NATURAL HISTORY OF POSTNATAL HUMAN CYTOMEGALOVIRUS INFECTION

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Abstract

Postnatal cytomegalovirus (HCMV) infection is well characterized in preterm infants, where it can lead to severe symptomatic infection. We analyzed the rate and route of transmission of postnatal HCMV infections in full-term babies during the first year of life. A cohort of 120 HCMV seropositive mothers and their 122 newborns were tested after delivery for HCMV DNA shedding in different bodily fluids. Postnatal HCMV infection was defined as the detection of $>2.5\times10^{2}$ HCMV-DNA copies/mL in infants' saliva swabs. Maternal neutralizing antibody serum titer, HCMV specific T-cell response, and HCMV glycoprotein B (gB) IgG on breastmilk were analyzed. HCMV shedding was detected in 67 of 120 mothers (55.8%), and 20 of 122 infants (16.4%) developed HCMV infection within the first three months of life. Six additional infants were infected during the first year, for a postnatal infection rate of 21.3%. Viral shedding was more frequent in breastmilk than saliva, urine and vaginal secretions, and the mothers of infected infants showed higher levels of HCMV-DNA in milk. No association was found between the antibody levels in serum or milk and maternal viral shedding, whereas a slightly lower frequency of HCMV-specific $CD4^{+}$ T-cells with long-term memory phenotype was observed in women with HCM-DNA-positive milk. About one out of five infants develop HCMV infection within the first year of life. Breastmilk appears the major route of transmission of the infection, maternal saliva have a minor role whereas the role of vaginal secretions is negligible.

INTRODUCTION

Human cytomegalovirus (HCMV) is the leading cause of congenital infections, occurring in 0.5-2% of pregnancies, with about 11% of live-born symptomatic infected infants. Congenital HCMV infection is more frequent after a primary HCMV infection during pregnancy than after non-primary infection (reactivation or reinfection).[1]Moreover, HCMV seems to be the principal cause of postnatal infection in the world. In fact, despite previous immunity, seropositive women may reactivate the infection in mammary gland and subsequently excretes the virus in the breastmilk.[2,3] In addition, HCMV shedding at delivery was observed in multiple bodily fluids,[4,5]among which vaginal secretions and saliva may represent potential routes of HCMV transmission to the newborn. It has been seen that HCMV reactivation during the first 3 months after birth occur in >95% of seropositive mothers.[6] However, in full term babies, transmission via breastfeeding is usually asymptomatic due to the protection given by maternal IgG antibodies transferred through the placenta, in particular after the 28^{th} week of gestation. However, it can lead to severe symptomatic HCMV-infection in preterm infants, in particular if the newborn is a very low birth weight or if is less than 32 weeks of gestational age.[6,7] Moreover reactivation in the genital tract can be considered a source of transmission for HCMV infection during delivery. Postnatal transmission is well characterized in pre-term newborns at high risk of symptomatic infection, while not much is known of the natural history of postnatal transmission in full-term, healthy newborns. Infants with postnatal HCMV infection usually have normal cognitive development and the infection does not affect the neurodevelopment during the first 6 years of live. However,

a study conducted in the United Kingdom assessed that HCMV infection was associated with suboptimal attention control at 8 years of age.[8,9]

This article will focus on the frequency HCMV of non-primary infection (NPI, namely HCMV reactivation) occurring post-delivery in a group of mothers of full-term infants and we will describe the source (in particular in breastmilk *vs* saliva and genital secretions) and the rate of transmission to their infants, during the first 6-12 months of life.

MATERIALS AND METHODS

Study design and participants

Between June 2017 and June 2022, 120 HCMV-seropositive women and their infants were enrolled at Fondazione IRCCS Policlinico San Matteo (Pavia, Italy). Maternal saliva swab, urine, vaginal swab and milk (when possible), and saliva from infants were collected after delivery (T0; median days since delivery 2, interquartile range -IQR- 1-3 days), and after three-six months (T1; median days since delivery 94, IQR 71-115 days). In a subgroup of 38 mother-infant pairs with a HCMV-DNA positive sample at T0 and/or T1, we also analyzed a subsequent time point, T2 (T2; median days since delivery 307, IQR 231-417 days), before the admission of the infant to a day care center.

HCMV-DNA quantification

Samples were tested for HCMV-DNA using Artus CMV PCR kit, (Qiagen, Hilden, Germany) after DNA extraction with EZ1 DSP virus kit (Qiagen) using EZ1 Advanced XL (Qiagen). We considered a cut-off for HCMV infection in infants of 2.5×10^2 HCMV-DNA copies/mL in saliva swabs. This cut-off was based on a previous study on congenital HCMV infection, in which among 45 newborns with HCMV-DNA positive saliva, the infection was confirmed only in cases with a level of DNA above 2.5×10^2 copies/mL.[10]

T-cell response

HCMV-specific T-cell response was analyzed in 15 women as previously described.[11,12] After the identification of memory T-cells by exclusion of naïve T-cells ($CD45RA^+CCR7^+$), $CD4^+$ and $CD8^+$ IFN-γ producing T-cells were determined, and among them the percentages of cells IL-$7R^+$ (long-term memory, LTM) or IL-2^+ were calculated.

ELISA

HCMV glycoprotein B (gB) IgG on breastmilk was tested by ELISA as previously described, with some minor changes.[13]Briefly, half-area 96-well microplates were coated with 25 µl/well of 3µg/ml gB (GSK plc, Brentford, United Kingdom) for 1 hour. Following incubation, plates were blocked for 2 hours with a solution of 80.5% PBS, 4% whey protein, 15% goat serum, and 0.5% Tween-20; then breastmilk (1:3 in PBS), was added to wells for 1.5 hours. After washing, the plates were incubated with horseradish peroxidase-labeled goat IgG to human IgG (SouthernBiotech, Birmingham, AL, USA) for 45' (1:4000 in PBS-skimmed milk 1%). Finally, plates were incubated for 25' with 30 mg/mL orthophenylendiamine (Sigma-Aldrich, St. Louis, MO, USA) before the addition of 4N sulphuric acid. Plates were read at 450nm.

Neutralization assay

HCMV neutralizing antibody (VR1814 for epithelial cells and AD169 for fibroblasts) were tested as reported. [14] The serum dilution inhibiting virus infectivity by 50% was considered the 50% neutralizing antibody titer.

Statistical analysis

Categorical variables were described as count and percentage and compared using Fisher's exact test. For numerical variables, the median value with IQR was reported and they were compared with the Mann-Whitney U-test. Comparison between more than two groups was performed using the Kruskall-Wallis test and Dunn's post test with correction for multiple comparisons. The Spearman R with its 95% confidence

interval (95%CI) was calculated for correlation analysis. A p-value <0.05 was considered statistically significant. Analyses were performed with GraphPad Prism version 8.3.0 (GraphPad software La Jolla, CA, USA).

RESULTS

Maternal HCMV reactivation after delivery and rate of postnatal HCMV infection in newborns.

Overall, 67/120 (55.8%) women developed a NPI post-delivery, in particular 24 (20%) at T0 and 43 (35.8%) subsequently at T1. HCMV-DNA was detected in 8/120 (6.7%) saliva swabs, in 13/117 (11.1%) vaginal swabs, in 6/116 (5%) urine and in 7/9 (77.8%) milk samples at T0. At T1 we found HCMV-DNA in 11/120 (9.0%) saliva swabs, 15/120 (12.5%) vaginal swabs, in 8/119 (6.7%) urine and in 49/83 (59.0%) milk samples (Fig 1.A). None of the 122 infants (two sets of twins) was positive for HCMV-DNA in saliva after delivery, thus excluding a congenital HCMV infection. Subsequently, 20/122 (16.4%) infants developed HCMV infection after birth at T1 (Fig 1.B). Infants with HCMV infection did not shown severe symptoms. Among maternal bodily fluids, HCMV-DNA load was higher in milk at T0 and at T1 compared with all the other samples of the mothers at both time points (p<0.001), with a median value of 2,551 (IQR: 6-26,467) copies/mL and 105 (IQR: 0-1,410) copies/mL, respectively (Fig 1.C).

To verify whether some characteristic of the infants recorded at delivery could be at the basis for a susceptibility to the HCMV infection, we compared them between infants with and without infection. No statistically significant difference was found in infants with HCMV infection compared with those without infection regarding the gestational age at birth, the type of delivery, the sex and the weight and length at birth, instead we observed a significant but small difference in the head circumference (p=0.03). (Table 1).

Table 1. Characteristics of infants with and without HCMV infection within the first three months of life

	Infected infants (n=20)	Non-infected infants (n=102)	*p Value*
Weeks of gestation at birth (median; IQR)	40; 39-40	40; 39-40	*0.83*
Type of delivery:			*0.76*
- *Natural delivery (number; %)*	16; 80%	82; 80%	
- *C-section (number; %)*	4; 20%	18; 18%	
Sex:			*0.15*
- *Male (number; %)*	7; 35%	55; 54%	
- *Female (number; %)*	13; 65%	47; 46%	
Weight *grams* (median; IQR)	3290; 3079-3378	3295; 3009-3513	*0.65*
Length *cm* (median; IQR)	51; 50-52	51; 50-52	*0.43*
Head circumference *cm* (median; IQR)	34; 33-34	35; 34-35	0.03
Mother with NPI (number; %)	18; 90%	50; 50%	<0.01

NPI, non-primary infection; IQR, interquartile range

A subgroup of 38 mother-infant pairs, in which a positive HCMV-DNA sample was found in T0 and/or T1, was followed for a longer period, until the admission of the infant to the daycare center, to verify whether infants of HCMV shedding mothers may be infected at a later time after birth but before entering in contact with other potential sources of HCMV (i.e. other infants attending the daycare center). Of the 38 infants, 12 had a primary infection diagnosed at T1 and were still shedding HCMV at T2 (Fig. 1D), while in six infants the infection was diagnosed subsequently at T2, leading to a cumulative number of 26/122 infants (Fig. 1.B) with HCMV infection at one year of age (21.3%). Of note, two mothers of the six infants were still breastfeeding and the milk samples were positive for HCMV-DNA.

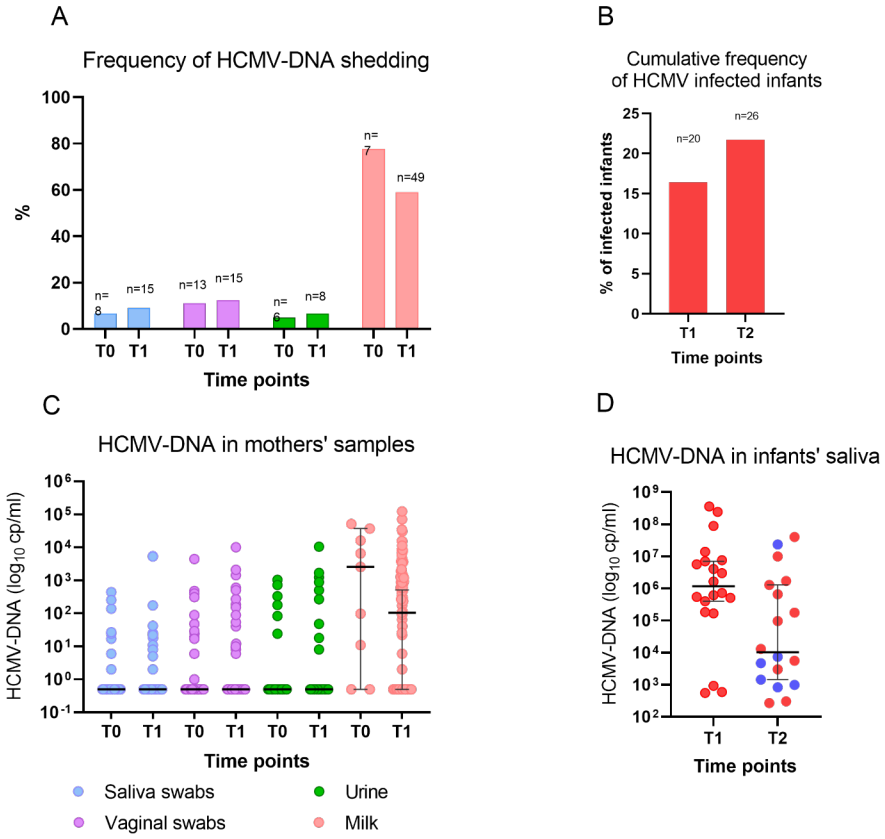

Figure 1. Frequency of HCMV-DNA detection (A) and HCMV viral loads in saliva, vaginal swabs, urine and milk of mothers (C). Frequency of HCMV infection in infants at T1 and T2 (B) and viral loads in saliva (blue dots represents infants who were infected later at T2) (D). HCMV, human cytomegalovirus

Source of postnatal HCMV infection

In order to understand the source of transmission of the virus to the infants, we compared HCMV shedding in the following bodily fluids between the transmitting and non-transmitting mothers: i) vaginal swabs at delivery (in women who had a natural childbirth); ii) milk at T1 and T2 (milk at T0 was not analyzed here due to the small number of samples collected); and iii) saliva swabs at the three time points. No significant difference were found in the viral load of vaginal swabs (p=0.65) and of saliva swabs (p=0.80) at delivery of transmitting mothers compared to non-transmitting ones (Fig 2.A and B). Conversely, at T1, we observed a significant difference in the viral load of both breastmilk (p<0.01) and saliva swab (p=0.01) a T2 (Fig. 2.C and D). In addition, viral load in both breastmilk and saliva swabs of transmitting mothers was significantly higher (p=0.03 and p=0.04, respectively), compared with non-transmitting mothers, also at T2 (Fig. 2.E and F). A significant although weak correlation between viral load in infants' saliva swabs and in saliva and milk of mothers was found at T1 (p<0.01, R= 0.31; 95%CI= 0,14-0,47 and p<0.01, R= 0.43; 95%CI= 0.23-0.59, respectively).

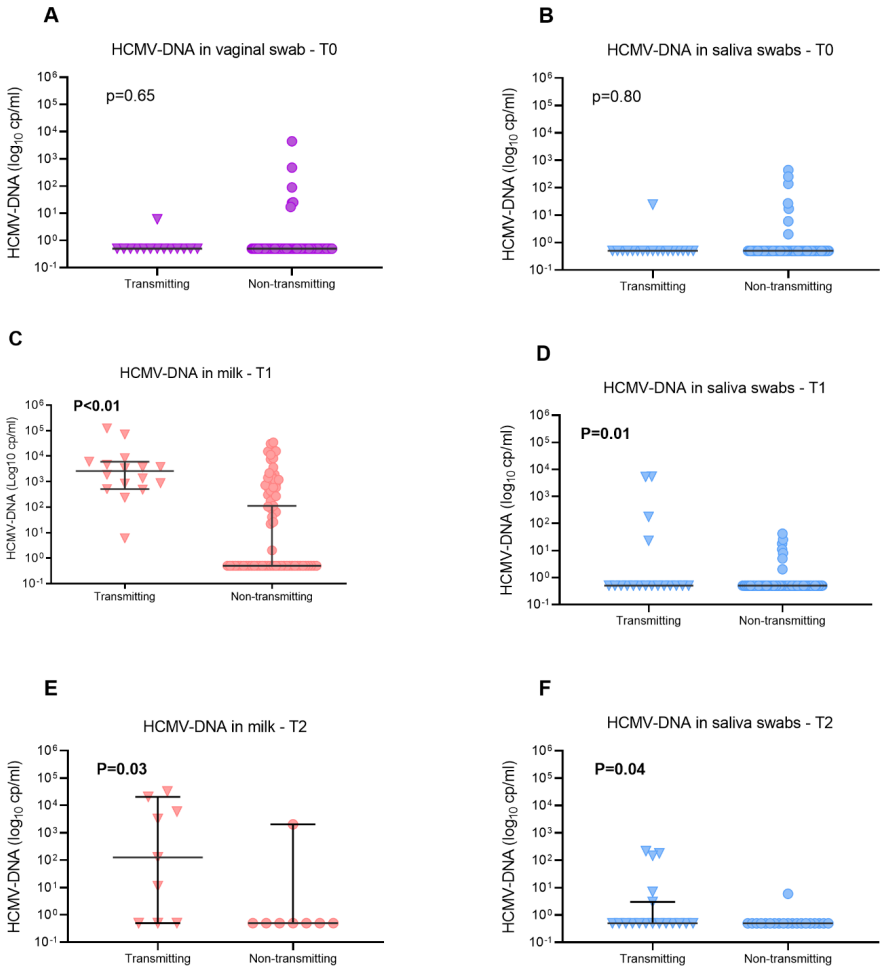

Figure 2. HCMV viral load in vaginal swabs (A) and in saliva swabs at delivery (B), in milk (C) and saliva swabs (D) at T1 and in milk (E) and saliva swabs (F) at T2, compared between transmitting mothers and non-transmitting mothers. HCMV, human cytomegalovirus

Of the 120 women analyzed, 92 (76.7%) breastfed their infant: in 85 (92.4%) of them milk was collected, while for seven (7.6%) women breastmilk was not available due to difficulties during the collection. These seven mother-infant pairs were excluded from the following analysis (two of these infants developed HCMV infection). The other 28 (23.3%) women used the formula. Overall, 113 mother-infant pairs, among which 24 infants developed HCMV infection, were analyzed. HCMV-DNA was found in milk of 52/85 (61.2%) breastfeeding mothers at T0 or T1. Among the 52 mothers with HCMV-DNA in milk, 21 (40.4%) had an infant with HCMV infection (12 of these 21 women were not shedding the virus in the saliva). One (3.6%) of the 33 breastfeeding mothers with HCMV-DNA-negative milk transmitted the infection to the infant, and she was shedding the virus through the saliva. Only 2/28 (7.1%) infants fed with formula had HCMV infection, and in this case one mother showed the highest level of HCMV-DNA in saliva swab of all the cohort (>5000 copies/mL), while the other did not shed HCMV-DNA in the saliva (Fig. 3). It was not possible to define a potential source of infection for this infant, who might have been infected by other family members. Despite the significant difference of viral load in saliva swabs of transmitting mothers compared to non-transmitting mothers, milk seems to be the major route of transmission: all but three infants with HCMV infection had breastfeeding mothers with presence of HCMV-DNA in milk, while maternal saliva was negative for HCMV-DNA for 13 infected infants.

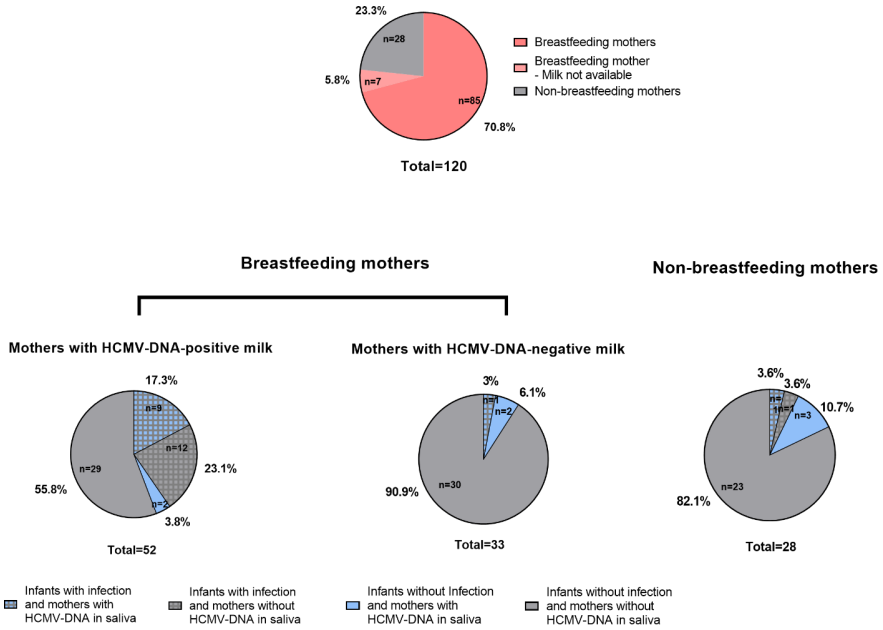

Figure 3. Route of transmission. Comparison between breastfeeding and non-breastfeeding mothers with and without HCMV-DNA-positive saliva swabs. Blue slides indicate mothers with HCMV-DNA-positive saliva swabs, squared pattern infants with HCMV infection and blank pattern infants without infection. HCMV, human cytomegalovirus

Maternal HCMV-specific immune response and HCMV reactivation in breastmilk.

HCMV-specific T-cells and antibody levels in blood and milk were determined in a subset of 8 women with and 7 women without HCMV-DNA in milk in order to investigate a potential association between maternal HCMV-specific immunity and HCMV shedding in milk. HCMV-specific LTM T-cells were determined, analyzing the $CD4^+$ and $CD8^+$ T-cells that expressed IL-7R, and produced IL-2 (Fig. 4). The percentage of $CD4^+IL\text{-}7R^+$ (p=0.04) and $CD8^+IL\text{-}7R^+$ (p=0.09) showed a trend toward lower values in mothers shedding HCMV in the milk.

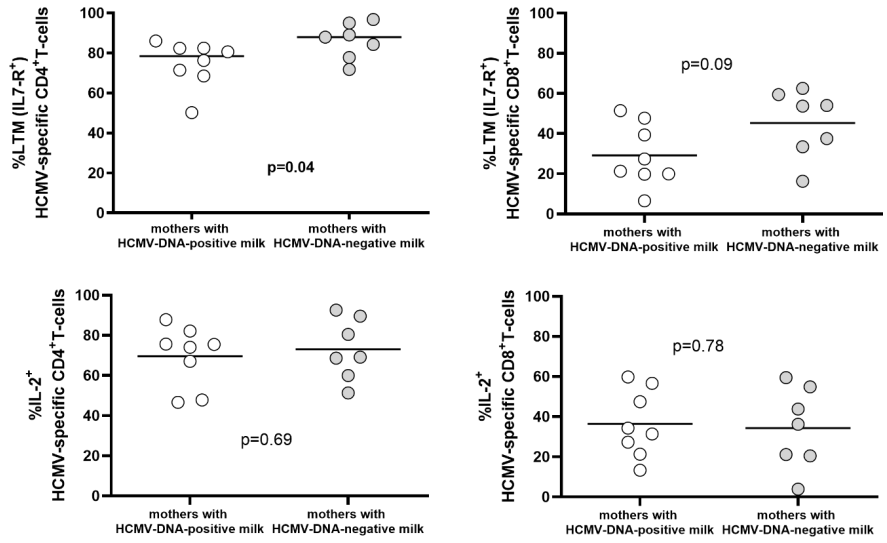

Figure 4. Percentages of HCMV-specific $CD4^+$and $CD8^+$ T-cells with LTM (long-term memory) and producing IL-2 in women with and without HCMV-DNA in milk. HCMV, human cytomegalovirus; IL7-R interleukin 7 receptor

In addition, the neutralizing activity of antibody against the infection of epithelial cells and fibroblast was compared between mothers with HCMV-DNA positive and negative milk. Neutralizing antibody titer against the infection of epithelial cells (p=0.03) or fibroblasts (p=0.07) was significantly higher in mothers who shed HCMV-DNA in milk (Fig.5).

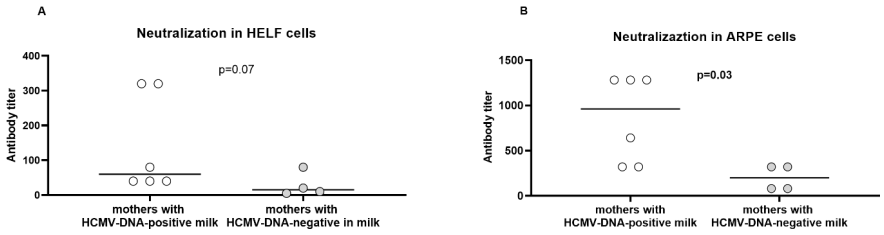

Figure 5. Serum antibody titers neutralizing HCMV (VR1814) infection of ARPE-19 cells (A) and HCMV (AD-169) infection of HELF cells (B) in women with and without HCMV-DNA in milk**.** HCMV, human cytomegalovirus; HELF, human lung fibroblast; ARPE-19, human retinal pigmented epithelial.

Previous studies showed anti-gB IgG titer higher in infants without HCMV infection, moreover, the anti-gB IgG titer was moderately associated with delayed HCMV acquisition.[15] Here the presence of anti-gB IgG was tested in breastmilk, due to the lack of infants' serum, to verify whether also IgG in milk could be protective against the acquisition of the infection in infants. Comparison between milk of transmitting and non-transmitting mother did not show a significant difference (p=0.84) (Figure 6.A). In addition, no significant difference was found while comparing HCMV-DNA positive or negative milk (p=0.35) (Figure 6.B).

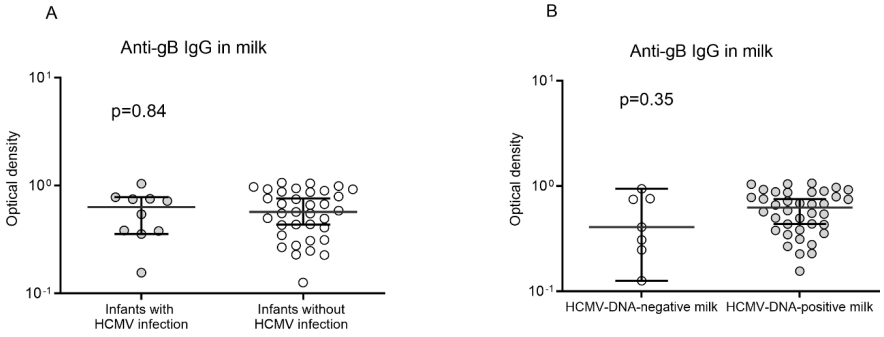

Figure 6. Comparison of anti-gB IgG in milk between transmitting and non-transmitting mothers (A) and between HCMV-DNA positive and negative milk. HCMV, human cytomegalovirus; gB, glycoprotein B

DISCUSSION

In this study, the frequency of postnatal HCMV infection was evaluated in a group of healthy infants born to HCMV-seropositive mothers. After a median time of three months after birth, 16.4% of enrolled infants (20/122) had HCMV infection, whereas within the first year of life, before admission to daycare centers, the rate of infection increased to 21.3% (26/122). Of the 120 mothers, 55.8% had a NPI after delivery, according to detection of HCMV shedding in bodily fluids. In particular, HCMV-DNA was detected more frequently and at higher levels in breastmilk (59% of women in whom breastmilk was collected shed the virus in this

compartment), whereas HCMV shedding in the other bodily fluid was detected in a lower percentage of women (12.5% women shed HCMV in vaginal secretions, 9% in saliva and 6.7% in urine).

It is known that HCMV reactivation occur during pregnancy, in particular in the genital tract.[4] Previous studies show that perinatal transmission can occur due to viral elimination through genital secretions,[16,17] but here no difference was seen in vaginal swabs HCMV-DNA load between transmitting and non-transmitting mothers, thus excluding the genital tract as a significant source of perinatal infection. Breastmilk transmission has been intensively studied in low-birth weight and preterm infants with a rate of acquisition of HCMV infection ranging from 5.7% to 60%.[17] Breastmilk seems to be the major route of transmission of the infection in the group of infants analyzed in this study, since viral load was higher in the milk of mothers of infected infants, and a significant correlation between viral load in maternal milk and infants' saliva was observed. Despite a significant difference of viral load was also found in saliva swabs of transmitting mothers compared to non-transmitting mothers, we could speculate that saliva does not represent a major source of infection for the infants. In fact, all but three of the infected infants were breastfed by mothers shedding HCMV in milk, whereas the mothers of 15/26 infected infants did not shed HCMV in saliva. Nevertheless, maternal saliva could be considered as a potential minor route of transmission, and the mother of one of the two infected child who was not breastfed showed the highest HCMV-DNA load in saliva (>5000 copies/mL). Viral shedding in saliva of mothers of infected infants could also be the effect of a local infection caused by an infant-to-mother transmission after primary infection of the child, as already described in a similar cohort of mother-infant pairs in Uganda.[18]

The prevalence of infection increases with age, and a previous study conducted in Italy showed that HCMV infects 56% of the population in the first 5 years of life.[16] Another study conducted in the USA showed that 31% infants of 0-47 months were HCMV-seropositive.[19] Analyzing the rate of infection at 1 year of life, we observed that 21.3% (26/122) of infants born to seropositive women develop a HCMV infection, although most of them are infected within the first 3-4 months. It is likely that HCMV infection during infancy occurs in two periods: a first period is within the first months after delivery, through contact with breastmilk, whereas a second period occurs after contact with other infants, especially when attending daycare centers: a French study reported a 51.9% prevalence of HCMV shedding in infants attending daycare centers.[20]

In our study, we observed that about 1 out of three infants of HCMV-shedding mothers developed HCMV infection. Different characteristics of the infants and of the pregnancy have been compared between infants with or without HCMV infection, looking for a susceptibility to the infection. Except for a small difference found in the head circumference, no difference in gestational age, weight and length at birth has been found, in line with the fact that our population is made of full term, healthy newborns.

Previous studies suggested a protective role of high level of anti-gB IgG in infants' serum for postnatal HCMV infection.[15]We could not analyze anti-HCMV antibody levels in infants' serum, but we investigated whether the presence of anti-HCMV antibody (namely anti-gB IgG) in breastmilk was associated with prevention of HCMV transmission. However, the levels in milk of these antibodies were not different between transmitting and non-transmitting mothers. Moreover, no significant difference was found comparing HCMV-DNA-positive milk with HCMV-DNA-negative milk, thus excluding any role in controlling local virus replication.

Conversely, the systemic HCMV-specific immune response appeared different between women who reactivated or not HCMV in breastmilk. In particular, women shedding HCMV in milk had a lower frequency of HCMV-specific T-cells with a LTM phenotype, and a higher titer of neutralizing antibodies. Higher level of neutralizing antibodies against epithelial HCMV infection was observed in a population of solid-organ transplanted host, compared with primary HCMV infection along with a viral load peak much higher.[21] Whether these characteristics indicate an inconsistent immune control, which may be at the basis of the HCMV reactivation from latency, or rather are a consequence of a greater immune stimulation induced by the HCMV reactivation itself, could be investigated in future studies. In line with this data, we also observed a higher anti-gB IgG level in pregnant women with compared to those without HCMV reactivation.[4]

The strength of this study is the comprehensive analysis of HCMV shedding in maternal bodily fluids and its relationship with postnatal HCMV infection of the newborns. Its major limitation is the lack of long-term follow-up for the infants whose mother were not shedding HCMV after delivery, which may have underestimated the actual rate of HCMV infection within one year of age. However, it is unlikely that a significant number of infections have been missed because of this design.

In conclusion, about 20% of infants born to HCMV seropositive women develop HCMV infection within the first year of life (mainly within the first three months). The major source of infection is breastmilk, in which about 60% of seropositive women shed HCMV, and one out of three infants receiving HCMV-positive milk develop HCMV infection. Conversely, maternal saliva represent a minor source of infection whereas the role of genital tract secretion is negligible. This data could be informative for future vaccination strategies, when considering the vaccination of infants.[22,23]

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Ethics Approval Statement

The study was approved by the Ethics Committee and Fondazione IRCCS Policlinico San Matteo Institutional Review Board (Procedure n° P-20170007596 and P-20190073479).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

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