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1 **Anti-cytomegalovirus activity in human milk and colostrum from mothers of preterm infants**

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

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

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62 **ABSTRACT**

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

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

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

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

KEYWORDS: antiviral activity; Holder pasteurization; immunoglobulins A.

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84 **What is Known**

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

90 **What is New**

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

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107 **Introduction**

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

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

132 **Methods**

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

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

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

149 *CMV inhibition assay.* Antiviral activity of individual milk fractions was determined by viral
150 inhibition assay on HFF-1. Cells were seeded in 96-well plates at a density of 5.0×10^3 /well in 100
151 µl of DMEM supplemented with 10% FBS. The next day, milk samples were serially diluted in
152 DMEM medium from 1/1 to 1/4096 parts, incubated with constant amount of GFP-coding CMV
153 (1000 PFU/well) at a multiplicity of infection (MOI) of 0.1 for 1 h at 37°C, and 100 µl of mixture
154 was inoculated on sub-confluent HFF-1 cells for 2 hours at 37°C, 5% CO₂. After three washing with
155 DMEM medium, the monolayers were overlaid with 1.2%-methylcellulose DMEM medium with 2%
156 FBS (100 µl). After 5-day-incubation at 37°C 5% CO₂ atmosphere, CMV infected cells were

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

162 *Cell viability assay.* Cell viability was assessed using the MTS assay as described in Cagno et al. (24)
163 (Supplemental Digital Content Text). The effect of breast milk fractions on cell viability at different
164 dilutions was expressed as a percentage of absorbance values of treated cells compared with those of
165 cells incubated with culture medium alone. The 50%-cytotoxic dilutions (CD₅₀) and 95% confidence
166 intervals (CIs) were determined with Prism 4 software.

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

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

182 device (Metalaredinox, Italy) (27) at “Donor Human Milk Bank” at Città della Salute e della Scienza
183 di Torino, Regina Margherita Hospital, Turin (28) (Supplemental Digital Content Text).
184 *Statistical analysis.* Statistical analysis was performed using Student’s t-test, ANOVA Analysis of
185 variance or F-test, as reported in legends of figures, on GraphPad Prism version 4.00 software.
186 Significance was reported for p-value <0.05.

187 **Results**

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

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

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

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

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

244 **Discussion**

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

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

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

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

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

283 time has been shown to reduce the viral concentration but it is not effective in complete elimination
284 of the virus (34). Although Holder pasteurization is the gold standard technique for safety, its
285 drawbacks are reductions of biological activities of protective milk's components, as growth factors,
286 lysozyme, immunoglobulins, lactoferrin and other enzymatic activities, some cytokines and vitamins
287 (35,36). In this context, we evaluated the impact of heat treatment at 62.5°C for 30 minutes on the
288 anti-CMV activity of human milk. Breast milk samples from preterm mothers were pooled to
289 reproduce the clinical settings of donor human milk banks. Our results showed that Holder
290 pasteurization significantly reduces anti-CMV activities of breast milk. As reported by other authors
291 and detailed in a recent review (36), among the factors that may influence the anti-viral activity of
292 human milk, those reported as highly affected by Holder pasteurization are immunoglobulins,
293 lactoferrin and lysozyme. Nevertheless, in a recent paper (37), we were not able to detect any
294 significant decrease in lysozyme activity following Holder pasteurization of human milk. Our data
295 further support the use of fresh colostrum for feeding newborns and/or the need to develop alternative
296 pasteurization techniques to gain better preservation of biological properties of human milk.

297 Overall, our findings are relevant for the management of preterm infants nutrition and health. The use
298 of fresh breast milk in preterm infants is highly recommended instead of preterm formula due to its
299 trophic effects and because it is associated to lower risk of morbidities (38,39). Even if clinical
300 evidence about the benefits of mother's own milk (MOM) versus pasteurized milk are controversial
301 (40,41) there are biological arguments to suggest donor human milk as second choice after
302 MOM (42,43). Of note, a recent meta-analysis reports that feeding raw MOM compared to feeding
303 pasteurized MOM protect against bronchopulmonary dysplasia in very preterm infants (41). Although
304 in CMV-IgG+ mothers viral reactivation during lactation can be detected already in colostrum, the
305 viral shedding begins with low viral DNA lactia (load <1000 copies/ml) and low virolactia within 10
306 days post-partum (44,45). For this reason, in clinical practice, the use of unpasteurized colostrum was
307 recommended, even in very preterm infants from CMV-IgG+ mothers (39). The results of our study
308 further support this indication by showing that colostrum from CMV-IgG+ mothers has a high

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

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

325

326 **Abbreviations**

327 CMV, human cytomegalovirus; IgA, immunoglobulins A; HFF-1, human foreskin fibroblasts;
328 DMEM, Dulbecco's Modified Eagle's Medium; FBS, foetal bovine serum; BAC, bacterial artificial
329 chromosome; GFP, green fluorescence protein; MOI, multiplicity of infection; MTS, 3-(4,5-
330 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; CD₅₀, the
331 50%-cytotoxic dilutions; CIs, 95% confidence intervals; CMV-IgG+, CMV-IgG positive; CMV-IgG-
332 , CMV-IgG negative; sIgA, secretory IgA; COL, colostrum; TM, transitional milk; MM, mature milk.

333

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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 \pm SEM; colostrum 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 \pm 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 \pm 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 colostrum 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 \pm 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 \pm SEM). On x-axis, the
464 dilutions of the samples tested are reported.

465

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 $\geq 95\%$ cell cytotoxicity

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

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