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Assays for Endogenous Components of Human Milk: Comparison of Fresh and Frozen Samples and Corresponding Analytes in Serum

Erin P. Hines, PhD, Jennifer L. Rayner, PhD, Randy Barbee, BS, Rae Ann Moreland, BS, Andre Valcour, PhD, DABCC, Judith E. Schmid, MS, and Suzanne E. Fenton, PhD

Abstract

Breast milk is a primary source of nutrition that contains many endogenous compounds that may affect infant development. The goals of this study were to develop reliable assays for selected endogenous breast milk components and to compare levels of those in milk and serum collected from the same mother twice during lactation (2-7 weeks and 3-4 months). Reliable assays were developed for glucose, secretory IgA, interleukin-6, tumor necrosis factor- α , triglycerides, prolactin, and estradiol from participants in a US EPA study called Methods Advancement in Milk Analysis (MAMA). Fresh and frozen (-20°C) milk samples were assayed to determine effects of storage on endogenous analytes. The source effect (serum vs milk) seen in all 7 analytes indicates that serum should not be used as a surrogate for milk in children's health studies. The authors propose to use these assays in studies to examine relationships between the levels of milk components and children's health. *J Hum Lact.* 23(2):144-156.

Keywords: human milk, cytokines, immunoglobulins, surrogate, biomonitoring

Epidemiologic studies have shown human milk to be a superior food for infants in that it provides optimal nutritional support, immunological benefits, and economic advantages. Breastfeeding has been shown to have protective effects against gastrointestinal (GI) and respiratory infections in the infant, to stimulate the proper development of the infant's immune system, and to decrease the infant's risk of autoimmune disease.^{1,2} A number of endogenous milk components, including hormones, cytokines, and immunoglobulins, may contribute to this development.³

Endogenous components such as cytokines are important in the physiology of the infant and the mother. For example, interleukin-6 (IL-6) has been shown to be crucial for the production of IgA in the mammary gland and possibly in the neonatal gut.⁴ Milk macrophages containing engulfed secretory IgA (sIgA) have been reported in the GI system,⁵ conferring on the infant passive immunity. Tumor necrosis factor- α (TNF- α , an important factor in mastitis) has been associated with β -casein and

whey acidic protein down-regulation in vitro,⁶ as well as differentiation of mammary epithelial cells. IgM has been positively correlated with lactation number or parity in bovines.⁷ IgE status in early life has been linked to development of atopy (including asthma) in later life, as theorized in the "hygiene hypothesis."⁸ Various other immunoglobulins provide passive immunity to the infant during early life stages. Furthermore, maternal hormones have been shown to be positively (prolactin) and negatively (estrogens) correlated with lactation status. A major problem with the ability to compare data across studies is the inconsistency with which the assays are run (various methods), as well as the variation in storage of the sample prior to measurement (fresh or frozen) and the methods of collection (glass or plastic; pumped or manual expression). Furthermore, it would be advantageous to determine breast milk-derived exposure of children to environmental and/or endogenous components of milk in a way that does not require milk (using a surrogate body fluid). However, it is not clear if that is possible using available methods.

The literature on the effect of freezing and thawing on preservation of endogenous components of human milk is limited. To develop milk collection and storage

protocols, as well as to compare the levels of endogenous compounds in blood and milk, the US Environmental Protection Agency (US EPA) conducted a study entitled Methods Advancement for Milk Analysis (MAMA), developed under the auspices of the National Children's Study (NCS). In the MAMA study, milk and serum from lactating women were collected to compare maternal levels of endogenous components in fresh and frozen milk to levels in the blood, either of which could act as a surrogate for estimating infant exposure. A long-term goal of our research is to assess the suitability of breast milk components for use as bioindicators of children's health. For this, baseline data are needed on levels of these components in human milk, changes over time during lactation, and reliable methods for collection and storage of breast milk in epidemiology studies. This report directly compares fresh and frozen breast milk and serum analytes, within and across donor women, at 2 time points during lactation and provides protocols that could be employed in the NCS or other

studies focused on the use of breast milk as an indicator of child nutrition, environmental exposure, or health risk assessment.

Methods

Human Subjects

The use of human subjects in the studies described was approved by the Institutional Review Board of the University of North Carolina's School of Medicine under IRB number 03-EPA-207. Each participant was briefed on the study goals, risks, and inclusion/exclusion criteria and signed an informed consent prior to participation. They also completed a comprehensive questionnaire (administered only at the first visit), designed to gain information on their dietary history, occupational situation, and environmental exposure.

Study Design

The women for the MAMA study were recruited by the Westat recruiting firm, an EPA contractor (Chapel Hill, NC), via newspaper advertisements, university e-mail publications, and fliers distributed to clinicians specializing in women's health or pediatrics. Nonsmoking primiparous or multiparous mothers (first, second, or third child) between the age of 20 and 38 years were recruited for this study. Mothers were not required to exclusively breastfeed for inclusion in this study. The women consented (verbally and in writing) and participated in the MAMA study at the EPA's Human Studies Facility clinic in Chapel Hill, NC, between December 2004 and July 2005.

One aim of the MAMA study was to determine and compare the levels of endogenous components (including cytokines, immunoglobulins, glucose, triglycerides, and hormones) in milk and serum of breastfeeding women. Thirty-four mothers were recruited for this study, and 28 successfully completed all stages of the study (success was determined as being able to provide the sample volumes we required at both clinic visits). The women fasted and refrained from breastfeeding their infant for at least 2 hours prior to collection, and samples were collected between 9:30 am and 2:00 pm. Standardized collection procedures were developed to ensure uniformity of sample and data collection (ie, the samples were collected in the same order for each donor, allowing for the same general amount of time since fasting began for each sample type). The mothers twice collected a total of 3 ounces of milk from both breasts, using a commercially available

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electric breast pump (N = 28 donors; Medela, Inc, McHenry, Ill). For the child's nutritional needs during the study visits, we asked that the mothers bring expressed milk with them to feed their child at the clinic.

Because we were interested in not only endogenous but also environmental components of milk in the MAMA study, we scheduled 2 time points for sample collection that would allow us to begin to understand clearance of those compounds that may be secreted into the milk. We also suspected that the comparison of early milk (that prior to 30 days of lactation) and mature milk (that past 2 months postpartum) would reveal differences in both endogenous and exogenous components. We believed that collection prior to 2 weeks was unrealistic, due to the volume of milk required for the study, and that the child would benefit substantially from that milk. The first visit was between 2 and 7 weeks postpartum, and the second visit was between 3 and 4 months postpartum (n = 31, first visit frozen milk samples; n = 21, second visit frozen milk samples; n < 34 due to assay timing constraints). Milk was expressed into bottles associated with the breast pump, divided into aliquots, and then stored in polypropylene tubes at 4°C or -20°C until the assays were run. Ten fresh milk samples were analyzed (8 second-visit samples and 2 first-visit samples).

The women also donated about 20 mL of blood, which was collected by an EPA nurse via venipuncture of the median cubital vein into heparinized agar-plugged vacutainer tubes. Blood samples were spun at 3000 rpm (revolutions per minute) for 15 minutes at room temperature, and serum was collected for analyses by a contracted laboratory, The Center for Esoteric Testing, Laboratory Corporation of America, Burlington, NC (LabCorp). The circumstance in which the individual samples were collected, including time of day and the amount of milk collected, was recorded in the collection log.

Assay Validation

To develop reliable assays for measurements of cytokines, hormones, and other endogenous components of breast milk, LabCorp performed 4 tasks for each assay: (1) development of a milk sample preparation method, (2) determination of assay precision of repeated measures, (3) evaluation of assay accuracy using spike recovery, and (4) determination of assay linearity using dilutional recovery.

Milk sample preparation. Nonpasteurized frozen human milk (not fit for human consumption, "test milk,"

Wake Med Milk Bank, Raleigh, NC) was used for assay development; it was prepared in 1 of 2 ways. "Mixed milk" preparation is whole milk that had not been separated and had been sonicated only to allow homogenous mixing of components. We refer to the "whey" preparation as the milk from which the top fat layer had been removed, following low-speed centrifugation ($8000 \times g$ for 10 minutes; the pellet was kept for a purpose unrelated to the focus of this article).

Assay precision. In these validations, intra-assay precision was assessed by running 20 replicates of 2 levels of the analyte in question within the same run. Interassay precision was also established by measuring analyte levels for 4 replicates of 2 concentrations of test milk sample per assay (which varied depending on the assay) for at least 5 separate assays/runs ($4 \times 2 \times 5 = 40$ samples). Precision was acceptable when there was less than a 15% coefficient of variation (CV) (chosen by the clinical laboratory based on previous experience).

Accuracy: Spike recovery. Nonpasteurized human test milk was run in triplicate as "blank." In triplicate, 3 known quantities of standard (provided within each individual assay, Table 3) were admixed 50:50 with milk and were analyzed for accuracy based on analyte recovery.

The theoretical concentrations were calculated as follows:

Theoretical Value = (Milk Value + Standard Value)/2, where,

Milk Value = the measured concentration after sample preparation and

Standard Value = the measured result of the standard/high test milk sample.

The theoretical and the actual values were used to calculate percent recovery:

Percent Recovery = (Actual Result/Theoretical Value) * 100

Reproducible accuracies from 80% to 120% were deemed acceptable.

Linearity: Dilutional recovery. To determine the linearity of a dilution recovery, 1 milk sample with a high level of the endpoint of interest was chosen for

each endpoint and assayed at neat and 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, and 10% concentrations, with samples diluted with appropriate materials as stipulated by the assay manufacturer. Theoretical yields were calculated and compared to actual results to give percent recoveries. Assigned concentrations were calculated as a percent of the mean measured value of the 100% concentration. An allowable total error of less than 10.0% was used. The statistical algorithm determined unambiguously whether the data set was linear within the allowable error.

Assay reliability. The data were evaluated using EP Evaluator, Release 3.0 statistical analysis software (David G. Rhoads Associates, Inc, Kennett Square, PA), which calculates the number of results (N), average value (mean), standard deviation (SD), and CV. All data reported in this manuscript came from human milk and serum assays that met the above criteria for linearity, precision, and accuracy.

Serum Measurements

Serum levels of IgG, IgM, IgA, IgE, glucose, triglycerides, estradiol, prolactin, IL-6, and TNF- α were measured for each MAMA study participant according to LabCorp's standard operating procedures for these assays, on the same day as or within 24 hours of their co-paired milk samples. As required for the individual assay protocol, the serum samples for TNF- α and IL-6 were frozen prior to shipping/measurement, whereas all other samples were shipped at 4 °C. Assays were run according to Table 1, and assay sensitivities are reported. Assays were run on single samples, and daily reference controls were used to standardize the individual machines, utilizing Westgard rules for acceptance or rejection of control material results.

Milk Assay Development

Cytokine enzyme-linked immunoabsorbent assays. Whey samples were used in measurements of IL-6, TNF- α , and sIgA. sIgA, IL-6, and TNF- α cytokine levels were measured using enzyme-linked immunoabsorbent assay (ELISA) kits (Immunodiagnostik from Immuno-Biological Laboratories [Minneapolis, Minn], R&D Systems [Minneapolis, Minn], and R&D Systems, respectively). The manufacturer's recommended procedure was followed, using 100 μ L of 1:200 dilution of whey for sIgA, 100 μ L for IL-6, and 200 μ L for TNF- α .

Table 1. Serum Endpoints Measured in the Methods Advancement in Milk Analysis Study

Analyte	Sample Volume (mL)	Current Serum Method	Serum Assay Sensitivity
Prolactin	250	Immunochemi-luminometric assay on Bayer Centaur	0.2 ng/mL
IgG, IgM, IgE, IgA	500	Turbidometric Immunoassay on Roche Integra	2.3 g/L
TNF- α	250	Immunochemi-luminometric assay on DPC Immulite 2000	4.0 pg/mL
IL-6	250	Immunochemi-luminometric assay on DPC Immulite 2000	5.0 pg/mL
Triglycerides	500	Enzymatic Assay on Roche Modular	4.0 mg/dL
Glucose	500	Enzymatic Assay on Roche Modular	2.0 mg/dL
Estradiol	500	Radioimmunoassay from DPC	2.5 pg/mL

Calibration curves were generated using recombinant sIgA, IL-6, and TNF- α standards, and from these curves concentrations of the human milk samples from the MAMA study were extrapolated.

Hormone radioimmunoassay. Estradiol, prolactin, and leptin in human milk were quantified using competitive radioimmunoassays (RIAs). The kit used for prolactin was from Orion Diagnostics IDS (Phoenix, Ariz), the ultrasensitive estradiol kit was from Diagnostic Systems Laboratories (Webster, Tex), and the leptin kit was from Linco Research (St Charles, Mo). Leptin linearity using spike recovery yielded non-linear data, and its measurement in neat milk provided data below the threshold of sensitivity. Thus, leptin was not measured in this study, due to inability to reliably measure leptin levels in milk (CVs and spike recoveries failed to meet the standards set for our assays).

Centrifuge-separated milk samples (500 μ L) were extracted with 4.0 mL diethyl ether and reconstituted with assay diluent prior to testing. Standards or specimens of human milk were assayed for prolactin (25 μ L of whey) and for estradiol (200 μ L of reconstituted extracted sample), pipetted in duplicate, and assayed per the manufacturer's specifications.

Table 2. Milk Endpoints Measured in the Methods Advancement in Milk Analysis Study

Analyte	Sample Preparation	LabCorp Current Method	Milk Assay Level of Detection
sIgA	Whey	Immuno-Biological Laboratories, Inc IBL sIgA ELISA reagent kit	13.4 ng/mL
IL-6	Whey	R&D Systems ELISA D6050 96 well plate IL-6 assay	0.7 pg/mL
Leptin	Mixed Milk & Whey	Linco Research HL-81K 250 RIA Human Leptin	0.5 ng/mL
Prolactin	Whey	Diagnostic Systems Laboratory (DSL) DSL 4500 RIA kit	0.5 ng/mL
TNF- α	Whey	R&D Systems DTA00C TNFa ELISA kit	1.6 pg/mL
Triglycerides	Mixed Milk	Roche Diagnostics Rinder Endpoint (GPO-PAP)	4.0 mg/dL
Glucose	Whey	Roche Diagnostics System P Module (Hexokinase)	2.0 mg/dL
Estradiol	Whey (sample extraction)	Diagnostic Systems Laboratory (DSL) 39100 Estradiol RIA	0.6 pg/mL

The amounts of estradiol and prolactin in human milk samples from the MAMA study were determined against a standard curve generated using recombinant human hormone within the range of 0 to 750 pg/mL for estradiol and 0 to 227 ng/mL for prolactin.

Milk Triglycerides

Results obtained for sonicated milk (diluted 1:10 in 0.9% saline) were compared to results obtained from saponified milk, following a previously described protocol⁹ that used an enzymatic method to measure total glycerol produced after saponification of the milk sample. It was determined that saponification was unnecessary, as the comparison revealed similar results. Triglyceride levels were measured on a Roche Diagnostics Modular automated chemistry analyzer (Indianapolis, Ind), using the serum Roche Diagnostics triglyceride reagent (50 mmol/L PIPES buffer base; 11876023).

Milk Glucose

Glucose was measured in whey (200 μ L) following the procedure of Arthur et al,¹⁰ using a Roche Diagnostics hexokinase-based reagent applied on the Roche Modular automated chemistry analyzer.

Assay Development

Assays for these same compounds measured in serum by LabCorp, except for the Igs (procedures shown in Table 1), were successfully developed for use in breast milk. The milk preparation methods and limit of detections (LOD and sensitivity) are listed in Table 2. The assays for leptin in milk were not successful and are not reported. The values and trends over time, fluid source, and milk storage conditions are reported below.

Statistical Analyses

Data from the MAMA study are presented as mean $\pm$ SEM values. All statistical analyses were done using SAS version 9.1.3 software (SAS Institute Inc, Cary, NC). For each outcome, variable univariate statistics were calculated with SAS Proc Univariate. For each compound, Spearman and Pearson correlations were calculated for all pairs of the concentrations in serum, and fresh and frozen milk, for visits 1 and 2 (Proc Corr); only correlations that showed significance are reported. Additionally, mixed-effects linear models (Proc Mixed) were used to investigate the relationships among fluid type (serum, fresh milk, frozen milk) and first and second visits while adjusting for subject by including subject as a random predictor. For calculations with a minority of the samples above LOD (detectable), only detectable samples were reported (milk TNF- α , milk estradiol, and serum IL-6). However, for calculation of percentiles, a value of "below detection" was used (LOD shown in Tables 1 and 2).

Results

MAMA Donor Characteristics

Detailed characteristics of the study population are provided in Table 3. Sixty-four percent of the samples were collected from mothers who exclusively breastfed at the first visit. Because the questionnaire was administered only at the first visit, this value is not available for the second visit. The ethnicity of the mothers is as follows: of the 34 participants, 29 were Caucasian, 3 were African American, 1 was Asian/Indian, and 1 was Hispanic/Latino. Nine percent of the mothers reported preeclampsia. Six percent of the mothers reported having

Table 3. Characteristics of Study Population

Characteristic	n or 0 ± SEM
Serum first visit, n	34
Second visit, n	30
Frozen milk first visit, n	31
Second visit, n	21
Fresh milk first visit, n	2
Second visit, n	8
Mother's age at first visit, y	30.8 ± 4.7
Infant's age at first visit, wk	5.0 ± 1.7
Second visit, wk	14.0 ± 1.0
Mother's stature at first visit, cm	165.4 ± 7.0
Mother's weight at first visit, kg	78.9 ± 22.5
Breastfeeding events/day	8.6 ± 2.3
Primiparous mothers	17/34

gestational diabetes. Three percent of the mothers reported excessive weight gain with their pregnancy, as informed by their clinician. Evaluation of the correlations of these outcomes and others with those assay levels reported in this study are under way and are outside the focus on these assay validation efforts.

Assay Detection Limits, Linearity, and Precision

The detection limits of the sIgA, IL-6, and TNF- α ELISAs were 1 mg/dL, 0.7 pg/mL, and 1.6 pg/mL, respectively. The within-run precision for sIgA was 4.9% and 3.9%; for IL-6, 3.2% and 2.6%; and for TNF- α , 6.1% and 13.1% (low- and high-level test samples, respectively). The dilutional testing showed that the sIgA, TNF- α , and IL-6 ELISA assays were linear. The sensitivity of the RIAs for estradiol and prolactin was 2.2 pg/mL and 0.5 ng/mL, respectively, and provided linear results in dilutional testing.

In the milk triglyceride assay, linearity studies on the sonicated milk samples indicated that milk samples diluted to 1261 mg/dL or less were within the linear range of measurement on the analyzer. The sample's coefficient of variation was found to be between 2.4% and 4.8% (low- and high-concentration test samples, respectively). In the milk glucose assay, the assay coefficient of variation was found to be between 4.6% and 5.6% (low- and high-test samples, respectively). Dilutional testing provided linear results.

Glucose

Milk glucose values ranged from 5 to 52 mg/dL and were significantly lower than serum levels of 61 to 140 mg/dL (Figure 1) ($P < .0001$). Mean serum glucose levels for visit 1 and visit 2 were similar (86.2 ± 2.2 and 82.3 ± 1.7 mg/dL, respectively) (Figure 1). Mean frozen

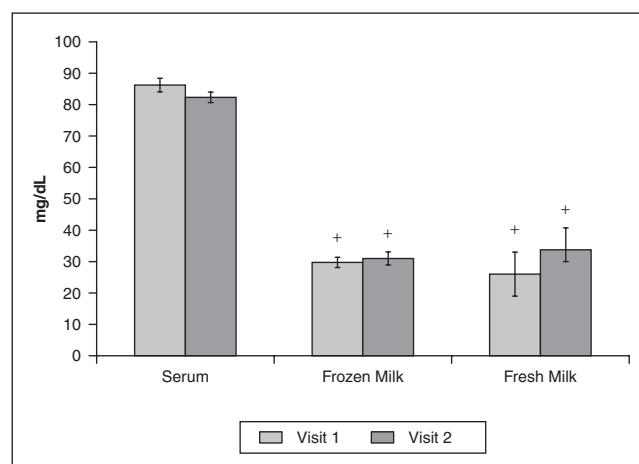


Figure 1. Serum and milk glucose. Error bars represent mean ± SEM. Serum glucose was significantly higher than milk glucose ($P < .05$). Glucose levels were not significantly different between visits.

milk glucose levels were also similar to one another at visits 1 and 2 (29.7 ± 1.6 and 31 ± 2.1 mg/dL, respectively). Mean fresh milk glucose levels were comparable to those in frozen milk. Milk glucose levels did not vary significantly between visits or in fresh versus frozen samples. The similarity between visits 1 and 2 glucose levels, independent of source, suggests that the 2-hour fast was adequate for repeatable results.

Triglycerides

Using a mixed model analysis, a source effect (serum, frozen milk, fresh milk) for triglycerides was detected, with serum levels significantly lower than frozen milk triglyceride levels ($P < .0001$) (Figure 2). Mean frozen milk triglycerides were significantly lower on visit 1 than visit 2 ($P < .05$). Serum values for visits 1 and 2 were correlated ($R = 0.59$, $P = .001$). For visit 2, frozen milk triglycerides correlated well with those of fresh milk ($R = 0.87$, $P = .006$).

IgA/sIgA

IgA was measured in serum (observed range 98-570 mg/dL), and the secretory form (sIgA) was measured in milk (observed range 18-232 mg/dL). Serum IgA ($R = 0.98$, $P < .001$) and frozen milk sIgA ($R = 0.64$, $P = .003$) values were correlated between visits 1 and 2. When comparing means within sources (serum, fresh milk, or frozen milk), there was no significant difference between visits. Mean serum IgA levels were 234.9 ± 19.7 and 246.1 ± 21.6 mg/dL for visits 1 and 2 (Figure 3), respectively. Frozen milk sIgA levels for visits 1 (65.3 ± 7.7 mg/dL) and 2 (57.1 ± 6.2 mg/dL) were not

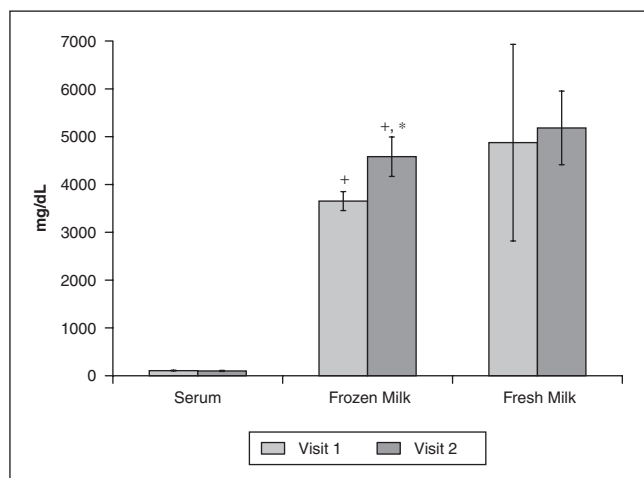


Figure 2. Serum and milk triglycerides. Error bars represent mean $\pm$ SEM. Serum triglycerides were significantly lower than milk triglycerides $^+(P < .05)$. Visit 2 frozen milk triglycerides were significantly higher than visit 1 frozen milk triglycerides $^{*}(P < .05)$. Frozen and fresh milk had similar triglyceride concentrations.

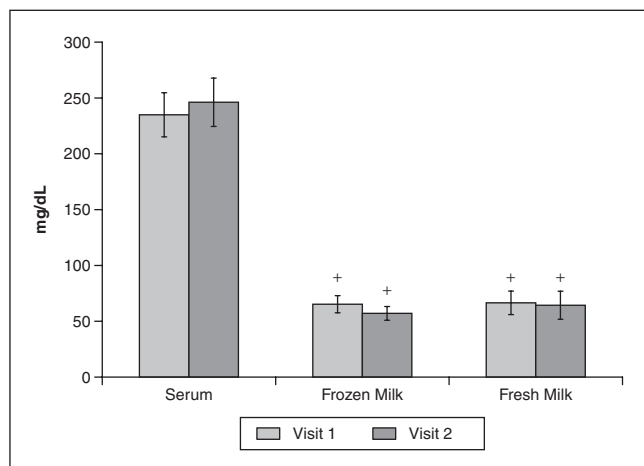


Figure 3. Serum IgA and milk sIgA. Error bars represent mean $\pm$ SEM. Serum IgA was significantly higher than milk IgA $^+(P < .05)$.

significantly different ($P > .05$) from fresh milk values from visits 1 (66.5 ± 10.5 mg/dL) and 2 (64.4 ± 12.6 mg/dL). Serum IgA levels were significantly higher than frozen or fresh milk sIgA values ($P < .0001$).

IgE

IgE was measured only in serum (not shown). The expected range, as reported by the clinical laboratory, was 0 to 158 IU/mL. Four MAMA study samples (3

individuals) fell outside of this range. One participant in the study had very high levels (> 900 IU/mL) on both visits. All other samples were less than 230 IU/mL. On the first visit, 4 donors were below detection limit. On the second visit, 1 donor remained below the detection limit. The correlation between visits was high ($R > 0.94$, $P < .001$) with Pearson or Spearman correlation. Mean serum IgE was 70.4 ± 30.7 IU/mL and 67.4 ± 31.3 IU/mL (visits 1 and 2, respectively).

IgG

IgG was measured in serum and not milk (not shown). IgG had a range of 512 to 1510 mg/dL (700–1600 mg/dL reference clinical range), but 4 samples (3 donors) fell below this normal range. IgG was significantly higher at visit 2 than visit 1 ($P < .001$). Mean serum IgG levels were 1039.5 ± 42.1 mg/dL (visit 1) and 1143.9 ± 43.8 mg/dL (visit 2).

IgM

IgM was measured only in maternal serum (not shown), and both Spearman and Pearson's tests show high correlation between IgM from visit 1 and visit 2 ($R = 0.93$, $P < .0001$), demonstrating highly reproducible values by a single mother over time. Mean serum IgM levels were 145.8 ± 11.6 mg/dL for visit 1 and 147.5 ± 13.6 mg/dL for visit 2.

Prolactin

Prolactin was measured in both milk and serum (Figure 4). Mean serum prolactin levels (Figure 4) were higher than the normal clinically reported range of 2.8 to 29.2 ng/mL for nonlactating adults. In our study, serum levels ranged from 7 to 156 ng/mL, even though most women had refrained from nursing their infants a similar length of time prior to donating fluid samples. Mean milk prolactin levels did not vary to the same extent as serum levels. When evaluating samples by source, fresh and frozen milk samples were significantly different. Fresh and frozen milk prolactin levels were significantly lower than serum levels of prolactin ($P < .05$). Frozen milk and serum prolactin levels at collection 1 were significantly higher than frozen milk and serum prolactin at collection 2 ($P < .05$ and $P < .01$, respectively), as expected.

Estradiol

Estradiol was measured in both serum and milk samples (Figure 5). The observed range for serum estradiol

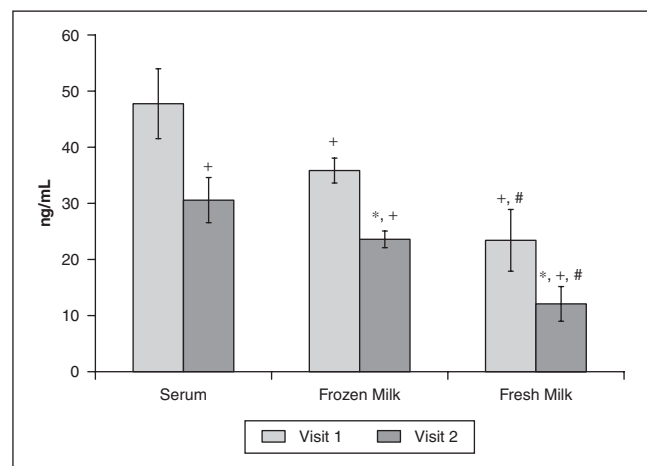


Figure 4. Serum and milk prolactin. Error bars represent mean $\pm$ SEM. Prolactin levels significantly decrease between visit 1 and visit 2 in all media $^+(P < .05)$. Fresh milk prolactin levels were significantly lower than frozen milk prolactin levels $^{\#}(P < .05)$. Serum prolactin was significantly higher than milk prolactin $^*(P < .05)$.

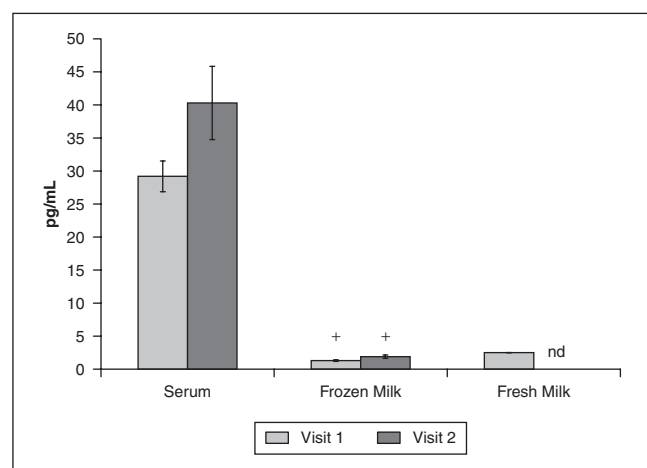


Figure 5. Serum and milk estradiol. Error bars represent mean $\pm$ SEM. Most frozen milk values were below the level of detection (LOD; 28/31 and 13/21 were $< \text{LOD}$ for frozen milk at visits 1 and 2, and 1/2 and 8/8 were $< \text{LOD}$ for fresh milk at visits 1 and 2, respectively). Serum estradiol was significantly higher than frozen milk estradiol $^+(P < .05)$. Serum estradiol at visit 2 was significantly increased over serum visit one ($P < .05$). nd = nondetectable samples.

was LOD to 130 pg/mL; the normal clinical serum range for nonlactating women was 18 to 528 pg/mL. The limit of detection was 15 pg/mL in serum and 2.2 pg/mL in milk. Mean serum estradiol levels were 29.2 ± 2.3 (visit 1) and 40.3 ± 5.5 pg/mL (visit 2) (Figure 5). Most

milk values were below the level of detection (28/31 and 13/21 from visits 1 and 2, respectively, were below LOD for frozen milk). Detectable estradiol in frozen milk ranged from 2.6 to 4.2 pg/mL at visit 1 and 2.3 to 4.5 pg/mL at visit 2. Serum estradiol levels were significantly greater than those of frozen milk ($P < .001$). Pairwise testing, using detectable estradiol levels, showed serum estradiol levels were significantly higher at visit 2 than visit 1 ($P < .05$).

TNF- α

TNF- α was measured in both serum and milk (Figure 6). In serum, the normal clinical range of TNF- α is from 0 to 8.1 pg/mL. Five MAMA samples were above the normal range (4 donors). Serum TNF- α levels were detectable in 27/34 visit 1 and 28/30 visit 2 samples, and ranged from LOD to 17.9 pg/mL. Visits 1 and 2 serum TNF- α levels were similar, 6.0 ± 0.3 pg/mL (visit 1) and 6.5 ± 0.5 pg/mL (visit 2), and were found to be correlated ($R = 0.49$, $P = .006$). Unlike serum, milk TNF- α was detectable in only a small fraction of the samples (10/33 visit 1 frozen, 0/2 visit 1 fresh, 9/21 visit 2 frozen, and 2/8 visit 2 fresh). In the samples above LOD, mean milk TNF- α concentrations were 16.9 ± 5.4 pg/mL (visit 1 frozen, $n = 10$), 14.7 ± 5.9 pg/mL (visit 2 frozen, $n = 9$), nondetectable (visit 1 fresh, $n = 2$), and 20.1 ± 11.6 pg/mL (visit 2 fresh, $n = 2/8$). The individual measurement range of frozen milk TNF- α values were from nondetectable to 64.0 pg/mL, which was well above the levels found in serum. Mean detectable frozen milk TNF- α was significantly higher than serum TNF- α levels ($P < .0001$).

IL-6

Serum and milk IL-6 concentrations were determined (Figure 7). The number of serum samples with detectable levels of IL-6 was 33% (visit 1) and 20% (visit 2), whereas IL-6 was detectable in the majority of the milk samples (100% visit 1 fresh, 96% visit 1 frozen, 68% visit 2 fresh, 75% visit 2 frozen). Detectable serum mean IL-6 values were 2.5 ± 0.1 (visit 1, $n = 11$) and 2.8 ± 0.6 pg/mL (visit 2, $n = 6$), well within the clinical reference range of 0 to 14 pg/mL. Frozen milk IL-6 levels were significantly higher than those in serum, indicating a source effect ($P < .001$). Serum IL-6 levels spanned the range of LOD to 6 pg/mL, whereas frozen milk IL-6 levels were from nondetectable to 76.8 pg/mL (fresh milk LOD-31.3 pg/mL).

Because it is difficult to compare serum and milk levels of these endogenous components in the current

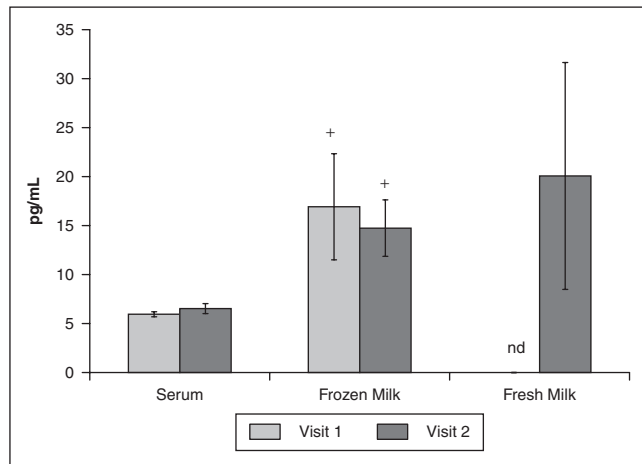


Figure 6. Serum and milk TNF- α . Error bars represent mean $\pm$ SEM. Detectable milk TNF- α levels were significantly higher than serum TNF- α levels ⁺($P < .05$). TNF- α was detectable in 27/34 (visit 1) and 28/30 (visit 2) serum samples, in 10/31 (visit 1) and 9/21 (visit 2) frozen milk samples, and 0/2 (visit 1) and 2/8 (visit 2) fresh milk samples. nd = nondetectable samples.

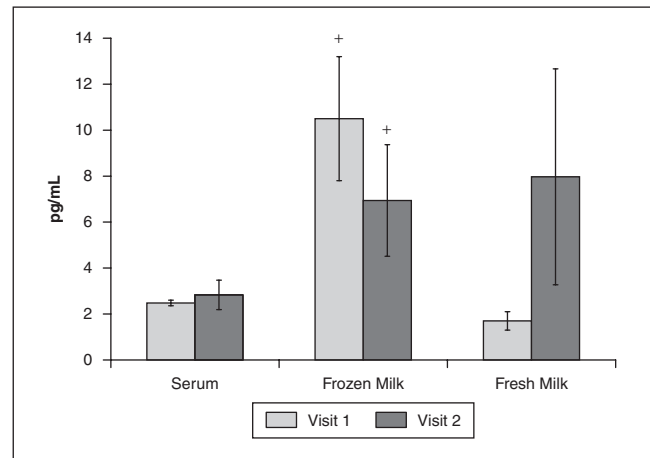


Figure 7. Serum and milk IL-6. Error bars represent mean $\pm$ SEM. Serum IL-6 was significantly lower than frozen milk IL-6 ⁺($P < .05$). IL-6 was detectable in 11/34 (visit 1) and 6/30 (visit 2) serum samples, in 30/31 (visit 1) and 19/21 (visit 2) frozen milk samples, and 2/2 (visit 1) and 6/8 (visit 2) fresh milk samples. Serum and fresh milk IL-6 were not significantly different.

literature, especially in humans, we have included the range of values detected in the MAMA study in Table 4. Furthermore, the 20 to 80 percentile range, or possibly an expected range, of these compounds in lactating, fasting, healthy women is shown for reference purposes.

Discussion

We report here the development of assays for use in breast milk with comparisons made to the serum levels of the same endogenous compounds, collected at 2 different times during lactation, as part of the US EPA MAMA study. The nutritive and immunological protective factors provided by breast milk and how these components can be affected by environmental factors may be measured in future studies such as the NCS and other studies focused on children's health. Thus, a crucial part of the MAMA study is the development of repeatable assays for endogenous components using minimal volumes of milk. Another goal was to compare the reported levels of endogenous components of milk to those in serum for decision making on whether to analyze the biomarker of interest in serum or milk, and whether storage (ie, freezing) affects those levels.

We found that the source from which a cytokine or endogenous component is measured affects the level of that analyte detected in 7 of the analytes tested. Serum levels of glucose, IgA, prolactin, and estradiol were

greater than milk levels of these components, whereas milk levels of triglycerides, IL-6, and TNF- α were substantially higher than those in serum. Therefore, these data suggest that serum not be used as a surrogate for determination of these analytes in breast milk. Successful assays were developed for the endpoints measured in this study, and frozen milk was an adequate media for our analytes. Alternatively, some components may degrade or be liberated during frozen storage (storage effect). Storage effects revealed that prolactin (first and second visits) was greater in frozen versus fresh milk. Furthermore, if future studies wish to evaluate cytokine delivery to the child, milk cytokine levels would need to be determined, as TNF- α was detectable in the majority of serum samples but only in a minority of the milk samples, whereas the converse was true for IL-6, which had detectable levels in the majority of its milk samples. Time of lactation effects in the study showed that visit 2 levels were significantly higher than visit 1 levels for serum estradiol, IgG, and frozen milk triglycerides; conversely, visit 1 levels were greater than visit 2 levels for prolactin in serum and frozen milk. Effects discernable in frozen milk but absent in fresh milk may be due to a smaller sample number in the fresh milk subpopulation.

The data from the MAMA study allowed us to determine if various endogenous endpoints are found at detectable levels in biological media (milk and serum).

Table 4. Selected Reference Percentiles of Serum and Breast Milk Endogenous Endpoint Concentrations for Lactating Women in the Methods Advancement in Milk Analysis Study

<i>Endogenous Endpoint, units</i>	<i>Minimum Value*</i>	<i>Maximum Value</i>	<i>Range 20th-80th Percentile*..**</i>
Serum glucose, mg/dL	61	140	76-91.5
Serum IgA, mg/dL	98	570	153.5-322
Serum IgG, mg/dL	512	1520	830-1322.5
Serum IgM, mg/dL	43	445	93-193
Serum IgE, IU/mL	LOD	1040	4-88.5
Serum triglycerides, mg/dL	34	270	62.5-178
Serum prolactin, ng/mL	7.1	156.2	18-51.85
Serum estradiol, pg/mL	LOD	130	21-46
Serum TNF- α , pg/mL	LOD	17.9	4.5-7.05
Serum IL-6, pg/mL	LOD	6	LOD-2.3
Frozen milk estradiol, pg/mL	LOD	4.5	LOD-2.7
Frozen milk prolactin, ng/mL	7.22	64.97	24.3-37.2
Frozen milk sIgA, mg/dL	18	232	39.5-76
Frozen milk IL-6, pg/mL	LOD	76.8	3.2-11
Frozen milk glucose, mg/dL	5	52	25-37.5
Frozen milk triglycerides, mg/dL	900	8580	3495-4810
Frozen milk TNF- α , pg/mL	LOD	21.84	LOD-13.8
Fresh milk estradiol, pg/mL	LOD	2.5	ND
Fresh milk prolactin, ng/mL	LOD	28.9	9.1-21.5
Fresh milk sIgA, mg/dL	19	142	41-72
Fresh milk IL-6, pg/mL	LOD	31.3	LOD-5.6
Fresh milk glucose, mg/dL	17	51	25.5-43
Fresh milk triglycerides, mg/dL	1190	8150	3335-7300
Fresh milk TNF- α , pg/mL	LOD	31.65	LOD-8.5

*LOD = level of detection.

**ND = not determinable.

A high percentage of the donor samples yielded detectable levels in the majority of the assays used. However, some immunity and cytokine markers, serum IL-6, and milk TNF- α were not detectable in a majority of donors, possibly because the mothers were healthy (low inflammation). Also, estradiol was not detectable in a majority of milk samples, possibly because of the fact the women donating early milk samples had likely not regained menstrual cyclicity.

IL-6 is a cytokine produced by adipocytes, epithelial cells,^{11,12} mononuclear cells,¹³ leukocytes,¹⁴ lymphocytes,¹⁵ and macrophages.¹⁶ IL-6 induces sIgA production⁴ and IgM production¹⁷ and activates T-cells. In human¹⁸ and bovine¹⁹ mammary epithelial cells, IL-6 has been shown to inhibit cellular proliferation, and lactoferrin (a milk protein) is able to inhibit LPS-dependent induction of IL-6 mRNA,²⁰ keeping IL-6 production in balance in the mammary gland. Weight loss in nondiabetic obese women is associated with a significant decrease in IL-6 production.²¹ Therefore, the variable levels of IL-6 in MAMA study milk samples may be related to weight loss in some but not all women in the early postnatal period.

The mean IL-6 concentration in milk (fresh and frozen) and the range of values in this study were comparable to other reports in the literature²²⁻²⁴ that also measured IL-6 using an ELISA. Serum concentration of IL-6 in a control population was reported to be 2.55 ± 0.3 pg/mL,²⁵ which was similar to the levels reported (Figure 7) in individuals in the MAMA study who had detectable levels of IL-6 (33% first visit, 20% second visit). Goebels et al²⁶ defined normal serum IL-6 levels to be in the range of 1.5 to 9 pg/mL. Similar to Sone et al's²⁷ work, we found that milk IL-6 levels were detectable in a higher percentage of milk samples (fresh and frozen) than serum samples. Milk IL-6 levels have been reported to increase after storage at 4°C and decrease after storage at -20°C.²⁸ We found no difference between frozen and fresh milk IL-6 levels (Figure 7), although our fresh samples were run more quickly than those previously reported,²⁸ to mimic the routine that a nursing mother may use for her child (24 hours or less of 4°C refrigeration). Furthermore, our results reaffirmed Saito et al's¹³ finding of higher levels of IL-6 in frozen milk than in serum, but our fresh milk and serum IL-6 levels were not significantly different. Our results suggest that frozen milk is a preferred media in which to measure IL-6 for future studies.

Increased milk TNF- α production has been associated with mastitis in cows²⁹ and humans.²⁴ A plethora of milk cells have been reported to produce TNF- α , including leukocytes,^{15,28} epithelial cells,¹¹ and macrophages,^{16,27} and milk TNF- α is found secreted and not intracellularly.²³ TNF- α is capable of stimulating IL-6 production in milk mononuclear leukocytes,^{30,31} which then feeds back to inhibit further TNF- α production.³² TNF- α has been reported to exist mainly in a bound form in milk,³³ but we saw little difference in fresh and frozen

milk TNF- α levels in MAMA study samples (freezing had a minor contribution). TNF- α has been shown to down-regulate β -casein and whey acidic protein production (major milk proteins⁶), and it has also been reported to modulate mammary ductal growth and differentiation.³³ In our study of self-reported healthy women, detectable levels of TNF- α were found in a minority of milk samples (both fresh and frozen) but in a majority of the serum samples. Goebels et al²⁶ defined normal serum levels for TNF- α as 3-27 pg/mL, which was consistent with reports by Hirokawa et al³⁴ and with our observed values. In our study, serum levels were significantly lower than detectable milk levels of TNF- α . Others have reported that the activation state of the TNF- α producing cells in milk versus blood is different, with milk cells already existing in the activated state and blood cells existing in a quiescent state,²⁷ which may explain the difference in scale of detectable TNF- α levels in lactating women. Because of the differences in both the percentage of women with detectable TNF- α and the higher levels in milk than serum, we recommend measuring TNF- α in frozen milk in future studies on milk composition.

Glucose is an important biomarker for children's and adult's health. This specific endpoint was chosen because it has relevance to nutritional status of the child and may be related to the obesity/diabetes health biomarkers chosen in future longitudinal health studies. Because diet trends affect glucose levels, our collection procedures involving fasting prior to collection were designed with this in mind. Furthermore, we specifically chose to evaluate glucose instead of lactose in milk so that the levels between milk and serum could be compared. Serum glucose levels were significantly higher than milk glucose levels. Glucose levels did not change between visits, demonstrating that the 2-hour period of fasting selected for this study was adequate to produce repeatable results and that milk glucose concentrations are not related to milk volume, as the amount of milk produced by these women at 3 to 4 months was increased from that at about 4 to 5 weeks. Glucose levels in fresh and frozen milk were not significantly different. However, serum was not an adequate surrogate for milk glucose.

Secretory IgA composes greater than 90% of the immunoglobulin population in milk.³⁵ However, infants are not able to produce their own sIgA until 4 to 12 months of age, and milk macrophages containing sIgA⁵ that can be released in the gut provide a neonatal source of IgA. Breastfed infants have increased levels of IgA

versus bottle-fed infants.^{36,37} Ammann and Stiehm³⁸ showed that colostral levels of sIgA in milk are higher than transitional milk. Comparison of 2 noncolostrum fluids in the MAMA study showed that both milk sIgA and serum IgA levels show no change over time, and IgA and sIgA were detectable in all serum and milk samples measured. Because serum levels of IgA were 5-fold higher than milk levels of sIgA (fresh or frozen), we recommend against using serum to predict milk sIgA levels in future studies. Pasteurization performed by US milk banks has been documented to decrease milk sIgA levels.³⁹ In our study, milk sIgA is not affected by cold storage temperature (4°C and -20°C), corroborating Liebhaber et al's⁴⁰ findings.

The National Research Council's review of hormonally active agents in the environment recommended further investigation into human exposure to natural and anthropogenic hormonally active agents be conducted to determine relative contributions of estrogen equivalents.⁴¹ In addition to those endocrine active compounds, the hormonal components of combined oral contraceptives (COCs), estrogens, and potentially progesterone have been shown to pass into breast milk.^{42,43} The World Health Organization (WHO) does not recommend the use of COCs during early lactation, unless other more appropriate methods are neither available nor acceptable.⁴⁴ COCs diminish the quantity of milk and decrease the duration of lactation^{45,46} but have not been shown to affect the growth of the child.⁴⁷ The results of various studies, including one by the WHO in Thailand and Hungary, found a 41% decreased volume of milk production in COC users.⁴⁴ There have been reports of the feminizing of breastfed male infants by mothers who take COCs.⁴⁸ The information in the MAMA study on use of oral contraception cannot discern all women who used COCs, as the questionnaire inquiring about COC use was administered only at visit 1. In this study, only 17% of frozen and 10% of fresh milk samples had detectable estradiol levels, and the milk levels of estradiol are 10-fold lower than the serum levels, indicating little if any transfer of endogenous estrogen to the infant. Estradiol values had a wider range in women the further postpartum they were, suggesting that the women began cycling again or COC use increased between visits 1 and 2; this trend is evident in visit 2, which has significantly higher mean serum estradiol values than visit 1. In future studies, collecting more information on COC use and cyclicity is recommended for better interpretation of data.

In the MAMA study, prolactin levels in milk decreased between visit 1 and visit 2 in all media (serum, frozen milk, and fresh milk). It is well established that prolactin levels increase during late pregnancy and early lactation, as nursing stimulates prolactin release from the pituitary, to maintain milk production.⁴⁹ The levels then decrease as lactation is established. As the women in this study consistently donated blood before breast milk, the moderate levels detected were anticipated (no large spikes in serum due to lactation-induced prolactin surges).

In this study, serum IgE levels were similar for a mother between visit 1 and visit 2, unexpectedly, even as the study progressed in its second visit into allergy season. Serum IgM levels were detectable in all samples and, like IgE, showed correlation in mothers between visit 1 and visit 2. Differing from this, serum IgG levels increase significantly between visit 1 and visit 2, possibly as the mothers recover from pregnancy-induced immunosuppression. The other serum immunoglobulins may recover to baseline levels after pregnancy more quickly than IgG. Immunoglobulins other than IgA make up less than 10% of total milk immunoglobulins and thus were not measured in that body fluid, but these Ig outcomes will be evaluated with IgA in our future analyses.

Each type of assay developed has distinct characteristics that may pose an advantage or act as a detriment, depending on the endpoint measured and the media in which the assay is performed. Cytokines, immunoglobulins, hormones, and nutritive factors in milk are essential for an infant's development and were detected without problem in both milk and serum. For the purpose of risk assessment or comparing outcomes across studies, the methods reported here will allow for reproducible milk analysis using small sample volumes. These biomarkers may be useful in determining the role of endogenous milk components in either the health or anomalous outcomes in breastfed children, or for establishing the extent that an environmental exposure alters milk composition. The effects of external signals or chemicals on the normal composition of milk are not known, nor do we understand what effects milk composition changes would have on a child's health. For 7 of the measured analytes in our study, we obtained significantly different results from milk and serum for the same endpoint, demonstrating the importance of monitoring milk content as it relates to children's health. Multiple analyses over time also are critical to draw conclusions in milk studies.

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Resumen

La leche materna es la principal fuente de nutrición que contiene muchos componentes endógenos que pueden impactar el desarrollo infantil. El objetivo de este estudio fue desarrollar pruebas confiables para componentes endógenos seleccionados de la leche materna y para comparar los niveles de estos en la leche y el suero colectados de la misma madre durante la lactancia (2-7 semanas y 3-4 meses). Se desarrollaron pruebas confiables para glucosa, IgA secretora, interleukina-6, factor de necrosis de tumor-alfa, triglicéridos, prolactina y estradiol de participantes en un estudio de la Agencia de Protección del Medio Ambiente (EPA) en Estados Unidos llamado Métodos Avanzados en el Análisis de la Leche Materna (MAMA). Se analizaron muestras de leche fresca y congelada (-20°C) para determinar los efectos de almacenamiento en los componentes endógenos analizados. Comparando los 7 componentes analizados en suero y leche se concluye que el suero no se debe usar como alternativa de la leche en estudios de salud infantil. Proponemos usar estas pruebas en estudios para examinar la relación entre los niveles de los componentes y la salud infantil.