Anti-Cytomegalovirus Activity in Human Milk and Colostrum From Mothers of Preterm Infants

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Manuela Donalisio[§¥], Massimo Rittà[§¥], Paola Tonetto[f], Andrea Civra[§], Alessandra Coscia[f], Marzia Giribaldi[#,£], Laura Cavallarin[#], Guido E. Moro[¤], Enrico Bertino[f], David Lembo[§*].

[§]*Laboratory of Molecular Virology, Department of Clinical and Biological Sciences, University of Turin, Italy*

[f]*Neonatal Intensive Care Unit, Department of Public Health and Pediatrics, University of Turin, Turin, Italy*

[#]*Consiglio Nazionale delle Ricerche-Istituto di Scienze delle Produzioni Alimentari, Grugliasco (To), Italy*

[£]*Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria, Centro di ricerca in ingegneria e trasformazioni agroalimentari, Turin, Italy*

[¤]*Italian Association of Human Milk Banks, Milan, Italy*

*Corresponding author: Prof. David Lembo

Department of Clinical and Biological Sciences

University of *Torino*, S. Luigi Gonzaga Hospital

Regione Gonzole, 10

10043, Orbassano, *Torino*, Italy

Phone: +39 011 6705484

Fax:+39 011 2365484

E-mail: david.lembo@unito.it

Declaration of Funding Source. This work was supported by a donation from Italian Association of Human Milk Banks (AIBLUD)

*Conflicts of interest.*There is no conflict of interest associated with the authors of this paper.

NUMBER OF FIGURES: 3

NUMBER OF TABLES: 1

SUPPLEMENTAL DIGITAL CONTENT (SDC): 3

Roles and responsibilities. D.L., P.T., G.M. and E.B. designed research; P.T., Alessandra C. provided essential materials (collected mothers' milk samples); M.D., M.R., Andrea C., L.C., M.G., conducted research; D.L., M.D., M.R. analysed results or performed statistical analysis; D.L., M.D., M.R., P.T., Alessandra C. wrote paper; D.L. had primary responsibility for final content.

[¥] M.D. and M.R. contributed equally to this work.

ABSTRACT

Objectives. This study aimed to investigate the anti-CMV activity of milk from seropositive and seronegative mothers of preterm infants and to analyze its changes throughout the different stages of lactation and after Holder pasteurization, a procedure adopted by donor human milk banks.

Methods. Eighteen mothers of preterm infants were enrolled in the study. Colostrum, transitional milk and mature milk samples were collected and tested for anti-CMV activity. Depletion of IgA from milk samples was carried out by Jacalin resin. Pools of milk samples were pasteurized according to Holder technique.

Results. All samples were endowed with anti-CMV activity, although to a different extent. In CMV IgG-positive mothers, colostra were significantly more active than the transitional milk and mature milk samples. Moreover, they were more potent than colostra from seronegative-mothers. IgA depletion in colostra from IgG-positive mothers resulted in a partial loss of anti-CMV activity. Holder pasteurization significantly reduced the antiviral activity.

Conclusions. Human milk is endowed with anti-CMV activity and its potency may vary depending on the stage of lactation and the serological status of the mother. This biological property could partially neutralize CMV particles excreted in the milk of CMV IgG-positive mothers thus reducing the risk of transmitting infectious viruses to the infant.

KEYWORDS: antiviral activity; Holder pasteurization; immunoglobulins A.

What is Known

- Human milk is highly beneficial in preterm infants but they are particularly vulnerable to human cytomegalovirus (CMV) infections potentially transmitted by the milk of CMV-seropositive mothers
- Holder pasteurization abrogates the risk of infection but affects some nutritional, trophic and immunologic properties of human milk

What is New

- Colostrum, transitional and mature milk from mothers of preterm infants are endowed with anti-CMV activity, although to a different extent
- Colostrum from CMV-seropositive mothers is the most potent but its antiviral activity is reduced by Holder pasteurization
- This study supports the use of unpasteurized colostrum even in very preterm infants from CMV-seropositive mothers

Introduction

The benefits of human milk are mediated by multiple nutritional, trophic and immunological components, able to promote infant's growth, maturation of its immature gut and also confer protection against infections (1,2). Among the immunological factors, maternal antibodies, lactoferrin, lysozyme, cytokines and lipidic compounds have been reported to exert antimicrobial and antiviral effects *in vitro* and contribute to control infections in neonates (3–6). If mother's own milk is unavailable, donor human milk from human milk banks is considered the next best alternative (7–10). Despite the nutritional and health benefits of breast milk, breastfeeding represents a main mother-to-child transmission route of several infections including the postnatal human cytomegalovirus (CMV) infection. Although this route of CMV transmission is not clinically relevant in term newborns, who are usually asymptomatic, the issue of CMV infection is of great concern for preterm infants (11,12). For this reason, the heat treatment of donor human milk by Holder pasteurization is an effective strategy to prevent CMV transmission to preterm infants (13).

CMV is commonly excreted in breast milk from seropositive women, and several factors including the extremely low birth weight or gestational age at birth (12), co-morbidities (14,15) and the precocity of the infection (16) increase the risk of disease after transmission of CMV. However, meta-analyses revealed low rates of symptomatic disease after transmission of CMV via breast milk to the preterm infant (17,18). This observation points towards a protective effect of some breast milk components against CMV infection to the breastfed infants (19–22). In this context, the main purposes of the present study were to evaluate the anti-CMV activity of breast milk from mothers of preterm infants, and analyze changes of anti-CMV activity throughout the different stages of lactation. Further aims of this study were to investigate the influence of maternal CMV serological status on anti-CMV properties of breast milk and the contribution of immunoglobulin A (IgA) to this antiviral activity. Finally, the study addressed the impact of Holder pasteurization on the antiviral properties of human milk.

Methods

Milk samples. Eighteen healthy mothers admitted to Sant'Anna Hospital (Città della Salute e della Scienza di Torino) for preterm delivery were enrolled in the study between October 2015 and December 2015. The study was approved by the local ethical committee; parents signed written informed consent. Colostrum (days 1-5 postpartum), transitional milk (days 6-14 postpartum) and mature milk (beyond day 15 postpartum) samples were longitudinally obtained. Samples were collected by an electric breast pump in disposable sterile polypropylene BPA-free bottles, in order to minimize the possibility of contamination, and immediately aliquoted and stored at -20°C until use. After freezing of samples, the aqueous fractions (defatted milk) were obtained by centrifugation of whole milk samples at 10,000 g for 1 hour at 4°C.

Cells. Human Foreskin Fibroblasts (HFF-1) (ATCC® SCRC-1041) at low-passage-number (less than 30) were grown as monolayers in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, Saint Louis, MO, U.S.A.) supplemented with 15% heat inactivated foetal bovine serum (FBS) (Sigma-Aldrich) and 1% antibiotic solution (Penicillin-Streptomycin™, Sigma-Aldrich).

Virus. A bacterial artificial chromosome (BAC)-derived HCMV strain Towne incorporating the green fluorescence protein (GFP) sequence was propagated on HFF-1 (23). CMV titres were determined on HFF-1 cells by plaque assay (Supplemental Digital Content Text).

CMV inhibition assay. Antiviral activity of individual milk fractions was determined by viral inhibition assay on HFF-1. Cells were seeded in 96-well plates at a density of 5.0×10^3/well in 100 µl of DMEM supplemented with 10% FBS. The next day, milk samples were serially diluted in DMEM medium from 1/1 to 1/4096 parts, incubated with constant amount of GFP-coding CMV (1000 PFU/well) at a multiplicity of infection (MOI) of 0.1 for 1 h at 37°C, and 100 µl of mixture was inoculated on sub-confluent HFF-1 cells for 2 hours at 37°C, 5% CO_2. After three washing with DMEM medium, the monolayers were overlaid with 1.2%-methylcellulose DMEM medium with 2% FBS (100 µl). After 5-day-incubation at 37°C 5% CO_2 atmosphere, CMV infected cells were

visualized as green fibroblasts using fluorescence microscopy and counted. Results were reported as percentages of fluorescent cells in comparison to controls. The inhibitory dilution of milk samples that reduced CMV infectivity by 50% (inhibitory dilution-50) was calculated by using the program PRISM 4 (GraphPad Software, San Diego, California, U.S.A.) to fit a variable slope-sigmoidal dose–response curve. All experiments were conducted in duplicate.

Cell viability assay. Cell viability was assessed using the MTS assay as described in Cagno et al. (24) (Supplemental Digital Content Text). The effect of breast milk fractions on cell viability at different dilutions was expressed as a percentage of absorbance values of treated cells compared with those of cells incubated with culture medium alone. The 50%-cytotoxic dilutions (CD_{50}) and 95% confidence intervals (CIs) were determined with Prism 4 software.

Jacalin-based Immunoglobulin A Depletion. Based on the suggestion that milk immunoglobulins may contribute to antiviral activity of colostrum, we evaluated the anti-CMV activity of colostra after removal of sIgA, the major immunoglobulin class present in the first stage of lactation (25). Jacalin is a plant-derived lectin that binds glycans in the hinge region of human IgA. A depletion technique based on Jacalin ability to specifically bind human secretory and serum IgA was used (20,26). Briefly, defatted colostrum underwent IgA depletion by gravity-flow affinity and size exclusion chromatography assay in polystyrene columns (Thermo Scientific, IL, U.S.A.) filled with Jacalin-agarose gel slurry (Immobilized Jacalin, Thermo Scientific, IL, U.S.A.). Column eluates were used for IgA quantitation and antiviral assays. Total amounts of IgA in defatted colostrum were quantified by enzyme-linked immunosorbent assay (Human IgA ELISA Kit; Abcam, Cambridge, U.K.) prior and after depletion. Results were reported as mean of two determinations.

Holder pasteurization of milk samples. To investigate the impact of heat treatment on antiviral properties of human milk, two pools of breast milk, each one from three preterm CMV-IgG positive (CMV-IgG+) mothers, were obtained (lactational stages: 8 – 27 days after delivery). The milk sample pools were pasteurized according to Holder technique (62.5°C for 30 minutes) by a HM pasteurizer

device (Metalarredinox, Italy) (27) at "Donor Human Milk Bank" at Città della Salute e della Scienza di Torino, Regina Margherita Hospital, Turin (28) (Supplemental Digital Content Text).

Statistical analysis. Statistical analysis was performed using Student's t-test, ANOVA Analysis of variance or F-test, as reported in legends of figures, on GraphPad Prism version 4.00 software. Significance was reported for p-value <0.05.

Results

Anti-CMV activity of human milk. The study group included 18 mothers of preterm infants admitted to Sant'Anna Hospital of Turin (Città della Salute e della Scienza di Torino). Gestational ages ranged from 23+3 to 32+0 (week+day). Out of 18 mothers, 12 were CMV-IgG+, and 6 were CMV-IgG-negative (CMVIgG-) within 3-month pre-delivery serologic determinations. The main clinical characteristics of the study group are reported in Table 1. Preliminary experiments were conducted to determine whether whole milk or its aqueous fraction was the most appropriate biological matrix for in vitro assays. As reported in Supplemental digital content Text, both whole milk and aqueous fraction matrices are endowed with similar antiviral activity but the lower impact of the aqueous fraction on cell viability prompted us to define it as the preferred biological matrix (Figure, Supplemental Digital Content 1). A first goal of our study was to investigate the potential anti-CMV activity of breast milk and variations in inhibitory activities according to different stages of lactation. Individual results are reported in Figure, Supplemental Digital Content 2, where the antiviral activity was expressed as the inhibitory dilution-50, i.e. the dilution of milk sample inhibiting the 50% of CMV infectivity. The antiviral assay revealed that all the samples of colostrum, transitional and mature milk exhibited net anti-CMV activity and a full inhibition of viral replication was still evidenced in the range of maximal dilutions from value 1 (1/1) to 0.0039 (1/256). Within each stage of lactation, milk samples exhibited a wide range of variation of anti-CMV activity, with inhibitory dilution-50 values ranging from 0.001 to 0.013 in colostrum, from 0.001 to 0.063 in transition milk, and from 0.001 to 0.029 in mature milk. Notably, as reported in Figure 1 (panel A), mean anti-CMV

activity of milk samples from the whole study group of 18 mothers appeared to differ according to the stages of lactation: colostrum samples exhibited the highest anti-CMV activity, and a significant difference between colostrum and transitional milk anti-CMVinhibitory dilution-50 values was found ($p<0.05$); the mean anti-CMV activity of mature milk was lower than that of colostrum but the difference did not reach statistical significance. Then, we investigated whether the anti-CMV potency could, at least to some extent, depend on the maternal CMV-IgG status. As reported in Figure 1B, colostrum samples from CMV-IgG+ mothers exhibited a clear tendency to greater anti-CMV activity than transitional or mature milk (mean inhibitory dilution-50 0.004 versus 0.016 and 0.015, respectively; $p=0.05$). By contrast, no significant difference in anti-CMV activity among the meaninhibitory dilution-50 of three lactational stages was reported in the subgroup of CMV-IgG- mothers. However, it must be noted that the limited number of CMV-IgG- mothers enrolled may affect the detection of possible significant differences in anti-CMV activity among the colostrum group and the transitional and mature milk groups (Figure 1C). Interestingly, when we compared the anti-CMV activity of CMV-IgG+ and IgG- groups for each stage of lactation, colostrum samples from CMV-IgG+ mothers exhibited higher antiviral activity than CMV-IgG-, with mean inhibitory dilution-50 values of 0.004 versus 0.009, respectively ($p<0.05$) (Figure 1D). By contrast, as reported in Figures 1E and 1F, no differences in antiviral activity of transitional and mature milk were reported between CMV-IgG+ and CMV-IgG-women. The observation that samples of colostrum from CMV-IgG+ mothers exhibited greater anti-CMV activity than CMV-IgG- mothers suggested that milk immunoglobulins could contribute to the overall antiviral activity of colostrum.

Anti-CMV activity of colostra after immunoglobulin A depletion. Since secretory IgA (sIgA) is the major immunoglobulin class present in human colostra (25), we investigated to which extent sIgA contribute to the anti-CMV properties of colostrum itself. To this purpose, colostra from eight randomly chosen mothers (4 CMV-IgG+ and 4 CMV-IgG-) were depleted of sIgA content by incubation with Jacalin gel slurry in pre-loaded chromatography columns, and antiviral assays on HFF-1 cells were performed on eluates. Jacalin is a lectin that binds IgA suited to remove them from

a biological matrix. Figure 2 shows that, when comparing antiviral activities in untreated versus IgA-depleted colostra, a significant decrease in anti-CMV activity was observed in the group of CMV-IgG+ colostra following IgA depletion (mean inhibitory dilution-50 values 0.005 versus 0.009 in untreated and IgA-depleted, respectively; $p<0.01$). By contrast, no difference in antiviral activity was observed in the CMV-IgG- subgroup following IgA depletion.

Anti-CMV activity of pasteurized milk. After having identified the anti-CMV activity of human milk, we assessed the impact of the Holder pasteurization on this biological property on two pools of breast milk from CMV-IgG+ mothers, as described in Materials and Methods. As reported in Figure 3, heat treatment resulted in a statistically significant reduction of the anti-CMV activity of pooled breast milk, with anti-CMV inhibitory dilution-50 values increasing from 0.016 to 0.054 in raw and Holder pasteurized milk, respectively ($p<0.0001$).

Discussion

While the protective role of some milk components against CMV infection has been described in the literature (3,5,29), the anti-CMV activity of human milk has not been explored so far. This study addressed this issue in the context of preterm infants, who are particularly vulnerable to CMV infections transmitted by human milk. The first notable finding was that all samples of colostrum, transitional and mature milk were endowed with antiviral activity against CMV, although to a different extent from sample to sample and from mother to mother. This variability is not surprising considering that several studies reported a high variability of human milk's composition between individuals and over lactation (30).

Interestingly, we observed that colostrum samples possessed the highest anti-viral potency. A similar antiviral pattern over time was previously described against Coxsackievirus B4 analyzing breast milk samples from term donors living in France and in Congo (20). Another interesting result of this study was obtained subdividing the milk samples in two groups according to the maternal IgG CMV-specific serostatus: in the group of CMV-IgG+ mothers, colostra were significantly more potent than

the transitional milk and mature milk samples in term of anti-CMV activity. In other words, the reduction of antiviral activity of milk samples was observed as the stage of lactation advanced. Furthermore, comparing the antiviral potency of the milk samples from CMV-IgG+ and IgG- mothers at the three stages of lactation we observed that the colostra from the seropositive mothers are more potent than their counterpart from seronegative donors. The last observations could be explained hypothesizing specific anti-CMV factors, that are more abundant in colostrum from seropositive mothers and whose concentration declines in transitional and mature milk. Indeed, immune factor concentrations during lactation have been shown to be higher in colostrum than in mature milk (3,31) and this is particularly true for sIgA (32). This hypothesis is supported by the experiments we conducted on IgA depletion in colostra from CMV-IgG+ mothers, where we demonstrated that depleted colostra were significantly less active against CMV then the undepleted counterpart. However, it must be noted that Jacalin binds only IgA1, leaving the question on the role played by IgA2 unanswered. If, on one hand, these findings indicate that specific IgA contribute to the overall antiviral activity of colostrum from seropositive mothers, on the other hand the antiviral activity in colostra from seronegative mothers and the residual activity in depleted colostra clearly indicate a partial contribution of sIgA suggesting that additional immune or non-immune factors also contribute to the overall antiviral property of human milk.

Notably, preterm breast milk has been shown to contain high concentrations of some immune proteins, as β-defensin 1, lysozyme, soluble CD14 receptor (31). Our findings stimulate further studies to identify yet unknown antiviral components of breast milk.

The Guidelines for Human Milk Banks recommend a heat treatment of human milk to limiting the risk of bacterial and viral infections transmitted by milk to extremely vulnerable newborns, such as preterm infants (28). Holder pasteurization of donor human milk, as well as of mother own milk in specific clinical situations, is currently the recommended pasteurization method to inactivate pathogens, as CMV (33). Alternatively, freezing of mother's milk at –20 °C for a certain period of

time has been shown to reduce the viral concentration but it is not effective in complete elimination of the virus (34). Although Holder pasteurization is the gold standard technique for safety, its drawbacks are reductions of biological activities of protective milk's components, as growth factors, lysozyme, immunoglobulins, lactoferrin and other enzymatic activities, some cytokines and vitamins (35,36). In this context, we evaluated the impact of heat treatment at 62.5°C for 30 minutes on the anti-CMV activity of human milk. Breast milk samples from preterm mothers were pooled to reproduce the clinical settings of donor human milk banks. Our results showed that Holder pasteurization significantly reduces anti-CMV activities of breast milk. As reported by other authors and detailed in a recent review (36), among the factors that may influence the anti-viral activity of human milk, those reported as highly affected by Holder pasteurization are immunoglobulins, lactoferrin and lysozyme. Nevertheless, in a recent paper (37), we were not able to detect any significant decrease in lysozyme activity following Holder pasteurization of human milk. Our data further support the use of fresh colostrum for feeding newborns and/or the need to develop alternative pasteurization techniques to gain better preservation of biological properties of human milk.

Overall, our findings are relevant for the management of preterm infants nutrition and health. The use of fresh breast milk in preterm infants is highly recommended instead of preterm formula due to its trophic effects and because it is associated to lower risk of morbidities (38,39). Even if clinical evidence about the benefits of mother's own milk (MOM) versus pasteurized milk are controversial (40,41) there are biological arguments to suggest donor human milk as second choice after MOM (42,43). Of note, a recent meta-analysis reports that feeding raw MOM compared to feeding pasteurized MOM protect against bronchopulmonary dysplasia in very preterm infants (41). Although in CMV-IgG+ mothers viral reactivation during lactation can be detected already in colostrum, the viral shedding begins with low viral DNAlactia (load <1000 copies/ml) and low virolactia within 10 days post-partum (44,45). For this reason, in clinical practice, the use of unpasteurized colostrum was recommended, even in very preterm infants from CMV-IgG+ mothers (39). The results of our study further support this indication by showing that colostrum from CMV-IgG+ mothers has a high

antiviral activity that may lower the risk of CMV transmission. Therefore benefits of fresh colostrum could outweigh possible risks deriving from maternal CMV reactivation.

Some hypotheses can be put forward on the biological role of the anti-CMV activity of human milk. Paradoxically, despite daily exposure to CMV, most preterm infants breastfed by CMV seropositive mothers do not become infected and do not develop clinical signs or severe diseases (17,18). A similar observation has been made for infants breastfed by HIV positive mothers (21). Among the several factors that could explain this paradox, we propose that the intrinsic antiviral activity of human milk may neutralize CMV infectivity or reduce the viral titer, thereby lowering the risk of acquiring a symptomatic infection. A second hypothesis is passive transfer of milk factors that protect the breastfed infant from exogenous CMV infections. From an evolutionary point of view, the plausibility of this hypothesis is supported by the fact that even the milk of seronegative mothers is endowed with anti-CMV activity, as reported by this study. To validate this hypothesis, specific studies, including digestomic analysis, are required to assess whether the anti-CMV activity of human milk remains intact after passing the digestive tract and specific factors are absorbed by the infant intestine. We believe that this study may stimulate further investigations to better understand the impact of antiviral factors in human milk and optimize the feeding guidelines for preterm infants.

Abbreviations

CMV, human cytomegalovirus; IgA, immunoglobulins A; HFF-1, human foreskin fibroblasts; DMEM, Dulbecco's Modified Eagle's Medium; FBS, foetal bovine serum; BAC, bacterial artificial chromosome; GFP, green fluorescence protein; MOI, multiplicity of infection; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; CD_{50}, the 50%-cytotoxic dilutions; CIs, 95% confidence intervals; CMV-IgG+, CMV-IgG positive; CMV-IgG-, CMV-IgG negative; sIgA, secretory IgA; COL, colostrum; TM, transitional milk; MM, mature milk.

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445 **Legends to figures**

446 Figure 1. *Anti-CMV activities of defatted breast milk of a cohort of 18 mothers.* (A) Anti-CMV
447 inhibitory dilution-50 values for colostrum (COL), transitional milk (TM) and mature milk (MM) are
448 reported as a mean ± SEM; colostra exhibited significantly higher anti-CMV activity than transitional
449 milk samples (ANOVA followed by Bonferroni post hoc test; * $p < 0.05$). Anti-CMV inhibitory
450 dilution-50 values are reported for CMV-IgG+ and for CMV-IgG- mothers as mean ± SEM (panel B
451 and C, respectively) (ANOVA followed by Bonferroni post hoc test; *$p = 0.05$). (D-F) Anti-CMV
452 activities of defatted milk samples of colostrum (D), transitional milk (E) and mature milk (F)
453 stratified on the basis of IgG-CMV maternal serostatus. Results are expressed as mean ± SEM of
454 inhibitory dilution-50 values (Student's *t*-test; * $p < 0.05$).

455 Figure 2. *Anti-CMV activity of IgA depleted colostrum.* Anti-CMV activity for untreated (white) and
456 IgA depleted (black) samples of colostra from CMV-IgG+ (left) and IgG- (right) mothers are
457 reported. Data are reported as anti-CMV inhibitory dilution-50 values as determined by viral
458 inhibition assay. Depletion of IgA was performed as described in Methods. Data are reported as mean
459 ± 95% C. I. Inhibitory dilution-50 values were compared using the sum-of-square F test, ** $p<0.01$.

460 Figure 3. *Impact of pasteurization on anti-CMV activity of human breast milk.* Milk samples
461 undergoing Holder pasteurisation (dot line) exhibited lower anti-CMV activity than the unpasteurised
462 (solid line) (sum-of-square F test, $p<0.0001$). On y-axis data are reported as percentage of inhibition
463 of viral infection in comparison to untreated cells (mean of duplicates ± SEM). On x-axis, the
464 dilutions of the samples tested are reported.

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466 **Legends to Supplemental Digital Contents**

467 Figure, Supplemental Digital Content 1. Cytotoxicity and anti-CMV activities of whole and defatted
468 breast milk. The reciprocal of the maximal milk dilutions associated with ≥ 95% cell cytotoxicity

(panel A) and the reciprocal of the maximal dilution associated with 95% inhibition of CMV infection (panel B) on HFF-1 cells are reported for whole (black bars) and defatted (white columns) breast milk samples (COL, colostrum; TM, transitional milk; MM, mature milk). Data are shown as mean from three mothers ± SEM of the inverse of dilution (Student's t-test;* $p<0.05$).

Figure, Supplemental Digital Content 2. Anti-CMV inhibitory dilution-50 values for colostrum (COL), transitional milk (TM) and mature milk (MM) of a cohort of 18 mothers are reported as single-point values.