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

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## ENGLISH SUMMARY

Cytomegalovirus (CMV) infection is a worldwide common infection that in a considerable proportion of individuals remains unnoticed. However, when the virus infects the fetus via the placenta during pregnancy, it can cause severe disease. The congenital CMV infection (cCMV) can induce a variety of clinical manifestations at birth (symptoms at birth), and of permanent long-term impairments (LTI). These clinical signs can also vary in severity, e.g. in symptomatic neonates they can range from elevated liver enzymes to microcephaly (having a smaller than normal head). The permanent long-term disabilities (ranging from neurological to visual impairments) can be progressive or reveal themselves later in childhood, for example late onset hearing loss, and in this context a retrospective diagnosis may be challenging. Importantly, of the total of infected neonates at birth, 13% are symptomatic at birth, and half of them will develop LTI. However, 13% of the asymptomatic neonates will still develop the same LTI; and since this group is much bigger than the symptomatic group, the majority of clinical cases come from the asymptomatic neonates. The latter is where the cCMV gets easily unnoticed because you do not see anything suspicious at birth. Summarizing, not all infected foetuses will have symptoms at birth or will develop long-term disabilities. At this moment, there is no prognostic marker available to anticipate which neonates, and when, will develop LTI, nor which type of disability. cCMV can occur at any time during pregnancy, and usually when it occurs early in pregnancy it can cause more severe disease because the fetus is still developing. cCMV can occur in mothers after their first infection with CMV during pregnancy (who were previously seronegative) or who were previously infected with CMV (seropositive). In the latter, the vertical transmission rate (i.e. CMV transmission from the mother to the fetus) is usually lower than in the seronegative pregnant women, because of the pre-existing immunity to CMV, but it is not clear whether this immunity also protects against severe clinical problems. These two variables, trimesters of maternal infection and pre-existing immunity, complicate studies on cCMV pathogenesis in the general population.

The aim of this thesis was to get new insights into cCMV pathogenesis, and to identify prognostic markers for short- and long-term clinical outcome, and correlates of protection for vaccine development. For this purpose, a retrospective nation-wide cohort of congenitally infected children and controls (i.e. non-infected children) was used. All children were born in The Netherlands in 2008, and dried blood spots (DBS) were used to diagnose cCMV. DBS are obtained by spotting whole blood from the heels of the neonate within one week after birth. These cards are routinely used for the screening of the rare genetic metabolic disorders for which a clinical, life-saving, intervention is available. DNA was extracted from these cards, and the presence of CMV DNA was evaluated in order to diagnose cCMV. Additionally, by quantification of CMV DNA, the viral load (which gives an indication on how much virus is present in the body) was determined. Of the children in this cohort, clinical data from birth till 6 years of age were available. Another specimen collected from this cohort, from both mothers and children, was represented by buccal swabs. DNA was extracted from buccal swabs for several genetic studies. In order to reach our goals, the following biomarkers



were evaluated and related to CMV viral load, symptoms at birth and LTI development at 6 years of age:

- markers for B cells,  $\alpha\beta$  and  $\gamma\delta$  T cells in DBS;
- gene expression profiles with specific attention to the immune system and inflammation pathways;
- data on essential amino acids, hormones, carnitines and enzymes in DBS;
- HLA (human leukocyte antigens) typing on buccal swabs of mothers and children with cCMV.

In the following paragraphs, the main findings of all chapters are presented. First, the above mentioned biomarkers in relation to cCMV infection itself and the viral load in DBS are presented. Then, the biomarkers in relation to symptoms at birth and LTI development.

### Congenital CMV infection

In order to study the effect of cCMV on different markers, we compared the biomarkers in DBS or buccal swabs in children with cCMV to those without cCMV (our control group). In our cohort, cCMV resulted in a reduced production of  $\alpha\beta$  T cells in the thymus, an increased number of  $\gamma\delta$  T cells, and increased production of B cells (**Chapter 2**). Additionally, cCMV induced exhaustion of T cells (i.e. a general state of cellular dysfunction usually induced by chronic infections and exposure to high viral loads) (**Chapter 4**). Of these markers, only the production of  $\alpha\beta$  T cells was not associated with CMV viral load. In **Chapter 4**, the gene expression profile on the DBS showed that gene expression of genes related to the innate immune response and NK cell activation was higher in children with a higher CMV viral load. The innate immune system, NK cells and T cells may play an important role in controlling CMV in the foetus, where the CMV-specific responses are still developing. Furthermore, a specific HLA of Class II in children (i.e. molecule that presents CMV components to immune cells), i.e. HLA-DRB1\*04, was associated with a better CMV viral control (**Chapter 6**). This positive effect on viral control may rely on the fact that a HLA Class II supports both arms of the immune response, cytotoxic and antibody responses. Furthermore, cCMV did not affect the neonatal metabolism in DBS, but a reduced level of essential aminoacids (components of proteins) was found in the subgroup of congenitally infected neonates that was premature (i.e. birth before the 37 weeks of gestation) compared to the prematurely born children without cCMV (**Chapter 3**). Although cCMV did not affect the neonatal metabolism, a higher CMV viral load induced an increase of C16 (**Chapter 3**). C16 is a fatty acid and the precursor to longer chain fatty acids, for which the outer structure of CMV is enriched. Therefore, the increase of C16 in the high viral load group may simply reflect the increased viral burden.

### Symptoms at birth

The different markers were also studied in relation to the presence of symptoms at birth, by comparing the markers in the cCMV group with symptoms to the cCMV group without. The only

markers related to symptoms at birth were found while studying the HLA-types of both mothers and children, with their specific combination. In **Chapter 5** and **Chapter 6** we assessed the role of HLA Class I and Class II, as well as the receptors for NK cells of the mothers, in relation to cCMV clinical outcome. We found that mothers with a specific HLA-G deletion/deletion genotype or mothers homozygous for HLA-C1, as well as mother-child pairs with HLA-E and HLA-C mismatches were associated with symptomatic disease (**Chapter 5**). The mothers that have the HLA-G deletion/deletion genotype probably have higher levels of the HLA-G protein that has immunosuppressive properties. The immunosuppression may lead to less viral control upon CMV infection during pregnancy and may lead to a higher viral burden of the placenta. A HLA mismatch exists when the mother does not have the same genetic HLA information as the fetus has, because some of the genetic HLA information derives from the father, which may be different from that of the mother. In **Chapter 5**, we demonstrated that in case of HLA-E or HLA-C mismatch the chance of a child with symptoms at birth was higher. A mismatch of HLA, such as HLA-E and HLA-C that are expressed in the placenta, may lead to maternal T cells that are capable of attacking the fetal tissue. Normally, this process is suppressed, but in case of a placental CMV infection, this balance may be disturbed leading to a T cell reaction towards the placenta and the fetus. Furthermore, HLA matching is necessary for an adequate immune response to virus-infected cells. Therefore, a HLA mismatch may lead to a worse immune reaction and less control of the viral infection, with higher placental and possibly fetal viral loads as a result. Finally, one of the most abundant cells in the placenta is the NK cell, which belong to the innate immunity and therefore react rapidly upon activation. These cells have the peculiar characteristic of having several activating and inhibitory receptors whose combination defines the degree of NK cells activation. The HLA-C molecules can bind to these receptors with different degrees of strength. In our cohort, in the group with symptoms at birth, the mothers more frequently missed the inhibitory type of HLA-C, and therefore the activating signals towards NK cells may prevail contributing to the already increased placental inflammation (**Chapter 5**). In **Chapter 6** the HLA-types that are not expressed in the placenta were studied. For these HLA-types no association with symptoms at birth was found. Summarizing, symptoms at birth appear to be related to genetic markers that contribute to high CMV viral load at maternal and placental level, due to a lack of viral control, and to a higher level of placental inflammation.

### Long-term impairments

In three chapters biomarkers are described that seem to be associated with LTI at 6 years of age. In **Chapter 2**, infected children that developed LTI had lower numbers of B cells. In addition, we found higher percentage of HLA-C non-inherited maternal antigens (NIMA) in those infected children that developed LTI (**Chapter 5**). The fetus is exposed to antigens that he/she did not inherit, the so-called NIMA, because during pregnancy and breast-feeding there is a mutual exchange of cells between mother and child. Small numbers of maternal cells seed in several fetal organs and persist at least until adulthood. However, instead of the initiation of a fetal immune response against these “foreign” maternal antigens, the fetus develops a long-lasting tolerance (NIMA effect). The NIMA effect is beneficial in the context of transplantation because it can improve graft survival.

However, during infections the NIMA effect may induce an indirect tolerance to the infection as well. Consequently an indirect tolerance to the CMV infection may induce an uncontrolled viral replication throughout childhood. Furthermore, in **Chapter 4**, we show that in children that did not develop LTI there was a higher expression of anti-inflammatory markers. This further suggests that part of the pathogenesis of LTI development can be attributable to an uncontrolled infection and inflammation.

The aim of this thesis was to identify prognostic markers for short- and long-term clinical outcome and correlates of protection for vaccine development. We did find some interesting biomarkers that, if confirmed in other cohorts, could be potential candidates for such goals. However, sometimes the differences were too small, and a striking biomarker was not found. On the other hand, we did find some clues towards a better understanding of cCMV pathogenesis (i.e. biological mechanisms that leads to disease). Understanding cCMV pathogenesis is fundamental for several reasons. First of all, it would help future research on prognostic markers. Second of all, it would support the introduction of innovative clinical strategies to prevent, or at least positively affect, the short- and long-term clinical outcome because we would understand the mechanisms for the progressive and permanent damage. Indeed, the way to a licensed vaccine is way too long to just patiently wait for it. However, in the end, vaccination should be the main goal in cCMV research because it could be the ultimate solution for prevention of vertical transmission during pregnancy, or disease development in the child. Finally, the identification of the aforementioned biomarker could help the future research for vaccine development.

To conclude, from the research presented in this thesis, several issues have emerged that should be taken into account in future research. The majority of congenitally infected neonates are asymptomatic at birth and have a good prognosis for a normal development, therefore the comparison of their immunological state with that of the symptomatic infected neonates with a worse long-term outcome, as well as those asymptomatic that develop the same LTI, is essential to determine what is happening in the children who are affected by cCMV. While doing that, we should also define new clinical strategies, and also study whether the current clinical strategies are beneficial for those asymptomatic neonates that develop the same LTI, and whether for this group the benefits of for example antiviral treatment outweigh the side effects. However, an even earlier goal should be the standardization of the definitions of symptomatic disease and LTI development without which no reliable conclusion can be made on cCMV pathogenesis.