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Potential of Breastmilk Analysis to Inform Early Events in Breast Carcinogenesis: Rationale and Considerations

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Abstract

This review summarizes methods related to the study of human breastmilk in etiologic and biomarkers research. Despite the importance of reproductive factors in breast carcinogenesis, factors that act early in life are difficult to study because young women rarely require breast imaging or biopsy, and analysis of critical circulating factors (e.g. hormones) is often complicated by the requirement to accurately account for menstrual cycle date. Accordingly, novel approaches are needed to understand how events such as pregnancy, breastfeeding, weaning, and post-weaning breast remodeling influence breast cancer risk. Analysis of breastmilk offers opportunities to understand mechanisms related to carcinogenesis in the breast, and to identify risk markers that may inform efforts to identify high-risk women early in the carcinogenic process. In addition, analysis of breastmilk could have value in early detection or diagnosis of breast cancer. In this article we describe the potential for using breastmilk to characterize the microenvironment of the lactating breast with the goal of advancing research on risk assessment, prevention, and detection of breast cancer.

Overview

Exposures during early adulthood are strongly implicated in the development of both early and late onset breast cancers: 1) menstrual and reproductive history influence risk of developing breast cancer throughout life [1]; 2) breast cancer incidence rates accelerate rapidly prior to menopause and then rise more slowly at older ages [2]; and 3) incidence of

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specific molecular breast cancer subtypes, such as estrogen receptor (ER) negative tumors (including many basal breast cancers), peak at early ages [2], with a relative predilection for affecting certain groups of women (African Americans and carriers of deleterious *BRCA1* mutations) [3]. Despite the importance of reproductive exposures in breast carcinogenesis, our understanding of these factors is limited because young women rarely require breast imaging or biopsy, and analysis of many critical circulating factors (e.g. hormones) is complicated by fluctuation during the menstrual cycle. Accordingly, novel approaches are needed to understand how events such as pregnancy, breastfeeding, weaning, and post-weaning influence breast cancer risk [4]. Defining mechanisms and markers related to critical early events in breast carcinogenesis could enable the discovery of improved approaches for risk assessment, prevention and detection of early onset tumors.

In this review, we discuss the potential value of studying breastmilk to understand early events in the pathogenesis of breast cancer. We describe methods for collecting, processing and testing breastmilk specimens and discuss related challenges, as summarized in Figure 1.

Etiologic Hypotheses Linking Pregnancy, Lactation and Post-weaning Remodeling to Breast Cancer Risk

Pregnancy is associated with expansion of breast epithelium. In rodent models, pregnancy is related to a marked increase in the number of breast stem cells [5]. Among women, childbirth is associated with a higher number of terminal duct lobular units (TDLUs) [6], the structures that produce breastmilk and give rise to nearly all breast cancers [7]. Women who give birth at earlier ages (<30 years) are at lower risk of developing breast cancer overall compared with women whose first birth occurs later in life or who remain nulliparous [8]. The time prior to a first birth may constitute a “window of vulnerability” during which carcinogenic exposures (such as lifestyle factors, environmental toxins and medications) produce greater risks. It is hypothesized that an early first pregnancy poses little cancer risk because the breast has accumulated few mutated cells, and the downstream protective effects of epithelial differentiation and elimination predominate [7]. However, the risk reduction associated with early age at first birth applies mainly to ER-positive cancers that develop after menopause [9]. Risk for a subset of aggressive early onset tumors, including many basal breast cancers, may be increased by pregnancy even at early ages, with this risk attenuated by breastfeeding [2]. This could reflect differential effects of pregnancy on stem/progenitor cells that give rise to luminal A (ER-positive) versus basal breast cancers [10,11].

Breast maturation and breastmilk secretion normally begin after progesterone levels fall post-delivery. Typically, small amounts of colostrum of about 37 mL/day are produced for 1–2 days postpartum until the onset of copious mature breastmilk production of about 1.5 L/day, signifying the final stage of breast differentiation [12–14]. After weaning, the breast undergoes extensive remodeling which returns the organ to its resting state, albeit one that differs from its pre-pregnancy constitution. Recent mechanistic studies have focused on the breast remodeling process to understand the pathogenesis of early onset breast cancer. Findings from rodent models have generated the “involution hypothesis”, which suggests that inflammation related to postpartum re-modeling promotes the development of

aggressive breast cancers [15]. Administration of non-steroidal anti-inflammatory agents has been shown to inhibit an inflammatory weaning process in mice, suggesting that women with highly inflammatory breasts might benefit from a short course of anti-inflammatory agents to reduce their breast cancer risk [16,17]. Future research evaluating the role of such changes in breast carcinogenesis could rely on analysis of inflammatory cytokines in breastmilk may enable the identification of women with an occult non-infectious mastitis who could participate in future research evaluating the role of inflammation in breast carcinogenesis.

UTILITY OF BREASTMILK FOR MOLECULAR ASSAYS

Analysis of epithelial cells

The value of using breastmilk as a risk assessment tool is predicated in part on the supposition that cells shed in breastmilk reflect the state of epithelial cells during the pivotal pregnancy-lactation cycle event, whose changes remain after weaning. Breastmilk collection provides a noninvasive method of studying cells shed by TDLU epithelium and other structures throughout the period of lactation. In comparison, random periareolar fine needle aspirates (RPFNA) and ductal lavage as used in cancer prevention research are expected to represent sampling of generalized proliferative changes that theoretically affect the entire breast field (add Fabian 2000, Fabian 2005, Arun 2007), and are useful despite their invasiveness in evaluating high-risk women regardless of age or pregnancy status. However, these methods are by their nature limited to representing parenchyma near the collection site, while breastmilk likely provides wider representation of breast epithelium throughout the entire lactating breast. [18]. Moreover, Lemay et al. (2013) demonstrated that RNA in breastmilk fat has its origins in milk-producing cells rather than other cell types, thus tissue or cell sampling may not be required for transcriptomic analyses.

Exfoliated epithelial cells in breastmilk can be analyzed by molecular methods to estimate the “load” of potentially deleterious genetic and epigenetic alterations that have accumulated in breast epithelium. For these studies, we have developed methods to isolate epithelial enriched fractions from whole breastmilk by low speed centrifugation and immunomagnetic bead separation. DNA extracted from epithelial enriched fractions can be used for DNA methylation profiling, detection of methylation in promoter regions of specific tumor suppressor genes (TSGs) [19,20] and potentially to identify deleterious mutations. In a targeted analysis of six TSGs in 102 breastmilk samples, there was significant variation in epithelial cell methylation among healthy women [19]. Further, in an analysis of breastmilk collected from 134 women who had a clinically indicated breast biopsy, there were greater average levels of DNA methylation in epithelial enriched fractions from the breast harboring the suspicious lesion as compared with the non-biopsied breast [20]. Epithelial cell enrichment is limited to samples of fresh milk in which the cell membranes are intact. In an effort to broaden the applicability of prior findings, we recently compared methylation profiles of DNA extracted from the epithelial enriched cell fraction with that of DNA extracted from 1 mL of previously frozen whole milk from the same sample. Early results indicate that the epithelial cell pattern is detectable in DNA extracted from whole milk (see Figure 2).

Analysis of the microenvironment

Information regarding the breast microenvironment, and potentially the state of the epithelium, can also be obtained by analysis of breastmilk for: 1) secreted proteins, including a wide range of cytokines, glycoproteins, transcription factors and enzymes, 2) leucocyte populations, and 3) levels of exogenous compounds including environmental pollutants and chemicals from personal care products that may accumulate in the fat or whey component of breastmilk [21–27]. A blood–breastmilk barrier partly isolates the breast microenvironment from the systemic circulation; therefore, concentrations of components in breastmilk may be more relevant to breast cancer risk than levels in blood [26]. Concentrations of pro-inflammatory factors such as IL-6 have been found to be detectable in breastmilk at different levels from that in serum collected at the same time [28]. TNFα tends to be higher in breastmilk than in serum [29], whereas adiponectin levels may be 10 times lower in breastmilk than in serum and the concentrations do not seem to be correlated [30]. Table 1 lists possible research targets in breastmilk.

The cytokine content of breastmilk has been of longstanding interest to researchers seeking to understand its effects on infant health. We have tested breastmilk for pro-inflammatory cytokines IL-1β, IFN-γ, IL-2, IL4, IL6, IL8, IL-12, IL-13 and TNF-α and angiogenesis factors TIE2, Flt-1, PlG-F, VEGF, VEGF-C, and VEGF-D using commercially available platforms for breast cancer risk factors. In a pilot study of breastmilk donated by 40 women (20 Caucasian and 20 African American), using multiplex ELISA electrochemoluminescence assays (Meso Scale Discovery, MSD, Gaithersburg, MD, USA), we found detectable levels (<5% undetectable) of all pro-inflammatory factors except IL-12. Factors Flt-1, PlG-F, VEGF-D, IL-1β, IFN-γ, IL6, IL8, and TNF-α had coefficients of variation of <25% while IL-10, IL-12 p70, IL-13, IL-2, and IL-4 had coefficients of variation ranging from 32.17 to 49.44. In another comparison of cytokines in breastmilk donated by a larger group of 292 women, intraclass correlation coefficients exceeded 90% for 13 of 15 pro-inflammatory markers; VEGFC and TIE2 had low ICC's: 0.48 and 0.42 respectively (unpublished).

COLLECTION OF BREASTMILK: PRACTICAL CONSIDERATIONS

Potential selection biases related to breastfeeding preferences are minimized because breastmilk can be collected from nearly all parous women with intact breasts, at least at early time points following birth. Since 2006, the Breastmilk Laboratory of the University of Massachusetts (KA, EB, AC, EP) have collected breastmilk from over 800 study participants, including 333 women with a history of breast biopsy, and 200 women who self-identified as Black or African American. Breastmilk research provides an opportunity for grassroots involvement of women in breastmilk collection and donation, often from home. National recruitment has been primarily internet-based and has aimed at collecting breastmilk from women with cancer or who are at increased risk of developing breast cancer. We follow women long-term for information on subsequent pregnancies and breastfeeding. Of 333 women with a history of a breast biopsy who donated milk, 27 (8%) of them were subsequently diagnosed with ductal carcinoma *in-situ* or invasive carcinoma (unpublished data), suggesting that research that compares molecular alterations in milk and tissue

biopsies may be feasible. For local recruitment of healthy lactating women, we have successfully used a combination of internet and printed advertisements, together with-in person recruitment efforts at lactation support meetings.

Most participants donated a breastmilk sample from each breast, completed a health and history questionnaire, and agreed to long-term follow-up. Administration of epidemiologic questionnaires to collect demographic information, reproductive history, including details about pregnancies, births, and medical history is critical because little is known about the determinants of levels of specific factors in breastmilk (Agarwal 2011). It is particularly important to query about time elapsed since birth, patterns of breastfeeding (including use of supplemental formula, water, and solid foods), menstrual history, and use of medications. We include a general health and reproductive history questionnaire and a milk-donation-specific questionnaire with each milk collection (see supplementary file S1).

Methods of collecting breastmilk vary around the world. In the U.S., an electric or hand pump is preferred, whereas manual expression may predominate in lower resource settings [31]. To our knowledge, it is unknown whether cellular composition of breastmilk varies by method of expression, however fat content may be increased with a combination of manual expression and electric pumping. Milk with high fat content, characteristic of breastmilk produced toward the end of a lactation session, is less effectively collected with pumping [32].

Strategies for processing breastmilk depend on the collection site and time to processing. Breastmilk donated at the laboratory can be processed fresh, enabling flexibility in processing and optimal preservation. However, using a central collection location poses challenges for targeted recruitment of specific populations. We have successfully collected and tested breastmilk from research participants across the United States using specially designed mailing kits (see supplementary file S2) that included labeled bags for breastmilk from each breast, small ice packs to be frozen the night before shipping, and a prepaid return mailing label with a phone number to call for at-home pickup of the mailing kit. Importantly, we asked the women to express their breastmilk on the morning of the pickup. Breastmilk frequently arrives at the lab with the ice pack still cool. Breastmilk shipped overnight and processed within 24–36 hours of collection generally contains adequate epithelial cells for molecular assays, but a smaller quantity compared to when breastmilk is processed within 3 hours of expression.

Figure 1 provides the sample processing workflow currently employed at the Breastmilk Laboratory of the University of Massachusetts Amherst. Immunomagnetic separation can be used to enrich samples for epithelial cells or deplete leucocytes. We have focused on epithelial cell enrichment using positive selection with an antibody targeting EPCAM (epithelial cell marker). We have also used negative selection with an antibody targeting CD45 to deplete leukocytes with similar results. It should be noted that positive and negative selection result in two populations of cells, neither of which is pure. Negative or positive selection of specific cell types can be performed optimally on fresh breastmilk, but as cells in breastmilk degrade, enrichment is compromised. Breast epithelial cells of pregnant or

recently pregnant women are fragile and easily stripped of cytoplasm, therefore rapid processing and gentle handling is important [33].

Breastmilk samples can be obtained from a single breast or from each breast and may be combined or kept separate; each procedure has strengths and limitations. A unilateral sample spares breastmilk from the contralateral breast for nursing the baby, however most women agree to provide bilateral samples. Collecting breastmilk separately from left and right breasts allows for side-specific analyses. This may help illuminate whether there are laterality differences, since asymmetric breastfeeding may induce changes in milk reflecting differential frequencies in nursing. Observational studies have demonstrated asymmetric cancer risk reduction found among women whose babies preferentially fed from one breast [34,35]. However, combining the breastmilk collected from each breast into a single sample is logistically easier, and is adequate for measuring environmental toxicants or exposures that are expected to be similar for both breasts.

Breastmilk composition changes over the course of the lactation event. In general, foremilk, or first breastmilk produced during a lactation session, is more dilute than hind breastmilk, collected toward the end of a session. The two types of breastmilk likely have different concentrations of immune markers and other constituents.[36,37] Therefore, if the goal is to obtain average characteristics per breast per woman, participants should be asked to empty their breasts completely and provide a sample from the entire mixed volume. Concentrations of immune markers can also vary by the time of day and length of time of breastfeeding [36]. Data suggest that a woman's perception of degree of breast fullness at specimen donation is related to the cellular and fat composition of breastmilk [38,39]. Other studies suggest that maternal nutrition status, even in periods of deprivation, is not strongly related to breastmilk composition [40]. Because the amount of breastmilk women produce in a donation session can vary depending on the time of day, the hours since last breastfeeding session, and how long women have breastfed, it is important to query about these factors. Even small amounts of colostrum or breastmilk can be adequate for analysis; we have been able to extract useful quantities of DNA from 1mL of breastmilk. Analysis of repeated donations of breastmilk from the same woman with complete annotation of sample donation characteristics would help elucidate the changes in biomarker levels associated with different lactation characteristics.

Preferred methods of collection, processing and storing breastmilk vary with study aims and logistical considerations (Table 1). Determining whether to pre-process breastmilk prior to freezing is critical. Breastmilk contains enzymes and bacteria that will alter its composition within a short time of being expressed. Cooling breastmilk may slow deterioration. While freezing breastmilk is optimal for preservation of molecular constituents, it precludes later separation/enrichment of specific cell fractions due to cell lysis during freeze-thaw cycles. However, sensitive assays for specific cell-free molecular targets (e.g. DNA mutations or gene specific methylation events) can be developed, and this issue can theoretically be overcome. Proteins, cytokines, and enzymes can degrade or be released by cells that rupture with thawing, leading to altered assay results [41]. Aliquoting breastmilk prior to freezing provides a source of samples that can be tested after a single thaw, which may minimize

artifacts. We routinely record prior freeze-thaw history for each sample and assess its effect on measured analytes.

MAINTAINING A BREASTMILK RESEARCH REPOSITORY: PRESERVATION METHODS

While we currently freeze breastmilk in aliquots for storage, a method of collecting and shipping breastmilk in a fixative that is compatible with planned laboratory assays would advance breastmilk research by reducing costs and logistical barriers associated with on-site donation, and expanding the pool of potential donors. An ideal fixative for breastmilk would be inexpensive, non-toxic, non-flammable and able to inactivate infectious agents, permitting routine shipping with few safety concerns in the home or during transport. In addition, the fixative would provide rapid, long-lasting cellular preservation with maintenance of surface antigens required for cell isolation/enrichment, and preserve macromolecules in a form that is compatible with planned assays. We have tested multiple fixatives at varying duration and temperature, including formalin, ethanol, and proprietary solutions used in detection of circulating tumor cells, but have not yet identified an optimal method of cellular preservation that is compatible with downstream epithelial cell enrichment. Overcoming the inherent fragility of cells in breastmilk has proved challenging for effective fixation and transport.

FUTURE DIRECTIONS

Epidemiologic studies with breastmilk data offer opportunities to identify breast cancer risk markers that may inform efforts to identify high-risk women early in the carcinogenic process, thereby providing opportunities for early intervention. Other topics related to breastmilk research include:

- Improving breastmilk stabilization, shipping and processing
- Characterization of analytes within and between women at different intervals post-partum, and in fore-versus hindmilk
- Refinement and validation of molecular assays: DNA, RNA, proteomics, cytokines and growth factors
- Validation of methods for detecting toxins
- Comparison of analyte levels in breastmilk and blood

Finally, we propose that an important next step in furthering breastmilk research would be the creation of a national breastmilk repository, with donated samples for research, as has been attempted on a regional scale [42], in the National Children's Study Vanguard (https://www.nichd.nih.gov/research/NCS/Pages/default.aspx) and in laboratories like that of University of Massachusetts Amherst. While donor breastmilk banks have been created for the purpose of distribution of breastmilk to babies whose mothers are unable to breastfeed [43], these banks must pasteurize breastmilk and thus may alter its constituents, limiting its utility for research. The repository would ideally recruit study participants across a wide spectrum of regional and sociodemographic groups, and from time points across the entire

nursing trajectory, including women who do not wish to breastfeed but would be willing to donate breastmilk for the first few days after birth. Long-term follow up of women is needed to obtain cancer outcomes, and to develop markers for early detection. To lessen sample sizes requirements, women at increased risk of breast cancer based on other factors (e.g. family history) could be recruited at a higher rate. The Komen Tissue Bank, which holds events to collect optimally processed, epidemiologically annotated breast tissue and blood samples from large groups of women, may provide a model for this effort [44] and a breastmilk bank would complement the goal of defining the “molecular histology” of the “normal” breast. These samples could provide ranges of healthy values for analytes that could be related to breast cancer risk factors and to outcomes related to breast cancer screening and development. While breastmilk can only be obtained from lactating women, most women (including almost 80% of women in the U.S. (Kirmeyer SE, Hamilton BE. Transitions between childlessness and first birth: Three generations of U.S. women. National Center for Health Statistics. Vital Health Stat 2(153). 2011.) experience at least one pregnancy resulting in a birth, and thus most women could easily provide a sample which does not involve biopsy or invasive procedures. Further, the bank would have value for research related to both breast and infant health, with the potential for providing early insights into the pathogenesis of diseases and approaches to reduce risk.

Breastfeeding has many established benefits for children and mothers, and increasing the percentage of women who breastfeed and the duration of nursing is a goal of Healthy People 2020 [45]. Breastfeeding seems to have especially strong benefits for reducing incidence of the basal-like breast cancers that strike early and are more lethal (Islami, 2015). Enhancing molecular epidemiologic research using breastmilk to enhance understanding of postpartum physiology and pathophysiology offers the potential to develop methods for reducing breast cancer risk and mortality.

All scientific work described complied with current, relevant U.S. law. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This work was supported in part by an NCI Bench-to-Bedside award (M.E.S., K.F.A.) and the NCI intramural research program. The authors would like to thank the women who have donated breastmilk to the University of Massachusetts Amherst Breastmilk Lab throughout the years.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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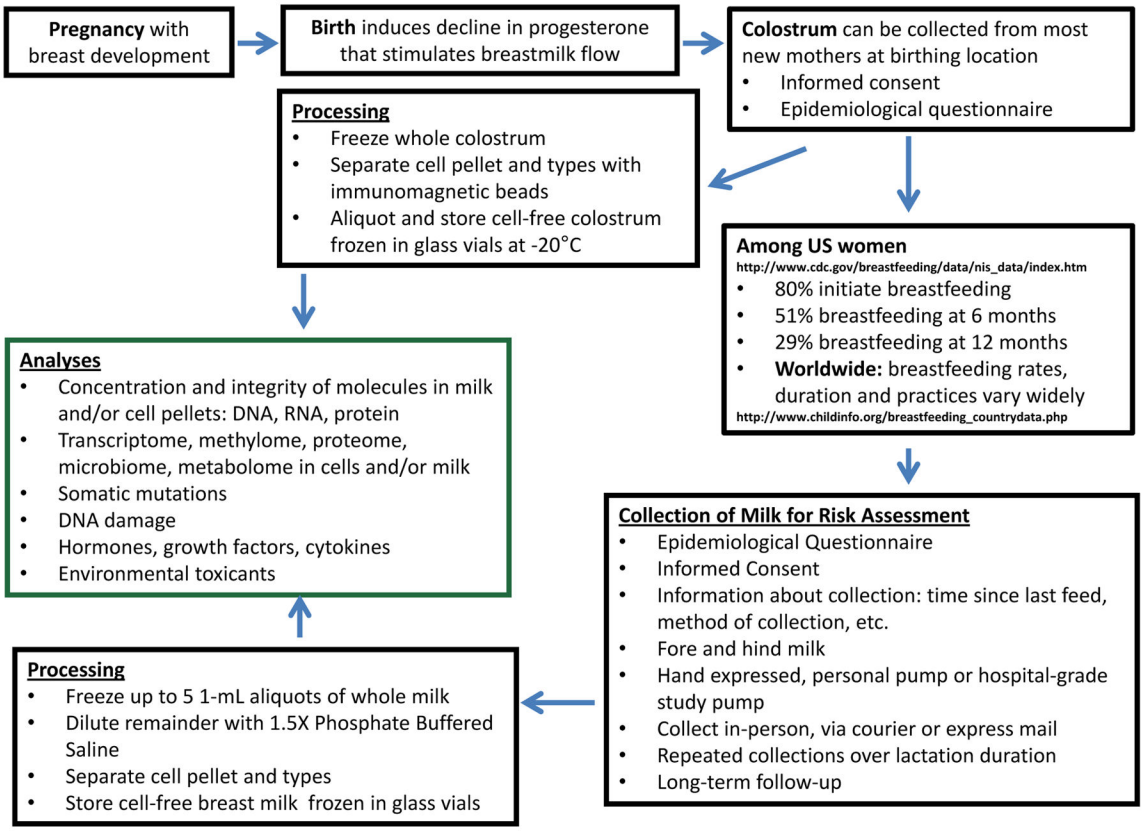

Figure 1. Breastmilk as a biospecimen

Most women who give birth can donate a few ounces of colostrum within 24 hours postpartum, and the sample can be conveniently collected at the birthing location. Differences in how and when the mature milk is collected, processed and stored can introduce biases in analysis. Standardization of collection and storage methods can reduce variability and can be adapted to optimize the targets of interest. The Breastmilk Laboratory at University of Massachusetts Amherst aims to process all breastmilk samples within a few hours of arriving at the lab. Whole breastmilk samples from each breast are aliquoted into acid washed glass vials and stored at −80°C, the remainder of the breastmilk is diluted to aid in the collection of the cells and the epithelial-enriched and depleted cell populations are obtained using immunomagnetic beads. Cell pellets and diluted breastmilk samples are archived at −80 and −20°C, respectively.

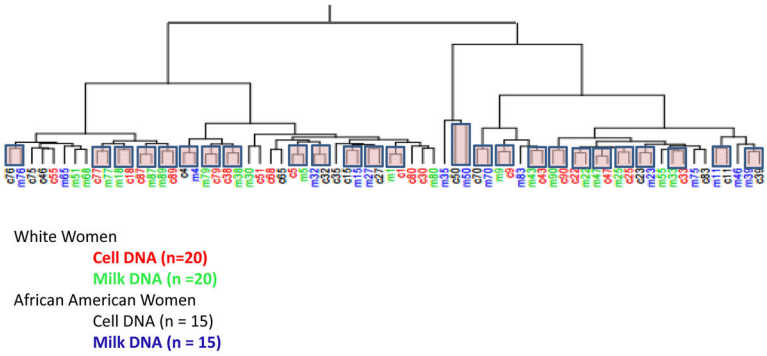

Figure 2. DNA in Breastmilk from Whole Milk versus Cell Pellet

Cluster analysis showing the grouping of methylation profiles of paired DNA samples extracted from 1 mL of whole milk and matching milk cell pellets from 15 African American women and 20 Caucasian women. The HM450 methylation data from the 70 DNA samples were analyzed by hierarchical cluster analysis; probes within 15bp of a repeat were excluded in the analysis. The boxes highlight the 26 out of 35 cell/milk pairs that clustered together.

Table 1

Considerations of components in human breastmilk that can be informative in epidemiologic studies

Target Molecule/Cell	Type of specimen	Measurement/Assay	Considerations for processing/interpretations	Ref
DNA	Can be extracted from whole fresh or frozen breastmilk	Somatic mutations DNA methylation & other epigenetic adducts	DNA may be from epithelial cells or leucocytes Reference databases may be useful in assigning a signal to specific cell type	(19, 20)
RNA	Can be extracted from “cytoplasmic crescents” in the fat of whole fresh or frozen breastmilk Can be isolated from whole breastmilk	Gene expression RT-PCR Microarrays RNASEQ	Special precautions are needed for extracting high quality RNA from the crescents RNA may be from epithelial cells or leucocytes Reference databases may be useful in assigning a signal to specific cell type	(26)
Epithelial cells	Need to be isolated from fresh breastmilk Immuno-magnetic separation with either positive or negative selection yields enriched populations	Flow cytometry Immunohistochemistry Extracted DNA and RNA can be used as above	Best if breastmilk is maintained at room temp or slightly cool and cells are isolated within 3 hours; but cells also have been isolated from breastmilk expressed 24 hours earlier Signals can be attributed to epithelial cells	(19, 20, 29)
Leucocytes	Need to be isolated from fresh breastmilk Immuno-magnetic separation with either positive or negative selection yields enriched populations Multiple subpopulations	Flow cytometry Immunohistochemistry Extracted DNA and RNA can be used similarly as above	Best if breastmilk is maintained at room temp or slightly cool and cells are isolated within 3 hours; but cells also have been isolated from breastmilk expressed 24 hours earlier Signals can be attributed to leucocyte populations	(29)
Cytokines	Can be measured in fresh breastmilk or previously frozen breastmilk Can be measured in whole breastmilk or whey (liquid portion with cells and fat removed)	Individual or multiplexed panels of cytokines can be measured with high sensitivity	Freeze/thaw cycles can alter measurements, either increasing or decreasing the detection of specific cytokines Prepare small aliquots of breastmilk to avoid freeze/thaw Analysis in each breast provides insight into local and systemic production and/or differential accumulation	(21, 36, 39)
Proteins	Can be measured in fresh breastmilk or previously frozen breastmilk Can be measured in whole breastmilk or whey (liquid portion with cells and fat removed) Can be measured in specific cell types	Proteome analyses using mass spectrometry to identify differentially expressed protein observed in 2-D gels	Analysis of whole breastmilk provides ability to identify secreted and intracellular proteins Need to remove high abundance proteins	(23)
Hormones & growth factors	Fresh or frozen breastmilk	Radioimmunoassays ELISAs	Small aliquots prepared from fresh or frozen breastmilk	(12, 21, 22)
Environmental pollutants and personal care products PCBs, PBDEs, PFCs, synthetic musks, chlorinated pesticides, bisphenol A, PAHs, dioxins	Measurements can be made on fresh or frozen breastmilk. Concentrations may differ with fat content-so best if both fore and hind breastmilk are collected	Breastmilk combined from both breasts is appropriate for analyses of most compounds Mass spectrometry Reporter assays for groups of compounds, e.g. estrogens	Both breasts expected to have similar exposure and therefore similar levels Care must be taken to protect the sample from contamination during analysis, e.g., introduction of BPA during laboratory handling	(24, 25)
Microbiome	Total DNA & RNA can be can be extracted from fresh or frozen breastmilk	DNA to assess microbes present DNASEQ RNA to assess active microbes		(27)

Target Molecule/Cell	Type of specimen	Measurement/Assay	Considerations for processing/interpretations	Ref
		RNASEQ		