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Maternal and child human leukocyte antigens in congenital cytomegalovirus infection

Roberta Rovito[a,*], Frans H.J. Claas[b], Geert W. Haasnoot[b], Dave L. Roelen[b], Aloys C.M. Kroes[a], Ann C.T.M. Vossen[a]

[a] Department of Medical Microbiology, Leiden University Medical Center, Leiden (LUMC), The Netherlands
[b] Department of Immunohematology and Blood Transfusion, Leiden University Medical Center (LUMC), Leiden, The Netherlands

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ABSTRACT

Congenital Cytomegalovirus infection (cCMV) is the most common cause of congenital infections worldwide causing permanent long-term impairment (LTI). cCMV immunopathogenesis remains largely unknown due to the complex interplay between viral, maternal, placental and child factors. The aim of this study was to determine the possible role of particular HLA antigens, of the number of HLA mismatches (mm) and non-inherited maternal antigens (NIMAs) in a large retrospective nation-wide cohort of children with cCMV and their mothers. HLA Class I (HLA-A, HLA-B and HLA-C) and HLA Class II (HLA-DR and HLA-DQ) were assessed in 96 mother-child pairs in relation to a control group of 5604 Dutch blood donors, but no significant differences were observed. Next, although these HLA antigens could not be assessed in relation to symptoms at birth, nor to LTI, due to the low number of cases, they could be evaluated in relation to CMV viral load. HLA-DRB1*04, and potentially HLA-B*51, was shown to have a protective role in the children as its frequency was increased in the low viral load group compared to the high viral load group, and this remained significant after correction. The number of HLA mm and of NIMAs were not associated to symptoms at birth nor to LTI or viral load. In conclusion, although none of the HLA alleles could be put forward as prognostic marker for long-term outcome, our findings give useful insights into cCMV pathogenesis, and identify potential HLAs that correlate with a better viral control.

1. Introduction

Congenital cytomegalovirus infection (cCMV) is the most common congenital infection in industrialized countries with an overall birth prevalence between 0.6 and 0.7% (Dollard et al., 2007; Kenneson and Cannon, 2007). Despite the considerable knowledge of cCMV clinical outcome, the multifactorial process that determines whether a neonate will have symptoms at birth or will develop permanent long-term impairment (LTI) is still poorly understood. In 12.7% of neonates, cCMV leads to symptoms at birth, such as petechiae, jaundice, hepatosplenomegaly, and microcephaly (Kenneson and Cannon, 2007; Dollard et al., 2007). An estimated 40–58% of these symptomatic neonates will develop LTI, such as hearing loss, cognitive and motor delay (Dollard et al., 2007). Importantly, of the 87.3% asymptomatic neonates at birth, around 13.5% will develop permanent sequelae (Dollard et al., 2007).

cCMV pathogenesis is the result of a complex multifactorial process that comprises maternal, placental, fetal and child factors. The nature of maternal infection and her immune response have an important role in cCMV and its outcome. Indeed, the risk of vertical transmission is 30–40% among women without prior CMV infection, while among previously exposed women this risk is at least 10-fold lower (Kenneson and Cannon, 2007). The risk of symptomatic CMV disease is mainly associated to maternal infection occurring in the first, and second trimester of pregnancy (Pass et al., 2006; Enders et al., 2011). Although previous studies have demonstrated a fetal and neonatal CMV-specific immune response, its role in controlling CMV disease still needs to be clarified (Vermijlen et al., 2010; Lidehall et al., 2013; Hassan et al., 2007).

The CMV-specific T cell mediated immune response is dependent of the HLA type of the individual (Goldrath and Bevan, 1999; Davis and Bjorkman, 1988). CMV-specific T cells restricted to certain HLA alleles may mount a more effective immune response than others. In transplantation, several HLA alleles have been shown to have a central role in CMV infection and disease (Du et al., 2007; Futohi et al., 2015; Acar et al., 2014; Bal et al., 2013; Chen et al., 2001). Importantly, pregnancy is considered a semi-allograft as the fetus can have HLA Ags that the

Abbreviations: cCMV, congenital cytomegalovirus infection; LTI, long-term impairment; DBS, dried blood spot; mm, mismatches; NIMAs, non-inherited maternal antigens; Ags, antigens

* Corresponding author at: Albinusdreef 2, 2333 ZA Leiden, The Netherlands.
E-mail addresses: R.Rovito@lumc.nl (R. Rovito), F.H.J.Claas@lumc.nl (F.H.J. Claas), G.W.Haasnoot@lumc.nl (G.W. Haasnoot), D.L.Roelen@lumc.nl (D.L. Roelen), A.C.M.Kroes@lumc.nl (A.C.M. Kroes), A.C.T.M.Vossen@lumc.nl (A.C.T.M. Vossen).

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mother does not have. The maternal immune system may recognize these Ags of paternal origin, as it has been demonstrated both in the maternal peripheral blood and in the placenta (Tilburgs et al., 2009; van Kampen et al., 2001). During pregnancy exchange of cells between mother and fetus will result in microchimerism (Maloney et al., 1999; Bianchi et al., 1996). The microchimeric cells carry Ags that the recipient does not have, which may result in sensitization of the mother or tolerance in the fetus (Bracamonte-Baran and Burlingham, 2015). However, different regulatory mechanisms are in place to prevent an allo-reactive immune response and create a tolerogenic environment for the undisturbed fetal development. For instance, decidual T cell activation is associated with a concomitant induction of functional T regulatory cells (Tilburgs et al., 2009). Furthermore, the extravillous trophoblasts do not express HLA-A, -B, -DR, -DQ and -DP (Moffett-King, 2002), but only HLA-C and non-classical HLA-E and −G.

The role of maternal and child HLA has mainly been shown in pregnancy complications such as pre-eclampsia, intrauterine growth retardation and recurrent spontaneous abortion (Faridi and Agrawal, 2011; Hiby et al., 2004), whereas its role during cCMV is unknown. When cCMV occurs, the placental tolerogenic environment may be altered, resulting in an enhanced alloreactivity (Tilburgs and Strominger, 2013), which in turn may affect cCMV outcome. The aim of this study was to assess the maternal and child HLA molecules in relation to cCMV, neonatal viral load and clinical outcome from birth till 6 years of age.

2. Materials and methods

2.1. Study population and clinical data

A previously described nationwide, retrospective cohort was used in this study (Korndewal et al., 2015b). The cohort was derived from a total group of 31,484 children, born in 2008 in the Netherlands, which was retrospectively tested for cCMV infection by PCR of CMV DNA in neonatal dried blood spot (DBS) at five years of age. In total, 156 children (0.5%) were diagnosed with cCMV. After approval by the Medical Ethics Committee of the Leiden University Medical Center, the parents of 125 congenitally CMV infected children and of 263 non-infected children gave written informed consent for the retrieval of clinical data. For this study, 104 children with cCMV and their mothers additionally provided buccal swabs for HLA typing. Two buccal swabs were retrieved from each subject (FLOQSwabs hDNA Free, 20-mm breaking point in 174.5 mm long dry tube, COPAN ITALIA SPA, Brescia, Italy). Children were defined as symptomatic at birth if they had one or more of the following signs or symptoms in the neonatal period: prematurity, being small for gestational age, microcephaly, hepato- or splenomegaly, generalized petechiae or purpura, hypotonia, abnormal laboratory findings (e.g. neutropenia or thrombocytopenia), cerebral ultrasound abnormalities, ophthalmologic abnormalities or neonatal hearing impairment. LTI was defined as the presence of impairment in one or more domains (hearing, visual, neurological, motor, cognitive and speech-language). The cCMV associated LTI in the original cohort has been described in detail (Korndewal et al., 2017). In brief, hearing impairment was defined as sensorineural hearing loss ≥40 dB; visual impairment was defined as a visual acuity below 0.3; neurological impairment included cerebral palsy, epilepsy, microcephaly, autism spectrum disorder and ADHD; motor developmental delay was based upon the physical therapist's report and if available on a score below the fifth centile in the Movement Assessment Battery for Children; cognitive developmental delay was defined as an intelligence quotient less than or equal to 70 if this was tested, or it was based on a diagnosis by a medical specialist; speech and language development were assessed by the speech therapist or speech and hearing centre. In this cohort maternal seroimmunity to CMV before birth was unknown, hence it was assumed that cCMV could have resulted from either maternal primary or secondary infection.

2.2. DNA extraction from buccal swabs and HLA typing

DNA was extracted from the buccal swabs by using QIAamp DNA (blood) mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction with minor modifications. The buccal swabs were incubated with PBS for 30 min at room temperature. The pre-incubation fluid was applied to one QIAamp spin column and eluted in 150 µl of Tris-EDTA-4 buffer. All mothers and children were DNA typed for the loci HLA-A (20 alleles), HLA-B (40 alleles), HLA-C (14 alleles), HLA-DR (14 alleles) and HLA-DQ (7 alleles). For class I, a commercially available assay was applied, LIFECODES HLA-A, B and C SSO Typing kits (Immucor, Norcross, GA, US). Data were analysed using MatchIT software (Immucor-Lifecodes). For class II, a locally developed SSO technique was used as previously described (Verduyn et al., 1993), and data were analysed using SCORE software (Wolfgang-Helmberg).

2.3. DNA extraction from DBS and qPCR of CMV

After a first initial CMV PCR screening performed at the National Institute for Public Health and the Environment, a second confirmatory PCR was performed at the Leiden University Medical Center (Korndewal et al., 2015b). DNA was extracted from DBS by using the QIAamp DNA minikit according to the previously described protocol (de Vries et al., 2012). For each test one full DBS was punched by using an automated DBS puncher (1296-071, Perkin Elmer-Wallac, Zaventem, Belgium). CMV DNA amplification of a 126-bp fragment from the immediate-early antigen region was performed using an internally controlled quantitative real-time PCR, as described previously (de Vries et al., 2009; Kalpoe et al., 2004), on the CFX96 Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands). The PCR was performed in triplicate, and the viral load expressed in IU/ml.

2.4. Statistical analysis

Data were analyzed by using the Statistical Package for Social Sciences (SPSS, version 23, Chicago, IL, USA). The frequency of the HLA alleles in the study group was compared to the frequency in 5604 healthy Caucasian Dutch blood donor by using the two-sided Fisher's exact test (van Rooijen et al., 2012). The p-values were corrected for multiple comparisons (pm) conform to the Šidák method (Sidák, 1967). Odds ratios and corresponding 95% confidence intervals were calculated according to the Woolf Haldane test (Haldane, 1956; Woolf, 1955). A large control group could lead to significant differences which are clinically irrelevant. Therefore p-values were standardized (ps) to a smaller sample size following the method of Good (Good, 1982). Next, the study group was split into two groups according to the median viral load measured on DBS, namely high and low viral load group. The choice of the median was dictated by the study design, which involved several HLA molecules potentially leading to lack of statistical power, not allowing further division of the total group, and by the fact that there is no commonly accepted cut-off to define high and low viral load. The frequency of the HLA alleles in the high viral load group was compared to the frequency in the low viral load group by using the same method without correction for the sample size, which was similar in both groups. A Mann-Whitney test was used to further assess the distribution of viral load in relation to the HLA alleles of interest. Next, the maternal and child HLA allele frequencies were tested for Hardy-Weinberg Equilibrium (HWE), stating that in absence of other influences the genotype frequency in a certain population remains constant from generation to generation (Hardy, 1908). This has been suggested as a measure of disease association when analysing the case group *per se* as, by definition, the control group has to hold the HWE (Namipashaki et al., 2015; Lee, 2003; Nielsen et al., 1998). The HWE was assessed for both maternal and child genotypes by using Pypop software 0.7.0. The chi-square test was used to evaluate the observed and expected number of HLA mismatches (mm), and non-inherited maternal antigens

Table 1
Characteristics and clinical outcome of study population.

	Overall n = 96	Congenital CMV infection Asympt.[a] n = 77	Sympt.[b] n = 19
Gender			
Male	57	44	13
Female	39	33	6
Gestational age (weeks)[c]	39(28–42)	39 (37–42)	36 (28–41)
Birth weight (g)[c]	3435 (900–5110)	3540 (2635–5110)	2800 (900–4170)
Long term impairment			
Hearing impairment[d]	3	2	1
Visual impairment[e]	2	2	0
Neurological impairment[f]	5	2	3
Motor impairment[g]	13	9	4
Cognitive impairment[h]	7	4	3
Speech/language problem[i]	18	10	8
One or more impairment[j]	27	16	11

[a] Asymptomatic at birth.
[b] Symptomatic at birth: premature (n = 11), dysmature (n = 2); microcephaly (n = 5); neonatal hearing loss (n = 1); abnormal cranial ultrasound (n = 1).
[c] Median and range.
[d] Sensorineural hearing loss.
[e] Optic nerve atrophy (n = 1), cortical visual impairment (n = 1).
[f] Cerebral palsy (n = 1), epilepsy (n = 1), microcephaly (n = 1), ADHD (n = 1), autism (n = 3).
[g] Motor impairment (fine, gross or balance) based on test or diagnosis or sensory processing disorder or developmental coordination disorder.
[h] Cognitive impairment based on test or diagnosis.
[i] Language impairment based on test or diagnosis, speech-impairment, oral motor skill difficulties or auditory processing disorder.
[j] Any long-term impairment, in one or more domains.

(NIMAs), in relation to symptoms at birth, LTI and viral load. With expected low values, a Fischer's exact test was used instead. A p-value < 0.05 was considered statistically significant.

3. Results

3.1. Study population and clinical data

A total of 96 mother-child pairs were successfully typed for at least one of the following HLA alleles, HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ. Eight mother-child pairs could not be typed because of lack of DNA quality and concentration. The clinical data of the congenitally infected children included in this study are shown in Table 1. Nineteen (20%) children had symptoms at birth. Of these children, 11 (58%) had LTI at the age of 6 years. Additionally, 16 (21%) asymptomatic children had LTI. Overall, 27 (28%) of the total group of children with cCMV developed any LTI. The control group consisted of 5604 randomly selected healthy Dutch blood donors who were previously genotyped (van Rooijen et al., 2012). In the control group, which was in HWE, 3318 (59%) were females and the mean (SD) age was 36.2 (8.7) years. No additional clinical information nor CMV serostatus were available. Therefore, it was assumed that 50% was CMV-positive at the moment of typing (Korndewal et al., 2015a). This group is considered to be a proper representation of the HLA gene distribution in the Dutch population (Schipper et al., 1996).

3.2. Analysis of maternal and child genotype frequencies

First, we evaluated whether any HLA alleles included in this study was associated with cCMV. For this purpose, the frequency of HLA Class I (A, B and C) and Class II (DR and DQ) alleles were analysed in 96 mother-child pairs, and compared to the HLA frequencies in 5604 Dutch healthy blood donors. Data are reported in Table S1 in Supplementary material. Initial uncorrected association analysis revealed an increased frequency of HLA-B*39 and HLA-DRB1*12 compared to the control group in the mothers (10.4% and 4.1% p = 0.0066 for HLA-B*39; 9.6% and 3.7% p = 0.0097 for HLA-DRB1*12). Whereas, in the children, an increased frequency of HLA-C*02 and HLA-DRB1*12, and a decreased frequency of HLA-C*16, compared to the control group were observed (17.9% and 10.3% p = 0.0254 for HLA-C*02; 9.6% and 3.7% p = 0.0097 for HLA-DRB1*12; 1.1% and 6.1% p = 0.0456 for HLA-C*16). After correction, none of the aforementioned associations remained significant.

Next, the study group was evaluated in more details, and the genotype frequencies of HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ from the mothers and their children were tested for the HWE. There was not enough discriminative power to determine if in the total group of alleles the HWE was maintained, both in the mothers and in the children. However, for HLA-DQ alleles, which are less polymorphic, the HWE could be assessed. For the mother, no deviations from the HWE were observed, whereas for the children a deviation was reported (Table 2). Additionally, all homozygote and heterozygote alleles for all loci were in HWE for both mothers and children (Table 2).

Finally, in order to evaluate whether any HLA allele included in this study was associated with CMV viral load, we split our study group in two according to the median viral load measured on DBS, which was 3.2 log (IU/ml), namely low (n = 47) and high viral load group (n = 48). The frequency of the HLA alleles of the high viral load group was compared to the frequency of the low viral load group, both in the mothers and in the children. In our cohort, the maternal viral load could not be assessed as maternal samples during pregnancy were not available, therefore the maternal HLA frequency was assessed only in relation to the child CMV viral load. Initial uncorrected association analysis revealed a few correlations (Table S2 in Supplementary material). In the mothers, HLA-C*03 was found increased in the high viral load group compared to the low (42.6% and 21.3% respectively, p = 0.0455). In the children, HLA-DRB1*04 was found decreased in the high viral load group compared to the low (10.6% and 37.0% respectively, p = 0.0034), as well as HLA-DQ8 (10.6% and 30.4%

Table 2
Hardy-Weinberg Equilibrium for HLA-Class I and HLA-Class II.

	Mothers		Children	
	Chi-square	p-value	Chi-square	p-value
HLA-A				
total[a]	N/A[d]	N/A	N/A	N/A
homozygosity[b]	0.99	0.319	1.15	0.283
heterozygosity[c]	0.20	0.654	0.23	0.632
HLA-B				
total	N/A	N/A	N/A	N/A
homozygosity	0.86	0.355	0.13	0.718
heterozygosity	0.07	0.787	0.01	0.912
HLA-C				
total	N/A	N/A	N/A	N/A
homozygosity	0.21	0.644	0.18	0.672
heterozygosity	0.04	0.843	0.04	0.845
HLA-DR				
total	N/A	N/A	N/A	N/A
homozygosity	0.01	0.913	0.76	0.382
heterozygosity	0.00	0.969	0.10	0.749
HLA-DQ				
total	0.88	0.830	8.80	0.032*
homozygosity	0.18	0.675	0.00	1.000
heterozygosity	0.04	0.837	0.00	1.000

[a] Hardy-Weinberg Equilibrium for all HLA-A allele frequencies included in this study.
[b] Hardy-Weinberg Equilibrium for the HLA-A homozygous alleles.
[c] Hardy-Weinberg Equilibrium for the HLA-A heterozygous alleles.
[d] N/A = not assessable, due to lack of statistical power the HWE could not be assessed.
* Significant p-value.

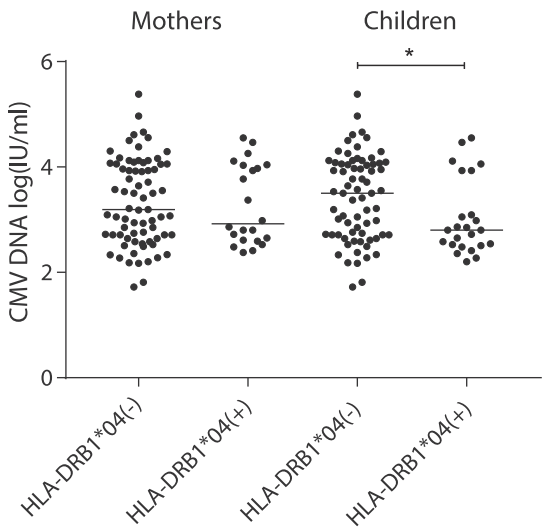

Fig. 1. Maternal and child HLA-DRB1*04 in relation to neonatal viral load measured in DBS (log(IU/ml)). *Horizontal bars represent medians.* *p = 0.017.

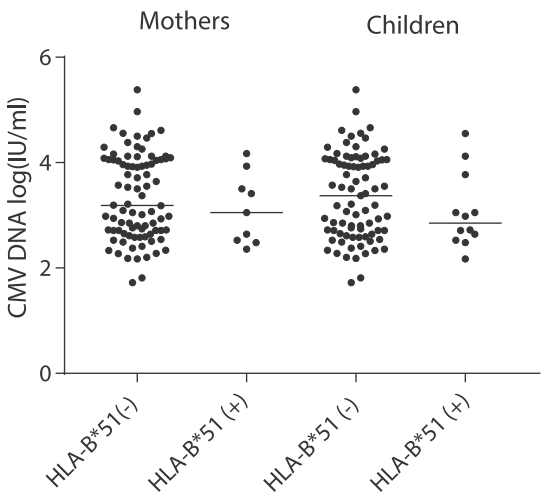

Fig. 2. Maternal and child HLA-B*51 in relation to neonatal viral load measured in DBS (log(IU/ml)). *Horizontal bars represent medians.*

respectively, p = 0.0218). However, given the high linkage between HLA-DR4 and HLA-DQ8, the increase of HLA-DQ8 percentage in the high viral load group may be considered as a secondary effect to the increase of HLA-DRB1*04. After correction for multiple comparison, only HLA-DRB1*04 remained significant (p = 0.0401) (Table S2 in Supplementary material). Therefore, a Mann-Whitney test was used to further assess the distribution of viral load between HLA-DRB1*04 positive and HLA-DRB1*04 negative children. The median viral load was significantly lower in the HLA-DRB1*04 positive children compared to the HLA-DRB1*04 negative children (p = 0.017), whereas this was not observed in the mothers (p = 0.627) (Fig. 1). Additionally, HLA-DRB1*04 was evaluated in relation to symptoms at birth and LTI, and no associations were found (data not shown). Furthermore, as several evidences have been supporting a protective role of HLA-B*51 during infections, we wondered whether the same trend could be observed in the context of cCMV. In the children, HLA-B*51 frequency was increased in the low viral load group compared to the high viral load group (17.4% and 4.3% respectively, p = 0.050) (Table S2 in Supplementary material), and the median neonatal viral load was slightly lower in HLA-B*51 positive children compared to the HLA-B*51 negative children (p = 0.220) (Fig. 2). These trends were not observed in the mothers (Table S2 in Supplementary material and Fig. 2). Finally, the frequency of the HLA alleles in relation to symptoms at birth (n = 19) and LTI (n = 27) could not be addressed due to the lack of statistical power.

3.3. Maternal-fetal HLA class I and class II mismatches in relation to cCMV clinical outcome

We next investigated whether maternal-fetal HLA mm were associated with a worse cCMV outcome. This was evaluated for HLA-A, HLA-B, HLA-DR and HLA-DQ as for HLA-C was already addressed (Rovito et al., 2018). For this purpose, mm were calculated on the basis of the presence of an Ag in the fetus, which was absent in the mother because the inherited paternal antigen was different from both maternal alleles (Table 3). The percentage of mm were compared between children symptomatic and asymptomatic at birth, as well as between children who developed LTI and those who did not. No differences in percentage of mm were observed in relation to symptoms at birth nor to LTI development (Table 4). Mm were additionally evaluated in relation to CMV viral load, by comparing mm percentage between low and high viral load groups, both in the mothers and in the children. No difference in the mm percentage were observed between high and low viral load groups (data not shown).

Table 3
Definitions of mismatches and NIMAs.

Genotype combinations[a]			
Maternal genotype	*Fetal genotype*	mm[b]	*NIMAs*[c]
a/b	a/a	no	yes
a/b	b/b	no	yes
a/b	a/c	yes	yes
a/b	c/b	yes	yes
a/a	a/b	yes	no
b/b	a/b	yes	no
a/a	a/a	no	no
a/b	a/b	no	no
b/b	b/b	no	no

[a] Combinations of maternal and child genotype by using 3 hypothetical genes (a, b, c).
[b] mm: mismatches, defined as the Ag that the child has but the mother does not have, because the inherited paternal antigen is different from both maternal alleles.
[c] NIMAs: non-inherited maternal antigens, defined as the Ag that the mother has and the child does not because the inherited paternal antigen differs from the non-inherited maternal antigen.

3.4. Non-inherited maternal HLA class I and HLA class II in relation to cCMV clinical outcome

We finally assessed whether NIMAs influenced cCMV clinical outcome. This was evaluated for HLA-A, HLA-B, HLA-DR and HLA-DQ as for HLA-C was already addressed (Rovito et al., 2018). For this purpose, NIMA was defined as an Ag in the mother, which was absent in the child because the inherited paternal antigen was different from the NIMA (Table 3). The percentage of NIMAs were compared between children symptomatic and asymptomatic at birth, as well as between children who developed LTI and those who did not. In our cohort, no differences in percentage of NIMAs were observed in relation to symptoms at birth nor to LTI development (Table 4). NIMAs were additionally assessed in relation to CMV viral load, but no differences were observed between high and low viral load groups (data not shown). Interestingly, HLA-DR4 has been associated with genetic susceptibility to rheumatoid arthritis, and, in HLA-DR4 negative patients, HLA-DR4 NIMAs was found increased, suggesting that the maternal presence of this allele may have a role (ten Wolde et al., 1993). Therefore, we wondered whether, in HLA-DRB1*04 negative infected children, HLA-DRB1*04 NIMAs could still influence CMV viral load (Fig. S1 in Supplementary material). However, no NIMAs effect was

Table 4
HLA mismatches and NIMAs in relation to cCMV clinical outcome.

	Symptoms at birth			Long-term impairments (LTI)		
	Sympt.[a] % n = 19	Asympt.[b] % n = 76[e]	p-value Chi	LTI (≥1)[c] % n = 26[f]	No LTI[d] % n = 69[g]	p-value Chi
HLA-A mm[h]	78.9	64.5	0.229	76.9	63.8	0.223
HLA-B mm	94.7	80.0	0.179̃	88.5	80.9	0.543̃
HLA-DR mm	63.2	80.0	0.137̃	68.0	79.7	0.236
HLA-DQ mm	47.4	64.0	0.185	52.0	63.8	0.302
HLA-A NIMAs[i]	89.5	72.4	0.145̃	80.8	73.9	0.487
HLA-B NIMAs	84.2	88.0	0.703̃	88.5	86.8	1.00̃0
HLA-DR NIMAs	78.9	80.0	1.00̃0	80.0	79.7	0.975
HLA-DQ NIMAs	63.2	57.3	0.645	56.0	59.4	0.766

[a] Symptomatic at birth.
[b] Asymptomatic at birth.
[c] Any long-term impairment, in one or more domains of impairments: hearing, visual neurologic, motor, cognitive, and speech-language.
[d] Absence of any long-term impairment.
[e] HLA-B, HLA-DR and HLA-DQ n = 75.
[f] HLA-DR and HLA-DQ n = 25.
[g] HLA-B n = 68.
[h] mm = mismatches, defined as the Ag that the child has but the mother does not have because the inherited paternal antigen differed from both maternal alleles.
[i] NIMAs = non-inherited maternal antigens, defined as the Ag in the mother which was absent in the child because the inherited paternal antigen differed from the NIMAs.-Fischer's exact test.

observed in our cohort (p = 0.486), suggesting that the protective effect of HLA-DRB1*04 is driven by the presence of this allele in the child. Finally, HLA-DRB1*04 NIMAs were not associated with symptoms at birth nor to LTI (data not shown).

4. Discussion

This study aimed to determine whether certain maternal and child HLA alleles play a role in cCMV. Therefore, HLA of Class I and Class II were assessed in relation to cCMV and cCMV clinical outcome in a large retrospective nation-wide cohort of children with cCMV.

The analysis of maternal and child genotype frequencies in relation to cCMV, which was performed comparing the HLA alleles frequencies to the frequencies in a control group of healthy blood donors, did not show any striking association with cCMV. However, for the purpose of this study different control groups may be needed. From the maternal point of view, a group of CMV infected women who did not transmit the virus to the fetus would be more appropriate. Whereas from the neonatal point of view, a group of neonates that was exposed to CMV but did not get infected would be more suitable. This can be quite difficult to achieve as these HLA studies require large study groups.

For the aforementioned reasons, we looked in more details into our study group by first evaluating the HWE, and then the HLA frequencies in relation to neonatal viral load. The HWE could be determined only for the less polymorphic HLA-DQ, which was not in HWE in the group of children. Whether HLA-DQ has a role in cCMV would need to be further assessed as it has shown to have a minor function in our cohort. Additionally, in the children, HLA-DRB1*04 appeared to have a protective role during cCMV as its frequency was increased in the low viral load group compared to the high viral load group, and the median neonatal viral load was lower in HLA-DRB1*04 positive children (Table S2 in Supplementary material and Fig. 1). This remained significant even after statistical correction. Interestingly, HLA-DR4 has been associated with genetic susceptibility to rheumatoid arthritis, and, in HLA-DR4 negative patients, HLA-DR4 NIMAs was found increased (ten Wolde et al., 1993). However, in our cohort, HLA-DRB1*04 NIMAs did not influence CMV viral load. Therefore, the protective effect of HLA-DRB1*04 is most likely driven by the presence of the allele in the children (Fig. S1 in Supplementary material). Furthermore, HLA-DRB1*04 was not associated with clinical outcome, suggesting that it may have a specific role in the viral control rather than CMV-related disease. Consequently, it may be assumed a similar trend of that observed between child HLA-DRB1*04 and neonatal viral load, if maternal viral load during pregnancy was available. Importantly, the impact of HLA-DRB1*04 in CMV infection has already been shown. In allogeneic hematopoietic stem cell transplantation, CMV reactivation was less frequent in HLA-DRB1*04 positive patients (Acar et al., 2014), and in kidney transplantation this allele protected from HBV, HCV and CMV infections (Gharesi-Fard et al., 2014). Additionally, as several evidences have been supporting a protective role of HLA-B*51 during infections, we wondered whether the same trend could be observed in the context of cCMV. Indeed, in kidney and allogeneic stem cell transplantation, HLA-B*51 has been shown to have a protective role towards CMV infection (Bal et al., 2013; Chen et al., 2001), while in infections, such as HIV, it was associated with an efficient immune control (Zhang, et al., 2011). In our cohort, HLA-B*51 appeared to have a trend towards a better viral control, as its frequency was increased in the children of the low viral load group (Fig. 2). However, to confirm a possible protective role of HLA-DRB1*04 and HLA-B*51 more studies in other cohort of congenitally infected children would be needed. In our cohort, CMV viral load was not correlated to symptoms at birth nor to LTI development at 6 years of age (Rovito et al., 2017b, 2017a). However, the predictive role of CMV viral load in blood for congenital CMV disease may differ depending on the timing of infection and whether there was a primary or recurrent maternal infection, which cannot be established in our cohort. Additionally, this cohort study, retrieved from a large population screening, does reflect a real population of newborns with cCMV in all its diversity, ranging from no symptoms at birth and no LTI, to symptoms at birth with severe LTI. Therefore, the predictive value of CMV viral load could not be assessed for the individual clinical outcomes. Importantly, the correlation between CMV viral load and clinical outcome has not been established yet as some studies have shown a correlation between CMV viral load and clinical outcome (Lanari et al., 2006; Forner et al., 2015), whereas others have not (Halwachs-Baumann et al., 2002; Binda et al., 2010; Ross et al., 2009).

Although the majority of the HLA molecules included in this study are not expressed at the placenta, an allogeneic response has been shown to occur due to the fetal microchimerism (Payne, 1957; van Kampen et al., 2001; van Kampen et al., 2002; Verdijk et al., 2004). Viral infections can increase the levels of pro-inflammatory cytokines and chemokines at the maternal-fetal interface, and intensify the decidual T cells influx (Constantin et al., 2007; Nancy et al., 2012). Here, the increased level of allogeneic maternal cells specific for fetal Ag that are not expressed by the trophoblast, may contribute to placental immunopathology by increasing the inflammation. The role of HLA mm in CMV infection has mainly been shown in transplantation. In allogeneic hematopoietic stem cell transplantation, CMV DNAemia, CMV disease and CMV resistance were associated with the presence of HLA mm (Sedky et al., 2014; Schonberger et al., 2010). Additionally, in kidney transplantation HLA mm were shown to be an important determinant of CMV-associated graft loss (Gatault et al., 2013). In the context of cCMV, HLA-A, HLA-B, HLA-DR and HLA-DQ mm, as well as NIMAs, were not significantly associated with clinical outcome, nor with CMV viral load. Even though the sample size may be a limiting factor in determining any significant HLA association, the HLA molecules expressed at the placenta, which are HLA-C, HLA-E and HLA-G, may exert a more important role in cCMV (Rovito et al., 2018). Interestingly, in uncomplicated pregnancies a significant correlation between the total number of HLA mm and an increased number of activated decidual T cells was reported (Tilburgs et al., 2009). However, HLA-C mm were the most influential in determining such an increase, whereas the other HLA mm contributed to a lesser degree (Tilburgs et al., 2009). In our cohort, a slightly higher percentage of HLA-A and HLA-B mm were

observed in the symptomatic group compared to the asymptomatic group, though not significant (Table 4).

Finally, some limitations characterise this study. First of all, potential effects of CMV on the expression of the HLA molecules included in this study could not be addressed. Indeed, CMV has developed strategies to evade host immunity and to establish latency, e.g. by down-regulating classical HLA molecules and up-regulating non-classical HLA (Gong et al., 2012; Onno et al., 2000; Huard and Fruh, 2000; Wilkinson et al., 2008). This could further influence the degree of HLA association with cCMV and cCMV clinical outcome. Second of all, the relatively small sample size may have limited the statistical power to detect any significant association. Third, due to the retrospective design of the study, cCMV diagnosis was performed by DBS testing, which in comparison with urine or saliva has been associated with limited sensitivity in some studies (Ross et al., 2014; Boppana et al., 2010; Inoue and Koyano, 2008). Urine specimens are commonly used for detection of cCMV due to the high viruria observed in congenitally infected infants, and this is still considered the gold standard method (Halwachs-Baumann et al., 2002). Urine and saliva are considered equally reliable for detection of cCMV (Yamamoto et al., 2006), and CMV viral load is usually lower in blood than in urine (Halwachs-Baumann et al., 2002). DBS can be considered a proper and reliable alternative specimen to fresh blood. Indeed, a positive correlation between CMV viral load measured on DBS and on whole blood, or on plasma, both in the context of cCMV and CMV in transplantation, has been previously shown (Christoni et al., 2012; Limaye et al., 2013). Importantly, even considering the relative reduction of CMV viral load on DBS during storage (Christoni et al., 2012), the specimens that could be potentially more affected from the aforementioned differences between blood and urine, or saliva, are those with low viral load. However, it is important to note that with the high sensitivity of our CMV PCR on DBS (estimated > 85%), high specificity (> 99.9%) and the cCMV birth prevalence of 0.5%, the chance of a CMV false-negative result is 1/1000 (Korndewal et al., 2015b). Therefore, the influence of the sensitivity of the CMV PCR on DBS on our conclusions can be considered negligible. Finally, the DBS specimens are available, almost, worldwide because they are collected for the screening of rare genetic metabolic disorders. This allowed us to perform a large-scale retrospective study for long-term cCMV outcome, which would be challenging in a prospective setting.

In conclusion, although none of the HLA alleles could be put forward as prognostic marker for cCMV or cCMV clinical outcome, our findings give useful insights into cCMV pathogenesis, and identify HLA-DRB1*04 and HLA-B*51 as potential HLAs correlating with a better viral control. Importantly, in view of the virus-host interaction at the maternal-fetal interface, the HLA expressed at the placenta may have a more substantial role in cCMV and cCMV clinical outcome (Rovito et al., 2018).

Conflict of interest statement

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jri.2018.01.002.

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