNA's. Het doel van deze rapportage is om bewustzijn te creëren bij andere onderzoekers die gebruik maken van NAF of van andere samples, zoals sputum en urine, die ook verschillende uiterlijke kenmerken kunnen hebben.

Conclusie

Met ons onderzoek streven we ernaar om biomarkers voor vroege opsporing van borstkanker te identificeren in NAF en bloed. Het tot stand brengen van dit idee in een onderzoeksproject vereist, naast een lange adem, een goed ontworpen protocol met verschillende cohorten. Vrouwen die aan het onderzoek hebben deelgenomen waren positief over de beperkte belasting van het afnemen van NAF en bloed. Beide werden als minder belastend ervaren dan de huidige medische beeldvorming (mammogram en borst-MRI). Het meten van onze doelbiomarkers, microRNA's, in NAF is uit ons onderzoek voor het eerst haalbaar gebleken. Daarnaast hebben wij met ons onderzoek laten zien dat kleur en helderheid van de NAF samples invloed hebben op de microRNA meting. Het werk dat beschreven wordt in dit proefschrift legt een basis voor toekomstige projecten. Deze projecten zullen zich richten op microRNA's in de context van hoge borstdichtheid en microRNA's voor vroege opsporing van borstkanker in lichaamsvloeistoffen. Tevens gaan wij drie verschillende technieken vergelijken om te onderzoeken welke het best toegepast kan worden voor de analyse van microRNA's in NAF samples. De eerste resultaten van deze projecten worden eind 2022 verwacht.

VI

Summary in Portuguese
Resumo em Português

Deteção precoce do cancro da mama

O cancro da mama é a forma mais comum de doença oncológica na população feminina. Nos Países Baixos, no ano 2019 um total de 17114 mulheres foram diagnosticadas com um tumor na mama. O cancro da mama também ocorre em homens e foi diagnosticado, nos Países Baixos, em 139 homens no ano 2019. Na maioria dos casos, o tratamento desta doença oncológica envolve tratamento cirúrgico, combinado ou não com radioterapia, e, dependendo do tipo de tumor mamário, terapia anti-hormonal, quimioterapia e/ou imunoterapia. Para limitar a intensidade desses tratamentos e os seus efeitos secundários, têm sido feitos vários esforços para melhorar a deteção precoce do cancro da mama.

Nos Países Baixos, o programa nacional de rastreio bianual do cancro da mama com mamografia foi implementado em 1989 para mulheres com idades entre os 50 e os 70 anos (mais tarde foi alterado para idades entre os 50 e 74 anos). Pouco depois foi iniciado, paralelamente, um programa de rastreio anual com mamografia ou ressonância magnética para mulheres que têm um risco elevado de desenvolver cancro da mama (exemplos são mulheres portadoras de alterações genéticas nos genes *BRCA1* ou *BRCA2*). As investigações que se focam na melhoria de métodos para a deteção precoce do cancro têm-se concentrado na identificação de biomarcadores, isto é, substâncias que, quando detetadas em níveis abnormais, podem indicar o desenvolvimento precoce do cancro. Exemplos de biomarcadores são fragmentos de DNA, proteínas e microRNAs. A deteção desses biomarcadores pode levar à deteção do processo que leva ao desenvolvimento de um tumor, antes de este se tornar visível. Isso tornaria possível iniciar tratamentos para interromper o crescimento de um tumor e, como tal, minimizar ou mesmo prevenir as doenças oncológicas, incluindo da mama.

Biomarcadores em biópsias líquidas para deteção precoce do cancro da mama

Com isso em mente, a nossa linha de investigação concentrou-se na análise de biomarcadores nas chamadas biópsias líquidas, nomeadamente no sangue e no líquido corporal que reside nos ductos da mama e que pode ser aspirado pelo mamilo (*nipple aspirate fluid* em Inglês, 'NAF'). Recolhemos amostras de NAF e sangue de três grupos diferentes de mulheres. Estes três grupos, também chamados de coortes, formam, em conjunto, a base dos nossos projetos de investigação. Os três grupos são 1) mulheres saudáveis (grupo de controlo), 2) mulheres diagnosticadas com cancro da mama invasivo e 3) mulheres com risco elevado de desenvolver cancro da mama, como as portadoras de alterações genéticas nos genes *BRCA1/2*.

Com o nosso estudo, as amostras do grupo de mulheres saudáveis vão ser comparadas com as amostras do grupo de mulheres diagnosticadas com cancro da mama invasivo. Com esta comparação, vai ser possível identificar quais os biomarcadores que estão mais ou menos presentes na situação de cancro da mama. Os biomarcadores identificados nesta análise vão permitir estabelecer um painel de biomarcadores para o cancro da mama. Este painel será depois testado no grupo de mulheres do terceiro coorte. Das mulheres que participaram neste coorte, foram recolhidas amostras de NAF e amostras de sangue anuais por um período máximo de 10 anos. Uma vez que algumas dessas mulheres desenvolveram cancro da mama ao longo desses 10 anos, as amostras colhidas antes do diagnóstico vão ser analisadas para determinar se o painel de biomarcadores já estava alterado meses ou anos antes do diagnóstico realizado por mamografia, ressonância magnética ou por sintomas (Figura 1).

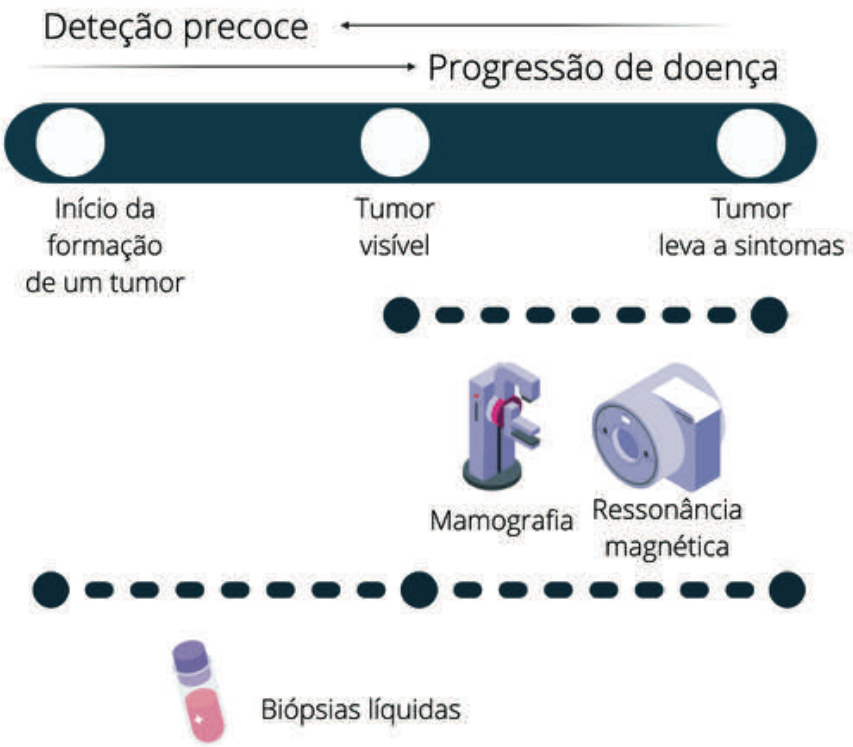


Figura 1. Representação esquemática do objetivo da nossa linha de investigação, nomeadamente a identificação de biomarcadores em biópsias líquidas para deteção precoce do cancro da mama. Desta maneira seria possível, por exemplo, uma deteção ainda antes de um tumor ser visível numa mamografia ou ressonância magnética.

Resultados descritos nesta tese

Para averiguar se as amostras de NAF (e sangue) podem ser uma ferramenta útil para a deteção precoce do cancro da mama, desenhámos um ensaio clínico em que amostras de biópsias líquidas dos três grupos de mulheres são adquiridas e armazenadas num biobanco. Alguns dos elementos a ter em conta para a boa execução de tal estudo são descritos nesta tese.

Na primeira parte da tese é resumida informação sobre a definição das amostras de NAF, a forma como são obtidas e quais os biomarcadores que já foram investigados com estas amostras. Nesta secção também são descritos os aspetos práticos sobre a condução de um ensaio clínico de longo prazo em que são adquiridas repetidamente amostras de NAF e sangue. Também é reportado o nível de desconforto, reportado pelas mulheres que participaram no ensaio clínico no processo de aquisição de NAF. Na segunda parte desta tese, são apresentadas análises dos biomarcadores escolhidos, nomeadamente, os microRNAs, em amostras de NAF e amostras de sangue.

Parte 1

Capítulo 2: Introdução sobre as amostras 'NAF'

No capítulo 2 é apresentada uma introdução sobre o NAF. NAF é um fluido corporal que reside nos ductos da mama, os quais terminam no mamilo. O cancro da mama desenvolve-se nas paredes desses ductos, tornando o NAF uma biópsia líquida com potencial para identificar biomarcadores de cancro da mama. O NAF pode ser obtido de forma simples, segura e repetida fazendo uso do spray nasal de oxitocina e uma bomba de aleitamento. Ao usar amostras de NAF, vários aspetos têm que ser levados em consideração, nomeadamente os pequenos volumes obtidos, a viscosidade das amostras e as diferentes colorações das amostras. Apesar de vários grupos de investigação no mundo se terem dedicado à investigação de amostras de NAF, o nosso grupo é o primeiro a assinalar a importância da coloração das amostras de NAF nas análises de biomarcadores (conforme descrito no capítulo 6). Neste capítulo também descrevemos as possíveis futuras aplicações clínicas diagnósticas de biomarcadores em NAF (ou sangue).

Capítulo 3: A perspetiva dos investigadores na condução do estudo

A realização de um estudo clínico de longo prazo como o nosso, no qual mulheres com risco elevado de desenvolver cancro da mama participaram anualmente, durante uma década, também leva a que os próprios investigadores tenham adquirido experiência e estabelecido instruções para futuros estudos. Descrevemos essas aprendizagens neste capítulo por meio de descrição de onze recomendações. Essas recomendações abrangem uma série de aspetos práticos

que vão desde o estabelecimento da equipa de investigação com especialistas em diversas áreas, desafios na obtenção de financiamento para iniciar e manter o estudo clínico, a importância da comunicação regular com os membros da equipa e as participantes no estudo, bem como aspetos a serem considerados devido às análises laboratoriais. As recomendações descritas não foram apenas descritas por nós, mas também compartilhadas com um grupo de investigação do hospital académico de Leiden (Leiden University Medical Center, LUMC), que lidera o estudo clínico TESTBREAST. Tal como nós, este grupo também se dedica à identificação de biomarcadores para o cancro da mama, especificamente em amostras de sangue adquiridas anualmente em mulheres com risco elevado de desenvolver cancro da mama.

Capítulo 4: A perspetiva das mulheres sobre a aquisição de amostras de NAF

Enquanto que no capítulo 3 descrevemos a perspetiva dos investigadores sobre a condução de um ensaio clínico, no capítulo 4 relatamos como as mulheres vivenciaram a colheita de NAF, analisando o nível de desconforto. Os nossos resultados mostraram que as mulheres sentiram significativamente menos desconforto pela aquisição de NAF (mediana de 1 numa escala de 0 a 10) em comparação com o nível de desconforto experienciado com uma mamografia ou ressonância magnética da mama (as quais tinham uma mediana de 5 e 3, respetivamente). Estes resultados confirmam os resultados publicados anteriormente sobre os níveis de desconforto em obter NAF, os quais se basearam em grupos de mulheres saudáveis e mulheres com risco elevado de desenvolvimento de cancro da mama. A colheita de amostras de sangue foi experienciada como a menos desconfortável (mediana de 0). Este era um resultado esperado, pois a colheita de sangue é um procedimento rápido e amplamente aceite. No entanto, desta maneira, este resultado foi pela primeira vez registado e usado para efeitos de comparação. Os nossos questionários mostram que quase todas as mulheres não teriam objeção em repetir a aquisição de NAF, tanto no contexto de futuros estudos científicos como no eventual uso desta amostra na prática clínica para deteção de cancro da mama.

Parte 2

Capítulo 5: O padrão normal dos biomarcadores “microRNAs” em NAF, tecido mamário, leite materno, plasma e soro

MicroRNAs são um dos muitos tipos de biomarcadores. Atualmente são conhecidos mais de 2000 microRNAs. Como os microRNAs estão associados ao desenvolvimento de doenças oncológicas, focámo-nos no estudo destes nas amostras de NAF. Neste capítulo estabelecemos pela primeira vez que é

possível detetar microRNAs em NAF. Descrevemos quais os microRNAs que estão presentes em amostras de NAF de mulheres saudáveis. Percebemos que em dados já publicados, os microRNAs mais abundantes em amostras de pessoas saudáveis muitas vezes não são relatados noutras publicações. É por isso que compilámos essas listas de microRNAs em várias amostras saudáveis, nomeadamente NAF, tecido mamário, leite materno, plasma e soro. A nossa hipótese era que a lista de microRNAs nas amostras de NAF se sobreporiam mais com a lista de microRNAs no leite materno e no tecido mamário, uma vez que estas são amostras específicas da mama. Surpreendentemente, os nossos resultados *quantitativos* não mostraram que havia muita diferença na sobreposição de microRNAs nessas amostras em comparação com as amostras de sangue (plasma e soro). Uma análise *qualitativa* das funções biológicas dos microRNAs encontrados nas amostras de NAF mostrou que a maioria desses microRNAs podem desempenhar um papel importante nos processos biológicos que levam à formação de tumores na mama.

Capítulo 6: A influência de diferentes cores de NAF no padrão normal de microRNA

As amostras de NAF têm colorações e turbidez diferentes. Neste capítulo investigámos se as diferentes colorações e de turbidez das amostras de NAF influenciariam o padrão de microRNA. Embora isso seja óbvio, essas características (colorações e turbidez) não foram incluídas anteriormente em (outras) análises de biomarcadores de amostras de NAF. A pesquisa descrita neste capítulo mostra que, de facto, existe uma relação entre a aparência das amostras de NAF, como coloração e turbidez, e a quantidade de microRNAs. O objetivo deste relatório foi alertar outros investigadores que utilizam NAF ou outras amostras, como por exemplo a urina, que também podem ter características de aparência diferentes.

Conclusões

A nossa linha de investigação, temos como objetivo identificar biomarcadores para a deteção precoce do cancro da mama em biópsias líquidas, nomeadamente NAF e sangue. A concretização desta ideia requer tempo, um protocolo de estudo bem elaborado e diferentes coortes. A grande maioria das mulheres que participaram no nosso estudo clínico não teriam objeções quanto à colheita de biópsias líquidas (NAF e sangue) para fins de estudos clínicos e deteção precoce do cancro da mama. A recolha destas amostras foi considerada menos desconfortável comparada com uma mamografia ou uma ressonância magnética. Demonstrámos pela primeira vez que a medição dos nossos biomarcadores alvo, os microRNAs, em NAF é viável. Além disso, mostramos com a nossa pesquisa que a coloração e a turbulência das amostras de NAF

influenciam em si os níveis de microRNAs. O trabalho apresentado nesta tese de doutoramento estabelece uma base para projetos futuros. Esses projetos irão concentrar-se na identificação de microRNAs no contexto de alta densidade mamária e na identificação de microRNAs na deteção precoce de cancro da mama em biópsias líquidas. Também iremos comparar três técnicas laboratoriais diferentes para investigar qual a mais adequada para a deteção de microRNAs em amostras de NAF. Os resultados desses projetos podem ser esperados no final de 2022.