

The Utility of Breastmilk for Genetic or Genomic Studies: A Systematic Review

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Abstract

This study synthesized scientific literature that applies genetic or genomic approaches to breastmilk. A literature search of PubMed was conducted in March 2012 using the key words "breast milk," "lactation," "genetic," "gene expression," and "epigenetic." Additional articles were identified/selected for evaluation with MeSH term searches, and a review of article reference lists was obtained from the search. The initial 657 abstracts retrieved from the literature search were reviewed, and 16 studies were selected for evaluation. Studies that examined the transmission of viruses/bacteria into breastmilk and/or measured concentration of specific proteins without examination of genetic material from milk were excluded. Data related to subjects, tissue, purpose, setting, gene/protein, approach (candidate versus genome-wide), platform, statistical analysis, and results were extracted. Gene expression and epigenetic/epigenomic study designs have been successfully implemented using breastmilk. A major weakness of both gene expression studies and epigenetic studies that examine breastmilk is the omission of maternal information known to influence milk composition. This review article is the first to synthesize evidence related to the application of breastmilk to evaluate RNA and epigenetic modifications. Additional research is needed that applies epigenetic analyses to human breastmilk samples. Findings from this review can be used for future research designs that use breastmilk for genetic analyses.

Introduction

BREASTMILK COMPOSITION is a constantly changing substance that is influenced by maternal lifestyle factors, including diet,¹ medications,² and exercise,³ among others. Unalterable maternal factors that also impact breastmilk composition include the time of day,⁴ number of days postpartum,^{5,6} and gestational week at delivery.⁷⁻¹¹ Breastmilk composition varies greatly between women who deliver prematurely and those who deliver at term, although the mechanism for this difference is poorly understood. There is also evidence of variation in breastmilk composition *between* women who deliver at the same gestational age,¹² suggesting that breastmilk may not be a uniform substance. For example, interleukin-10, an anti-inflammatory cytokine present in mature human milk, is influenced by gestational age and is found in lower levels among preterm infants.¹³ Additionally, evidence of undetectable interleukin-10 in the milk of women whose infants developed necrotizing enterocolitis¹⁴ suggests that milk variability may also influence neonatal outcomes.

Variable levels of protective components may be driven by genetic or genomic factors, and the procurement of breastmilk is a noninvasive way to examine these factors. Epithelial cells,

which contain both RNA and DNA, make up 50–90% of cell types found in human breastmilk.¹⁵ Analyses of RNA and DNA from human milk provide a platform to better understand the mechanism for compositional variability and neonatal outcomes.

A potential link between breastmilk variation and subsequent neonatal outcomes has been minimally explored at both the protein and gene levels; however, recent work has examined breastmilk profiles with particular attention paid to gene expression and, more recently, epigenetics. Exploring the potential genetic mechanism for breastmilk variation between women may lead to the improvement of neonatal diet through breastmilk optimization, including (1) donor breastmilk fortification, (2) maternal dietary supplements, and (3) maternal lifestyle changes. The collection of breastmilk is a noninvasive way to understand gene regulation in mammary epithelial cells that may explain a potential mechanism for outcome disparities among breastfed infants. Despite evidence that has focused on breastmilk variability at the protein level, a critical review and synthesis of the literature are needed that address genetic or genomic approaches to investigate the mechanism for these differences. This systematic review will describe and critique the recent science that has examined breastmilk using genetic or genomic approaches. This review

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can be used as a reference by investigators who hope to examine human breastmilk using a genetics or genomics.

Data Collection Method

We used the PubMed database to identify studies that examined human breastmilk using a genetic or genomic approach, including gene expression, candidate gene, and epigenetic analyses. The key words used were "breast," "milk," "lactation," "milk fat globule," "gene," "genetics," "expression," "epigenetic," "epigenomic," "methylation," "gestational age," and "preterm." The following combinations of key words were used: "breast" AND "milk" AND "gene" AND "expression"; "lactation" AND "gene" AND "expression"; "breast milk" AND "genetics" AND "gestational age"; "breast milk" AND "genetics" AND "preterm"; "milk fat globule" AND "genetic"; and "milk fat globule" AND "gene expression." We limited the literature search, which covered literature through May 2012, to the English language and articles involving human subject research and excluded articles related to human immunodeficiency virus, cytomegalovirus, and cancer. We relaxed the inclusion criteria for epigenetic studies to include both human and non-human studies, as few have examined breastmilk from an epigenetic approach.

After completing the literature search, we reviewed abstracts of retrieved articles for relevance, excluding duplicate articles, review articles, and those that did not address the use of genetic approaches to examine breastmilk. We also reviewed PubMed e-mail updates and reference lists of selected articles to identify additional studies. After independent review, we met to discuss findings and synthesize results. We extracted from gene expression studies data related to maternal information, RNA isolation method, type of milk, gene product of interest, data collection platform, and results. From epigenetic studies, we extracted the following data: maternal information, epigenetic modification of interest, gene(s) of interest, data collection platform, and results. This information was summarized in tabular format.

Results

We reviewed 35 articles of the initial 657 results. Of these 35 articles, 16 were included in the final analysis. Of those excluded, 10 articles used milk to examine protein levels, four used human mammary epithelial cells, two were animal gene expression studies, one only evaluated DNA damage, one article used milk only to identify epithelial cells, and one article quantified DNA to assess feasibility of DNA adduct evaluation. There were 16 articles that fulfilled our inclusion criteria and were included in this review. Of these, 13 were gene expression articles, and the remaining three were epigenetic studies. Table 1 includes a summary of the results and characteristics of the articles that examined breastmilk at the gene expression level. Table 2 includes a summary of the results and characteristics of the articles that examined breastmilk from an epigenetic approach. Although we encountered many studies that examined maternal DNA polymorphisms with regard to breastmilk properties and/or milk protein concentration measured with enzyme-linked immunosorbent assay, we did not use these studies in the current review. Only articles that examined epigenetics or gene expression using DNA or RNA isolated from breastmilk were included.

Discussion

We conducted this systematic review in order to critique and synthesize scientific literature that used DNA or RNA found in breastmilk in their methodology. Breastmilk is a unique tissue source; therefore it is ideal to investigate dynamic templates such as epigenetic changes to the DNA or mRNA levels. The resulting articles are overwhelmingly gene expression related. This is appropriate because our focus of this review is on the utility of breastmilk as a biospecimen, and expression studies require the tissue of interest. Few articles in this review are epigenetically focused, likely because of the relatively new technologies available for epigenetic analyses.

Use of breastmilk in gene expression studies

The methodological article by Lindquist et al.¹⁶ documents one of the first approaches of RNA isolation from human breastmilk to examine β -casein mRNA using a northern blot. Most of the studies examined in this review incorporated reverse transcription (RT)-polymerase chain reaction (PCR) to examine gene expression of epithelial cells from breastmilk, including genes for cytokines,¹⁷ defensins,¹⁸ interleukin-18,¹⁹ and interferon- γ .²⁰ One study incorporated PCR with a western blot to examine M-ficolin expression.²¹ Andersson et al.²² acknowledged that many cell types, including leukocytes and macrophages, exist in breastmilk. To ensure that epithelial cell gene expression data were obtained, they performed a Southern blot on commercially available human mammary epithelial cells and found identical fragments from human milk cells. Obermeier et al.²³ subjected both whole epithelial cells and isolated RNA from milk to RT-PCR in order to determine if RNA isolation is a necessary step. The investigators concluded that the cell fraction from fresh human milk is an appropriate model to examine glucose transporter gene expression, and this was confirmed with Southern and western blotting.

As technology advanced, the available platforms to examine gene expression became more efficient and comprehensive. In addition to RT-PCR, spectrophotometry allowed Alcorn et al.²⁴ to quantify total RNA concentration extracted from breastmilk to examine transporter genes implicated in drug disposition. Another study²⁵ used a microarray approach on one breastmilk sample that focused on cytokine-related genes and found the gene for osteopontin was the most highly expressed gene among those tested. A western blot examined osteopontin expression differences among colostrum, early, and mature milk. RT-PCR was performed on remaining milk donor samples to confirm high osteopontin gene expression. Three additional studies, all performed by the same investigators, also used a microarray platform to examine milk fat globule gene expression throughout the day,²⁶ when different pumping protocols were used,²⁷ and after administration of recombinant human growth hormone.²⁸ Maningat et al.²⁷ used a traditional nonparametric approach to analyze results from one subject's milk that was subjected to the microarray platform, and RT-PCR confirmed these results using milk from 10 other donors. The remaining microarray study²⁸ used a traditional nonparametric analysis approach using GeneSpring GX 9 (Agilent Technologies, Santa Clara, CA).

TABLE 1. GENE EXPRESSION STUDIES THAT EXAMINE MRNA FROM BREASTMILK

Reference (year)	Maternal information	RNA isolation method	Fresh vs. frozen milk	Gene product	Platform	Results
Alcorn et al. ²⁴ (2002)	n = 6 healthy women, 1–11 months postpartum	RNeasy	Fresh	Drug transporter genes	RT-PCR	Lactating MEC had fourfold higher RNA levels of OCT1, OCTN1, PEPT2, CNT1, CNT3, and ENT3.
Andersson et al. ²² (1996)	n = 1, 9 days postpartum; 200 mL provided (50 mL used)	Unclear	Fresh	PTHrP	RT-PCR	mRNA encoding three PTHrP variants is present in human milk cells.
Frankenberger et al. ²¹ (2008)	n = 3; additional information not provided; 20–50 mL	TRI-Reagent	Fresh	M-Ficolin	RT-PCR Confirmed with western blot	Macrophage M-ficolin expression from breastmilk was lower than in blood monocyte M-ficolin expression.
Lindquist et al. ¹⁶ (1994)	Additional information not provided; 50 mL	Unclear	Fresh and frozen	β -Casein	PCR Northern blot	PCR amplification of β -casein gene was successfully performed on mRNA and genomic DNA from breastmilk. Freezing milk degraded β -casein mRNA.
Maningat et al. ²⁷ (2007)	Microarray: n = 1, additional information not provided. RT-PCR: n = 10 healthy women 18–35 years old, 6–12 weeks postpartum, term singletons, exclusively breastfeeding, standard diet, pumping protocol; 10 mL	RNeasy; switched to Trizol (less RNA degradation)	Fresh	α -Lactalbumin	Microarray of MFG RNA from one subject; QRT-PCR confirmed array results. Breastmilk from the 10 subjects enrolled in the pumping study was subjected to RT-PCR.	RT-PCR confirmed microarray results: GHR and IGF1 were undetectable in up to 2 μ g of MFG RNA. IGF1-R was expressed at low levels using 1–4 μ g of RNA. α -Lactalbumin is abundant in the MFG, and its expression may be regulated by pumping.
Maningat et al. ²⁶ (2009)	n = 5 healthy women, uncomplicated pregnancies, 18–35 years old, exclusively breastfeeding, 6–12 weeks postpartum, standard diet; 10 mL	TRizol	Fresh	Global	Microarray of MFG RNA	1,029 genes were influenced by the time of day milk was expressed. Genes implicated include those involved in cell development, growth, proliferation, and cell morphology.

(continued)

TABLE 1. (CONTINUED)

Reference (year)	Maternal information	RNA isolation method	Fresh vs. frozen milk	Gene product	Platform	Results
Maningat et al. ²⁸ (2011)	n = 5 healthy women, 18–35 years old, 6–12 weeks postpartum, singleton, term, uncomplicated birth, exclusively breastfeeding, BMI ≤ 27 kg/m ² , standard diet, administration of rhGH; 10 mL	TRizol	Fresh	Global	Microarray of MFG RNA	681 unique gene probes experienced altered expressions following rhGH administration. These networks are involved in cell cycle, DNA replication, recombination and repair, and cancer. Genes influenced by circadian cycles were not altered by rhGH.
Nagatomo et al. ²⁵ (2004)	n = unknown, healthy mothers, healthy term infants. Microarray analysis performed on pooled cDNA of milk cells from colostrum, early, and mature milk; unknown volume	ISOGEN	Fresh	240 Cytokine-related genes	Microarray to first determine highly expressed genes of cytokines/growth factors. Confirmed with RT-PCR, OPN gene expression quantification.	240 cytokine-related genes are highly expressed. OPN ranked highest. OPN mRNA levels in early or mature milk were more than 3 times higher than in colostrum. Late mature milk had the highest OPN mRNA levels of all periods.
Obermeier et al. ²³ (2000)	1.5–8 months postpartum; 30 mL	Guanidine thiocyanate method or milk epithelial cells were directly subjected to RT-PCR without RNA isolation.	Fresh	Glucose transporters	Total RNA was prepared and subjected to RT, and nested-PCR was performed. Milk epithelial cells were directly used for RT-PCR without prior RNA isolation.	Cells that are isolated from fresh breastmilk are an acceptable source for investigating gene expression.
Srivastava et al. ¹⁷ (1996)	Healthy women delivering preterm (<37 weeks) or term (37–42 weeks); colostrum, transitional, and mature milk, collected by pump or manual expression; unknown volume	Guanidine thiocyanate method	Fresh	Cytokines	RT-PCR	Maternal cells in breastmilk expressed mRNA for MCP-1, IL-8, TGF-β ₁ , TGF-β ₂ , M-CSF, IL-6, and IL-1β. mRNA not detected in maternal cells from breastmilk included that for IL-2, IL-10, IFN-γ, and TNF-α. RANTES was weakly expressed. There was variability between individual women, gestational age at delivery, and breastmilk type.

(continued)

TABLE 1. (CONTINUED)

Reference (year)	Maternal information	RNA isolation method	Fresh vs. frozen milk	Gene product	Platform	Results
Takahata et al. ¹⁹ (2001)	<i>n</i> = 116 milk samples; demographics not provided but complications reported; colostrum; unknown volume	MagExtractor MFX-2000	Fresh	IL-18	Semi-QRT-PCR of 7 colostrum samples	There was no correlation between IL-18 mRNA levels and IL-18 protein concentration in milk. There was also not a clear correlation between IL-18 milk mRNA and IL-18 monocyte mRNA.
Takahata et al. ²⁰ (2003)	<i>n</i> = 127 (preterm <i>n</i> = 39, term <i>n</i> = 55); <i>n</i> = 6 milk samples; colostrum, early, and mature milk; manual breast pump; unknown volume	ISOGEN	Unclear	MIG and IP-10	Semi-QRT-PCR	IP-10 and MIG are expressed in breastmilk. Unclear if this was conducted in preterm/term or different milk types.
Tunzi et al. ¹⁸ (2000)	<i>n</i> = 6 healthy women, 5 days–7 months postpartum; unknown volume	TRizol	Unclear	HBD-1 and HBD-2	RT-PCR	All milk samples expressed the HBD-1 mRNA transcript.

BMI, body mass index; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; GHR, growth hormone receptor; HBD, hemoglobin δ ; IFN- γ , interferon- γ ; IGF, insulin-like growth factor; IGF1-R, insulin-like growth factor receptor 1; IL, interleukin; IP-10, interferon- γ -inducible protein of 10 kDa; M-CSF, macrophage colony-stimulating factor; MCP1, monocyte chemoattractant protein 1; MEC, mammary epithelial cell; MFG, milk fat globule; MIG, monokine induced by interferon- γ ; OCT, organic cation transporter; OPN, osteopontin; PCR, polymerase chain reaction; PEPT2, peptide transporter 2; PTHrP, parathyroid hormone-related protein; QRT, quantitative reverse transcription; RANTES, regulated upon Activation, normal T-cell expressed, and secreted; rhGH, recombinant human growth hormone; RT-PCR, reverse transcription–polymerase chain reaction; TGF- β , transforming growth factor, β ; TNF- α , tumor necrosis factor α .

TABLE 2. EPIGENETIC STUDIES THAT EXAMINE DNA FROM BREASTMILK

Reference (year)	Maternal information	Epigenetic modification	Gene(s) of interest	Platform	Results
Browne et al. ²⁹ (2011)	n = 134 lactating women with history of biopsy for a suspicious lump; volume varied	Methylation	RASSF1, SFRP1, GSTP1	Bisulfite conversion and PCR amplification	Mean methylation scores for RASSF1 and GSTP1 were higher in women with breast biopsy history.
Qin et al. ³² (2012)	n = 32 healthy women who delivered term; 3 milk samples: (1) within 10 days of delivery, (2) 2 months after lactation started, and (3) weaning period; unknown volume	Methylation	KLK6	Bisulfite conversion and PCR amplification	KLK6 methylation varied among women and with breastfeed duration. Methylation intensity was not associated with protein level.
Wong et al. ³⁰ (2010)	n = 102 healthy, exclusive breastfeeders, 19–45 years old, breastfeeding for 30–820 days, no history of breast cancer; at least 10 mL with varied volumes	Methylation	PYCARD, CDH1, GSTP1, RBP1, SFRP1, RASSF1	Pyrosequencing	There was significant relationship between breast cancer risk and those who had a biopsy and methylation scores for CpG sites in CDH1, GSTP1, SFRP1, and RBP1. Regardless of reproductive past, RASSF1 methylation was positively correlated with age.

CDH1, cadherin 1, type 1, E-cadherin (epithelial); GSTP1, glutathione S-transferase π 1; KLK6, kallikrein-related peptidase 6; PCR, polymerase chain reaction; PYCARD, pyrin domain and caspase recruitment domain containing; RASSF1, Ras association domain family member 1; RBP1, retinol binding protein 1, cellular; SFRP1, secreted frizzled-related protein 1.

Use of breastmilk in epigenetic studies

An epigenetic mechanism is a biochemical alteration to the DNA that does not change the sequence but does influence gene expression. This relatively new field has shown great promise in diseases with multifactorial origins because these epigenetic alterations are greatly influenced by the environment. Despite the influence of environmental factors on breastmilk composition, only three studies were found that used an epigenetic approach to examine breastmilk, and all had an oncology focus.

Breastmilk provides a potentially rich source of maternal genetic information. Breastmilk collection is noninvasive, and there is great potential in this practice for individualized screening of breast cancer risk. Two of the epigenetic studies included in this review used methylation analyses to examine promoter regions of tumor suppressor genes known to influence breast cancer risk.^{29,30} The remaining epigenetic study examined the methylation of the promoter region of KLK6, which is down-regulated in breast cancers.³¹ Wong et al.³⁰ were able to attain a sufficient quantity of DNA for methylation analyses and used pyrosequencing to attain mean overall methylation concentrations for six genes of interest. Browne et al.²⁹ also used pyrosequencing to attain methylation concentrations for three genes of interest in women with a

history of a breast biopsy. Similarly, pyrosequencing was used to quantify methylation intensities of KLK6 in healthy women at three time points to examine the extent of epigenetic regulation on protein levels at three stages of lactation.³²

Limitations of gene expression studies that use breastmilk

RNA isolation techniques varied, and some articles completely omitted this important information. One investigator switched the isolation method technique during the study from RNeasy[®] (Qiagen, Düsseldorf, Germany) to TRizol[®] (Life Technologies, Carlsbad, CA) due to RNA degradation.²⁷ Additionally, the use of fresh versus frozen breastmilk should be explicit. Lindquist et al.¹⁶ subjected both fresh and frozen breastmilk to northern blotting and found that the frozen samples experienced β -casein mRNA degradation. Most articles examined in this review used fresh milk that was immediately processed; however, there were a few articles that omitted this important information.^{18,20} When samples are placed on ice, RNA degradation occurs and increases the longer samples remain there.³³ One study²⁴ placed milk samples on ice, although the authors noted that samples were processed within 1 hour. Three studies reported placing milk samples on ice but failed to report the elapsed time between

this and processing.^{26–28} Four studies described “immediately” processing milk samples.^{17,19,21,25} Five articles did not report any information on sample preservation before processing.^{16,18,20,22,23} It is important that the way in which breastmilk samples were preserved and time to processing are reported, as this may impact the RNA quality and final conclusions.

Breastmilk composition and subsequent gene expression are influenced by many factors, including circadian patterns,²⁶ diet,¹ frequency of pumping,²⁷ gestational age at delivery,⁷ and number of days since delivery. Most of these studies failed to inform the reader about the time of day milk was expressed, the type of pump used, and how frequently the mothers were pumping. Gestational age also contributes to breastmilk composition, as protective factors in preterm breastmilk include an increase in levels of immunoglobulins,⁷ fatty acids,³⁴ and cytokines.⁸ Although most of the articles in this review included subjects who were described as healthy and delivered term babies, some included preterm deliveries. The phenotypic definition of preterm was variable: some defined preterm as less than 37 weeks,¹⁷ others defined preterm as less than 36 weeks,¹⁹ whereas others who included preterm deliveries did not provide exclusion criteria.²⁰ Other factors that influence gene expression and/or breastmilk composition that were largely overlooked include mixed fore and hind milk, maternal drug/alcohol use, differences in pumps used (or manual expression), maternal diet, and omission of maternal demographics, maternal health, and obstetrical complications.

Limitations of epigenetic studies that use breastmilk

The use of both methylated and unmethylated controls should be used when applying methylation analyses to ensure bisulfite-conversion efficiency. Wong et al.³⁰ and Qin et al.³² appropriately used both methylated and unmethylated controls, although it is unclear if Browne et al.²⁹ did the same. It is unclear if Wong et al.³⁰ collected milk from subjects with a history of a breast biopsy. Wong et al.³⁰ reported a minimum breastmilk volume for analysis (10 mL) but did not reveal how many women, if any, were unable to produce this volume. Browne et al.²⁹ did not reveal a minimum breastmilk volume requirement for analysis but did report a range from 56 mL to 86 mL. Qin et al.³² reported KLK6 methylation is not associated with protein levels; however, DNA isolation was performed on samples from only 32 women, and a large portion of these were from weaning milk for which the time of collection varied greatly. All three epigenetic studies asked subjects to provide breastmilk samples with breast pumps *or* manual expression. The method of milk collection should be identical among subjects because resulting breastmilk volumes vary between methods,³⁵ potentially implicating both gene expression and epigenetic regulatory mechanisms.

Conclusions

Breastmilk is an appropriate source of RNA or DNA when conducting a gene expression or epigenetic study. Although techniques for RNA isolation and the volume of breastmilk collected varied, the yield was generally sufficient to conduct gene expression studies. If a gene expression approach is used, frozen breastmilk should be avoided because of RNA

degradation. Multiple platforms have been applied to examine mRNA in breastmilk, including RT-PCR, microarrays, and western blots. When the epigenome is examined, regardless of tissue source, it is important to use methylated and unmethylated controls to ensure efficient bisulfite conversion. Many lifestyle factors are known to influence breastmilk composition and are therefore contributors to gene methylation and should be accounted for in the analyses.

Only four of the 16 articles used a nonparametric genome-wide approach to examine gene expression. The mechanisms that contribute to breastmilk variability remain elusive; therefore, genome-wide evaluations are needed to better understand breastmilk genetics. Most of the articles examined a specific pathway or physiological process, and few had a direct clinical application. Although physiological and pathway-related studies are important, more clinically relevant studies are needed.

Investigators can utilize the findings from this review to design future genetic or epigenetic studies that use breastmilk. First, research addressing a more standard approach to breastmilk collection and RNA isolation is needed. Steps to mitigate this include reporting volume of breastmilk collected, RNA isolation technique, and RNA yield. Second, research that explores breastmilk genetics in any capacity should report factors known to impact milk composition and subsequent gene expression. A better understanding of the mechanism that underlies breastmilk variability may lead to approaches that optimize donor breastmilk, a practice that is becoming increasingly popular as an increasing number of premature infants become viable because of neonatal advances. Lastly, epigenetic approaches to explore breastmilk show great promise and may provide a way to capture environmental influences on human milk. A molecular examination of breastmilk is applicable to all researchers in lactation science, including behaviorists and traditional bench scientists, as knowledge gained on the mechanisms for milk variability will move the science forward.

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