Human cytomegalovirus (CMV) is a major cause of morbidity in fetuses following intrauterine infection. The glycoprotein (g) envelope trimeric gH/gL/gO and pentameric gH/gL/pUL128/pUL130/pUL131A complexes are required for CMV entry into fibroblasts and endothelial/epithelial cells, respectively, and both are targets for neutralizing antibodies. The role of sequence variability among viral strains in the outcome of congenital CMV infection is controversial. Variation in the CMV UL75 gene encoding glycoprotein H (gH), the UL115 (gL), the UL74 (gO), and the UL128 locus (UL128L) encoding three structural proteins (pUL128, pUL130, and pUL131A) was determined in 82 newborns with congenital CMV infection and 113 infants with postnatal or unproven congenital CMV infection. Genotyping was performed by sequencing analysis of PCR-amplified fragments and the PCR-restriction fragment length polymorphism (RFLP) method, and the viral load was measured by quantitative real-time PCR. The obtained results demonstrated that (1) different CMV variants and mixed CMV infections can be detected in newborns infected congenitally; (2) the gH1 genotype, UL130 variant 6, and UL131A variant 1 were associated with some signs/symptoms within cohort of pediatric patients, mainly consisting of infants with symptomatic CMV infection. The results revealed that pUL130, pUL131A, and gH polymorphisms seemed to be associated with the outcome of CMV infection in infants.
splenomegaly, thrombocytopenia, petechiae, jaundice, and seizures.\textsuperscript{46,47} Moreover, approximately 15% of newborns with asymptomatic cCMV infection at the birth develop long-term neurological sequelae in the next years of life.\textsuperscript{48} The highest risk of severe symptoms in the fetus and newborn exists in cases of primary maternal infection in the first-trimester.\textsuperscript{49} However, natural immunity is not protective against CMV infection, and the majority of infected newborns are born to mothers who are already seropositive.\textsuperscript{51} Primary or nonprimary maternal infection, including reactivation of the latent virus or reinfecction with a different strain, can lead to crossing the placenta and infecting the fetus. Approximately 20–30% of nonimmune women who are infected during pregnancy transmit the virus to their offspring, whereas the rate of transmission following nonprimary infection is between 0.6–1%.\textsuperscript{57}

CMV replicates in different cells, including epithelial cells, endothelial cells, fibroblasts and smooth muscle cells, which are the predominant targets.\textsuperscript{52} This broad cell tropism facilitates both systemic spread within the host with efficient proliferation of the virus and interhost transmission. A number of factors can affect the interaction between the host immune system and the virus, and the major determinants of CMV disease appear to be host factors. The polymorphisms in the CMV genes encoding envelope glycoproteins, important targets of the host immune response, could potentially be associated with variability in the control of virus infection. Sequence variability among the viral strains may affect viral dissemination, disease severity, and immune response.\textsuperscript{53} However, placental transmission appears to be independent of a specific virus strain, and different CMV genotypes are detected in infected neonates.\textsuperscript{14–19} An association between CMV variants in surface proteins and clinical outcome appears to be independent of a specific virus strain, and different CMV genotypes are more frequently in congenital than in postnatal patients and are associated with an increased risk of specific disease outcomes. Because mainly symptomatic patients were enrolled in the study, our findings may not pertain to recent studies have revealed findings concerning pentamer structure, location of epitopes for neutralizing antibodies and potential binding sites for cell surface receptors.\textsuperscript{57} These data suggest that receptor binding triggers a conformational change in the pentamer, allowing it to interact with gB and initiate the membrane fusion process. The dimer gH/gL is thought to act as an intermediary, transmitting the fusion trigger to gB.\textsuperscript{66–67} It is suggested that complexes containing gH/gL play a key role in host cell tropism.\textsuperscript{52–54,68} Moreover, high expression of the pentamer on the epithelial cell surface leads to the interference of virus entry into cells, possibly through sequestration of cell surface receptors, providing strong evidence for a cell-specific receptor.\textsuperscript{58} High levels of pentamer expression have been associated with an increase in cell-association of the virus and with cell-to-cell transmission.\textsuperscript{59} Pentamer proteins are the dominant target of the most potent neutralizing antibodies, highlighting their critical role in CMV infection.\textsuperscript{51,52} While antibodies that target gB and gH/gL prevent infection of all cell types, antibodies specific to the pentamer are a thousand-fold more potent than antibodies against gB or gH/gL complex specifically in neutralizing CMV infection in epithelial and endothelial cells.\textsuperscript{52,53} Moreover, antibodies against pentamer are capable of protecting cytomegalovirus against CMV infection.\textsuperscript{53} A response against pentamer in seronegative pregnant women with primary infection is associated with a lower risk of transmission of the virus to the fetus.\textsuperscript{54} These findings make the gH/gL/pUL128-131A complex a promising vaccine candidate.

In this study, we determined the distribution of CMV genes that code proteins forming trimer and pentamer complexes in infants with congenital and postnatal infection. We show that some genomic variants are detected more frequently in congenital than in postnatal patients and are associated with an increased risk of specific disease outcomes. Because mainly symptomatic patients were enrolled in the study, our findings may not pertain to...
Results

Study population and clinical outcome. One hundred and ninety-five pediatric patients with CMV DNAemia were enrolled in the study. The patients were selected based on clinical diagnosis and diagnostic markers. Congenital CMV infection was confirmed in 82 newborns (positive CMV DNA in urine ≤ 21 day of life), and the remaining 113 children were classified as having postnatal or unproven congenital CMV infection (pCMV). Among infants in pCMV group, negative CMV DNA in urine ≤ 21 day of life was found in 83% cases. The average age at which the newborns with cCMV infection were examined was 9.6 days (median age 8.0 days; range 1–21 days), while in the group of infants with pCMV infection, it was 3.4 months (median age 3.0 months; range 1–11.5 months). The demographic and clinical characteristics of congenitally or postnatally infected children are summarized in Table 1. Almost all examined patients demonstrated cytomegaly symptoms, and this high frequency of symptomatic infection was due to selection bias. The most prevalent symptoms in CMV-infected children from both groups were neurological dysfunction and hematological disorders (especially anemia and thrombocytopenia). In addition, newborns with CMV infection usually demonstrated CNS damage.

Table 1. Demographic and clinical characteristics of study subjects with CMV infection. cCMV, congenital CMV infection; pCMV, postnatal or unproven congenital CMV infection; aP, Fisher’s exact test; bValues are the number of infants (%); cP, Mann-Whitney U test with correction for continuity; dValues are the number of infants with symptomatic CMV infection (%); eNeurological dysfunction e.g. tremor, hypotonia/hypertonia, or poor sucking reflex; fCNS damage; central nervous system damage e.g. cystic lesions, microcephaly, intracranial calcification, or ventriculomegaly; gHematological disorders e.g. anemia, thrombocytopenia, thrombocytosis, neutropenia, or leukocytosis; IUGR, intrauterine growth restriction.

| Characteristics                          | Type of infection | P*  |
|-----------------------------------------|------------------|-----|
|                                         | cCMV             | pCMV|     |
| Total No.                               | 82               | 113 |     |
| Mean ± SD age                           | 9.6 ± 6.1 days   | 3.4 ± 2.2 months |     |
| Median (range) age                      | 8 (1–21) days    | 3 (1–11.5) months |     |
| Gender number; n (%)b                   |                  |     |
| Female                                  | 39 (47.6)        | 43 (38.1) | 0.186 |
| Male                                    | 43 (52.4)        | 70 (61.9) |     |
| Mean ± SD birth weight (g)              | 2 763 ± 651      | 2 884 ± 874 | 0.06* |
| Mean ± SD gestational age at birth (weeks) | 37.3 ± 4.6      | 37.1 ± 3.8 | 0.79* |
| Symptoms/signs; n (%)                   |                  |     |
| Neurological dysfunction²               | 60 (75.9)        | 58 (54.2) | 0.003 |
| CNS damage²                            | 58 (73.4)        | 24 (22.4) | < 0.0001 |
| -Cystic lesions                         | 45 (57.0)        | 11 (10.3) | < 0.0001 |
| -Microcephaly                           | 21 (26.6)        | 3 (2.8)   | < 0.0001 |
| -Intracranial calcification             | 28 (35.4)        | 6 (5.6)   | < 0.0001 |
| -Ventriculomegaly                      | 25 (31.6)        | 10 (9.3)  | 0.0002 |
| IUGR                                    | 32 (40.5)        | 10 (9.3)  | < 0.0001 |
| Hearing loss                            | 31 (39.2)        | 10 (9.3)  | < 0.0001 |
| Ocular defects                          | 19 (24.1)        | 11 (10.3) | 0.015 |
| Liver damage                            | 10 (12.7)        | 9 (8.4)   | 0.463 |
| Hepatitis                               | 6 (7.6)          | 21 (19.6) | 0.022 |
| Hepatomegaly                            | 16 (20.3)        | 14 (13.1) | 0.228 |
| Splenomegaly                            | 16 (20.3)        | 10 (9.3)  | 0.053 |
| Hepatosplenomegaly                      | 13 (16.5)        | 7 (6.5)   | 0.053 |
| Pneumonia                               | 3 (3.8)          | 19 (17.8) | 0.005 |
| Petechiae                               | 16 (20.3)        | 2 (1.9)   | < 0.0001 |
| Jaundice                                | 19 (24.1)        | 38 (35.5) | 0.109 |
| Hematological disorders²               | 51 (64.6)        | 72 (67.3) | 0.755 |
| -Anemia                                 | 42 (53.2)        | 60 (56.1) | 0.766 |
| -Thrombocytopenia                       | 23 (29.1)        | 8 (7.5)   | 0.0001 |
| Anti-CMV serologic status; n (%)⁵       |                  |     |
| IgG-positive, IgM-negative              | 46 (56.1)        | 62 (54.9) | 1.080 |
| IgG-positive, IgM-positive              | 32 (39.0)        | 42 (37.2) | 0.891 |
| IgG-negative, IgM-negative              | 0 (0)            | 6 (5.3)   | 0.083 |
| No data                                 | 4 (4.9)          | 3 (2.6)   | 0.457 |

all CMV-infected infants. These studies confirm the findings that various CMV variants can be vertically transmitted. The results support the hypothesis that variability in pentamer genes is an important factor that affects clinical sequelae following CMV infection.
infants with pCMV infection, was gO1 (approximately 91%). The gO4 genotype was detected more frequently in those from pCMV group (P < 0.0001). Similarly, the median blood virus load was significantly higher in children with cCMV infection than in children with pCMV infection compared to the children who had pCMV infection (P < 0.0001), unilateral or bilateral hearing loss (P < 0.0001), ocular defects (P = 0.015), thrombocytopenia (P = 0.0001), and petechiae (P < 0.0001) occurred more commonly after cCMV infection. Some complications, such as pneumonia and hepatitis were more common in infants in pCMV group (P = 0.005 and P = 0.022, respectively).

Viral load. The CMV DNA was detected in 94 samples obtained from 82 newborns with cCMV infection and in 125 samples obtained from 113 infants with postnatal or unproven congenital CMV infection. The viruria was found in all newborns with cCMV infection and in 83/113 (73.5%) of infants from pCMV group after 21 days of life. The CMV DNA concentration in blood samples obtained from congenitally infected newborns ranged from 2.1 × 10^1 to 3.2 × 10^5 copies/mL (median 4.2 × 10^3 copies/mL; mean 3.8 × 10^4 ± 8.6 × 10^4 copies/mL), while in urine samples, it ranged from 3.1 × 10^2 to 8.7 × 10^5 copies/mL (median 1.2 × 10^5 copies/mL; mean 3.4 × 10^4 ± 1.2 × 10^4 copies/mL). In infants from pCMV group, the viremia levels ranged from undetected to 5.3 × 10^3 copies/mL (median 3.6 × 10^3 copies/mL; mean 2.7 × 10^4 ± 1.4 × 10^4 copies/mL), whereas the viruria levels ranged from undetected to 2.2 × 10^2 copies/mL (median 1.6 × 10^3 copies/mL; mean 3.5 × 10^4 ± 2.4 × 10^4 copies/mL). In both patient groups, the CMV DNA concentration in urine was significantly higher than in blood samples (P < 0.0001, Wilcoxon test). The concentration of CMV DNA in urine samples was higher among the children with cCMV infection compared to the children who had pCMV infection (P < 0.0001; Mann–Whitney U-test). Similarly, the median blood virus load was significantly higher in children with cCMV infection than in those from pCMV group (P = 0.0002; Mann–Whitney U-test).

Prevalence of CMV variants. The trimer gH/gL/gO gene products were amplified for all examined pediatric patients (Table 2). Genotyping of gH was performed by analysis of nested PCR (nPCR)-amplified fragments and the two genomic variants were identified by different amplicon size lengths. The applied PCR-restriction fragment length polymorphism (PCR-RFLP) method allowed the detection of major gL or gO genotypes (Fig. 1). Genotypes determined by RFLP were sequenced, and no discrepancies were found. In contrast, the UL128 locus gene products were amplified and sequenced successfully for 79/82 (96.3%) newborns with cCMV infection and 101/113 (89.4%) infants in pCMV group (Table 3).

The distribution of the gH genotypes were similar in both patient groups (P > 0.05). To explore the data, one hundred and five infants with CMV infection, including 28 cases of cCMV and 77 cases of pCMV, were examined for the presence of UL75 genotypes as described in our previous study [17]. No significant advantages of the gH1 genotype in congenital cases and gH2 in postnatal cases were observed. The gH1 genotype was detected in 54/82 (65.9%), while the gH2 in 46/82 (56.1%) newborns with CMV infection (Table 2). Among children with pCMV infection, the gH1 genotype was found in 63/113 (55.8%), while the gH2 genotype was found in 74/113 (65.5%) cases. The nucleotide sequence analysis confirmed the gH genomic variants previously identified by a nPCR analysis. Mixed infections with both gH1 and gH2 genotypes were detected in 18/82 (22.0%) patients with cCMV infection and in 24/113 (21.2%) cases with pCMV infection.

The UL115 gene encoding gL had a low proportion of nucleotide and amino acid variability in clinical isolates (5–9% and 1.4–2.5%, respectively), while low sequence conservation for UL74 (gO) was observed (sequence variability: 20–50% and 19.4–26.4%, respectively). All obtained amino acid sequences are illustrated in Fig. 2A–C. The gL3 genotype was prevalent in congenital infections, whereas this genomic variant was less common in postnatal infection (P < 0.0001). The most prevalent gO genotype in both groups, newborns with cCMV infection and infants with pCMV infection, was gO1 (approximately 91%). The gO4 genotype was detected more frequently in

| Gene     | Genotype | Prevalence of CMV genotypes, n (% | pCMV | cCMV | P     |
|----------|----------|-----------------------------------|------|------|-------|
| UL74     | gO1      | 75 (91.5)                         | 103  | 91.2 | 1.000 |
|          | gO2      | 7 (8.5)                           | 4    | 3.5  | 0.207 |
|          | gO3      | 5 (6.1)                           | 6    | 5.3  | 1.000 |
|          | gO4      | 20 (24.4)                         | 12   | 10.6 | 0.018 |
|          | mixed    | 26 (31.7)                         | 13   | 11.5 | 0.0009|
| UL75     | gH1      | 54 (65.9)                         | 63   | 55.8 | 0.183 |
|          | gH2      | 46 (56.1)                         | 74   | 65.5 | 0.233 |
|          | mixed    | 18 (22.0)                         | 24   | 21.2 | 1.000 |
| UL115    | gl1      | 40 (48.8)                         | 46   | 40.7 | 0.307 |
|          | gl2      | 23 (26.8)                         | 36   | 31.8 | 0.637 |
|          | gl3      | 50 (61.0)                         | 31   | 27.4 | <0.0001|
|          | gl4      | 23 (28.0)                         | 37   | 32.7 | 0.532 |
|          | mixed    | 46 (56.1)                         | 34   | 30.1 | 0.0004|
| UL74-UL75-UL115 | mixed | 62 (75.6) | 55 | 48.7 | 0.0002|

Table 2. Distribution of CMV gH/gL/gO genotypes in infants infected congenitally (n = 82) or postnatally (n = 113). n, number of isolates with CMV genotype; cCMV, congenital CMV infection; pCMV, postnatal or unproven congenital CMV infection; *P, Fisher’s exact test.
congenital compared to postnatal infection ($P = 0.018$). Congenital infection with mixed gL and gO genotypes was detected in 56.1% and 31.7% cases, respectively. Mixed pCMV infections were detected in 30.1% (gL) and 11.5% (gO) of infants. The children exhibited mixed infection with two gO genotypes, especially gO1 and gO4 or gO3, and with two or three distinct CMV gL genotypes. Among examined infants, mixed infections were identified mostly with viral gL1-gL3 (29 cases), gL2-gL3 (22 cases), gL3-gL4 (14 cases), and gL1-gL3-gL4 (5 cases) genomic variants. Analysis of the gH/gL/gO genotypes revealed that multiple CMV strains were detected in congenitally infected patients. Unexpectedly, considering all trimer gH/gL/gO genotypes, mixed infections were more commonly detected in congenital than in postnatal infections (75.6% vs. 48.7%; $P = 0.0002$).

The obtained UL128, UL130, and UL131A gene sequences were clustered into thirteen (named from 1 to 13), nineteen (1–19), and seven (1–7) variants, respectively. An amino acid sequence alignment of the UL128, UL130, and UL131A products is shown in Fig. 3A–C. The UL128L showed a low degree of variability. The ratios of sequence variability of UL128 ranged from 3 to 9% at the DNA level and from 0.6 to 2.3% at the amino acid level. The overall variability of the UL130 ranged from 2 to 10% at the DNA level and from 0.5 to 2.8% at the amino acid level, whereas the variability of the UL131A was 2–4% at the DNA level and 0.8% at the amino acid level.

Several viral variants were identified in newborns with cCMV infection, indicating that all variants of the virus could be passed from mother to child. The UL128 variant 3 was identified most commonly in both pediatric patient groups (43.0% and 44.6%), and no statistically significant association with the UL128 variant distribution among children with congenital or postnatal CMV infection was observed (Table 3). The UL130 variant 6 was prevalent in newborns with cCMV and infants in pCMV group (46.8% and 43.6%, respectively). The UL131A variant 8 was identified more commonly in cCMV than pCMV patients ($P = 0.027$). Analysis of the UL131A region in clinical isolates revealed that the variant 1 was prevalent in both patient groups, and it occurred more frequently in congenital than postnatal infections (79.7% vs. 58.4%; $P = 0.002$). In contrast, the UL131A variant 4 was found in 20.3% newborns with cCMV and in 34.7% infants in pCMV group ($P = 0.045$). Some mixing of pUL128, pUL130, and pUL131A gene variants was observed, but the frequency was ≤ 10% among isolates.

**Linkages among components of the gH/gL/gO complex.** Potential linkage disequilibrium between the gH, gO, and gL genotypes was investigated in isolates obtained from all pediatric patients. The analysis included only those isolates for which the genotypes were determined (Table 4). One hundred and thirteen of 122 isolates with the gH1 genotype had also a gO1 genotype (92.6%), 2 had gO2 (1.6%), 1 had gO3 (0.8%), and 6 had gO4 (4.9%). Among 123 isolates with gH2 genotype, 112 had also a gO1 genotype (91.1%). Mixed CMV infections with various gO genotypes were detected in 25/122 (20.5%) isolates with gH1 genotype and in 18/123 (14.6%) isolates with gH2 genotype. The prevalent gO1 genotype was not associated with gH genomic variant ($P > 0.05$). Analysis of the Spearman’s rank correlation coefficients ($\rho$) and associated $P$-values showed no significant correlation between the gH, gL, and gO genotypes, including gH-gO ($\rho = 0.02$), gH-gL, and gL-gO ($\rho = 0.12$) paired groups (Spearman’s correlation test $P > 0.05$). In addition, there was no significant association between the gH1, gO1, and gL1 genotypes (Cohen’s kappa coefficient $\kappa = -0.07$; $P > 0.05$). We observed a weak negative correlation between pUL130 and pUL131A variants ($\rho = -0.18$; $P < 0.05$). No significant association was found between other gene pairs encoding the gH/gL/gO complex ($\rho$-values from $-0.05$ to 0.11; $P > 0.05$). In the examined patients we also observed no conformity between the gH, gL, pUL128, pUL130, and pUL131A variants ($\kappa = -0.05$; $P > 0.05$).

**CMV gene variability is associated with an increased risk of specific disease outcomes.** In the patients carrying the gH1 genotype, hearing loss was diagnosed with higher incidence compared to those with the gH2 genotype ($P = 0.023$; Table 5). As was previously found in a smaller group of cases17, infants carrying the gH2 genotype were diagnosed with deafness at a lower incidence compared to those with the gH1 genotype ($P = 0.004$). In addition, gH2-infected children exhibited an increased risk of developing purpuric and petechial
rashes. The gH2 genotype was associated with a diminished risk of hearing loss and psychomotor retardation in infants in adjusted and unadjusted models (Table 5).

Infection with gH1 genotype was also associated with a three-fold increased risk of neurological dysfunction and microcephaly \( (P < 0.05) \). Infection with the gH2 genotype was associated with a decreased risk of hearing loss and ventriculomegaly \( (P = 0.007 \text{ and } P = 0.024, \text{ respectively}) \), though this genotype was observed only in a small number of the symptomatic infants (data not shown).

The UL130 variant 6 was detected in 62.5% of newborns with IUGR \( (P = 0.013) \) and was associated with at least a three-fold increased risk of this symptom (Table 5). The risk of ocular defects was more than three-fold increased following congenital infection with this variant \( (P = 0.024) \). Infection with the UL131A variant 1 was associated with a four-fold increased risk of IUGR, neurological dysfunction, and hepatitis \( (P = 0.001, P = 0.025, \text{ and } P = 0.021, \text{ respectively}) \) and with at least a two-fold increased risk of CNS damage \( (P = 0.005) \). In contrast, gL2-infected patients exhibited a decreased risk of developing hematological disorders \( (P = 0.018 \text{ for neonates with cCMV infection}; P = 0.018 \text{ for all pediatric patients}).

### Table 3. Distribution of CMV UL128L (UL128, UL130, and UL131A) variants in infants infected congenitally or postnatally. n, number of infants with CMV variant; cCMV, congenital CMV infection; pCMV, postnatal or unproven congenital CMV infection; \(^aP\), Fisher’s exact test.

| Gene  | Variant | Prevalence of CMV variants, \( n (%) \) | \( P^a \) |
|-------|---------|----------------------------------------|--------|
|       |         | cCMV                                  | pCMV   |
| UL128 | 1       | 10 (12.7)                              | 6 (5.9) | 0.186 |
|       | 2       | 2 (2.5)                                | 8 (7.9) | 0.189 |
|       | 3       | 34 (43.0)                              | 45 (44.6) | 0.880 |
|       | 4       | 7 (8.9)                                | 5 (4.9) | 0.372 |
|       | 5       | 13 (16.5)                              | 16 (15.8) | 1.000 |
|       | 6       | 7 (8.9)                                | 18 (17.8) | 0.127 |
|       | 7       | 3 (3.8)                                | 2 (1.9) | 0.655 |
|       | 8       | 1 (1.3)                                | 2 (1.9) | 1.000 |
|       | 9/10    | 1 (1.3)                                | 1 (0.9) | 1.000 |
|       | 11      | 3 (3.8)                                | 1 (0.9) | 0.321 |
|       | 12      | 0                                      | 1 (0.9) | 1.000 |
|       | 13      | 1 (1.3)                                | 2 (1.9) | 1.000 |
|       | Mixed   | 3 (3.8)                                | 7 (6.9) | 0.516 |
| UL130 | 1       | 6 (7.6)                                | 13 (12.9) | 0.331 |
|       | 2       | 2 (2.5)                                | 2 (1.9) | 1.000 |
|       | 3       | 3 (3.8)                                | 3 (2.9) | 1.000 |
|       | 4       | 2 (2.5)                                | 9 (8.9) | 0.116 |
|       | 5       | 1 (1.3)                                | 7 (6.9) | 0.081 |
|       | 6       | 37 (46.8)                              | 44 (43.6) | 0.763 |
|       | 7       | 6 (7.6)                                | 13 (12.9) | 0.331 |
|       | 8       | 11 (13.9)                              | 4 (3.9) | 0.027 |
|       | 9/10    | 5 (6.3)                                | 1 (0.9) | 0.088 |
|       | 11      | 0                                      | 1 (0.9) | 1.000 |
|       | 12      | 0                                      | 2 (1.9) | 0.505 |
|       | 13      | 2 (2.5)                                | 0       | 0.191 |
|       | 14/15/16| 1 (1.3)                                | 0       | 0.439 |
|       | 17      | 2 (2.5)                                | 1 (0.9) | 0.583 |
|       | 18      | 1 (1.3)                                | 2 (1.9) | 1.000 |
|       | 19      | 2 (2.5)                                | 0       | 0.191 |
|       | Mixed   | 8 (10.1)                               | 2 (1.9) | 0.023 |
| UL131A| 1       | 63 (79.7)                              | 59 (58.4) | 0.002 |
|       | 2       | 0                                      | 3 (2.9) | 0.257 |
|       | 3       | 0                                      | 1 (0.9) | 1.000 |
|       | 4       | 16 (20.3)                              | 35 (34.7) | 0.045 |
|       | 5       | 0                                      | 2 (1.9) | 0.505 |
|       | 6       | 1 (1.3)                                | 0       | 0.439 |
|       | 7       | 1 (1.3)                                | 2 (1.9) | 1.000 |
|       | Mixed   | 2 (2.5)                                | 1 (0.9) | 0.583 |
CMV glycoprotein polymorphisms and viral load. The viral load in the blood and urine samples correlate with the infection of some CMV genotypes. The gL2 and gO3 genotypes were associated with a low shedding of CMV in urine ($P = 0.005$ and $P = 0.008$, respectively; Wilcoxon test). The viral load in the blood and urine samples did not correlate with the presence of the other CMV variants.

Figure 2. Amino acid alignments of gH (A), gL (B), and gO (C) in clinical isolates from children with congenital and postnatal CMV infection.
Discussion

Several studies have analyzed sequences from CMV clinical isolates and revealed that genomic variability caused additional infectivity or immunomodulation functions. The increasing evidence suggests a correlation of clinical importance of CMV genetic diversity with pathogenesis. To assess whether the variability in the CMV genes encoding trimeric and pentameric complexes are associated with congenital transmission, we compared the frequencies of variants in children with and without confirmed congenital CMV infection. Our study focused on the variation in the all CMV genes encoding envelope protein complexes, gH/gL/gO and gH/gL/

Figure 3. The phylogenetic analysis of UL128 (A), UL130 (B), and UL131A (C) from clinical isolates of newborns with congenital CMV infection and infants with postnatal or unproven congenital CMV infection. The conserved cysteine residues are in box.
similar and ranged between 92% and 96% in isolates from Italian patients. The overall identity of these CMV genes was sequence identity at the aa level in the UL128, UL130, and UL131A proteins, respectively, in comparison to the characteristic of CC- and CXC-chemokines, respectively. Our findings showed 96–99%, 84–97%, and 97–99% identity in the UL128, UL130, and UL131A genes, respectively, than that by CMV-specific hyperimmune globulin. The pentamer gH/gL/pUL128-131A complex was 1000-fold and 10-fold higher, respectively, than that by CMV-specific hyperimmune globulin. The pentamer gH/gL/pUL128-131A complex and the gH/gL complex was 1000-fold and 10-fold higher, respectively, than that by CMV-specific hyperimmune globulin. The pUL128/pUL130/pUL131A complex is also the target of the most potent neutralizing monoclonal antibodies against the UL128L complex and the gH/gL complex. No significant difference was detected between the CMV variants determined on the basis of the UL128L and the signs of infection in Chinese infants. The UL130 variant 6 showed a few amino acid substitutions such as P40L, L60I, and S78L that were undetected in common CMV variants. It was found that a 2-bp insertion (TT) led to a frameshift mutation in the UL130 (Towne strain) and a 1-bp insertion (A) in UL131A (AD-169 strain) genes leading to a frameshift mutation that introduces in-frame translational termination codons or splicing are predicted to ablate gene functions, insertions, and deletions that affect one or more genes. These mutations impact the growth properties and tropism of the virus. Moreover, these changes have likely impaired the immunogenicity, particularly with respect to induction of epithelial cell and endothelial cell neutralizing antibodies.

The findings of Maidji et al. support a potential role of endothelial cells in CMV transmission from the uterus to endovascular cytotrophoblasts. The syncytiotrophoblast constitutes a barrier to vertical transmission, and first-trimester chorionic villi are largely resistant to CMV infection, whereas cytotrophoblasts and other villous cells are susceptible. It is suggested that virions transmitted to cytotrophoblasts could then spread the infection to the placenta and to fetal blood vessels in the villus core. Considering the structure of the human placenta as an active barrier against infection, the pentamer gH/gL/pUL128-131A complex is of utmost importance in vertical CMV transmission. The pUL128/pUL130/pUL131A, when assembled with the gH/gL heterodimer to form the pentameric complex, is necessary for entry into endothelial and epithelial cells, as well as for CMV transmission to leukocytes. The pentamer complex mediates virus entry into epithelial and endothelial cells by endocytosis and fusion. The entry process into fibroblasts is independent of the proteins of the pentamer complex, and the UL128L genes may be lost during long-term cultivation in human fibroblasts. Thus, this genetic locus is supposed to play an important role in cell tropism. Recent studies of the virus tropism concentrated on the UL128L of wild-type CMV strains. The pUL128/pUL130/pUL131A complex is also the target of the most potent neutralizing antibodies following natural infection. The neutralization potency of CMV infection in epithelial cells by monoclonal antibodies against the UL128L complex and the gH/gL complex was 1000-fold and 10-fold higher, respectively, than that by CMV-specific hyperimmune globulin. The pentamer gH/gL/pUL128-131A complex is of interest in the CMV vaccine field.

Table 4. Distribution of genotypes of the gH, gL, and gO in isolates from children with CMV infection.

| gH genotype | No. of isolates with gH1 and gO genotype | No. of isolates with gH2 and gO genotype |
|-------------|----------------------------------------|----------------------------------------|
|             | 1           | 2           | 3           | 4        | mixed   | 1           | 2           | 3           | 4        | mixed   |
| 1           | 21          | 0           | 1           | 2        | 6       | 17         | 3           | 0           | 1        | 4       |
| 2           | 15          | 1           | 0           | 0        | 4       | 16         | 2           | 0           | 1        | 2       |
| 3           | 7           | 0           | 0           | 0        | 1       | 7          | 0           | 0           | 0        | 1       |
| 4           | 16          | 1           | 0           | 1        | 3       | 14         | 0           | 1           | 0        | 4       |
| mixed       | 29          | 0           | 0           | 3        | 11      | 41         | 0           | 1           | 1        | 7       |

The pentamer complex may be complexed either with pUL128L forming the pentamer complex, or gO, forming the gH/gL/gO complex. Greater amounts of pentamer gH/gL/pUL128/pUL130/pUL131A complex may result in there being less gH/gL available for the formation of trimer gH/gL/gO complex. Two viral factors, UL148 and US16, were identified to impact the composition of gH/gL complexes in CMV strains. Moreover, the expression levels of UL128-131A and gO seem to influence the abundance of gH/gL/pUL128-131A and gH/gL/gO complexes.
disease. The present results performed on larger groups of pediatric patients confirmed our earlier findings that no correlation exists between gO type and CMV infection in infants. The distribution of the gO genotypes in major gO genotypes, with two of them divided in five sub-genotypes. However, no relationship was observed between gO genotype and the outcome of CMV infection in infants. The distribution of the gO genotypes in clinical isolates from different disease settings confirmed that no correlation exists between gO type and CMV disease.

Most of congenital infections occur during nonprimary maternal infection, and it has been estimated that approximately three-quarters of congenital CMV infections occur in the setting of recurrent maternal infection during pregnancy. A previously acquired maternal CMV infection does not provide complete protection against infection of the fetus, but it reduces this risk. It was found that antibodies targeting the CMV pentameric complex can block infection of trophoblasts and restrict cCMV transmission. It was observed that antibodies elicited far more neutralizing antibodies than gB. Antibodies to anti-UL128L complex are increased in the groups of non-transmitting mothers. The increase in antibodies after CMV infection in pregnant women is associated with a decreased risk of congenital infection, suggesting that anti-UL128L complex antibodies play a critical role in protection against transmission to the fetus. However, it is unknown if antibodies specific for pentamer complex can block infection of trophoblasts and restrict cCMV transmission. It was observed that these antibodies inhibit infection of term cytrophoblasts, while no inhibition in the first-trimester cytrophoblast progenitor cells was found. This study has a number of strengths, but also some limitations. This is the first study to focus on the genes encoding the gH/gL/pUL128-131A complex of CMV in infants infected congenitally or postnatally. The main strength of the present study is the clinical evaluation of children with a CMV infection and important clinical implications. We have found significant associations of CMV variants with specific clinical symptoms. It should be underlined that we have no universal CMV screening for newborns and virion infectivity. There are at least eight genotypes of gO that differ by 10 to 30% of amino acids. Used set of primers was adequate for amplifying a region of the UL74 gene that was digested for identification of the five major gO genotypes, with two of them divided in five sub-genotypes. However, no relationship was observed between gO genotype and the outcome of CMV infection in infants. The distribution of the gO genotypes in clinical isolates from different disease settings confirmed that no correlation exists between gO type and CMV disease. The present results performed on larger groups of pediatric patients confirmed our earlier findings that no correlation exists between gO type and CMV infection in infants. The distribution of the gO genotypes in clinical isolates from different disease settings confirmed that no correlation exists between gO type and CMV disease.

| Gene | Genotype/variant | Symptom/sign | n (%) | P | OR (95% CI) | P | OR (95% CI) |
|------|------------------|--------------|-------|---|-------------|---|-------------|
| UL75 | gH1 Neurological dysfunction | 44/60 (73.3) | 0.021 | 3.3 (1.2–9.1) | 0.027 | 3.3 (1.1–9.5) |
|     | gH2 Petechiae     | 13/16 (81.3) | 0.033 | 4.3 (1.1–16.6) | 0.023 | 6.5 (1.3–33.0) |
| UL115 | gL2 Hematological disorders | 14/51 (27.5) | 0.025 | 0.1 (0.0–0.7) | 0.032 | 0.1 (0.0–0.8) |
| UL130 | 6 IUGR | 20/32 (62.5) | 0.013 | 3.2 (1.3–8.2) | 0.006 | 3.9 (1.5–10.2) |
|     | 6 Ocular defects | 13/19 (68.4) | 0.024 | 3.5 (1.6–10.5) | 0.021 | 3.8 (1.2–11.7) |
| UL131A | 1 Neurological dysfunction | 29/60 (48.3) | 0.025 | 4.5 (1.2–17.2) | 0.026 | 4.6 (1.2–17.8) |
|     | 1 Hematological disorders | 35/51 (68.6) | 0.032 | 0.2 (0.1–0.9) | 0.034 | 0.2 (0.1–1.1) |

Table 5. CMV variants as prognostic markers for the risk of symptoms in infants with cCMV or pCMV infection. n, number of genotype/variant cases among symptomatic patients; OR, odds ratio; 95% CI, 95% confidence interval; cCMV, congenital CMV infection; pCMV, postnatal or unproven congenital CMV infection; aAdjusted analysis was carried out for CMV DNA copy number in urine samples; bNeurological dysfunction e.g. tremor, hypotonia/hypertonia, or poor sucking reflex; cHematological disorders e.g. anemia, thrombocytopenia, thrombocytosis, neutropenia, or leukocytosis; dCNS damage e.g. cystic lesions, microcephaly, intracranial calcification, or ventriculomegaly; IUGR, intrauterine growth restriction.
in our country. As pCMV infection was diagnosed in children above three weeks of age, it is possible that, apart from the postnatally infected majority, there are also congenitally infected infants (with no clinical findings at birth) in this group.

In summary, this report demonstrates that a relationship between the CMV variant in trimeric and pentameric complexes, including gH, pUL130 and pUL131A, and clinical outcome exists. This study suggests that the CMV variant may be one of the virological markers of congenital CMV disease.

Methods

Patients. A total of 195 children with CMV infection were enrolled from February 2008 to April 2011 and again from January 2015 to August 2018 at the Children's Memorial Health Institute, Warsaw, and the Polish Mother’s Memorial Hospital Research Institute in Lodz, Poland. The geographical origins of the patients were in half the Central Poland (Warsaw, Lodz and the surrounding areas) and all Poland. Patients were divided into two groups: cCMV group, which represent infants who were CMV-positive in urine samples collected ≤ 21st day of life, and pCMV group, which represented infants with the postnatal acquisition of CMV (CMV-negative in urine samples ≤ 21st day of life, seroconversion > 21 day of life) or probable postnatal CMV infection/unproven cCMV infection (unavailable result of CMV DNA in the urine ≤ 21st day of life). Blood and/or urine samples were obtained from 82 newborns with cCMV infection and from 113 infants in pCMV group. One hundred and five children with CMV infection, including 28 cases of cCMV and 77 cases of pCMV, were earlier examined for the presence of UL75, as described in our previous study.17 CMV infection was confirmed by the presence of CMV-specific antibodies and/or CMV DNA detection in whole blood and/or urine samples. Congenital infection was confirmed within the first 2–3 weeks of life by CMV DNA detection in the urine samples. When the samples were collected after 3 weeks of life, infants were classified as having postnatal or unproven congenital CMV infection. The children were classified as having symptomatic infection with any clinical manifestations (jaundice, petechiae, IUGR, hepatosplenomegaly, hepatitis, cholestasis, bearing loss, microcephaly, neurological dysfunction, CNS damage in neuroimaging [ultrasound and/or magnetic resonance imaging], chorioretinitis, pneumonia) or laboratory findings, including thrombocytopenia, granulocytopenia, and anemia. Due to selection bias, 96.3% of newborns and 94.7% of infants with CMV infection in this study were symptomatic. Serum samples were assessed for anti-CMV IgG and IgM antibodies with the use of ELISA AxSYM CMV IgM and IgG (Abbott Laboratories, Abbott Park, IL, USA) or CLIA LIASON CMV IgM and IgG assays (DiaSorin, Saluggia, Italy). The demographic and clinical characteristics of pediatric patients with CMV infection were summarized in Table 1. The study protocols were approved by the Bioethics Committee of the Medical University of Lodz (RNN/278-279/16/KE, RNN/120/09/KE) and the Ethics Committee of the Polish Mother’s Memorial Hospital Research Institute (90/KBE/207). Parents or guardians provided written informed consent to participate in this study on behalf of the children. All experiments were performed in accordance with relevant guidelines and regulations.

DNA extraction. Total genomic DNA was extracted from peripheral blood and urine samples using a QiAamp DNA Blood Mini Kit or QiAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of DNA were assessed using a 2000c UV-VIS Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Quantification of CMV DNA. The CMV DNA copy numbers in DNA isolates were determined using a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) as previously described.22,23 DNA was amplified using primers specific for the CMV gB (UL55) gene. The sensitivity of the assay was determined to be approximately 2 × 10^5 copies/mL. A negative control without template DNA was included in every amplification run.

Genotyping of three genes encoding the gH/gL/gO complex of CMV. The UL75 gene (gH) was amplified by nPCR by using two sets of primers, as described elsewhere.17 The genomic variants gH1 and gH2 were identified by different amplicon size lengths (240 bp and 237 bp, respectively). The identification of UL74 (gL) and UL115 (gO) was performed by nPCR-RFLP with the primers 5′-TAACGGGCGCTTGTTTACGT-3′ (UL74) and 5′-CAGCAAAACGACCAAGAATCAG-3′ (UL74) and 5′-GACGCAGGCCGGCGTTTTGATCACG-3′ and 5′-CGTGCAGAGCTTGTATGCTGCC-3′ (UL115) in the first round and primer sets designed in our Laboratory or described previously by Stanton et al.14 The sequences of all primers used in PCR assays are listed in Table 6. Each run consisted of an initial denaturation at 95 °C for 1 min following by 45 consecutive cycles of denaturation at 95 °C for 3 min and different annealing temperatures (68 °C for UL74 and 55 °C for UL115) for 30 s, extension at 72 °C for 1 min, and the final extension at 72 °C for 10 min. The PCR was performed in a Veriti 96 Well Thermal Cycler (Applied Biosystems). Each PCR was performed in a volume of 50 μl as follows: 0.5 μg (blood) or 0.2 μg (urine) template DNA (5 μl), 5 μl 10 × DreamTaq™ Buffer (20 mM Tris-HCl, pH 8.0; 1 mM DTT, 0.1 mM EDTA, 100 mM KCl, 0.5% Nonidet P40, 0.5% Tween 20, 50% glycerol), 4 μl 2.5 mM dNTP, 0.5 μl gene-specific primers (100 pmol/μl each), 0.25 μl DreamTaq™ polymerase (5 U/μl, Fermentas, Glen Burnie, MD, USA), and 34.75 μl nuclease-free water. DNA isolates from MRC-5 cells (ATCC CCL-171, American Type Culture Collection, Rockville, MD, USA) infected with the CMV strains AD-169 (ATCC VR-538), Towne (ATCC VR-977) or Davis (ATCC VR-807) was used as positive controls in each of PCR runs, and nuclease-free water was used as a negative control. The nPCR products were digested with the restriction enzymes HpaII (UL74), Eco24I (BanII), and Cse1 (Hgal) (UL115) (Fermentas, Hanover, MD, USA) according to the manufacturer’s recommendations. The restriction enzymes and the CMV genotypes identified by different fragment lengths are described in Table 7. Briefly, a digestion with HpaII was used to characterize the five major gO genotypes, while double digestion with Eco24I and Cse1 was used to identify the four gl genotypes. The nPCR products and the digested
DNA were analyzed using a QIAxcel DNA Screening Kit and a QIAxcel system (Qiagen) and the AL420 method. The QX Alignment Marker 15 bp/1 kbp and QX DNA Size Marker 50–800 bp were included in the analysis. Randomly selected samples (50–60 samples per each gene) were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and the 96-capillary 3730xl DNA Analyzer (Applied Biosystems).

**CMV UL128 locus genotyping.** To successfully amplify the genes encoding the CMV UL128L, nPCR (UL130 and UL131A) and hemi-nested PCR (UL128) assays were used. The sequences of all primers used in UL128L genotyping are listed in Table 6. The reaction mixture for UL128L and UL131A gene amplifications consisted of 0.5 μg (blood) or 0.2 μg (urine) template DNA (5 μl) and 45 μl reaction solution, as described above, while for the UL130 gene, it was as follows: 5 μl template DNA, 10 μl KAPA HiFi Fidelity Buffer, 1.5 μl 10 mM KAPA dNTP, 0.15 μl gene-specific primers, 1 μl KAPA HiFi polymerase (1 U/μl, KAPA Biosystems, Boston, MA, USA), and 32.2 μl nuclease-free water. The UL128 and UL131A reactions were performed at 95 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, annealing at 55 °C for UL128 and 57 °C for UL131A for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The PCR was performed in a Veriti® 96 Well Thermal Cycler (Applied Biosystems). The PCR parameters for UL130 were as follows: 95 °C for 3 min and 35 cycles each at 98 °C for 20 s, at 59 °C for 15 s, and at 72 °C for 15 s with a final extension at 72 °C for 5 min. Positive and negative controls were included in the PCR assays, as described above. The amplicons were verified by the QIAxcel capillary.

### Table 6. Primer sequences and amplicon lengths obtained in nested PCR assays in the CMV genes. bp, base pairs; F, forward starter; R, reverse starter; nF, nested-forward starter; nR, nested-reverse starter.

| Gene  | Forward primer | Reverse primer | Amplicon length (bp) | References |
|-------|----------------|----------------|----------------------|------------|
| UL74  | TAACGGGGCGCTTGTTTACGT | GACGCAAAACGACCAATCAG | 868                  | This paper74 |
| UL75  | TGTTACGGACTCTCGTCTTG | GACGGGACTTTTGAACCCG | 334                  | 17         |
| UL115 | TGGCGGATAAACCAGCTATC | ATGTTGCACCGCTGGACGG | 636                  | This paper |
| UL128 | TGTTACGGACTCTCGTCTTG | ATGTTGCACCGCTGGACGG | 910                  | 61         |
| UL130 | TGTTACGGACTCTCGTCTTG | ATGTTGCACCGCTGGACGG | 2182                 | 59         |
| UL131A| TGTTACGGACTCTCGTCTTG | ATGTTGCACCGCTGGACGG | 2182                 | 59         |

### Table 7. Restriction enzymes and length of the restriction fragments. bp, base pairs.

| Gene | Restriction enzyme | Genotype | Length of the restriction fragments (bp) |
|------|-------------------|----------|----------------------------------------|
| UL74 | HpaII             | gO1      | 390                                    |
|      |                   | gO2      | 134, 203                               |
|      |                   | gO3      | 134, 271                               |
|      |                   | gO4      | 110, 203                               |
| UL115| Eco24I            | gL1      | 40, 90, 100, 108, 133, 150             |
|      |                   | gL2      | 40, 100, 133, 150                      |
|      |                   | gL3      | 100, 133, 165, 225                     |
|      |                   | gL4      | 90, 100, 133, 165                      |
electrophoresis system (Qiagen) and were sequenced using an Applied Biosystems 3730xl sequencer (Applied Biosystems).
CMV sequences were aligned to the reference sequences deposited in the NCBI using BLAST.

**Statistical analysis.** The gene variant distributions and the association between them and the outcome of CMV infection were analyzed using Fisher's exact test or the chi-square test. A nonparametric Mann–Whitney U-test with correction for continuity and Wilcoxon rank-sum test were performed to calculate the P-values for the relationship between the viral load and infection outcome. Pairwise correlations between gene variants were evaluated with Spearman's rank correlation coefficient (ρ). A logistic regression model was used to evaluate the association between the specific CMV variants and clinical characteristics of patients, adjusting for potential confounders, e.g., DNAemia level. The data were analyzed using descriptive statistics, including the median, range, and 95% confidence intervals (CIs). Analyses were performed using the SPSS statistical software package for Windows 24.0 (SPSS, Chicago, IL). A P-value < 0.05 was considered as statistically significant.

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**References**

1. Griffiths, P., Baraniak, J. & Reeves, M. The pathogenesis of human cytomegalovirus. *J. Pathol.*** **235**, 288–297 (2015).

2. Cannon, M. J., Schmid, D. S. & Hyde, T. B. Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. *Rev. Med. Virol.* **20**, 202–213 (2010).

3. Boppana, S. B., Ross, S. A. & Fowler, K. B. Congenital cytomegalovirus infection: clinical outcome. *Clin. Infect. Dis.* **57**, S178–181 (2013).

4. Kenneson, A. & Cannon, M. J. Review and meta-analysis of the epidemiology of congenital cytomegalovirus (CMV) infection. *Rev. Med. Virol.* **17**, 253–276 (2007).

5. Wang, C., Zhang, X., Bialek, S. & Cannon, M. J. Attribution of congenital cytomegalovirus infection to primary versus non-primary maternal infection. *Clin. Infect. Dis.* **52**, e11–e13 (2011).

6. Boppana, S. B., Pass, R. F., Britt, W. J., Stagno, S. & Alford, C. A. Symptomatic congenital cytomegalovirus infection: neonatal morbidity and mortality. *Pediatr. Infect. Dis. J.* **11**, 93–99 (1992).

7. Britt, W. J. Maternal immunity and the natural history of congenital human cytomegalovirus infection. *Viruses*** **10**, e405 (2018).

8. Drollard, S. C., Grosse, S. D. & Ross, D. S. New estimates of the prevalence of neurological and sensory sequelae and mortality associated with congenital cytomegalovirus infection. *Rev. Med. Virol.* **17**, 355–363 (2007).

9. Dahl, H. H. *et al.* Etiology and audiological outcomes at 3 years for 364 children in Australia. *PLoS One*** **8**, e59624 (2013).

10. Enders, G., Daiminger, A., Bäder, U., Exler, S. & Enders, M. Intrauterine transmission and clinical outcome of 248 pregnancies with primary cytomegalovirus infection in relation to gestational age. *J. Clin. Virol.* **52**, 244–246 (2011).

11. de Vries, J. J. *et al.* The apparent paradox of maternal seropositivity as a risk factor for congenital cytomegalovirus infection: a population-based prediction model. *Rev. Med. Virol.* **23**, 241–249 (2013).

12. Sinzger, C., Digel, M. & Jahn, G. Cytomegalovirus Cell Tropism in Human Cytomegalovirus. Current Topics in Microbiology and Immunology (eds Shenk, T. E. & Stinski, M. F.) 63–83 (Springer, 2008).

13. Chou, S. Molecular epidemiology of envelope glycoprotein H of human cytomegalovirus. *J. Infect. Dis.* **166**, 604–607 (1992).

14. Pignatelli, S. *et al.* Intrauterine cytomegalovirus infection and glycoprotein N (gN) genotypes. *J. Clin. Virol.* **28**, 38–43 (2003).

15. Pignatelli, S. *et al.* Cytomegalovirus gN genotypes distribution among congenitally infected newborns and their relationship with symptoms at birth and sequelae. *Clin. Infect. Dis.* **51**, 33–41 (2010).

16. Paradowska, E. *et al.* Distribution of cytomegalovirus gN variants and associated clinical sequelae in infants. *J. Clin. Virol.* **58**, 271–275 (2013).

17. Paradowska, E. *et al.* Cytomegalovirus glycoprotein H genotype distribution and the relationship with hearing loss in children. *J. Med. Virol.* **86**, 1421–1427 (2014).

18. Arav-Boger, R. Strain variation and disease severity in congenital CMV infection – in search of a viral marker. *Infect. Dis. Clin. North. Am.* **29**, 401–414 (2015).

19. Paradowska, E. *et al.* Human cytomegalovirus UL55, UL144, and US28 genotype distribution in infants infected congenitally or postnatally. *J. Med. Virol.* **87**, 1737–1748 (2015).

20. Patt, S. K. *et al.* Genotypic and mixed infection in newborn disease and hearing loss in congenital cytomegalovirus infection. *Pediatr. Infect. Dis. J.* **32**, 1050–1054 (2013).

21. Zhou, M., Yu, Q., Wechsler, A. & Ryckman, B. J. Comparative analysis of gO isoforms reveals that strains of human cytomegalovirus differ in the ratio of gH/gL/gO and gH/gL/UL128-131 in the virion envelope. *J. Virol.* **87**, 9680–9690 (2013).

22. Van, H. T. *et al.* Genetic linkage among human cytomegalovirus glycoprotein N (gN) and gO genes, with evidence for recombination from congenitally and post-natally infected Japanese infants. *J. Gen. Virol.* **89**, 2275–2279 (2008).

23. Rasmussen, L., Geissler, A., Cowan, C., Chase, A. & Winters, M. The genes encoding the gCII complex of human cytomegalovirus exist in highly diverse combinations in clinical isolates. *J. Virol.* **76**, 10841–10848 (2002).

24. Issacson, M. K. & Compトン, T. Human cytomegalovirus glycoprotein B is required for virus entry and cell-to-cell spread but not for virion attachment, assembly, or egress. *J. Virol.* **83**, 3891–3903 (2009).

25. Vanarsdall, A. L., Ryckman, B. J., Chase, M. C. & Johnson, D. C. Human cytomegalovirus glycoproteins gB and gH/gL mediate epithelial cell-cell fusion when expressed either in cis or in trans. *J. Virol.* **82**, 11837–11850 (2008).

26. Boppana, S. B., Rivera, L. B., Fowler, K. R., Mach, M. & Britt, W. J. Intrauterine transmission of cytomegalovirus to infants of women with preconceptional immunity. *N. Engl. J. Med.* **344**, 1866–1871 (2001).

27. Wille, P. T., Wisner, T. W., Ryckman, B. & Johnson, D. C. Human cytomegalovirus (HCMV) glycoprotein b promotes virus entry in trans acting as the viral fusion protein rather than as a receptor-binding protein. *MBio*, 4, e00332–13 (2013).

28. Vanarsdall, A. L., Howard, P. W., Wisner, T. W. & Johnson, D. C. Human cytomegalovirus gH/gL forms a stable complex with the fusion protein gB in virions. *PLoS Pathog.* **12**, e1005564 (2016).

29. Ryckman, B. J. *et al.* Characterization of the human cytomegalovirus gH/gL/UL128-131 complex that mediates entry into epithelial and endothelial cells. *J. Virol.* **82**, 60–70 (2008).

30. Ciferri, C. *et al.* Structural and biochemical studies of HCMV gH/gL/gO and Pentamer reveal mutually exclusive cell entry complexes. Proc. Natl. Acad. Sci. USA **112**, 1767–1772 (2015).

31. Hahn, G. *et al.* Human cytomegalovirus UL131-128 genes are dispensable for virus growth in endothelial cells and virus transfer to leukocytes. *J. Virol.* **78**, 10023–10033 (2004).

32. Kabanova, A. A. Platelet-derived growth factor-alpha receptor is the cellular receptor for human cytomegalovirus gHgLgO trimmer. *Nat. Microbiol.* **1**, 16082 (2016).
33. Stegmann, B. et al. A derivative of platelet-derived growth factor receptor alpha binds to the trimer of human cytomegalovirus and inhibits entry into fibroblasts and endothelial cells. PLoS Pathog. 13, e1006273 (2017).
34. Wu, Y. et al. Human cytomegalovirus glycoprotein complex gH/gL/gO uses PDGFR-alpha as a key for entry. PLoS Pathog. 13, e1006281 (2017).
35. Gerna, G., Percivalle, E., Perez, L., Lanzavecchia, A. & Lilieri, D. Monoclonal Antibodies to Different Components of the Human Cytomegalovirus (HCMV) Pentamer gH/gL/pUL128 and Trimer gH/gL/gO as well as Antibodies Elicited during Primary HCMV Infection Prevent Epithelial Cell Syncytium Formation. J. Virol. 90, 6216–6223 (2016).
36. Zhou, M., Lancy, J. M. & Ryckman, B. J. Human cytomegalovirus gH/gL/gO promotes the fusion step of entry into all cell types, whereas gH/gL/UL128-131 broadens virus tropism through a distinct mechanism. J. Virol. 89, 8999–9009 (2015).
37. Malito, E., Chandramouli, S. & Carli, A. From recognition to execution-the HCMV pentamer from receptor binding to fusion triggering. Curr. Opin. Virol. 31, 43–51 (2018).
38. Vanarsdall, A. L., Wisner, T. W., Lei, H., Kadavuska, A. & Johnson, D. C. PDGF receptor-alpha does not promote HCMV entry into epithelial and endothelial cells but increased quantities stimulate entry by an abnormal pathway. PLoS Pathog. 8, e1002905 (2012).
39. Stegmann, C., Rothemund, F., Lab Sampaio, K., Adler, B. & Sinzger, C. The N terminus of human cytomegalovirus glycoprotein O is important for binding to the cellular receptor PDGFRα. J. Virol. 93, e00138–19 (2019).
40. Wang, D. & Shenk, T. Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism. J. Virol. 79, 10330–10338 (2005).
41. Wang, D. & Shenk, T. Human cytomegalovirus virion protein complex required for epithelial and endothelial cell tropism. Proc. Natl. Acad. Sci. USA 102, 18153–18158 (2005).
42. Adler, B. et al. Role of human cytomegalovirus UL131A in cell type-specific virus entry and release. J. Gen. Virol. 87, 2451–2460 (2006).
43. Gerna, G. et al. Dendritic-cell infection by human cytomegalovirus is restricted to strains carrying functional UL131-128 genes and mediates efficient viral antigen presentation to CD8+ T cells. J. Gen. Virol. 86, 275–284 (2005).
44. Nogalski, M. T., Chan, G. C., Stevenson, E. V., Collins-McMilen, D. K. & Yurochko, A. D. The HCMV gH/gL/UL128-131 complex triggers the specific cellular activation required for efficient viral internalization into target monocytes. PLoS Pathog. 9, e1003463 (2013).
45. Ryckman, B. J., Jarvis, M. A., Drummond, D. D., Nelson, J. A. & Johnson, D. C. Human cytomegalovirus entry into epithelial and endothelial cells depends on genes UL128 to UL150 and occurs by endocytosis and low-pH fusion. J. Virol. 80, 710–722 (2006).
46. Sathiyamoothy, K., Chen, J., Longnecker, R. & Jardetzky, T. S. The complexity in herpesvirus entry. Curr. Opin. Virol. 24, 97–104 (2017).
47. Stampfer, S. D. & Heldwein, E. E. Stuck in the middle: structural insights into the role of the gH/gL heterodimer in herpesvirus entry. Curr. Opin. Virol. 3, 13–19 (2013).
48. Chandramouli, S. et al. Structural basis for potent antibody-mediated neutralization of human cytomegalovirus. Sci. Immunol. 2, 12 (2017).
49. Ryckman, B. J., Chase, M. C. & Johnson, D. C. HCMV gH/gL/UL128-131 interferes with virus entry into epithelial cells: evidence for cell type-specific receptors. Proc. Natl. Acad. Sci. USA 105, 14118–14123 (2008).
50. Murrell, I. et al. The pentameric complex drives immunologically covert cell–cell transmission of wild-type human cytomegalovirus. Proc. Natl. Acad. Sci. USA 114, 6104–6109 (2017).
51. Fouts, A. E., Chan, P., Stephan, J. P., Vandlen, R. & Feierbach, B. Antibodies against the gH/gL/UL128-130/131 complex comprise the majority of the anti-cytomegalovirus (anti-CMV) neutralizing antibody response in CMV hyperimmune globulin. J. Virol. 86, 7444–7447 (2012).
52. Macagno, A. et al. Isolation of human monoclonal antibodies that potently neutralize human cytomegalovirus infection by targeting different epitopes on the gH/gL/UL128-131A complex. J. Virol. 84, 1005–1013 (2010).
53. Chiuppesi, F. et al. Vaccine-derived neutralizing antibodies to the human cytomegalovirus gH/gl pentamer potently block primary cytopathoblast infection. J. Virol. 89, 11884–11898 (2015).
54. Lilieri, D. et al. Fetal human cytomegalovirus transmission correlates with delayed maternal antibodies to gH/gL/UL128-130–131 complex during primary infection. PLoS One. 8, e99863 (2013).
55. Baldanti, F. et al. Human cytomegalovirus UL131A, UL130 and UL128 genes are highly conserved among field isolates. Arch. Virol. 151, 1225–1233 (2006).
56. Dargan, D. J. et al. Sequential mutations associated with adaptation of human cytomegalovirus to growth in cell culture. J. Gen. Virol. 91, 1535–1546 (2010).
57. Wilkinson, G. W. et al. Human cytomegalovirus: taking the strain. Med. Microbiol. Immunol. 204, 273–284 (2015).
58. Murphy, E. et al. Codiabination of the postnatal and clinical strains of human cytomegalovirus. Proc. Natl. Acad. Sci. USA 100, 14976–14981 (2003).
59. Murphy, E. & Shenk, T. Human cytomegalovirus genome. Curr. Top. Microbiol. Immunol. 325, 1–19 (2008).
60. Lilieri, D., Kabanova, A., Lanzavecchia, A. & Gerna, G. Antibodies against neutralization epitopes of human cytomegalovirus gH/gL/UL128-130–131 complex and virus spreading may correlate with virus control in vivo. J. Clin. Immunol. 32, 1324–1331 (2012).
61. Vogel, J. U. et al. Role of human cytomegalovirus genotype polymorphisms in AIDS patients with cytomegalovirus reinitis. Med. Microbiol. Immunol. 202, 37–47 (2013).
62. Sijmons, S. et al. High-throughput analysis of human cytomegalovirus genome diversity highlights the widespread occurrence of gene-disrupting mutations and pervasive recombination. J. Virol. 89, 7873–7895 (2015).
63. Sun, Z. R. et al. Structure characterization of human cytomegalovirus UL131A, UL130 and UL128 genes in clinical strains in China. Genet. Mol. Res. 8, 1191–1201 (2009).
64. Maidji, E., Cavalcetto, N., Raimondo, S., Geuna, S. & Gribaudo, G. virus of the human cytomegalovirus US6 protein abrogates virus entry into endothelial and epithelial cells by reducing the virion content of the pentamer. J. Virol. 91, e02033–16 (2017).
65. Li, G., Nguyen, C. C., Ryckman, B. J., Britt, W. J. & Kamil, J. P. A viral regulator of glycoprotein complexes contributes to human cytomegalovirus cell tropism. Proc. Natl. Acad. Sci. USA 112, 4471–4476 (2015).
66. Li, G., Cavalcetto, N., Raimondo, S., Geuna, S. & Gribaudo, G. virus of the human cytomegalovirus US6 protein abrogates virus entry into endothelial and epithelial cells by reducing the virion content of the pentamer. J. Virol. 91, e02033–16 (2017).
67. Li, G., Nguyen, C. C., Ryckman, B. J., Britt, W. J. & Kamil, J. P. A viral regulator of glycoprotein complexes contributes to human cytomegalovirus cell tropism. Proc. Natl. Acad. Sci. USA 112, 4471–4476 (2015).
68. Lanzavecchia, A. & Gerna, G. Maternal immune correlates of protection from human cytomegalovirus transmission to the fetus after primary infection in pregnancy. Rev. Med. Virol. 27, 2 (2017).
69. Zydek, M. et al. HCMV infection of human trophoblast progenitor cells of the placenta is neutralized by a human monoclonal antibody to glycoprotein B and not by antibodies to the pentamer complex. Viruses 6, 1346–1364 (2014).
70. Paradowska, E. et al. Detection of cytomegalovirus in human placental cells by polymerase chain reaction. APMIS. 114, 764–771 (2006).
73. Paradowska, E. et al. TLR9 -1486T/C and 2848C/T SNPs Are Associated with Human Cytomegalovirus Infection in Infants. PLoS One. 11, e0154100 (2016).
74. Stanton, R., Westmoreland, D., Fox, J. D., Davison, A. J. & Wilkinson, G. W. Stability of human cytomegalovirus genotypes in persistently infected renal transplant recipients. J. Med. Virol. 75, 42–46 (2005).
75. Grosjean, J. et al. Direct genotyping of cytomegalovirus envelope glycoproteins from toddler’s saliva samples. J. Clin. Virol. 46, S43–48 (2009).
76. Murrell, I. et al. Impact of sequence variation in the UL128 locus on production of human cytomegalovirus in fibroblast and epithelial cells. J. Virol. 87, 10489–10500 (2013).

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Author contributions
E.P. conceptualized the study and supervised the study, analyzed the data, and drafted the manuscript. A.J. and M.S. performed experiments and analyzed the data. B.K. performed the ELISA experiments. Material from patients was organized by J.C.K., B.K., K.D.F., M.W.L. and T.W.G. Clinical information were prepared by J.C.K. and M.W.L. Critical manuscript revisions were done by A.J., J.C.K., K.D.F., B.K., M.S., M.W.L. and T.W.G.

Competing interests
The authors declare no competing interests.

Additional information
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