

Cytomegalovirus (CMV) Enzyme-Linked Immunosorbent Spot Assay but Not CMV QuantiFERON Assay Is a Novel Biomarker To Determine Risk of Congenital CMV Infection in Pregnant Women

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Cytomegalovirus (CMV) enzyme-linked immunosorbent spot (ELISPOT) and CMV QuantiFERON assays were examined as potential biomarkers predictive of congenital CMV (cCMV) transmission. Fifty-seven pregnant women with primary CMV infection and 23 with nonprimary CMV infection were recruited in the study. Maternal age, CMV IgG avidity, viremia, and viruria were also included among the potential predictors. Spearman's statistical correlation analysis revealed a positive correlation between the CMV ELISPOT and CMV QuantiFERON assay results ($P < 0.001$), but only the CMV ELISPOT assay correlated with cCMV ($P < 0.001$). cCMV was positively correlated with maternal viremia and viruria ($P < 0.05$) and negatively correlated with CMV IgG avidity ($P < 0.01$). Maternal age and CMV QuantiFERON assay results were not statistically associated with cCMV. CMV-specific cell-mediated immunity detected by the CMV ELISPOT assay plays a critical role in cCMV.

Congenital cytomegalovirus (cCMV) infection affects about 0.7% of newborns worldwide (1–3). The clinical CMV-related sequelae at birth are highly variable and related to maternal serostatus and the time of onset of congenital infection during pregnancy (4–9). Whenever clinically evident, CMV-induced damages include sensorineural hearing loss (SNHL), visual impairment, delayed psychomotorial development, and retardation (10–13). Understanding the risk factors and biomarkers associated with the maternal transmission of CMV infection represents a leading priority for both diagnosis and clinical management of cCMV. Recently, it was shown that maternal CMV cell-mediated immunity (CMI) plays a critical role in determining cCMV (14–16). Several assays are available to assess CMV-specific CMI, and the large majority of these assays are based on interferon gamma (IFN- γ) release assays (IGRAs) (17–20). In this study, two IGRAs that detect CMV-specific CMI, the CMV enzyme-linked immunosorbent spot (ELISPOT) and CMV QuantiFERON assays, were compared for their prediction of cCMV. Both the CMV ELISPOT and CMV-QuantiFERON assays detect IFN- γ produced by antigen-stimulated peripheral blood mononuclear cells (PBMCs). The main differences between the assays include the antigen stimulus composition, with stimulation of CD8⁺ T-cell responses in the CMV QuantiFERON assay (21) and stimulation of both CD4⁺ and CD8⁺ T-cell responses in the CMV ELISPOT assay (22, 23). Moreover, the CMV QuantiFERON assay detects IFN- γ in a volume of ~1 ml of whole blood, while the CMV ELISPOT assay detects IFN- γ secreted by ~2 × 10⁵ PBMCs (22, 23). Recent studies suggest that the CMV ELISPOT and CMV QuantiFERON assays may display large variability on an individual basis (24, 25).

In order to have a more comprehensive view of the maternal factors associated with cCMV, this study also investigated maternal parameters such as maternal age, viremia, viruria, and CMV immunoglobulin G (IgG) avidity.

(The data in this study were partly presented at the Congenital CMV Conference, Brisbane, Australia, 2015.)

MATERIALS AND METHODS

Patients. Eighty pregnant Caucasian women were referred from January 2012 to January 2013 to the Padua Reference Center for Gestational and Congenital Infections for suspected infection and potential risk for the fetus. Patients and patient specimens were previously described in other studies (15, 25). The Padua Reference Center represents the main referral hub for congenital infections, serving about 950,000 women ranging in age from 15 to 45 years in the Veneto region (National Statistic Institute [ISTAT] data [see <http://www.istat.it/en/veneto>]). Patient exclusion criteria were (i) women with preexisting or acquired immunodeficiency or (ii) women exhibiting primary CMV infection after the 20th week of gestation. The median age of the pregnant women was 31 years (range, 17 to 42 years). These cases were classified as primary CMV infection (57 women) and nonprimary CMV infection (23 women). Primary maternal CMV infection was defined by (i) seroconversion in previously seronegative mothers or (ii) detection of maternal CMV immunoglobulin M (IgM) and concomitant low maternal CMV IgG avidity (<25%). Nonprimary CMV infection was defined by the presence of CMV viruria in already CMV IgG-positive pregnant women and detection of CMV IgG avidity of >45% within the 14th week of gestation. All serologic and molecular tests were performed at the Padua General Hospital Microbiology and Virology Diagnostic Laboratory. In primarily infected pregnant women, the estimated timing of CMV infection occurred within a median of 6 weeks of gestation (range, 0 to 20 weeks), and CMV ELISPOT and CMV QuantiFERON assays were performed within a median of 8 weeks

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TABLE 1 Spearman pairwise correlation analysis^a

Variable	Correlation coefficient						
	Maternal age	Maternal CMV ELISPOT assay result	Maternal CMV QuantiFERON assay result	Maternal CMV IgG avidity	CMV detection in maternal blood	CMV detection in maternal urine	CMV transmission to newborn
Maternal age	1						
Maternal CMV ELISPOT assay result	0.04	1					
Maternal CMV QuantiFERON assay result	-0.1053	0.3629***	1				
Maternal CMV IgG avidity	-0.0196	-0.3101**	-0.0958	1			
CMV detection in maternal blood	-0.0996	0.3195**	0.1429	-0.6042***	1		
CMV detection in maternal urine	-0.2228*	0.0912	-0.0399	0.0755	0.1699	1	
CMV transmission to newborn	-0.0177	0.4357***	-0.1131	-0.3075**	0.2565*	0.2537*	1

^a Negative values indicate negative correlations. Maternal age, CMV ELISPOT assay result, CMV QuantiFERON assay result, and CMV IgG avidity were quantitative variables, while CMV viremia and viruria and CMV transmission to the newborn were expressed as binary variables (0 or 1). Significance is indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

(range, 2 to 17 weeks) after CMV infection. Of the women experiencing primary CMV infection, 16/57 (28%) transmitted the infection to the fetus, 19/57 (33%) had episodes of viremia, and 43/57 (75%) had viruria. Of the 23 nonprimary infections, no cases of CMV viremia were reported, all women experienced CMV viruria, and no cases of congenital transmission occurred. Fetal or newborn CMV infection was assessed by CMV DNA detection in amniotic fluid at 20 to 21 gestational weeks of age or in urine at birth (26, 27). The Padua General Hospital Ethical Committee approved the study. The participants provided written informed consent.

Detection of maternal CMV DNA in blood and urine, CMV IgM and IgG, CMV IgG avidity, CMV QuantiFERON, and CMV ELISPOT tests. CMV IgM and CMV IgG (Siemens Immulite) and CMV IgG avidity (Tecnogenetics) tests were performed according to the manufacturers' instructions. CMV DNA detection in blood, amniotic fluid, and urine was performed by using real-time quantitative PCR (28). The lowest limit of CMV DNA detection is <1,000 copies/ml of whole blood. In primary and nonprimary CMV infections, CMV ELISPOT and QuantiFERON assays were performed after confirmation of infection. CMV ELISPOT and QuantiFERON tests were performed at the same time.

Blood was drawn into 10-ml tubes containing sodium citrate (CMV ELISPOT assay), and ~3 ml was collected into 3 CMV QuantiFERON tubes (positive control, negative control, and CMV, with ~1 ml/tube), according to the manufacturers' instructions. CMV QuantiFERON tubes (Qiagen) were kept overnight at 37°C, and samples were then processed according to the manufacturer's instructions. PBMCs were isolated from whole blood by Ficoll density gradient centrifugation and used for the CMV ELISPOT assay (Autoimmune Diagnostika) as previously described (29). Freshly isolated PBMCs were stimulated with commercially available peptides spanning the highly immunogenic CMV pp65 (ppUL83) protein (Autoimmune Diagnostika). In order to avoid false-negative responses due to peptide misrecognition, the whole CMV lysate was included as an internal control, as described previously (29).

Statistical analysis. Data including maternal age, maternal CMV ELISPOT assay result, maternal CMV QuantiFERON assay result, maternal IgG avidity, maternal CMV detection in blood and urine, and CMV transmission to the newborn were analyzed by using Spearman's pairwise correlation analysis. For statistical analysis, viremia, viruria, and CMV transmission were considered qualitative categorical binary variables (0 [yes] or 1 [no]). Maternal age, CMV ELISPOT assay result, CMV QuantiFERON assay result, and CMV IgG avidity were quantitative continuous variables. The Spearman rank method was employed to analyze the correlation of CMV ELISPOT and QuantiFERON assay results with the time of CMI assay determination to assess if the time of assay deter-

mination (2 to 17 weeks) introduced any bias for the CMI assays and thus for determination of cCMV. P values of <0.05 were considered statistically significant. To determine the diagnostic performance of the CMV ELISPOT and CMV QuantiFERON assays, a receiver operating characteristic (ROC) analysis was performed. The area under the curve (AUC) indicates the performance of the diagnostic test. Youden's index was employed to determine the optimum diagnostic cutoff point (30). Statistical analysis was performed by using STATA 14 software. R software (open-source software; R Foundation [see <http://www.r-project.org/>]) was used for translating the numerical data reported in Table 1 and Fig. 1. The

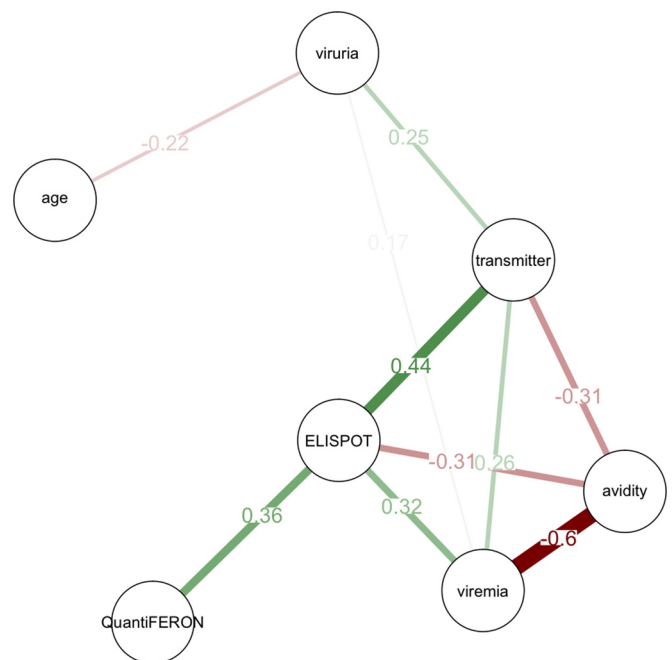


FIG 1 Graphical representation of the factors associated with congenital CMV transmission. Green lines indicate a positive correlation, while red lines indicate a negative correlation. The thickness of the lines and intensity of the color are directly proportional to the significance. The numbers indicated within the connecting lines represent the correlation coefficients. The distance between nodes is proportional to the degree of correlation.

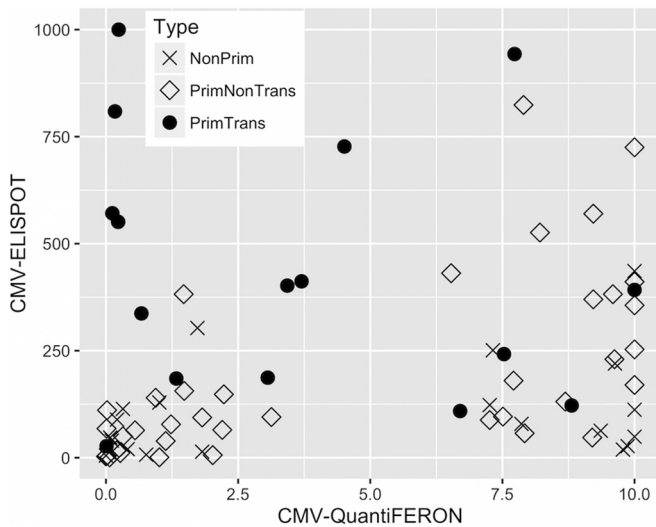


FIG 2 Scatterplot distributions of CMV ELISPOT and CMV QuantiFERON assay results for transmitting (PrimTrans) and nontransmitting (PrimNon-Trans) pregnant women with primary CMV infection and pregnant women with nonprimary CMV infection (NonPrim). Detection by the CMV ELISPOT assay was limited to 1,000 spots/ 2×10^5 PBMCs, while detection by the CMV QuantiFERON assay was limited to 10 IU/ml.

distance between the nodes is decreased when the nodes are highly correlated.

RESULTS

In order to examine the factors associated with cCMV, a pairwise Spearman statistical correlation analysis was performed, and congenital transmission, maternal age, CMV ELISPOT assay result, CMV QuantiFERON assay result, CMV IgG avidity, and CMV DNA detection in maternal blood and urine were considered (Table 1). CMV transmission to the newborn (Fig. 1) was positively associated with the maternal CMV ELISPOT assay result (correlation coefficient, 0.4357; $P < 0.001$) and detection of CMV DNA in maternal blood and urine (correlation coefficients, 0.2565 and 0.2537, respectively; $P < 0.05$ for both) and negatively associated with maternal IgG avidity (correlation coefficient, -0.3075 ; $P < 0.01$). Remarkably, the CMV ELISPOT assay result was positively correlated with the CMV QuantiFERON assay result and viremia (correlation coefficients, 0.3629 and 0.3195, respectively; $P < 0.001$ and $P < 0.01$, respectively) and negatively correlated with CMV IgG avidity (correlation coefficient, -0.3101 ; $P < 0.01$). CMV IgG avidity was negatively correlated with CMV viremia (correlation coefficient, -0.6042 ; $P < 0.001$). The factors not statistically correlated with cCMV were maternal age (correlation coefficient, -0.0177) and CMV QuantiFERON assay result (correlation coefficient, -0.1131).

The different patterns of CMV ELISPOT and CMV QuantiFERON assay results for transmitting and nontransmitting mothers with primary infection and for mothers with nonprimary infection are shown in Fig. 2. As previously described (25), primary infections display statistically significantly higher CMI than nonprimary infections. To estimate the diagnostic performance and determine the diagnostic thresholds for cCMV, a ROC analysis was performed for the CMV ELISPOT assay, the CMV QuantiFERON assay, and the combination of both the CMV ELISPOT and CMV QuantiFERON assays (Fig. 3).

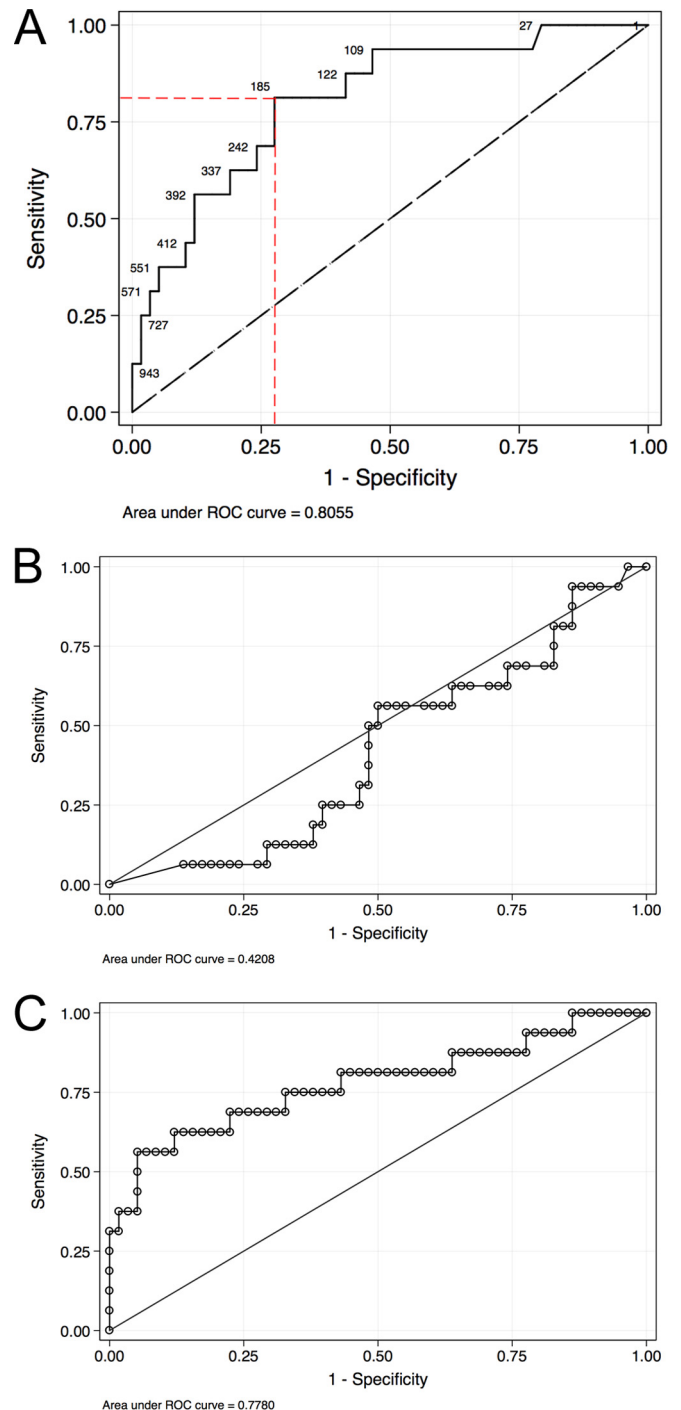


FIG 3 Receiver operating characteristic (ROC) analysis of the CMV ELISPOT assay alone (A), the CMV QuantiFERON assay alone (B), and the combination of both the CMV ELISPOT and CMV QuantiFERON assays (C). Panel A also shows Youden's index with the highest specificity and sensitivity.

The CMV ELISPOT assay had an AUC value of 0.805, and Youden's index was calculated to be 185 spots/ 2×10^5 PBMCs for the CMV ELISPOT assay (Fig. 3A). Youden's index defines the point with the highest sensitivity and specificity, generally used for cutoff determination. This result was previously described (15). The CMV QuantiFERON assay had an AUC of 0.42, below the

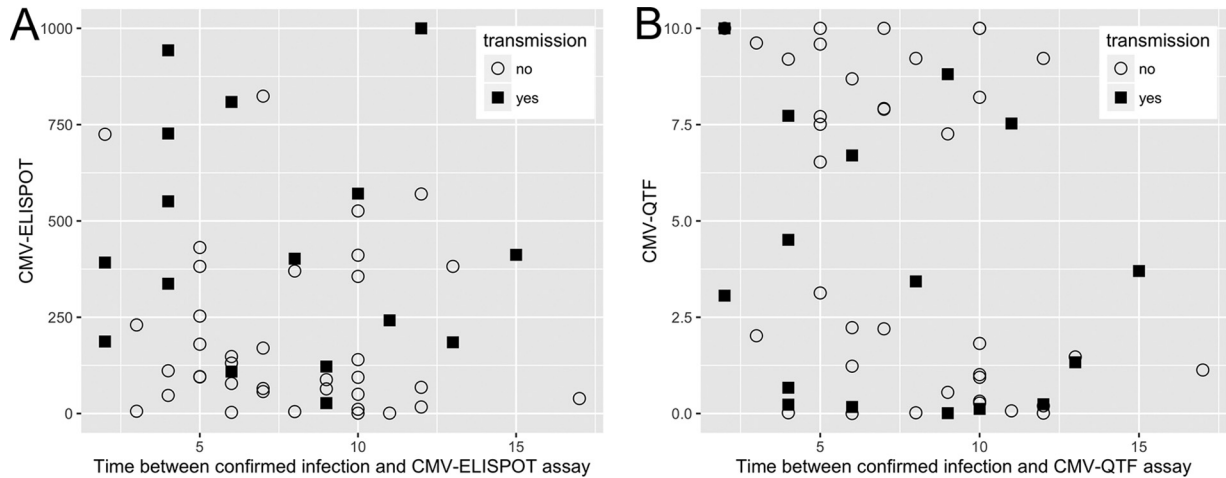


FIG 4 Scatterplot distributions of the CMV ELISPOT (A) and CMV QuantiFERON (B) assay results over time (weeks) after infection.

reference (AUC = 0.5) and thus a null value (Fig. 3B). For this reason, a CMV QuantiFERON assay cutoff was not determined. The combination of both the CMV ELISPOT and CMV QuantiFERON assays had an AUC value of 0.778 (Fig. 3C) and thus did not improve the overall diagnostic performance. Since the CMV ELISPOT and CMV QuantiFERON assays were executed within 8 weeks (range, 2 to 17 weeks) after confirmation of infection, we investigated whether the time of assay execution may have influenced the robustness of the immune response, introducing a bias into the analysis of cCMV. Figure 4 shows the plots of CMV ELISPOT and CMV QuantiFERON assay results for transmitting and nontransmitting mothers and the time of assay execution. The correlation of both assays was investigated by using the Spearman rank method regression. The test *P* values were 0.2277 (Spearman's rho = -0.1638) for the CMV ELISPOT assay and 0.0653 (Spearman's rho = -0.2481) for the CMV QuantiFERON assay. For both assays, the *P* values were not significant, indicating that both the CMV ELISPOT and CMV QuantiFERON assay results were independent of the time of the assay. The effect of CMV IgG avidity on cCMV was also investigated: Fig. 5 shows the kernel density and fraction of CMV IgG avidity patterns in transmitting

versus nontransmitting mothers, showing that CMV IgG avidities of between 2 and 20% were associated with cCMV.

DISCUSSION

This study, conducted on 80 pregnant women with primary and nonprimary CMV infections, evaluated the CMV ELISPOT and CMV QuantiFERON assays as potential biomarkers associated with cCMV. Other known biomarkers, such as maternal viremia, viruria, CMV IgG avidity, and age, were also included in this study. This study showed that the CMV ELISPOT assay result was strongly associated with an increased risk of cCMV. Other factors associated with cCMV were low IgG avidity and virus detection in blood and urine. Maternal age does not influence the risk of cCMV. The CMV QuantiFERON assay result correlated with the CMV ELISPOT assay result but not with cCMV. If both the CMV ELISPOT and CMV QuantiFERON assays are used to determine the risk of cCMV, the combined diagnostic performance is lower than that of the CMV ELISPOT assay alone. The optimal cutoff for cCMV in the CMV ELISPOT assay was 185 spots/2 × 10⁵ PBMCs. This study and a previous one (15) showed an increased rate of cCMV in the presence of low CMV IgG avidity and a robust ma-

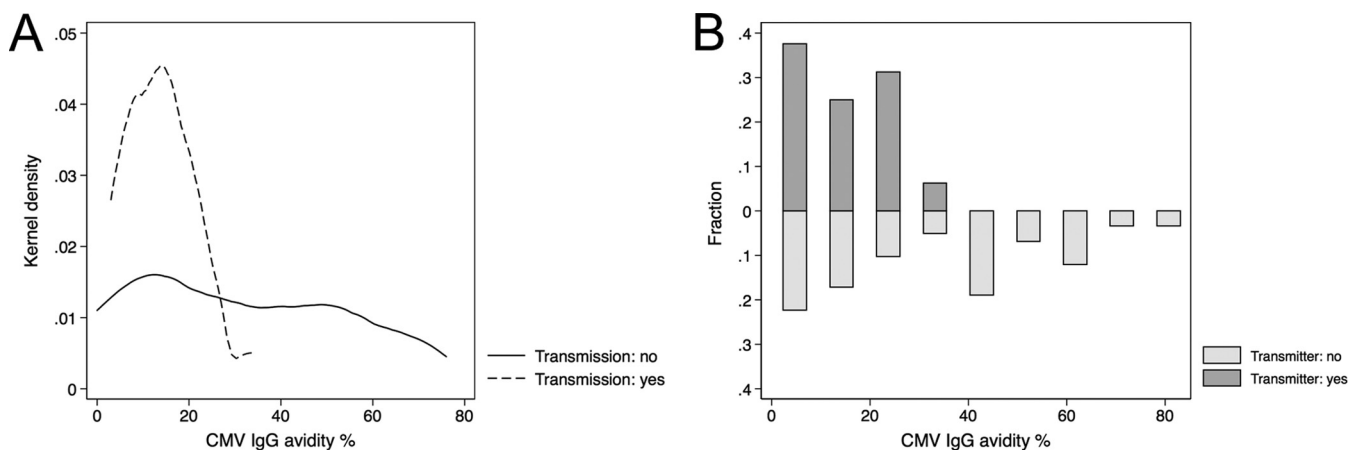


FIG 5 CMV IgG avidity in transmitting and nontransmitting CMV-infected pregnant women. (A) Kernel density distribution; (B) fraction.

ternal T-cell response to CMV; thus, cCMV may be a consequence of an imbalanced Th1/Th2 response to CMV. In particular, we speculate that an altered CMV-specific CD4⁺ T-cell response, detected by the CMV ELISPOT assay but not by the CMV QuantiFERON assay, may promote cCMV. A second hypothesis is that cCMV may be a consequence of a proinflammatory state at the placental level caused by a large number of circulating CMV-specific activated T cells secreting IFN- γ . Under proinflammatory conditions, the placenta may express atypical receptors and molecules, facilitating CMV transplacental passage (31–34). The third hypothesis is that the strong T-cell response observed for cCMV may be directly related to a preceding protracted or high-level viremia: evidence from the transplant field suggests that high viral loads in blood promote higher-level T-cell responses (35). The latter hypothesis would be technically difficult to assess for pregnancy since T-cell assays are performed after the occurrence of CMV infection.

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D.A., A.S., G.F., and C.M. analyzed the data and wrote the manuscript. A.S. and G.F. collected and analyzed the CMV ELISPOT assay and CMV QuantiFERON assay data. G.F. and N.G. collected the clinical and diagnostic data. C.M. performed the statistical analysis. G.P. and N.G. supervised the study.

We declare no conflicts of interest.

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