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Original article

Human cytomegalovirus non-primary infection during pregnancy: antibody response, risk factors and newborn outcome

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ABSTRACT

Objectives: Human cytomegalovirus (HCMV) non-primary infections can occur in pregnant women and may result in congenital infection. Comprehensive studies investigating the frequency, characteristics, risk factors and immune response of non-primary infection in pregnancy are missing, while the rate of vertical transmission is not known.

Methods: HCMV non-primary infection was investigated prospectively in 250 pregnant women. Blood and urine samples as well as saliva and vaginal swabs were collected at 13, 21 and 31 weeks of gestation and at delivery. HCMV-DNA and specific IgG and IgM levels were determined.

Results: Overall, 105/250 pregnant women (42.0%) developed non-primary infection. HCMV-DNA was detected more frequently in vaginal secretions (84/250 of the women, 33.6%) than in urine (35/250, 14.0%), saliva (26/250, 10.4%) and blood (7/250, 3.0%). The rate of HCMV non-primary infection increased significantly with the progression of pregnancy (from 12.9% in the first trimesters of gestation to 21.9% at delivery, $p < 0.01$). IgM was detected in 25/250 of the women (10.0%), with no association with non-primary infection, while anti-gB IgG was significantly higher ($p < 0.01$) in women with non-primary infection. Age and close contact with children were not associated with non-primary infection. No woman with non-primary infection transmitted the infection to the fetus (95% confidence interval of transmission rate: 0–3.5%).

Discussion: Although HCMV non-primary infection is frequent during pregnancy, the rate of congenital infection as a consequence of non-primary infection is likely to be $\leq 3.5\%$. **Paola Zelini, Clin Microbiol Infect 2022;28:1375**

Introduction

Human cytomegalovirus (HCMV) is a major cause of congenital infections, occurring in 0.5–2% of pregnancies [1], and in 20% of the cases it results in sensory and developmental impairment [2]. HCMV infection in pregnant women can occur by primary or non-primary infection, i.e. reactivation of the latent virus or reinfection with a different strain. The risk of congenital cytomegalovirus infection (cCMV) is around 40% after primary infection and is much lower, but not absent, during non-primary infection [3]. Few studies have been performed to investigate the rate of HCMV non-primary infection during pregnancy [4,5]. Whether non-primary infection in seropositive pregnant women are mostly due to reactivations or reinfections remains undefined. An annualized rate of 9% reinfection has been reported in highly immune pregnant women in Brazil, with a higher rate of reinfection and transmission to the fetus in mothers caring for young children [6]. The natural history of and immune response to HCMV non-primary infection in immunocompetent individuals are largely

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unknown. In this study, we evaluated the rate and risk factors for non-primary infection during pregnancy in an Italian population, and estimated the rate of cCMV.

Materials and methods

Study design

We designed a prospective observational study to evaluate the frequency of non-primary infection during pregnancy (primary endpoint). Risk factors, antibody response, newborn parameters and the rate of cCMV were the secondary exploratory endpoints. Pregnant women with previously documented HCMV-IgG positive and IgM-negative results attending the Obstetrics and Gynaecology Clinics of our institution for routine antenatal screening were enrolled at the first visit (at the end of the first trimester of gestation). Women with no documentation of HCMV serostatus were tested before enrolment according to the routine screening protocol of our Institution. HCMV-specific IgG avidity is not routinely tested but was determined at enrolment for study reasons to exclude women with low IgG avidity indicating a recent primary infection.

Enrolled women were followed up at about 20 and 30 weeks of gestation and at delivery. At each time point, blood and urine samples and saliva and vaginal dry swabs were collected. At delivery, the newborn's saliva swab was collected and tested as screening for cCMV (to be confirmed, in case of a positive result, on a urine sample). Anamnestic data of the mothers (including contact with children <36 months) and newborn clinical parameters at birth were collected. Non-primary infection was diagnosed as the detection of HCMV-DNA in bodily fluids in a woman with pre-conception immunity, as previously defined for both transplant recipients and pregnant women [7–9].

Thirty pregnant women with HCMV primary infection were also analysed for the presence of HCMV-DNA in bodily fluids at 1, 2, 3, 6 and 12 months after infection. Diagnosis was based on the following criteria: HCMV-specific IgG seroconversion, HCMV-specific IgM antibody kinetics, low IgG avidity index and HCMV DNA-aemia. The primary infection occurred in 23 women in the first trimester (gestational weeks median: 5; range 1–12), and in seven women in the second trimester (gestational weeks median: 16; range 13–23).

The study was approved by the local ethics committee and all subjects gave written informed consent.

HCMV-DNA quantification

Freshly collected or thawed dry swabs (FLOQSwabs, Copan, Brescia, Italy) were resuspended in 1 m of phosphate-buffered saline and DNA was isolated with the EZ1 DSP Virus Kit (Qiagen) from swab resuspension medium and urine using the EZ1 Advanced XL instrument (Qiagen). DNA was isolated from whole blood using the QIAamp DNA Mini kit. HCMV-DNA was quantified by real-time PCR using the Artus CMV RG PCR kit (Qiagen) and QiAgility and Rotor-Gene Q instruments (Qiagen). HCMV-DNA was expressed as copies/mL blood, urine or swab resuspension medium.

HCMV- and antigen-specific antibody assays in HCMV-seropositive pregnant women

HCMV-specific IgG avidity was determined by the LIAISON CMV Avidity II assays (DiaSorin, Saluggia, VC, Italy). Anti-HCMV IgG and IgM were determined by ELISA based on cell lysate (Euroimmun anti-HCMV ELISA IgG, Euroimmun anti-HCMV ELISA

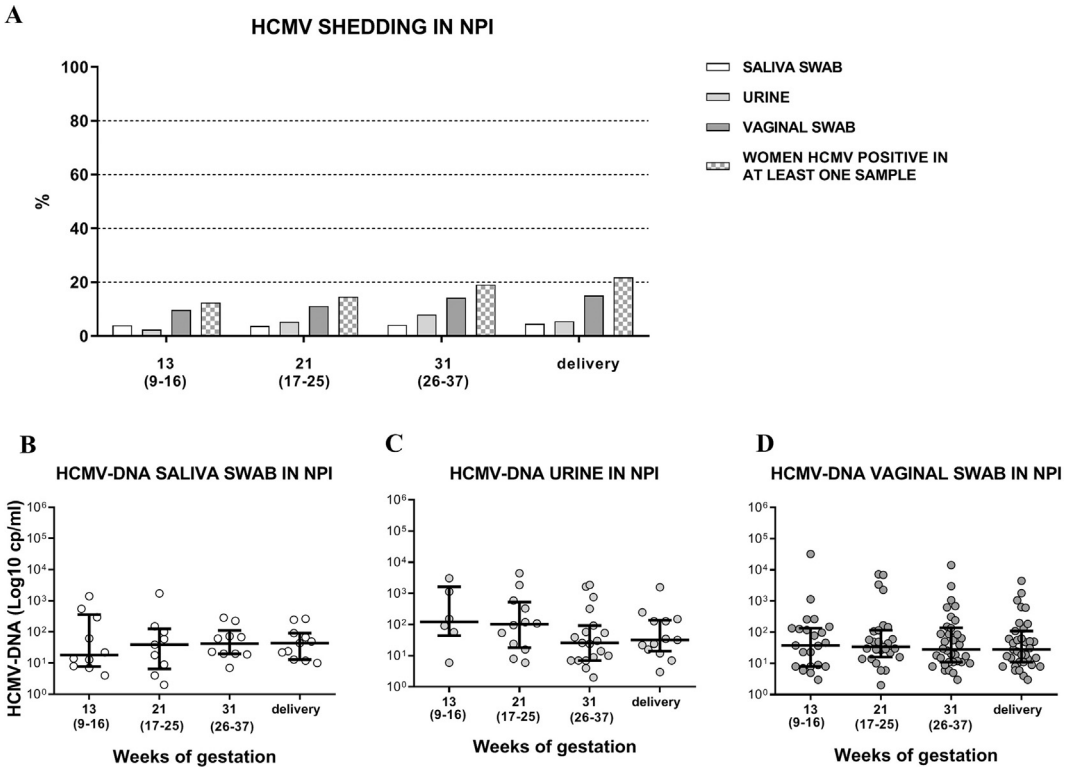

Fig. 1. (A) Rates of HCMV DNA detection in different bodily fluids in pregnant women with pre-conception HCMV infection. HCMV viral load in (B) saliva swabs, (C) urine and (D) vaginal swabs in pregnant women with HCMV non-primary infection (NPI).

IgM, Euroimmun AG, Luebeck, Germany) or on recombinant proteins (Euroimmun anti-HCMV gB ELISA IgG, Euroimmun anti-HCMV p52 ELISA IgM, Euroimmun AG). IgM specific for HCMV recombinant antigens (pp28, p52 and p150) was also determined by chemiluminescence with LIAISON CMV IgM II assays (DiaSorin). Mikrogen RecomLine immunoblotting assay (Mikrogen GMBH, Neuried, Germany) and serum neutralization assay [10] was used in some dubious cases to confirm or exclude previous HCMV infection.

Statistical analysis

The frequency and 95% CI of HCMV non-primary infection during pregnancy (primary endpoint), HCMV detection in the different compartments and in the three trimesters of pregnancy, and cCMV, were calculated. For numerical variables, median value with range and/or interquartile range was reported. Categorical variables were compared with Fisher's exact test or the chi-squared test for trend, and continuous variables were compared with the Mann–Whitney U test or with the Wilcoxon signed-rank test (for paired data). For comparison of three or more groups, the Kruskall–Wallis analysis of variance test and Dunn's *post hoc* test with correction for multiple comparisons were used. A p value < 0.05 was considered statistically significant. Analysis was performed with GraphPad Prism version 8.

Results

Study subjects

Between 2016 and 2019, 304 consecutive pregnant women were enrolled, but 49 of them were lost to follow-up (maternal and newborn samples were not collected at delivery). Among the 255 women completing the study, 5/250 (2.0%) were found to be HCMV seronegative according to Euroimmun-IgG ELISA performed in serum samples collected throughout follow-up (all five cases), notwithstanding a documented IgG positivity before pregnancy. These women were also tested with Liaison CMV IgG II, which was positive in three out of cases, while immunoblotting confirmed the absence of previous HCMV infection and neutralization assays were negative (see Table S1).

Frequency of HCMV non-primary infection and HCMV shedding in bodily fluids

Bodily fluids were obtained at a median time (range) of 13 (9–16), 21 (17–25) and 31 (26–36) weeks of gestation and at delivery. Overall, 105/250 (42.0%; 95% CI 35.8–48.4%) pregnant women developed non-primary infection. HCMV-DNA was detected in saliva swabs of 26/250 (10.4%), in the urine of 35/250 (14.0%) and in vaginal swabs of 84/250 (33.6%) woman (Fig. 1A). HCMV-

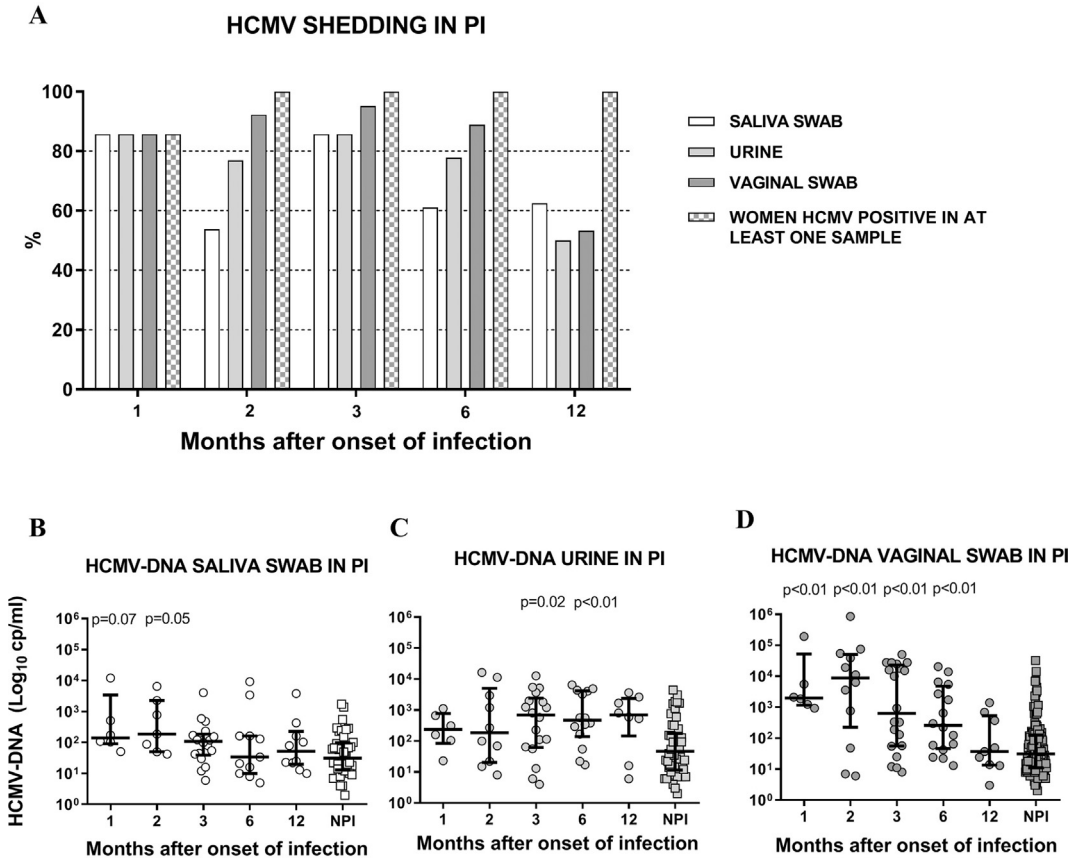

Fig. 2. (A) Rates of HCMV DNA detection in different bodily fluids in pregnant woman with HCMV primary infection (PI). HCMV viral load, HCMV viral load in (B) saliva swabs, (C) urine, and (D) vaginal swabs in pregnant women with HCMV PI (1–12 months after infection) or non-primary infection (NPI). Levels of HCMV DNA for NPI detected at different time-points during pregnancy are grouped together. Kruskall-Wallis analysis of variance test and Dunn's *post hoc* test with correction for multiple comparisons were used for statistical analysis. Only p values ≤ 0.10 were reported.

DNA was more frequently detected in vaginal swabs ($p < 0.01$). As pregnancy was progressing, the rate of HCMV detection increased significantly ($p < 0.01$), particularly in vaginal swabs.

In detail (see Table S2), 32/250 (12.8%) women showed a continuous shedding throughout pregnancy, whereas 28/250 (11.2%) women showed HCMV shedding only at delivery. HCMV-DNA was detected in a single bodily fluid in 65/250 (26%) women, whereas HCMV-DNA was detected in multiple bodily fluids in 40/250 (16.0%) women.

The HCMV viral load was not significantly different between saliva swabs, urine and vaginal swabs, even if higher HCMV-DNA values (>10 000 copies/mL) were observed in vaginal swabs (Fig. 1B–D). Although it was not foreseen in the original study protocol, rapid viral isolation (please see supplementary material for methodological details) was performed on three HCMV-DNA-positive vaginal swabs (HCMV-DNA range 16–64 copies/mL): all of them were positive (1–10 p72-positive fibroblasts), indicating the presence of infectious virus.

Finally, HCMV was detected in the blood of 8/250 (3.2%) women (median level 57, range 1–291 copies/mL), all of whom were positive for HCMV in one or more bodily fluid.

HCMV detection in primary vs non-primary infection

All 30 women with primary infection were still positive for HCMV-DNA in at least one bodily fluid 12 months after the onset of infection (Fig. 2A). HCMV-DNA was higher in saliva swabs of primary infection woman at 1 and 2 months after infection (Fig. 2B), as well as in the urine of primary infection woman at 3 and 6 months after the onset of infection than in non-primary infection women ($p < 0.02$, Fig. 2 B,C). Finally, the HCMV load was higher in vaginal swabs of primary infection women up to 6 months after the onset of infection ($p \leq 0.01$, Fig. 2D). HCMV in the blood was detected in 17/21 (81%) women tested, with a median level of 90 (30–18 450) copies/mL.

Antibody response in pregnant women with or without non-primary infection

All women showed high anti-HCMV IgG avidity at enrolment with the exception of one who had intermediate avidity. Anti-HCMV IgG was positive throughout follow-up in all women (two cases showed intermittent borderline results). Anti-gB IgG was positive in 242/250 (96.8%) women (seven of whom showed intermittent borderline results) and negative (or borderline) in 8/250 (3.2%) women. Anti-HCMV IgM (Euroimmun) was consistently positive in 7/250 (2.8%) women, and showed intermittent positive results in 18/250 (7.2%) women. Anti-p52 IgM was consistently positive in 2/250 (0.8%) women and showed intermittent positive results in 3/250 (1.2%) women. Anti-HCMV IgM according to the Liaison assay was consistently positive in 3/250 (1.2%) women and showed intermittent positive results in 3/250 (1.2%) women (Fig. 3). The three assays adopted for IgM analysis were concordantly positive in 2/68 (2.9%) positive samples, whereas 12/68 (17.6%) samples were concordantly positive for two IgM assays (Table S3). Although participating women were enrolled based on previously documented IgM-negative results, some of the samples collected at enrolment were IgM positive when subsequently analysed with the study tests.

Anti-HCMV IgG, IgM and anti-p52 IgM were not significantly different in women with or without non-primary infection, and only one out of eight women with HCMV-DNA in blood was positive for IgM (Euroimmun assay). In contrast, anti-gB IgG was significantly higher in non-primary infection women at all time points (p 0.06 recruitment, $p < 0.01$ during follow-up) (Fig. 4).

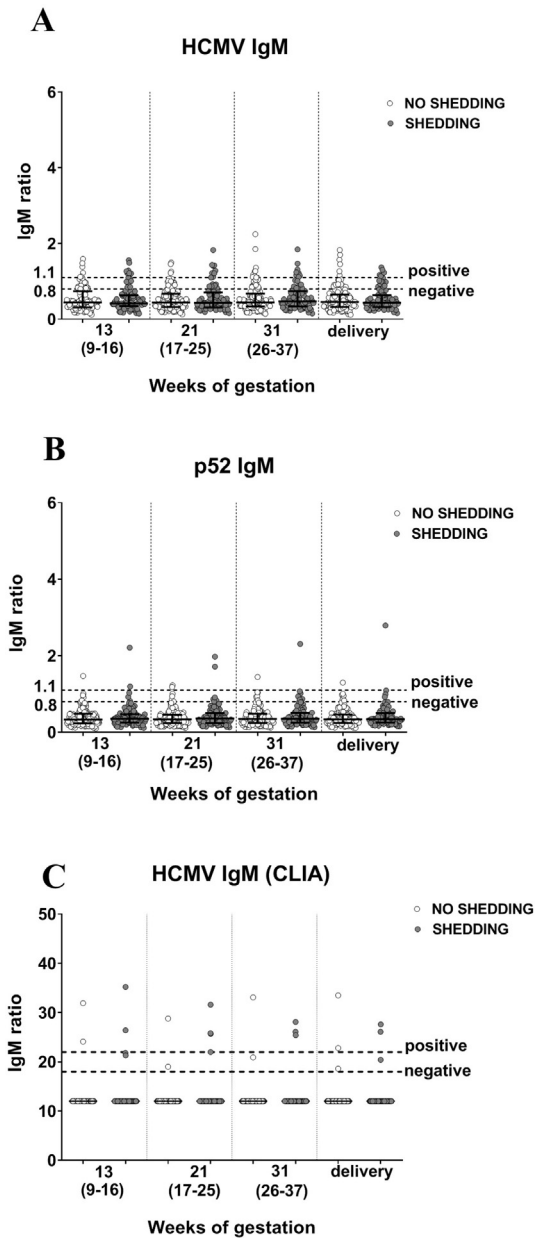

Fig. 3. Levels of IgM (A) anti-HCMV and anti-p52 IgM (B) by ELISA and levels of IgM anti-HCMV antigens (pp28, p52, pp150) (C) by chemiluminescence immunoassay (CLIA) in seropositive pregnant women with or without non-primary HCMV infection (NPI) at all times.

Risk factors associated with non-primary infection and pregnancy outcome

The median age was similar in women with or without non-primary infection. Close contact with young children (<36 months) in the family or for occupational reasons was not significantly different in women with or without non-primary infection (Table 1). All the other parameters analysed were similar in the two groups of women. None of the 105 women with non-primary infection transmitted the infection to the fetus (95% CI 0–3.5%), nor did women without non-primary infection, while 11/30 women with primary infection (36.7%; 95% CI 21.9–54.5%) did. All the newborn parameters examined were not significantly different between women with or without non-primary infection (Table 1).

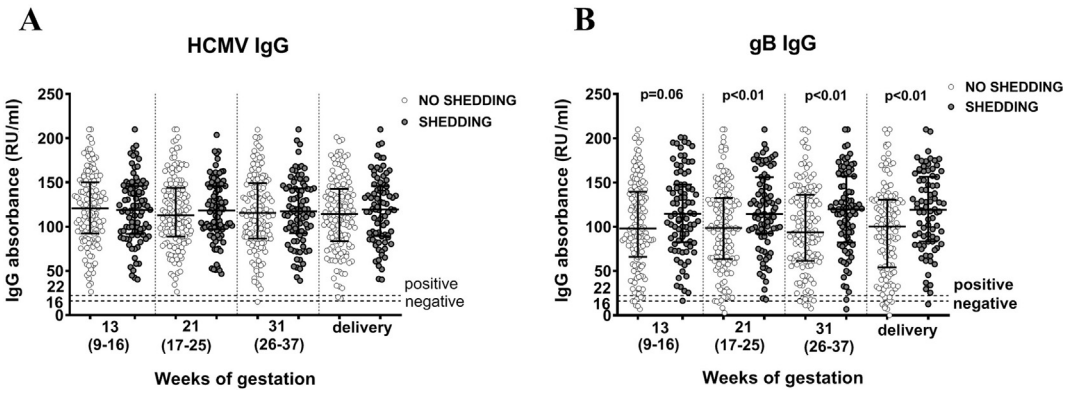

Fig. 4. Levels of IgG (A) and IgG anti-gB (B) by ELISA in seropositive pregnant women with or without non-primary HCMV infection (NPI) at all times. Kruskall–Wallis test and Dunn's *post hoc* test with correction for multiple comparisons were used for statistical analysis. Only p values ≤ 0.10 were reported.

Table 1
Analysis of potential risk factors for HCMV non-primary infection in seropositive pregnant women

Parameter	Lost to follow-up	Women with infection	Women without infection	p
Mother, *n*	49	105	145	
Age, years (median, range)	33 (19–41)	32 (19–49)	33 (22–44)	0.21
Delivery (median, range)	0 (0–3)	1 (0–2)	0 (0–3)	0.20
Abortion (median, range)	0 (0–4)	0 (0–3)	0 (0–3)	0.56
Multiple pregnancy, *n* (%)	1 (2.0)	4 (3.8)	4 (2.8)	0.72
Immunodeficiency, immunosuppression, autoimmune disease, *n* (%)	3 (6.1)	8 (7.6)	14 (9.7)	0.65
Smoking, *n* (%)	6 (12.2)	5 (4.8)	15 (10.3)	0.15
Diabetes, *n* (%)	0 (0.0)	1 (1.0)	3 (2.1)	0.64
Hypertension, *n* (%)	0 (0.0)	2 (1.9)	3 (2.1)	1.00
Mothers of children of <36 months, *n* (%)	10 (20.4)	30 (28.6)	30 (20.7)	0.18
Contact with children <36 months for occupational reasons, *n* (%)	1 (2.0)	4 (3.8)	3 (2.1)	0.76
Newborn, *n*[a]	NA	95	130	
Weeks of gestation at birth (median, range)	NA	40 (35–43)	40 (34–42)	0.47
Sex (male/female), *n* (%)	NA	37/58 (38.9/61.1)	66/64 (50.8/49.2)	0.10
Birth weight, g (median, range)	NA	3185 (2300–4465)	3270 (1980–4395)	0.95
Birth length, cm (median, range)	NA	50 (45–51)	51 (44–58)	0.96
Head circumference, cm (median, range)	NA	34 (31–37)	34 (30–38)	0.24
Apgar 1' (median, range)	NA	9 (4–10)	9 (1–10)	0.15
Apgar 5' (median, range)	NA	10 (7–10)	10 (8–10)	0.70

Statistical analysis was performed between women with or without infection. Categorical variables were compared with Fisher's exact test, and continuous variables were compared with the Mann-Whitney U test.
[a] Data were not available for 25 newborns.

Discussion

Major results of our study indicate that (a) about 40% pregnant women develop HCMV non-primary infection, as revealed by HCMV-DNA detection, mainly in vaginal secretions; (b) close contact with young children is not a risk factor for non-primary infection; (c) non-primary infection is not associated with the presence of HCMV-specific IgM; (d) the rate of cCMV appears to be much lower than that observed during primary infection.

The observed frequency of non-primary infection is in line with that reported in previous studies (reviewed in [4]). A more recent study conducted in Brazil [5] also reported 35% of pregnant women with HCMV non-primary infection, with a median viral load that was grossly 1 Log_{10} higher than in our subjects. However, this study did not find an increase in viral detection with the progression of pregnancy, and observed a higher rate of HCMV detection in saliva. Results of our study are more similar to the other reports showing an increase in HCMV detection during gestation, especially in the genital tract [11–15]. Due to the higher rate of positivity, a vaginal swab would be the sample of choice to investigate non-primary infection in pregnant women. However, we did not observe an association between HCMV shedding and cCMV, and therefore we do not believe that monitoring for HCMV in bodily fluids has to be used in routine clinical practice, nor that particular counselling should be provided to HCMV-seropositive pregnant women in European countries.

Another major difference of our study is the lack of association between HCMV non-primary infection and close contact with young children, which was reported in Brazil [5]. Whether or not cCMV in seropositive pregnant women is mainly a consequence of the reactivation of a latent strain or of reinfection with a new strain remains undefined. Recent studies have argued that maternal reinfection plays a significant role [6,16]. One of the most important routes of transmission of HCMV is via contact with young children as they actively shed the virus [17,18]. The association of HCMV non-primary infection and close contact with young children suggested that HCMV reinfection was the basis for non-primary infection in the Brazilian population. On the contrary, in our population, reactivation of the latent virus may more likely to be the cause of non-primary infection. The different socio-economic environment of the two populations may be the basis for the contrasting results observed.

Detection of HCMV-specific IgM in the presence of high-avidity IgG or increase in HCMV-specific IgG levels have been used markers of non-primary infection [19]. However, our data indicate that IgM is not a reliable diagnostic marker. In our cohort, 25/250

(10%) women showed the presence of HCMV-specific IgM at low levels (according to the assay that provided the highest positivity rate), either occasionally or consistently, but their detection was not associated with non-primary infection. Detection of p-52-specific IgM, which was shown to be more specific and restricted to the acute phase of infection [20], was even less frequent. In line with our data, a study conducted in Israel reported that IgM was not a useful diagnostic tool of non-primary infection in seropositive mothers delivering a cCMV newborn [21]. HCMV-specific IgM may persist at low levels for several months (or even years) after the onset of primary infection [20,22]. Thus, detection of low-level HCMV-specific IgM in the presence of high-avidity IgG does not exclude that a HCMV primary infection occurred some months before pregnancy.

Another caveat is also the fact that IgG determination may provide false-positive results [23], as observed in 5/255 (2.0%) women of our study. Had a congenital infection occurred in any of these women, a maternal non-primary infection could have been erroneously diagnosed.

The overall IgG response to HCMV was not different between women with or without non-primary infection. Strikingly, higher levels of antibodies specific for glicoprotein B (gB) was associated with non-primary infection. The higher levels of anti-gB antibodies may be a consequence of a greater immune stimulation induced by the non-primary infection. Intriguingly, higher levels of anti-gB antibodies were observed in pregnant women with primary infection after delivery of a congenitally infected newborn than in woman who did not transmit the infection [24,25].

The limitations of this study were the lack of genomic or serological analyses to distinguish between reinfection and reactivation, and the small sample size for the definition of the rate of cCMV. In addition, a high percentage of enrolled women (16%) were lost to follow-up.

No cCMV was observed in the 105 cases of non-primary infection (95% CI 0.0–3.5%). Although our study was not powered for the definition of cCMV rate, this result is in line with the hypothesized 0.2–3% transmission rate of non-primary infection [26]. This is about 10–100 times lower than that observed in primary infection (30–40%), indicating that maternal immunity is largely protective. While waiting for an HCMV vaccine, the implementation of a prevention strategy in Italy (and other developed countries) based on pre-conception serological screening to identify women at risk for primary infection and their information about hygienic and behavioural measures to reduce it [27] appears justified.

Transparency declaration

The authors have no conflict of interest to declare. This study was funded by the “Fondazione Regionale per la Ricerca Biomedica” (grant no. FRRB 2015-0043), the “Ministero della Salute”, Ricerca Corrente (grant no. 8053615).

Author contributions

P.Z. conducted the study; P.Z. and P.d.A. analysed data; M.D.C., L.F., D.C. performed virological analyses; P.d.A., A.S.a. performed serological analyses; V.M., S.P., C.A. enrolled subjects and collected data; M.F., A.A., G.M., A.S.p. performed clinical follow-up; D.L. conceived the study and drafted the manuscript; all the authors critically revised the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2021.09.013.

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