Pathogenesis of congenital cytomegalovirus infection : finding prognostic markers and correlates of protection

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CHAPTER 1

GENERAL INTRODUCTION

1.1. CYTOMEGALOVIRUS INFECTION: FROM GLOBAL INFECTION TO NEGLECTED PUBLIC HEALTH CONCERN

Cytomegalovirus (CMV) infection is without a doubt a global and endemic infection. In the USA, Australia and Europe the CMV seroprevalence varies between 36% and 77%, while in developing countries this percentage often reaches 100% (Fig. 1) (1). In countries with low to moderate seroprevalence, age is a predictor of seropositivity since the chance of exposure to CMV continues throughout life. As a rule of thumb, the socioeconomic status may be a reflection of factors that contribute to the exposure to CMV, such as crowded living conditions or numerous infants. Although in a considerable proportion of immunocompetent individuals CMV infection is subclinical, it can still cause a variety of clinical manifestation among which the mononucleosis like syndrome in young individuals is the best characterized (2). CMV has also been suggested as a co-factor in the pathogenesis of inflammatory, autoimmune and vascular disease, such as atherosclerotic coronary artery disease (3-6), as well as a risk-factor for all-cause mortality in large population-based cohorts in the USA and Europe (7, 8). However, the severe morbidity caused by CMV in immunocompromised individuals and neonates, and the following long-term disability in children, accounts for the true burden of disease of CMV infections.

Despite the considerable knowledge of the contribution of CMV to mortality and morbidity in immunocompromised individuals, the disease burden of congenital CMV infection (cCMV) is less often acknowledged (9). One of the reasons of this discrepancy may lie in the epidemiology of congenital infection, which altered the dogma of pre-existing immunity to CMV in pregnant women protecting against vertical transmission. Indeed, the rate of cCMV is higher in highly seropositive countries, i.e. developing countries, where CMV is acquired very early in life. At 3 months of age two third of the infants are estimated to be infected, and 85% by the first year of life (10, 11). Here, fewer women of child-bearing age are seronegative compared to developed countries, and the rate of cCMV ranges from 1% to 5%, or even higher (11-13). In developed countries, ~50% of child-bearing age women is seronegative with a lower rate of cCMV, between 0.6% and 0.7% of live births (14-17) (Fig. 2). This discrepancy is most likely due to the fact that the seroprevalence reflects the size for the viral reservoirs in a population, though the characteristics of CMV infection make the modes of transmission and acquisition difficult to determine (see "The ancient virus" paragraph) (9). In general, when a seronegative mother gets infected during pregnancy the vertical transmission rate is 30-35%, whereas in seropositive mothers this rate is estimated to be 1.2% (18-20). Although recent observations highlighted the fact that this may be higher due to re-infections with a new viral strain (21). However, the main contributor to the worldwide number of cCMV cases is represented by seropositive mothers.

Among congenitally infected children, 12.7% are estimated to be symptomatic at birth, with the most common symptoms being petechiae, jaundice, hepatosplenomegaly, thrombocytopenia, chorioretinitis, and microcephaly. An estimated 40-58% of these symptomatic children will develop permanent sequelae, such as hearing loss, mental retardation, and developmental delay. Importantly, of the 87.3% neonates that are asymptomatic at birth, ~13.5% will also develop permanent sequelae (16, 17) (Fig. 3). A similar frequency of symptomatic congenital infections has

been shown in mothers with primary infection and in mothers with secondary infection (being either reactivation or reinfection) (12, 22, 23), as well as similar severity of symptoms (12, 24, 25). Overall, a quite high percentage of congenitally infected neonates, estimated to be 17%, will have permanent sequelae. The majority of children with permanent sequelae come from the group that

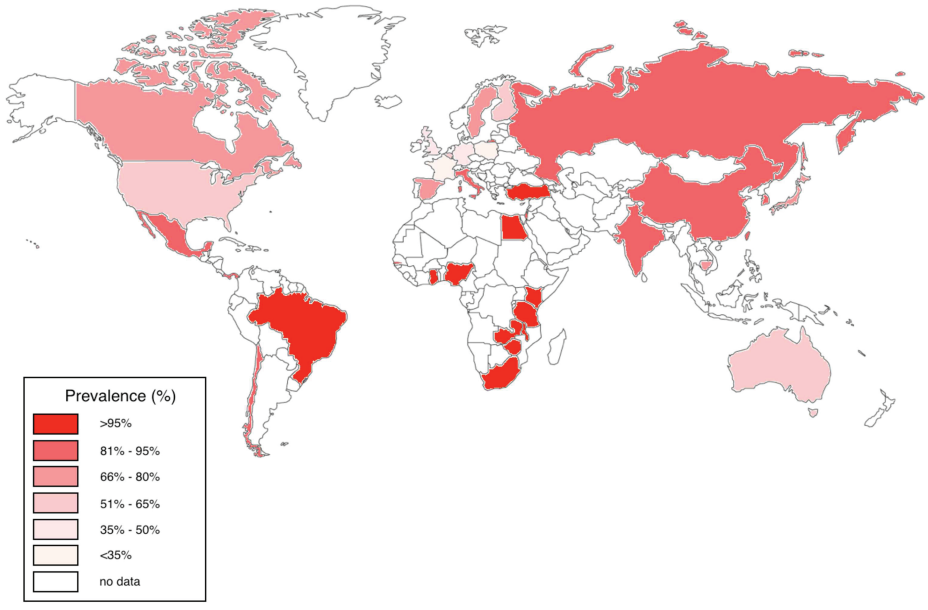

Figure 1. Worldwide CMV seroprevalence rates in adults. Reprinted with permission from (1).

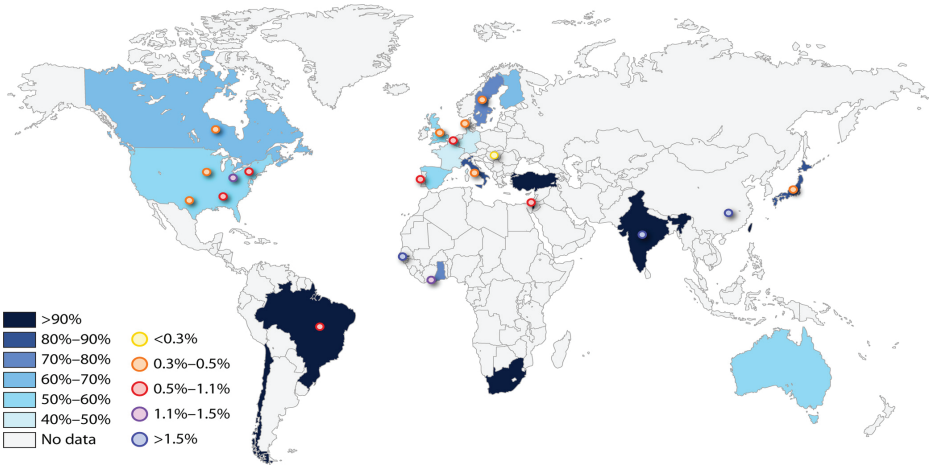

Figure 2. Worldwide CMV seroprevalence rates among women of reproductive age, and birth prevalence of congenital CMV infection. Shades represent CMV seroprevalence in women of child-bearing age, circles represent congenital CMV birth prevalence. Reprinted with permission from (9).

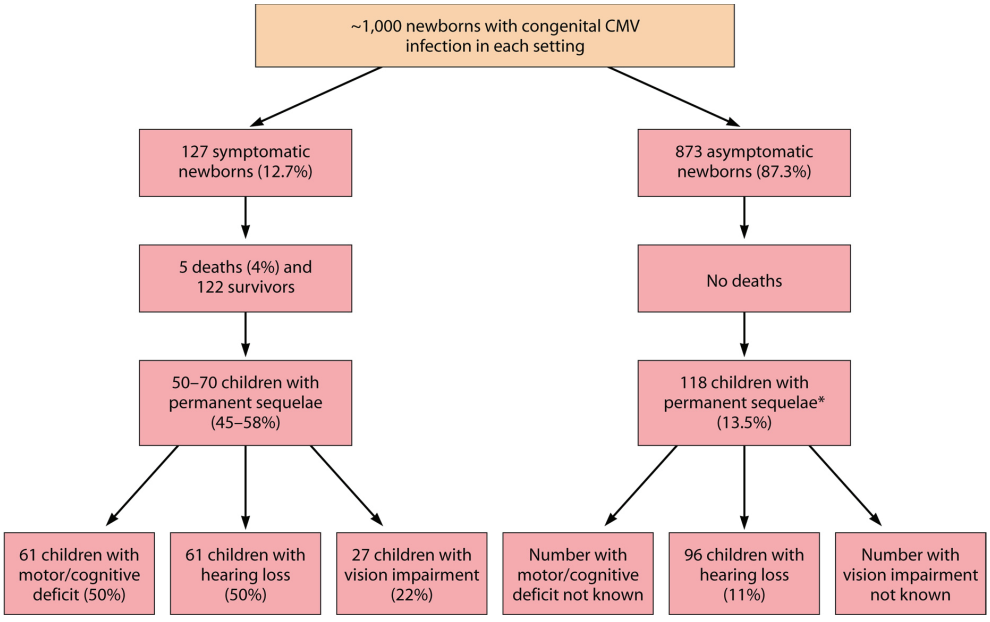

Figure 3. Estimates of the prevalence of congenital CMV infection and sequelae. Reprinted with permission from (9).

is asymptomatic at birth. In this group there are no clinical signs that would point towards a CMV infection in the neonate, therefore the infection may easily remain unnoticed, and the late onset of sequelae makes a retrospective diagnosis rather challenging.

To conclude, CMV is the most common cause of congenital infection worldwide, a much less exotic infection than newly emerging infections such as Zika virus infections, but overall a much greater global problem (16, 17).

1.2. THE ANCIENT VIRUS

Cytomegalovirus (Fig. 4) is the largest and structurally most complex member of the family of human *herpesviridae*, which includes several DNA viruses whose hallmark is latency. The pathogenic *herpesviridae* for humans are divided into three subfamilies based on structural and replicative properties. CMV belongs to the sub-family of β-*herpesvirinae*, which share a common long replicative cycle (~36-48 hours in permissive cells). The common ancestor of α-, β- and γ-*herpesvirinae* is believed to date 400 million years ago (Mya) while the β-*herpesvirinae* made their appearance only 50 Mya thereafter (350 Mya) (26). Importantly, the typical large inclusion-bearing cells produced by CMV were first reported in 1881 in a kidney of a stillborn infant with congenital syphilis (27), considerably later than their first appearance. CMV is 200 nm of diameter and has four distinct morphologic units: the *core* which contains 230 kilobase pairs of linear double-stranded DNA; the proteic *nucleocapsid* which consists of 162 capsomers (28); an amorphous layer called *tegument*

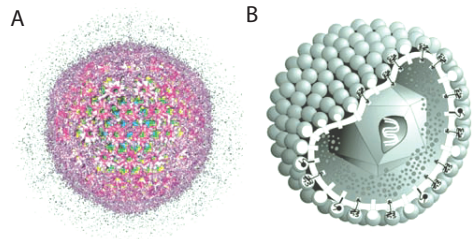

Figure 4. Human Cytomegalovirus. A) Three-dimensional reconstruction of icosahedrally portion of human CMV. B) virtual three-dimensional model showing the various CMV components. Reprinted with permission from (47).

which contains several viral proteins and RNA (29), and a double-stranded lipidic layer called *envelope*, where viral glycoproteins are embedded. The massive size of the genome is accompanied by a complex genomic organization and post-transcriptional modifications. The tegument is the most heterogeneous structure in CMV, and exerts several functions for viral replication, e.g. blocking of the cellular response that degrades entering DNA, and enhancing IE (immediate early) gene transcription (30-32). It also contains the immunodominant target of T cells and Abs, pp65 (33-36). Several glycoproteins have been described, and the pentameric complex gH/gL/gUL128-131 has been shown to elicit neutralizing Abs against several CMV strains (37-40). The initial binding between CMV and the target cell occurs via viral glycoproteins and cellular proteoglycans, followed by binding with more specific receptors and fusion/endocytosis (41-43). CMV has a broad tropism as it can be detected in fibroblasts, endothelial cells, epithelial cells, monocytes/macrophages, smooth muscle cells, stromal cells, neuronal cells, neutrophils, and hepatocytes (44-46). This suggests that the cellular receptors may be multiple, even though the primary targets are epithelial cells and monocytes. After these first events, the nucleocapsid is transported to the nucleus where the IE genes are expressed. IE1 (or pp72) is a phosphoprotein used as a target for detection of CMV infected cells, while IE2 is a transactivating protein responsible for activation of other viral genes (24). Next, the early, or β-genes, are expressed, which mainly contain viral proteins necessary for the viral genome synthesis, such as the viral DNA polymerase (24). Finally the late, or γ-genes, are expressed and these mainly encode structural proteins. The viral DNA is produced in the nucleus as concatemers, and during the packaging is cleaved and incorporated into the nucleocapsid. Though the mechanism is not completely understood, the nucleocapsid leaves the nucleus and reaches the cytoplasm as a partially tegumented viral particle. The final maturation occurs in a specialized cytoplasmatic compartment called *assembly compartment* (100). The virus egression is believed to occur via cell lysis in certain cell subtypes, e.g. fibroblasts, or via a non-completely understood mechanism of exocytosis. *Latency* is accomplished through viral genome circularization in the cellular nucleus, forming a so-called *episome* (108), and is mainly established in monocytes, CD34+ progenitors and endothelial cells. Consequently, in the host there might be a low level of productive infection with recurrent viral secretion, and several pro-inflammatory signals can induce such reactivation (106-109). This finding distinguishes acute from chronic infection. Acute infection

is characterized by an intense viral replication and shedding that lasts months, while the chronic infection is characterized by alternating replications and concomitant shedding.

1.3. TRANSMISSION OF CYTOMEGALOVIRUS

By definition the infectivity of CMV is much lower than of other viruses that have an airborne transmission (48). Therefore a close contact between a susceptible person with infected secretions is necessary for an efficient transmission. The transmission can occur via direct person-to-person or indirect contact, and sources of virus include oropharyngeal secretions, cervical and vaginal excretions, semen, breast milk, tears, urine, feces, and blood (49). CMV can therefore be transmitted horizontally through saliva, sexual contact, transplantation or breast-feeding, and vertically through the placenta. Despite the necessity of a close contact, the high seroprevalence in several countries suggests that CMV keeps spreading within a population, causing frequent infections. One of the most important contributing factors may be the high and chronic viral shedding of certain subgroups, such as congenitally and perinatally infected children, that can shed the virus for years, whereas in children and adults with primary infection this lasts ~6 months (50, 51). In addition, CMV can irregularly, and unpredictably, contribute to this frequent infection via secondary infections. In developed countries, where ~50% of women of child bearing age are seronegative, the most important source of infection is young children, possibly through their urine and saliva (52). Susceptible children are mainly infected through breast-feeding or other contacts with infected mothers and children, and only a small percentage via contact with the maternal cervical excretion during delivery (53). If infection occurs through breast-feeding, the presence of CMV in breast milk tends to be a bit later than immediately after birth, and CMV becomes detectable in the child only 6-8 weeks after the first exposure to CMV in the breast milk (54-56). Approximately 37-59% of infants breast-fed by a seropositive mother are eventually infected (54, 57).

1.4. CMV IMMUNE RESPONSE

The clinical impact of cCMV is the result of the interplay between virus and host in a stepwise process that starts in the mother, reaches the placenta and the fetus to then continue in the children throughout their childhood. In the following paragraphs these compartments will be discussed separately, although the processes within *one mother-child pair* are obviously non-separable. The general CMV immune response will be discussed first as it helps understanding the changes occurring during pregnancy, and in the fetus.

1.4.1. General CMV immune response

Following binding of CMV to the target cell, an alteration of the host gene expression occurs (58-60), with a rapid activation of the innate defence, possibly through TLR-2 and gB, that leads to a pro-inflammatory environment, co-stimulatory molecules, DC maturation, and IFN-α/β production (59, 61). The primary aim of such a response is to control the infection, while modulating the adaptive immune response. After an initial local replication, CMV spread and dissemination is most likely cell-associated, occurring mainly through monocyte/macrophages. When infected

circulating monocytes enter host tissues, they differentiate into macrophages that can sustain viral r eplication (62).

Upon acute primary CMV infection, a high pool of CD8+ T cells with a broad non-selective repertoire against several CMV Ags is produced. In healthy individuals, CMV DNAemia has a peak at 2-3 weeks after primary infection, and it decreases in 4-5 weeks after the primary infection (63). Cytotoxic T cells, with polyclonal TCRs, appear 2-3 weeks post-infection, and at ~5-8 weeks post-infection only a few sub-populations will reach the memory pool (63). This time approximately coincides with the resolution of primary infection. The memory compartment produced upon CMV infection is much bigger than that produced in response to other viruses, and it increases with age reaching 5% of all peripheral CD8+ T cells for a single CMV epitope (64). This phenomenon is called *memory inflation* and it may be attributable to the intermittent virus reactivation, and/or reinfection, that boost the T cell response.

Unfortunately, data on the CD4+ T cell response during primary CMV infection in immunocompetent individuals is not available as the majority of studies focused on latent infection. In latency the frequency of CD4+ T cells specific for CMV in healthy donors was 1-2% of all peripheral CD4+ T cells (65). However, some important conclusions drawn from transplantation studies may apply to the general population; though one should keep in mind that the immunosuppressive therapy might slightly change the kinetics of infection. In seronegative individuals that received a renal transplant from seropositive donors, viremia was detected ~25 days after transplantation, and CD4+ T cells appeared ~7 days after the first detection of viremia to then reach ~2.5% of the total of peripheral CD4+ T cells and again decrease to low level in the following ~2-3 months (65). Additionally, even though the detection of CMV DNA was similar, the peak viral load and the duration of viremia was higher and longer in those who had symptoms (66). Interestingly, in immunocompetent children an impaired CD4 response was associated with longer viral shedding in both urine and saliva (67).

Although it is commonly recognised that Abs have an important role during CMV infection, the majority of studies focused on the T cell response, and very little is known on the B cell compartment, and its kinetics during CMV infection. The protective effect of high Abs titers has been shown in several target populations (68-72). E.g. in immunocompetent individuals, a primary CMV infection induced an early and remarkable Abs response regardless of the clinical outcome, however, in those individuals with a more severe manifestation a deficient neutralizing Ab response was observed (69). Unfortunately, the real mechanism through which these Abs act in vivo still needs to be elucidated.

This remarkable CMV-specific immune response has an important feature, which is T and B cell *exhaustion*, though most studies focused on T cell exhaustion (73). T and B cell exhaustion is a state of dysfunction induced by chronic infections and prolonged exposure to high viral loads (73-75), and it is characterised by poor effector functions, expression of inhibitory molecules and specific transcriptional state (76). However, a certain degree of reversibility of this state has been shown through e.g. block of PD-1 (77). Importantly, exhaustion is a different concept than *anergy* or senescence. *Anergy* is considered to be a state of non-responsiveness that is initiated rapidly upon

first encounter with the Ag. Whereas, *senescence* is considered to be the terminal differentiation of a cell that loses the proliferative potential, and is typical of a latent infection.

1.4.2. Maternal CMV immune response

During pregnancy, the need to maintain the balance between fetal tolerance and antiviral immunity complicates the aforementioned immune response to CMV. Pregnancy does not seem to considerably alter the CMV-specific cell-mediated immune response compared to that of immunocompetent non-pregnant women (78, 79). However, in pregnant women who transmitted CMV to their fetus (transmitter), the lymphoproliferative response to CMV was lower and delayed (78, 79). Additionally, the duration of viremia was similar between transmitter and non-transmitter women (79). For what concerns the humoral response, Abs against gB were found to be higher during delivery in transmitter mothers, though the level of neutralizing Abs was lower (68). Additionally, primary CMV infection increased the expansion of activated and atypical memory B cells, enriched for CMV-specific cells, compared to the chronic infection. In the latter, the frequencies were similar to those of healthy adults, suggesting that the expansion does not last long (73), though the presence of memory B cells ensures a quick Abs production upon reactivation/reinfection.

1.4.3. Placental CMV immune response

The maternal immune system at the decidua level is mainly characterized by decidual natural killer cells (dNK), around 10% of leukocytes are T cells, both αβ and γδ subsets, while the frequency of B cells is low (80-82). At the maternal-fetal interface the majority of trophoblast cells are in direct contact with maternal cells, and fetus-specific maternal immune cells have been shown both locally and peripherally (83, 84). In normal conditions it does not damage pregnancy as several mechanisms are in place to prevent rejection of the fetal semi-allograft. Extravillous trophoblast cells do not express HLA-A, -B, -DR, -DQ and –DP (85), but they do express HLA-C and the non-classical HLA-E and HLA–G. HLA-C and HLA-E prevent maternal NK cell-mediated cytotoxicity through binding with killer cell immunoglobulin-like receptors (KIRs) expressed on dNK. Whereas, HLA-G modulates the response of different cellular subsets including dNK, antigen-presenting cell (APC), T cells, and B cells (86, 87). The complex local maternal-fetal immune cross-talk differs from the peripheral immune system of both mother and child (88). Indeed, immune cells can be generated locally with a different function than the one acquired at the periphery. For example, CD8+ T cells express significantly lower levels of perforin and granzyme-B, dendritic cell (DC) are arrested in a tolerogenic state, and dNK cells can be generated locally (88, 89). Viral infections may increase the levels of pro-inflammatory cytokines, chemokines, and the influx of T cells in decidual tissues (90, 91). In this situation, the regulatory mechanisms might not be able to efficiently inhibit the allogeneic lymphocytes (89), which could damage the placenta. CMV replication occurs in the decidua, endothelial cells and endovascular cytotrophoblast (92-94). Even though maternal macrophages and NK cells may limit the viral replication in the uterine wall (93), once CMV infects the cytotrophoblasts there is an extensive change in molecule expression that hampers their migration and invasion (92, 95-98). Women with primary CMV infection and a symptomatic fetus had

thicker placentas than women with asymptomatic foetuses; additionally the latter placentas were thicker than those of women with secondary infection (99). Furthermore, the infected placentas are characterized by an hypoxic-like environment that attempts to induce compensatory mechanism by increasing the area of the fetal part of the placenta in order to increase the oxygen influx, and this may contribute to the thickening as well (100, 101). However, more data are needed on the impact of cCMV on the placental immune cross-talk and on the decidual tissues in relation to outcome.

1.4.4. Fetal and neonatal CMV immune response

How CMV reaches the fetus from the placenta is largely unknown, but maternal Abs affinity probably plays a role in the chance of fetal infection. In case of high avidity IgG, viral replication in the cytotrophoblast is limited and therefore the virus spreads less to the other layers of the placenta (93, 102-104); with low avidity IgG the viral infection is not prevented and therefore viral transport is facilitated. The placental alterations mentioned in the previous paragraph clearly induce a placental dysfunction that could hamper fetal development, and in turn contribute to the development of symptoms at birth. Upon fetal infection, the induction of a CMV-specific immune response has been shown. The majority of available data refer to αβ T cells. In congenitally infected children, there is a strong response of CD8+ T cells (10, 67, 105-110), during which they acquire a late differentiation phenotype (10, 105, 106, 111), and a restricted number of clones is generated (105). CMV-specific CD8+ T cells have been detected as early as 22-28 weeks of gestation (105, 108). During the first year of life, the number of CMV-specific IFNγ-producing CD8+ T cells increases, as well as the repertoire of CMV peptides against which they react (10, 110). Expansion of CD4+ T cells, as well as of γδT cells (112), has also been described in the context of cCMV (113). γδT cells with antiviral activity have been detected as early as 21 weeks of gestation (112). These might have a more important role in early life as they develop earlier than αβ T cells (114). Despite the oligoclonal expansion of fetal CD4+ and CD8+ T cells, similar to that in adults, their functionality and cytokines response was lower, and they showed a typical exhaustion phenotype (74). This is similar to what happens in adults after primary or chronic infection, but the magnitude of this phenomenon is more intense during fetal life (114), most likely because of the higher viral loads the foetuses are exposed to (63). This exhaustion of T cells in the foetuses may be responsible for the prolonged CMV viral excretion in these children which can last up to 5 years (50, 74). In general, also after postnatal CMV infection the viral excretion is longer, ~2 years, than in immunocompetent adults, and this suggests a limited control of the infection in early life (50, 51, 67, 114). Unfortunately, little information is available on fetal B cell immunity during cCMV. IgM positive B cells have shown to emerge in the peripheral circulation as early as 12 weeks of gestation (115), and CMV infected foetuses can produce IgM (116-118), but the antiviral activity and the role in CMV disease control have not been evaluated yet (119). The evidence of NK cell activation in the context of cCMV is scarce. An expansion of NKG2C+ NK cells was observed in infants with cCMV, and was particularly marked in symptomatic cases (120).

1.5. PROGNOSTIC MARKERS FOR cCMV CLINICAL OUTCOME

One of the main problem with cCMV is that, despite the extensive knowledge on the clinical outcome and despite being the most common congenital infection leading to a variety of permanent disabilities, the question whether a child will be symptomatic at birth or will develop LTI remains largely unanswered. This is just because cCMV pathogenesis is largely unknown. A prognostic marker for clinical outcome would help identifying subgroups of patients that would benefit from certain clinical interventions, as well as giving more insights into cCMV pathogenesis.

In the following paragraphs, the available prognostic markers are classified into different subgroups according to the main goals of prediction: vertical transmission of CMV, symptoms at birth and LTI development, and whether these measurements were performed in the mother, in the fetus or in the neonate. Whereas, the first paragraph is focused on what is known on the viral genomic variability in relation to clinical outcome.

1.5.1. CMV genomic variability

Several viral proteins have the capacity to modulate the host immune response (listed below). Therefore, the first attempt was to predict outcome by means of these immunomodulatory molecules.

- *UL144:* non-functional truncated TNF-α-like receptor gene;
- *US28:* functional β-chemokine receptor which binds and sequesters extracellular chemokines;
- *Envelope glycoproteins:* gB (UL55), gN (UL73), gH (UL75) have been associated with polymorphisms that may contribute escaping the immune response;
- *UL146, UL147:* α-chemokines, vCXCL-1 and vCXCL-2.

Some studies have related *UL144* (121, 122), *UL55 (gB)* (121, 123-125), *UL73 (gN)* (123, 126, 127), *UL146 (vCXCL-1)* and *UL147 (vCXCL-2)* (128, 129) to outcome, whereas others have not. For *US28* (121, 122) and *UL75 (gH)* (130, 131), there seems to be no clear association with cCMV outcome. Additionally, infection with multiple strains have been demonstrated in several populations: at the maternal-fetal interface (94), in congenitally infected newborns (132, 133), in immunocompetent and immunocompromised adults and young infants (134-138). Few studies have evaluated mixed infection in relation to clinical outcome, with some contradicting results (121, 130). However, in murine studies, and in immunocompromised patients, the mixed infection has been associated to enhanced pathogenicity (139-141). And unique genotypes are found in different compartments (133). How a mixed infection occurs in the fetus is largely unknown as it may occur as a single event with multiple strains, as multiple transmission moments or both mechanisms. To conclude, there are no strong evidence for either genomic variability nor for multiple strain infection in relation to short-term or long-term outcome.

1.5.2. Mothers

1.5.2.1. Predicting vertical transmission of CMV

Pregnant women who transmitted CMV to their fetus showed a lower and delayed lymphoproliferative response to CMV (78, 79). In these cohorts, the primary maternal infection occurred in different trimesters of gestation. The trimester of maternal primary infection is a determinant for vertical transmission, and the risk of transmission increases with the month of gestation (142, 143). In cases of infection in the first trimester, IgG avidity may be considered a prognostic marker for vertical transmission. A low avidity IgG (suggesting recent infection) is associated with 36% transmission, whereas intermediate avidity (timing of infection unclear) with 6% transmission (144). Contradicting results have been shown for maternal IgM, as it is used in different definitions of primary maternal infection. Indeed, when used as a marker for primary maternal infection the vertical transmission occurred only when IgM were present (145), while in another study of congenitally infected foetuses IgM could be detected only in 56% of mothers most likely because the group of mothers was characterized by both primary and secondary infection (though not stated clearly) (146). Overall, maternal seropositivity may be considered a marker for transmission. When a seronegative mother gets infected during pregnancy the vertical transmission rate is 30-35%, whereas in seropositive mothers this rate is lower (18-20). Concerning the virus, though very high viral load, evaluated by means of DNA quantification in AF, gave 100% of vertical transmission, very low or negative viral load, did not exclude it (145, 147). Different gB genotypes did not seem to correlate with transmission (148). Importantly, if the ultimate goal was to predict vertical transmission, all the mothers would need to be screened.

1.5.2.2. Predicting symptoms at birth

The gestational age at the onset of maternal primary infection is perhaps the most recognised factor related to cCMV outcome. If cCMV occurs in the first trimester the outcome is more severe, e.g. with neurological involvement, whereas if it occurs in the third trimester the symptoms, if any, are mild (149). Additionally, CMV DNA in AF was assessed in relation to maternal primary infection. Even though it seems that higher viral loads are related to a worse outcome at birth (145, 150-152), some studies have highlighted the importance of stratifying for onset of maternal primary infection. The CMV DNA in AF was significantly correlated to a worse outcome at birth if the maternal infection occurred relatively early (145, 149), whereas others have not found a correlation (153). Only few studies have evaluated the role of CMV DNA in AF of a group of mothers with both primary and secondary infection, and no correlation was found (147, 148). These findings may be due to different factors. First of all, primary maternal infection may result in higher AF viral load than secondary infection, as the maternal CMV-specific immune response is still developing and may not be able to control viral replication as efficiently as when a previous infection occurred. Second of all, a correlation between AF viral load and gestational age at the time of sampling has been shown (147, 148, 151), therefore when maternal primary infections occur early in gestation there might be an accumulation of viral DNA in AF that results in higher viral load (149).

1.5.2.3. Predicting LTI development

This has not been extensively explored, as after birth the immune system of the child may have a more important role in controlling CMV infection and disease. However, a trend towards higher risk of LTI development was shown for those infected neonates born from mothers with primary infection occurring in the first trimester of gestation (followed-up annually till 6 years of age) (149). This may be because earlier maternal primary infection infections are associated with symptoms at birth, and symptoms at birth are associated with LTI development. Whereas, CMV DNA in AF did not seem to correlate with LTI (149).

1.5.3. Fetus

1.5.3.1. Predicting symptoms at birth

Several markers, mainly quantified in cord blood, have been explored with the goal to predict clinical outcome, and these can be divided into different categories (virological, immunological, metabolic and imaging). In the majority of the cases, these markers have been quantified in the context of maternal primary infection. In case of secondary maternal infection this was specified.

Virological (markers related to viral components)

- *Antigenemia (detection of viral proteins in blood):* viral proteins are more indicative of a productive infection compared to DNA quantification, therefore they are associated with symptoms at birth (149, 152);
- *DNAemia (detection of viral DNA in blood):* the results are not conclusive in predicting symptoms at birth as some have found an association while others have not (149, 152, 154);
- *Viremia (detection of virus in blood):* the results are not conclusive in predicting symptoms at birth as some have found an association while others have not (149, 152).

Immunological (marker related to the host immune system)

- *Platelet counts:* lower platelet count has been consistently associated with symptomatic disease at birth (152, 154), and when combined with fetal ultrasound seemed to predict better (154);
- *IgM:* shown to be related to symptomatic disease at birth (149, 152);
- *White blood cells count:* higher levels were found in symptomatic (152);
- *% lymphocytes:* higher in the CMV+ foetuses and in those who were symptomatic at birth (152);
- β-*2 microglobulin:* higher in CMV+ foetuses and in those who were symptomatic at birth (152);
- *CD4+/CD8+:* even though the ratio is higher in CMV- no correlation was found in those CMV+ with symptoms at birth (152);
- *% CD3+HLADR+*: though higher in CMV+ there was no correlation with those who developed symptoms at birth (152).

Metabolic (markers related to the host metabolism)

- *Plasma aminotransferase:* aspartate aminotransferase (AST) increased in the symptomatic, whereas this was not observed for alanine aminotransferase (ALT) (152, 154, 155);
- *Gamma-glutamyl-transpeptidase (GGT):* no correlation was found in any of the studies, even though it was found higher in CMV+ foetuses (152, 154, 155).

Imaging

- *Ultrasound:* if fetal infection is not established, ultrasound may predict symptoms, whereas, if fetal infection is diagnosed, ultrasound imaging may be associated with a poor outcome (154, 156). In the absence of ultrasound abnormalities, a minority of neonates were symptomatic (154). The absence of abnormal ultrasound does not exclude the presence of abnormalities because some of them may appear later than when the ultrasound is performed;
- *MRI (Magnetic Resonance Imaging):* no correlation was found between MRI findings and symptoms in the first 11 months of life (157). This technique is more accurate and can show secondary lesions, even in the absence of ultrasounds abnormalities. Type of maternal infection not clear.

1.5.3.2. Predicting long-term impairment

The study of markers in fetus in relation to LTI development did not show any meaningful findings, though only few markers have been explored: CMV DNA, viremia, IgM, and ultrasound/MRI (149, 157). In particular, for imaging the main conclusion was that a normal ultrasound or MRI correlates with a normal development (157).

1.5.4. Neonate

1.5.4.1. Predicting symptoms at birth

Measuring markers at birth may have advantages over measuring them in the fetus because the material is more accessible and still reflects the situation during pregnancy, at least in the last phase of pregnancy. Therefore, a big overlap between markers evaluated in the fetus and in the neonates was observed, and a similar categorization is presented (virological, immunological and metabolic). In the majority of the cases, these markers were quantified in the context of maternal primary infection. In case of secondary maternal infection this is specified.

Virological

- *Antigenemia:* as in the fetus, higher levels are associated with symptomatic disease (158, 159);
- *DNAemia:* as in the fetus, contradicting results were shown (149, 158-161). In general, in all studies a big overlap between symptomatic and asymptomatic was observed, usually with

high viral load in both groups, and very low only in the asymptomatic. Therefore, what could be reliably concluded from these data is the lower risk of symptomatic disease in very low viral load (160). Interestingly, in a study of CMV DNA quantified in DBS, with an unknown type of maternal infection, higher viral loads correlated with a higher risk of SNHL (162). Importantly, the relationship between CMV DNA and symptoms is not linear, and this has been demonstrated not only in DBS (161-163), suggesting that the damage is evident after a certain accumulation of viruses;

- *CMV DNA in cerebrospinal fluid (CSF):* only found in symptomatic neonates (164);
- *CMV DNA in urine:* no correlation was found even though the viral load is usually higher in urine compared to blood ($10^{\wedge 5}$ vs $10^{\wedge 3}$), and persist longer (164).
- *Viremia:* associated with symptoms at birth (159);
- *Virus clearance (urine or blood):* the role of viral clearance in the clinical outcome remains not completely understood. Indeed, no difference in clearance from blood or urine was found between symptomatic and asymptomatic (161, 165). And there are some evidence of a shorter duration of excretion of CMV in urine in those neonates that had SNHL and progressive SNHL (165).

Immunological

- *IgM:* the results are discordant as some have not found a correlation (149, 159), whereas some have (166, 167);
- *β2-microglobulin and proteins:* found increased in symptomatic that developed LTI (168) (type of maternal infection not clear).

Metabolic

- *Platelet count + ALT:* with maternal infection not clear, if platelet count <100000/mm^3 + high ALT >80 UI/ml association with symptoms at birth (hearing loss) (169).

1.5.4.2. Predicting LTI development

In the majority of the cases, these markers were quantified in the context of maternal primary infection. In case of secondary maternal infection this was specified.

Virological

- *Antigenemia:* no correlation was found with respect to long-term outcome (149);
- *DNAemia:* the results are contradicting as some studies have not found a relation between load at birth in the neonatal blood and long-term outcome (149), whereas others have (170). A correlation between high loads in asymptomatic developing LTI compared to asymptomatic not developing LTI was observed (161).

Immunological

- *IgM:* no correlation was found between IgM in neonatal blood and LTI development (149);
- β*2-microglobulin and proteins:* higher in the CSF of symptomatic that developed LTI (168).

Clinical

- *Severe symptoms at birth (central nervous system (CNS) involvement):* are associated with higher risk of SNHL and cognitive deficits in a follow-up of ~4 years (maternal infection was not taken into consideration) (171). Clinical predictors are only available for symptomatic children;
- *Symptoms at birth*: studies have shown that even in case of not so severe symptoms at birth, symptomatic neonates have higher risk of developing LTI (17).

Imaging

- *CT (Computed Tomography):* abnormalities in symptomatic infected neonates are predictive for a worse long-term outcome (172), and in association with the presence of microcephaly at birth is predictive of cognitive LTI (173). In the latter, where all individuals were symptomatic, the absence of microcephaly in presence of CT abnormalities was associated with an intermediate outcome, whereas in absence of microcephaly and CT abnormalities with a good outcome.
- *MRI:* even though the spectrum of MRI abnormalities is extremely broad, there are certain abnormalities associated with a poor long-term outcome (age at MRI ~2 years and follow-up ~5 years) (174).

To conclude the section on prognostic markers for cCMV clinical outcome, there is no clear evidence of a biomarker that could potentially be used to define subgroups of patients that would benefit from certain clinical interventions. Several factors may have contributed to these discrepancies. First of all, the clinical definitions used for symptomatic neonates, or LTI development, are extremely diverse across studies. Different immunopathologies may underlie different outcomes. Second of all, in the majority of the cases a maternal primary infection was considered, therefore what happens in mothers with secondary infection is largely unknown. Third, it is almost impossible to determine the gestational age at vertical transmission.

1.6. CLINICAL STRATEGIES

Finally, even if we had "The Biomarker" allowing the identification of such subgroups, what, how, and to whom we offer certain clinical interventions that would solve/improve short- and long-term disabilities remain an open question. Such complex problem needs stratification according to the goals of the clinical intervention and to the target population of such an intervention. As a *primary prevention*, we would like to prevent maternal infection as cCMV would not occur at all, and this could be potentially achieved with a vaccine. As a *secondary prevention*, we would

like to prevent vertical transmission for the same aforementioned reason. However, there is no currently effective vaccine registered (175, 176), and there is no effective treatment that reduces vertical transmission, either antiviral or hyperimmunoglobuline (177, 178). As a *tertiary prevention*, we would like to prevent, or at least positively affect, the short- and/or long-term impairments. Provided that all infected neonates have been identified, e.g. through maternal and/or neonatal screening. Some studies have shown a certain degree of beneficial effect of a prenatal treatment with hyperimmunoglobuline or valacyclovir, however further studies are necessary (100, 179-182). Some other studies have focused on postnatal treatment of symptomatic neonates with (val) ganciclovir, and have shown an improvement of the hearing, language/receptive components (183-185). However, it still needs to be evaluated whether such treatment is beneficial for mildly affected or asymptomatic neonates who can still develop permanent impairments.

Importantly, several observations would need to be taken into consideration for the use of a future vaccine in the cCMV settings. Additionally to the mothers, infants and adolescents could be potential targets as well, depending on our goals. If given to infants it would prevent females to get infected and transmit the virus to the future foetuses. If given to toddlers we would reduce the transmission to mothers, though this would not be complete as the transmission from adults to adults may still occur. If given to adolescent girls we would prevent transmission to the fetus, similarly to when we give it to women, but with a different vaccine duration needed. The vaccine may as well be used in seropositive setting in order to boost pre-existing immunity.

As the way to a licensed vaccine seems to be long, the tertiary prevention sounds like a good compromise. However, in order this system to work a maternal/neonatal screening is necessary. The neonatal screening can be selective or universal. In the first case, CMV screening is reserved to those neonates that failed the hearing screening, or for those who have a specific clinical picture at birth. Therefore, all asymptomatic would be missed, as well as some symptomatic whose clinical signs are rather general and difficult to attribute to cCMV. In the second case, CMV screening is meant for all newborns, and is defined as "The systematic application of a test to identify asymptomatic individuals at risk of a specific disorder in order to prompt further investigation or preventative action. Benefits must outweigh harms." cCMV meets many criteria of the universal screening. However, the universal screening would identify those asymptomatic for whom no clear beneficial intervention plan is available. This would increase parental stress, alter the familiar dynamics (186), force treatments whose benefits are not established yet, and induce unnecessary medical exams (187). This problem would be considerably resized if predictors for clinical outcome were available.

Concluding, understanding the pathogenesis of cCMV would allow to figure out why certain children develop LTI and others do not. In turn, this would provide the necessary biomarkers to predict outcome and stratify patients according to the risk. On one hand we could define who benefits most from certain clinical interventions; and on the other hand we could define correlates of protection to be used in future vaccine trials.

1.7. OUTLINE ON THE THESIS

The aim of this thesis was to determine potential prognostic markers for short- and long-term clinical outcome, and to determine potential correlates of protection. The first would allow the identification of subgroups of children that would benefit from certain clinical interventions; whereas the latter could be useful for future vaccine development. These goals were achieved by using samples from a large retrospective nationwide cohort of children, with (n=125) and without (n=263) cCMV, born in The Netherlands in 2008 and their mothers.

In **Chapter 2** the immune system of the neonates, with and without cCMV, is assessed by means of quantification of several immunological markers extracted from dried blood spots (DBS) and correlated to the presence of cCMV, short- and long-term clinical outcome, and CMV viral load in DBS. These immunological markers refer to the number of γδ T cells, αβ T cells and B cells, with particular attention to B cell replication.

In **Chapter 3** the metabolism of the neonates, with and without cCMV, is assessed in relation to the presence of cCMV, short- and long-term clinical outcome, and CMV viral load. This is achieved by the analysis of the metabolic markers normally extracted from DBS after birth for the screening of the rare genetic metabolic disorders. The metabolic markers included are essential amino acids, hormones, carnitines and enzymes.

In **Chapter 4** the gene expression profile of the neonates, with and without cCMV, is assessed in relation to long-term clinical outcome, and CMV viral load. The RNA is extracted from DBS and sequenced by using the Illumina platform. Data analysis is performed with particular attention to the neonatal immune system.

In **Chapter 5** data on maternal and child HLA that are expressed at the placenta are presented. HLA-C, HLA-E, and HLA-G are determined from 96 mother–child pairs with cCMV in relation to short- and long-term clinical outcome, and CMV viral load. The mothers are additionally typed for killer cell immunoglobulin-like receptors (KIRs) to get more insights into NK cells response. The typing is performed on DNA extracted from buccal swabs from children and their mothers.

In **Chapter 6** data on maternal and child HLA that are not expressed at the placenta are presented. HLA-A, HLA-B, HLA-DR and HLA-DQ are determined from 96 mother-child pairs with cCMV in relation to a control group of 5604 Dutch blood donors and in relation to CMV viral load. The typing is performed on DNA extracted from buccal swabs from children and their mothers.

In **Chapter 7** a brief summary of the findings of each chapter is presented first, with particular attention to the mechanisms of cCMV pathogenesis and its clinical implications. Second, the main findings are integrated in one final model, with special emphasis on the future directions.

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