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HUMAN CONGENITAL CYTOMEGALOVIRUS INFECTION: CHARACTERISTICS AND PATHOGENESIS OF FETAL BRAIN DAMAGE

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ABSTRACT

Human cytomegalovirus (HCMV) causes congenital neurological lifelong disabilities. The study analyzed 10 HCMV-infected human fetuses at 21 weeks of gestation to evaluate the characteristics and pathogenesis of brain injury related to congenital human CMV (cCMV) infection. Specifically, tissues from cortical and white matter areas, subventricular zone, thalamus, hypothalamus, hippocampus, basal ganglia and cerebellum were analysed by: *i)* immunohistochemistry (IHC) to detect HCMV-infected cell distribution, *ii)* hematoxylin-eosin staining to evaluate histological damage and *iii)* real-time PCR to quantify tissue viral load (HCMV-DNA). Viral tropism was assessed by double IHC to detect HCMV-antigens and neural/neuronal markers: nestin (expressed in early differentiation stage), doublecortin (DCX, identifying neuronal precursor cells) and neuronal nuclei (NeuN, identifying mature neurons).

HCMV-positive cells and viral DNA were found in the brain of 8/10 (80%) fetuses. For these cases, brain damage was classified in mild (n=4, 50%), moderate (n=3, 37.5%) and severe (n=1, 12.5%) based on presence of *i)* diffuse astrocytosis, microglial activation and vascular changes; *ii)* occasional (in mild) or multiple (in moderate/severe) microglial nodules and *iii)* necrosis (in severe). The highest median HCMV-DNA level was found in the hippocampus (212 copies/5ng of humanDNA [hDNA], range: 10-7,505) as well as the highest mean HCMV-infected cell value (2.9 cells, range: 0-23), followed by that detected in subventricular zone (1.8 cells, range: 0-19). This suggests a preferential HCMV tropism for immature neuronal cells, residing in these regions, confirmed by the detection of DCX and nestin in 94% and 63.3% of HCMV-positive cells, respectively. NeuN was not found among HCMV-positive cells and was nearly absent in the brain with severe damage, suggesting HCMV does not infect mature neurons and immature HCMV-infected neuronal cells do not differentiate into neurons. HCMV preferential tropism in immature neural/neuronal cells delays/inhibits their differentiation interfering with brain development processes that lead to structural and functional brain defects.

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CHAPTER 1

Cytomegalovirus

1.0 Cytomegalovirus

1.1 Introduction

By the early 1950s, cases of a lethal congenital infection characterized by cells with typical intranuclear inclusions, had been described. Even if its viral etiology was not yet known, for this clinical condition, Wyatt et al. suggested the term “CID, generalized cytomegalic inclusion disease” [1,2]. Later, the etiological agent was independently isolated by Smith in 1956, Rowe and coworkers in 1956, and by Weller et al in 1957, who proposed the name "cytomegalovirus" [1].

1.2 Virion structure

Human cytomegalovirus (HCMV), or human herpesvirus 5, is a highly species-specific virus, belonging to the beta-herpesviridae subfamily, that is ubiquitous with worldwide distribution [3,4,5]. HCMV infection in healthy adults is usually asymptomatic or causes a mild mononucleosis-like syndrome. However, in immunocompromised hosts and infected fetuses, HCMV causes significant morbidity and mortality [3,6]. In particular, congenital human cytomegalovirus (cCMV) infection is also a leading non genetic cause of sensorineural hearing loss (SNHL) and neurodevelopmental delays [4,7,8].

The mature viral particle of HCMV has a diameter of 150–200 nm and share with other herpesviruses a common architecture (Figure 1) [3,9]. The double-stranded DNA genome is contained within an icosahedral capsid made up of 162 capsomeres, surrounded by an envelope derived from portions of the host cell membrane enriched in viral glycoproteins to control attachment and entry into cells [3,9,10]. In addition, a particularly thick tegument (or matrix) layer of virus encoded proteins lies between the genome-containing capsids and the viral envelope. Proteins within this layer, when the viral envelope fuses with the cell membrane, are released into the cell upon entry, playing a key role in viral entry, gene expression, and immune evasion [3,10]. HCMV is sensitive to heat, low pH and lipid-dissolving agents. It needs to be stored at a minimum of –70° C in order to maintain its infectivity; in fact, the half-life of HCMV is approximately of 60 min at 37° C and is unstable at –20° C [9].

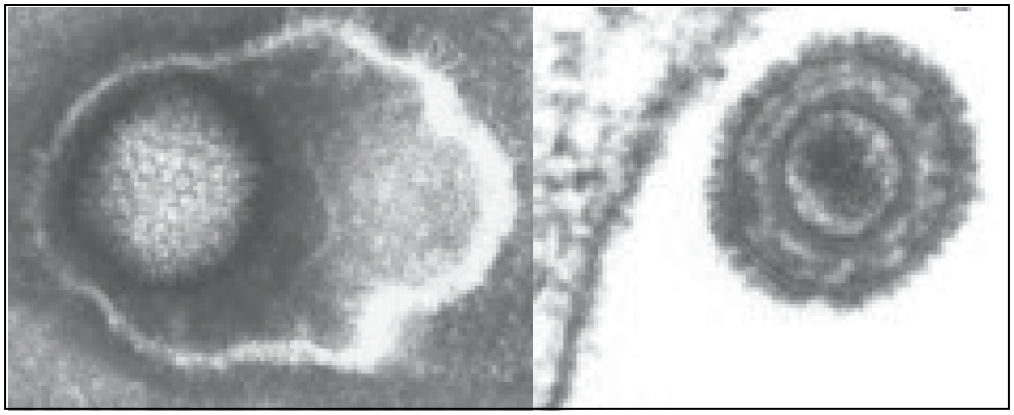


Figure 1. Electron microscope images of HCMV particles (from Schottstedt V et al. Transfus Med Hemother. 2010, 37(6): 365-75. [9]).

The HCMV icosahedral **capsid** contains viral genome and is composed of four core proteins: *major capsid protein* (MCP, encoded by UL86) including most pentons and hexons, triplexes composed of two subunits, *triplex subunit 1* (TRI1, encoded by UL46 gene or minor capsid protein) with *triplex subunit 2* (TRI2, encoded by UL85 or minor capsid protein binding protein), and the *smallest capsid protein* (SCP, encoded by UL48A). For HCMV replication, all capsid proteins are necessary. In particular, during both encapsidation and release of viral DNA, one specialized penton composed of the portal protein (PORT, encoded by UL104) acts as a channel together with two principal subunits of the terminase, subunit 1 (TER1, encoded by UL89) and subunit 2 (TER2, encoded by UL56). All pentons are capped by a capsid vertex-capping complex composed of UL77 and UL93 and the proteins encoded by UL51 and UL52 likely provide stability [3]. Capsid assembly takes place in the infected cell nucleus, as immature capsid or procapsid with virus-encoded scaffolding proteins, which are later digested by the action of the viral protease, clearing the way for viral DNA packaging. Subsequent nuclear tegumentation, nuclear egress, cytoplasmic tegumentation and envelopment steps are essential for maturation of nucleocapsids into infectious virions [3,11]. During HCMV infection, as for other herpesviruses, different capsid forms are present in the host cell nucleus: A-, B-, and C-capsids. C-capsids represent DNA-containing nucleocapsids that appear to be the closest in form to mature virions, and are considered precursors to infectious virus in the process of

maturing; while, A- and B-capsids represent abortive forms that appear to have failed to complete encapsidation [3,12].

The **tegument** layer located between the lipid envelope and the icosahedral protein capsid generally appears to be unstructured or amorphous by electron microscopy and electron tomography visualization of herpesvirus virions, although it contains proteins associated with the capsid. [13].

Most tegument proteins are phosphorylated (thus sometimes named with the prefix phosphoprotein, pp), and many are highly immunogenic. In addition to viral proteins, within the tegument of HCMV virion, approximately 70 cellular proteins have been found. [3,13]. All of these carry out different activities. During the infection, these proteins deliver the viral genome to the nucleus, guiding nucleocapsid translocation to nuclear pore complexes through microtubules (such as pUL47, pUL48 and pp150). In addition, the tegument proteins control nucleocapsid stability during assembly and final envelopment from the nucleus to the steps in egress (such as pp28) [13]. The pp71 tegument protein is the only component that has a key role in activating the viral immediate early (IE) genes expression needed to start of a lytic infection [3,13]. Finally, pp65 (lower matrix protein, UL83 gene product) is the most abundant tegument protein in HCMV virions. This protein, which is highly immunogenic, is a target of MHC class I - restricted CD8 and MHC class II CD4 T-cell responses. After viral entry, pp65 localizes to the nucleus of infected cells where it plays an immunomodulatory role mitigating the interferon-like cellular response to viral infection. During natural infection, pp65 is also abundantly contained in the virus-infected cells and may be transferred to neutrophils that come into contact with HCMV-infected cells. The detection of pp65 with quantitates the number of HCMV-infected leukocytes in peripheral blood neutrophils by antigenemia diagnostic assay, has been widely used for monitoring HCMV infection guiding therapy in patients at risk of developing HCMV disease [3,13,14].

The HCMV virions are surrounded by a lipid bilayer, termed the **envelope**, that is similar in structure and composition to host cell membranes, though contains virus-coded transmembrane glycoproteins. These include glycoprotein B (gB), gH, gL, gM, gN, and gO. The site of final envelope acquisition of the HCMV particles have not conclusively identified, however different studies show that this layer derives from endoplasmic

reticulum-Golgi intermediate compartment or endosomal membranes [3,15]. Among the glycoproteins of envelope, most likely contribute to modulation of the host cell response to infection, while some are involved in attachment and entry. Three glycoprotein complexes (gc) play a key role in the attachment, entry and maturation of HCMV into the host cell: gB (gcI), gM:gN (gcII) and gH:gL (gcIII). In particular, gB and gH:gL contribute to attachment and entry, whereas gM:gN in maturation. These glycoproteins, which during infection, accumulate in the inner portion of the cell membranes, are the main target of HCMV neutralizing antibodies. gB mediates binding to heparan sulfate proteoglycan and is a fusion protein that allows the entry directly at the plasma membrane (as occurs in fibroblasts) or through the endocytic route (as occurs in epithelial and endothelial cells). In addition, gB contributes in viral cell-to-cell spread and cell-cell fusion leading to syncytia. The complex gH:gL is involved together with gB in the cell fusion step. However, the cellular receptor(s) for gB are still being investigated and it is known that HCMV attachment does not require dedicated subgroup-specific receptor recognizing envelope glycoprotein [3]. Besides the glycoproteins that control the viral entry in any cell type, in certain settings, trimeric complex, gH:gL:gO and pentameric complex, gH:gL:pUL128:pUL130:pUL131A facilitate HCMV attachment. In particular, the pentameric complex acts on epithelial and endothelial cell types, suggesting its important role in viral pathogenesis [3]. In clinical samples, different HCMV strains are identified on the basis of genetic polymorphism in envelope glycoproteins, including gB, gO and gN. Currently, HCMV genetic variability is being investigated in correlation with viral tropism and “pathogenic potential” [16,17].

1.3 Genome organization and viral replication

HCMV has the largest genome among human herpesviruses, which consists of around 235 kb linear double-stranded DNA encoding 165 genes [8,18]. The genomic architecture is organized as two regions of unique sequences, unique long (UL) and unique short (US), both flanked by two sets of inverted repeats, terminal/internal repeat long TRL/IRL and internal/terminal repeat short IRS/TRS, (Figure 2). UL and US regions can be inserted in

both directions between repeats, giving rise to four genome isomers that coexists in an equimolar mixture [19,20].

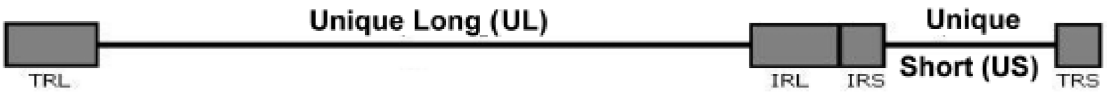


Figure 2. Structure of HCMV genome (adapted from McGregor A et al. Expert Opin Drug Metab Toxicol. 2011, 7(10):1245-65 [20]).

HCMV productively infects numerous cell types in the human host (lytic infection), as well as it establishes a latent infection in bone marrow myeloid progenitor cells. The outcome of infection, lytic or latent, is dependent on viral IE gene expression, that leads to expression of early and late genes [21]. In fact, following viral entry in host cells, nucleocapsid is translocated through cytoplasmic microtubules to the nucleus where viral DNA is released and three kinetic classes of genes, IE (or α), delayed early (DE or β) and late (L or γ) genes, are sequentially expressed on the basis of a coordinately regulated synthesis over the course of lytic replication cycle (Figure 3) [3,21]. During the course of infection, the transcription of viral genome is performed by host cell RNA pol II and related transcription machinery, although transcription is regulated via virus encoded transactivator. The expression of major IE gene is due to enhancers on the viral genome as well as to the tegument protein pp71.

The viral DNA synthesis depends on DE gene expression, encoding ppUL84 which constitutes ppUL84:IE2-p86 complex. This complex allows the activity of the oriLyt promoter. OriLyt is a 3 kb-long DNA element located in the HCMV genome, where DNA synthesis starts. This region site between the UL57 and UL69 genes is separated into two regions: essential region I, which contains a bidirectional UL84/IE2-responsive promoter and essential region II, which contains an RNA stem-loop region to which UL84 binds. This region is also rich in other direct and inverted repeat sequences. After being delivered to the nucleus, viral DNA destined for synthesis circularizes [3]. HCMV DNA replication starts as theta form, subsequently switching to circularised molecule, which is the template for amplification by rolling circle-based mode, that produces concatemers of the genome in head-to-tail. A viral protein complex, terminase (pUL56 and pUL89) then cleaves

concatemeric HCMV DNA into unit-length genomes for DNA packaging [3,11]. Finally, to HCMV replication, other proteins are required: UL44 (polymerase-associated factor), UL54 (polymerase), UL57 (single-stranded DNA-binding protein), UL70 (primase-associated factor), UL102 (helicase) and UL105 (primase) [22].

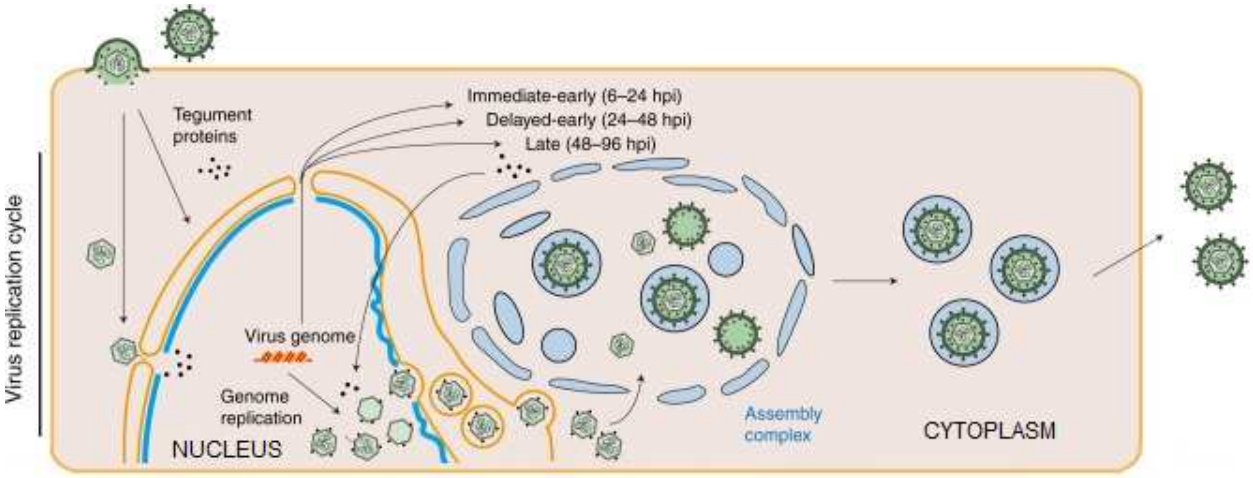


Figure 3. HCMV temporal replication cycle: immediate early, delayed early and late gene expression hours post-infection (hpi) (adapted from Murray LA et al. Nat Commun. 2018, 9(1):4967 [22]).

The packaging of newly synthesized viral DNA into preformed capsids occurs in the nucleus. During translocation of nucleocapsid from the nucleus to the cytoplasm, tegument proteins are added sequentially, providing stability and directing nucleocapsid trafficking to sites of envelopment. The release of mature virion from the host cell occurs by exocytosis [3]. The replication cycle of HCMV requires 48 - 72 hours to reach the release of progeny and the switch from early phase to late phase dependent on type of cell host. In fact, IE gene expression is sensitive to cell cycle status, such that cells in S, G2, or M phases do not produce IE proteins until cells return to G1 [3].

1.4 Epidemiology and transmission

HCMV is ubiquitous, with a global estimated seroprevalence equal to 83% in the general population, ranging from the highest seroprevalence of 90% in the World Health Organisation (WHO) Eastern Mediterranean region to the lowest 66% in the WHO European region. [23]. The worldwide HCMV distribution varies greatly, depending on the

socio-economic standards of countries; in fact, the prevalence in industrialised regions is lower than in developing areas, where it may reach 100%. In addition, the prevalence increases with age. In industrialised countries, HCMV infection occurs in two phases of life: a first peak is reached in the first 2–3 years and a second lower peak is reached in adolescence and early adults, between the ages of 16 and 30 years, as a result of sexual contacts. Afterwards, the portion of seropositive individuals increases up to 50 –70% with age. [9,24]. Primary infection is associated with viral shedding in saliva, urine, cervical secretions, semen, breast milk and other body fluids [25]. Transmission of HCMV can occur by several routes: horizontal transmission (viral shedding in close-contact settings, sexual transmission, organ transplantation, via blood products, i.e. transfusions) or vertical transmission (transplacental, breastfeeding and perinatally for contact with infected maternal genital tract) [9,24].

1.5 Pathogenesis

As mentioned in the previous section, HCMV has two life cycle phases: a productive phase (or lytic phase), where new virions are produced, and a latent phase characterized by the absence of new virion production as well as by a restricted gene transcription profile [26]. In fact, following primary productive infection, HCMV is able to establish a lifelong latent infection, from which may periodically reactivate to form new virion that can be transmitted to other susceptible individuals [27]. The direct contact with body fluids from a person who is shedding HCMV is required for viral transmission [28]. Active HCMV infection could be caused by: *i)* primary infection, when the infection occurs in a HCMV-naive host; *ii)* endogenous reactivation from latency in HCMV infected individuals, and *iii)* exogenous reinfection in HCMV-seropositive individuals who experience infection by a different strain [29].

Primary HCMV infection in humans initiates on mucosal epithelium at a portal of entry; oral cavity is likely the main site of viral acquisition and from which it spreads again [3,30]. The development of a leukocyte-associated viremia then determines a systemic phase of the infection, which can last for long periods, especially in immunocompromised subjects, in which it was well-documented [3,31]. In all clinical conditions related to HCMV

infection, the viral dissemination mediated by interaction between endothelial cells (ECs) and circulating leukocytes, plays a key role [3,31,32]. During acute infection, different cell types, such as ECs, epithelial, fibroblast and hematopoietic cells in peripheral blood and bone marrow, become infected [3]. In particular, it has been shown that ECs are natural sites of HCMV infection, through which the virus may be spread to different districts of the body. In fact, HCMV-infected ECs spread the virus along the vessel walls, invading the lumen and sometimes detaching from the vessel and reaching distant body sites. However, the main role of ECs in the mechanism of viral pathogenesis is due to the bidirectional interaction with circulating leukocytes, polymorpho-nuclear leukocytes (PMNL) and monocytes (leukocytes), that allow to transfer HCMV from infected ECs to uninfected leukocytes and *vice versa* [3,31,32]. In fact, infected ECs realised leukocyte chemoattractant factors, in particular cellular chemokines (IL-8 and Gro-α) and viral α-chemokines (encoded by UL146). After leukocytes attraction, the adhesion is mediated by the interaction between the ICAM-1 (intercellular adhesion molecule) expressed by ECs and its two specific ligands: LFA-1 (lymphocyte function-associated antigen-1) and Mac-1 (macrophage-1 antigen), present on PMNL and monocytes, respectively. Transfer of HCMV and viral material, from ECs to leukocytes, is mediated by transitory microfusion events between plasma membranes of adjacent involved cells (Figure 4) [3,31-33]. HCMV in PMNL and monocytes is passively transported since viral replication seems to be blocked at the IE gene transcription. Monocytes may also migrate to organ tissues, where after differentiating into macrophages they become permissive to virus replication. In immunocompetent individuals the host immune response following active HCMV infection is generally effective at stopping virus replication and dissemination. However, virus is never cleared by the host and persists for life in cell types where latency can be established, such as peripheral monocytes and CD34+ progenitors in the bone marrow [31,34].

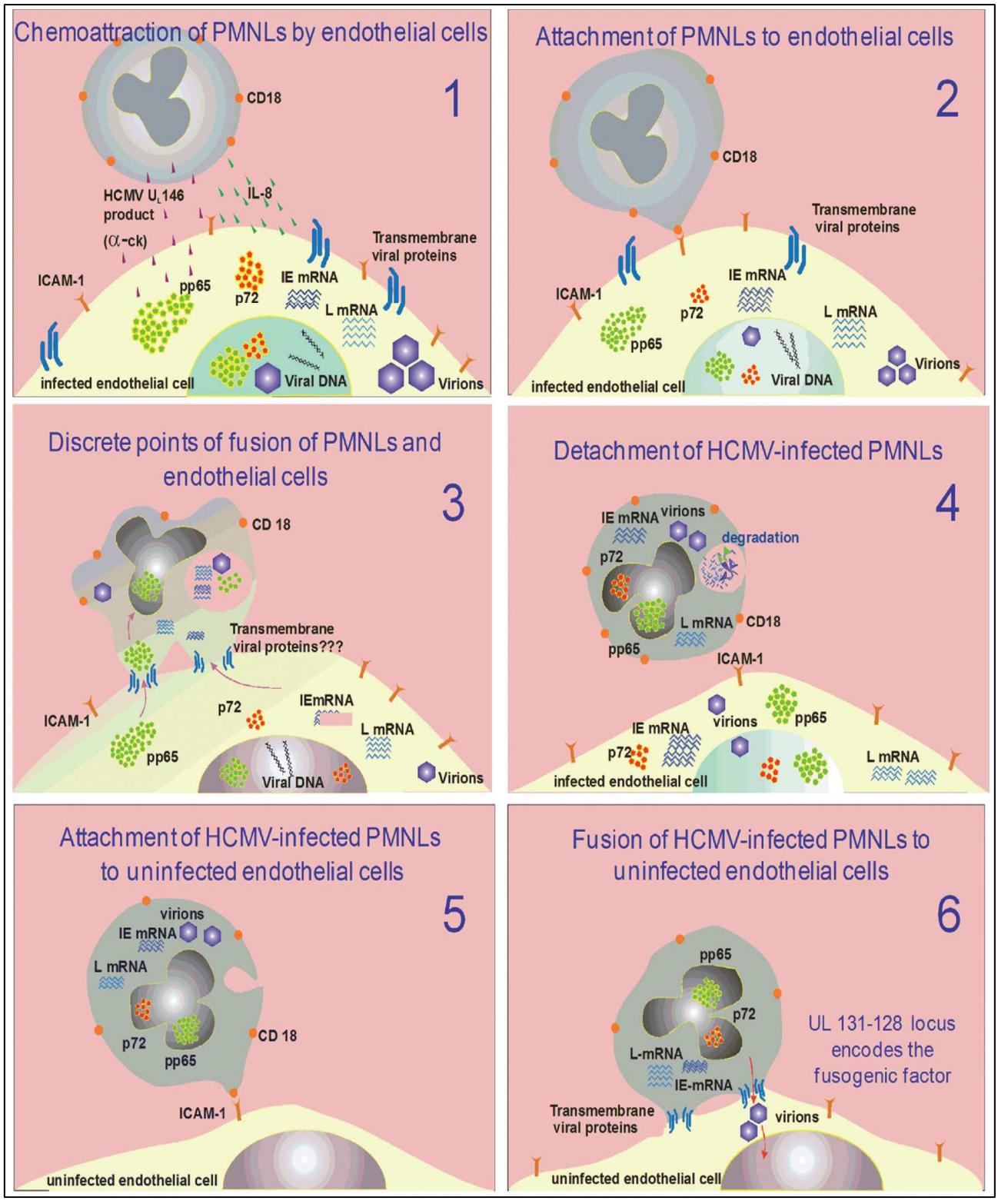


Figure 4. Transfer of HCMV from infected ECs to PMNLs and *vice versa* (from Gerna G et al. J Gen Virol. 2017, 98(9):2215-34 [34]).

1.6 Clinical manifestations of HCMV infection

HCMV infection in the immunocompetent host is generally asymptomatic, or in some cases, it may result in a mononucleosis syndrome with fever, myalgia, lymphadenopathy and hepatomegaly. HCMV lead to serious opportunistic infection in immunocompromised individuals, such as those with advanced HIV (human immunodeficiency virus) infection, transplant recipients or very premature infants. The most common manifestation of HCMV disease in HIV patients is retinitis, which is characterized by hemorrhagic retinal necrosis and it occurs in 85% of cases. Between 10 to 50% of solid organ transplant recipients develop symptomatic disease during the post-transplant period. The infection can manifest as HCMV syndrome characterized by fever illness with leukopenia or as tissue-invasive disease (hepatitis, enterocolitis, pneumonitis, encephalitis, nephritis, myocarditis, chorioretinitis, cystitis, or pancreatitis). In hematopoietic stem cell transplantation**,** primary HCMV infection occur in 30% of cases while reactivation in approximately 80% of patients and the most frequent symptoms correlated to HCMV infections are: esophagitis or gastritis, enterocolitis; pneumonitis, hepatitis, retinitis and encephalitis [3,19]. However, the most severe clinical manifestations are those related to cCMV infection that involve the central nervous system (CNS). In fact, in this setting, HCMV infection causes severe morbidity and mortality in newborns and it is the leading infectious cause of deafness and neurodevelopmental abnormalities [35].

CHAPTER 2

Congenital cytomegalovirus infection

2.0 Congenital cytomegalovirus infection

2.1 Magnitude of cCMV infection

cCMV infection is the most common congenital infection worldwide and it is an important public health problem in pediatric populations. In fact, cCMV infection causes neurodevelopmental delay and it is the leading non-genetic cause of SNHL in the developed countries. In many parts of the world, the number of children with cCMV-related disabilities is similar to or higher than those with better-known conditions, such as Down syndrome or spina bifida. Nevertheless, cCMV remains largely unrecognized respect to other childhood disability conditions for which, screening during pregnancy, are routinely performed [4,36,37]. For cCMV infection there is no maternal or neonatal screening programmes and parent and clinician awareness about of infection during pregnancy is very limited [36].

2.1.1 Epidemiology of cCMV infection

The global prevalence of cCMV infection ranging from 0.2% to 2.0% (average of 0.64%) of pregnancies [36]. Several studies showed that there is a relationship between the maternal seroprevalence and the incidence of cCMV infection. In particular, in developed countries the incidence of cCMV infection, as well as the maternal seroimmunity, is lower than in poor countries. Nevertheless, within a geographic region the maternal seroprevalence varies widely among women with different age, race, culture and socioeconomic status [38,39]. The incidence of cCMV infection is 0.15-2.0% in Europe, around 0.5-1.8% in Africa and in Asia, 0.42-1.4% in north America and about 1.8% in south America [38]. Considering the number of live birth and the cCMV prevalence reported by countries, a number of cCMV infections approximately equal to 0.12 million and 0.7-4.5 million per year was estimated in developed areas and poor countries, respectively [40].

Transplacental transmission of HCMV infection may occur following the viremic phase during both primary or non-primary maternal infection. In the latter setting, the infection

can result by reactivation of endogenous latent virus or through reinfection with a new HCMV strain [41]. Considering the reinfection in seroimmune women, both seronegative and seropositive mothers have the same probability of acquiring HCMV infection. In fact, this risk increases by exposure to people excreting virus; i.e. through caring for children (they represent the leading source of HCMV for women of reproductive age) or sexual activity [39,42]. The HCMV transmission to fetus could occur during viremic phase, probably when infected leukocytes across the placenta, where efficiently viral replication occurs. Subsequently, HCMV-infected cells thought the amniotic fluid could be swallowed by the fetus, in whose oropharynx, the virus replicates until it reaches the fetal circulation [38]. Despite the mechanism by which the virus reaches the fetus may be similar in primary infection as in that non-primary, it is well documented that in primary HCMV infection the risk of mother–child transmission is greater than in non-primary infection. Transmission to the fetus occurs in 14.2–52.4% (average of 32.4%) of primary maternal infections, versus 1.1–1.7% (average of 1.4%) of non-primary infections, suggesting a protective role of pre-existing maternal antibodies [35,40,43,44]. Nevertheless, based on the high seroprevalence of HCMV in adults, it is estimated that more than two-thirds of HCMV-infected infants are born to already seropositive women. Consequently, non-primary infections likely contribute to more cases of cCMV disease [40]. In this latter setting, the transmission appears mostly to be related to maternal infection caused by a new HCMV strain; although further studies are needed on this issue to define the exact frequency of HCMV reinfection and the rate of uterine transmission [39]. Despite maternal immunity contribute to reduce the probability of mother-child transmission; the severity of cCMV disease in the infected newborn seems similar following primary or non-primary maternal infections [40,45-48]. The vertical transmission appears to increase with older gestational age at infection, while the outcome is more severe when infection occurs during the early stages of pregnancy (first trimester) [4,49-51].

2.1.2 cCMV infection and clinical manifestations

Most of infants with cCMV infection (85%-90%) have no signs or symptoms at birth, while the remaining 10%-15% is symptomatic. Among these, the around 10% die in the newborn

period for fulminant illness, mostly due to disseminated intravascular coagulation, hepatic dysfunction or bacterial superinfection [38,8]. Among symptomatic newborns the clinical manifestation of cCMV infection at birth can range from mild/transient findings to multiple organ system involvement, which includes in most cases the reticuloendothelial and the CNS (Table 1) [8]. As reported by Boppana et al., the main physical findings observed are petechial rash, jaundice and hepatosplenomegaly followed by neurological abnormalities, such as microcephaly and lethargy.

Finding	Infants With Abnormality, %
Clinical findings	
Petechiae	76
Jaundice	67
Hepatosplenomegaly	60
Microcephaly	53
Intrauterine growth retardation	50
Chorioretinitis/optic atrophy	20
Purpura	13
Seizures	7
Laboratory findings	
Elevated AST (>80 U/L)	83
Conjugated hyperbilirubinemia (direct bilirubin >4 mg/dL)	81
Thrombocytopenia (<100 000/mm^3)	77
Elevated CSF protein (>120 mg/dL)	46

Abbreviations: AST, aspartate aminotransferase; CSF, cerebrospinal fluid.

Table 1. Main clinical and laboratory findings detected in infants with symptomatic cCMV infection (from Boppana S.B. et al. Clin Infect Dis. 2013, 57 4: S178-81 [8]).

This wide spectrum of clinical manifestation reflects the ability of HCMV to spread in fetal organs resulting in a disseminated HCMV infection. In a previous study performed on tissues from 34 fetuses with cCMV infection, HCMV-positive cells were detected in different fetal organs: pancreas, lung, kidney, liver, brain and heart, that were involved in

100%, 87%, 87%, 71%, 55% and 44% of cases, respectively [52]. Hepatosplenomegaly, as well as jaundice and petechiae usually are due to abnormalities of the reticuloendothelial system and are, in most of cases, transient. On the contrary, the neurological deficits evident at birth persist for life [49]. In fact, in symptomatic cCMV infected newborns, the risk of long-term neurodevelopmental disabilities is high (around 50-90%). These long-term sequelae include microcephaly, motor deficits, encephalic palsy, mental retardation, seizures, ocular abnormalities, SNHL and learning disabilities [53]. Predictive findings of adverse neurologic outcome, in symptomatic cCMV infection, include microcephaly, chorioretinitis and the presence of other early neurologic signs and the detection of brain abnormalities by cranial computed tomography (CT) within the first month of life [8]

Finally, although 85%-90% of newborns with cCMV infection are asymptomatic at birth, about the 10-15% will develop long-term neurodevelopment sequelae, such as SNHL (prevalently), cognitive impairment and retinitis (Table 2) [8].

	Affected children, %	
Sequelae	**Symptomatic**	**Asymptomatic**
Overall incidence	50–90	10–15
Hearing loss	50–60	7–15
Cognitive deficits	50–70	~ 4
Microcephaly	35–40	~ 2
Ocular abnormalities	25–50	~ 3
Seizures	15–20	~ 1
Motor deficits (mild to moderate)	25–30	< 1
Motor deficits (severe)	15–25	< 1

*Rates reflect a range of incidence data reported in the pediatric literature.

Table 2. Long-term sequelae in children with cCMV infection symptomatic and asymptomatic at birth (from Schleiss MR. Curr Treat Options Neurol. 2008,10(3):186-92 [53]).

It is well documented that SNHL is the most common sequela of cCMV infection occurring in 30-65% of newborns symptomatic at birth and in 7-15% of infants with asymptomatic infection. Approximately half of SNHL is late-onset or progressive. However, the mechanisms of pathogenesis behind HCMV-SNHL are still unclear [39,54]. In a previous study involving 20 HCMV-infected human fetuses at 21 weeks; HCMV-positive cells were detected by immunohistochemistry in the inner ear of the 45% of cases. In particular, the marginal cell layer of the stria vascularis was always infected in the positive inner ears, followed by infection in the Reissner's membrane. In addition, HCMV-antigens were found in sensory cells of the utricle and crista ampullaris into the vestibular labyrinth. The clinical consequences of these finding are still unknown [39,54].

2.2 Role of the placenta in mother-to-child transmission of HCMV infection

The mechanisms by which the HCMV crosses the placenta are still unknown. The placenta supplies oxygen, nutrients and immunoglobulin (Ig) G to the fetus by maternal circulation. This hematogenous organ is composed of a fetal part, known as chorionic villi (that contain fetal blood vessels) and a maternal portion named basal decidua (attached to the uterine wall) [55]. This structure is connected to the fetus via the umbilical cord, and contacts maternal blood within the intervillous blood space. The exchange between fetal compartment and maternal blood occur through differentiated cytotrophoblasts, (on the villous surface) that fuse into multinucleate cells, named syncytiotrophoblasts. Data obtained by chorionic villus explants and animal models, showed that, different cell types into uterine-placental interface may support the HCMV replication in first-trimester of pregnancy: fibroblasts, cytotrophoblasts, ECs, etc. (Figure 5) [55,56]. Based on these findings, various routes by which the virus spreads from the uterine tissue to placenta, and then to the fetus were proposed. One way involves the syncytiotrophoblast layer, that directly contacts the maternal blood space, where HCMV can be carried through leukocytes or may infect the ECs of blood vessels [56,57]. The syncytiothrophoblast layer may allow the passage of HCMV to underlying stratum of cytotrophoblast stem cells, that support the viral replication and then the HCMV spread to fetal ECs [57]. The cytotrophoblast layer

may be also infected, by its direct interaction with HCMV-infected cells in uterine wall, such as leukocytes and ECs [49,55-57]. Finally, it was hypnotized that HCMV exploits maternal IgG to cross the placenta via transcytosis, as IgG-virion complexes, using the Fc receptor expressed on the surface of syncytiotrophoblasts. It is postulated that, on the fetal side, villus core macrophages may quickly neutralize IgG-virion complexes formed of high-avidity neutralizing antibodies, while immune-complexes of low avidity IgG allow virus to escape the macrophages and infect the fetus. Therefore, in this model, the timing of HCMV infection respect to the pregnancy is critical determinant of protection [49,55].

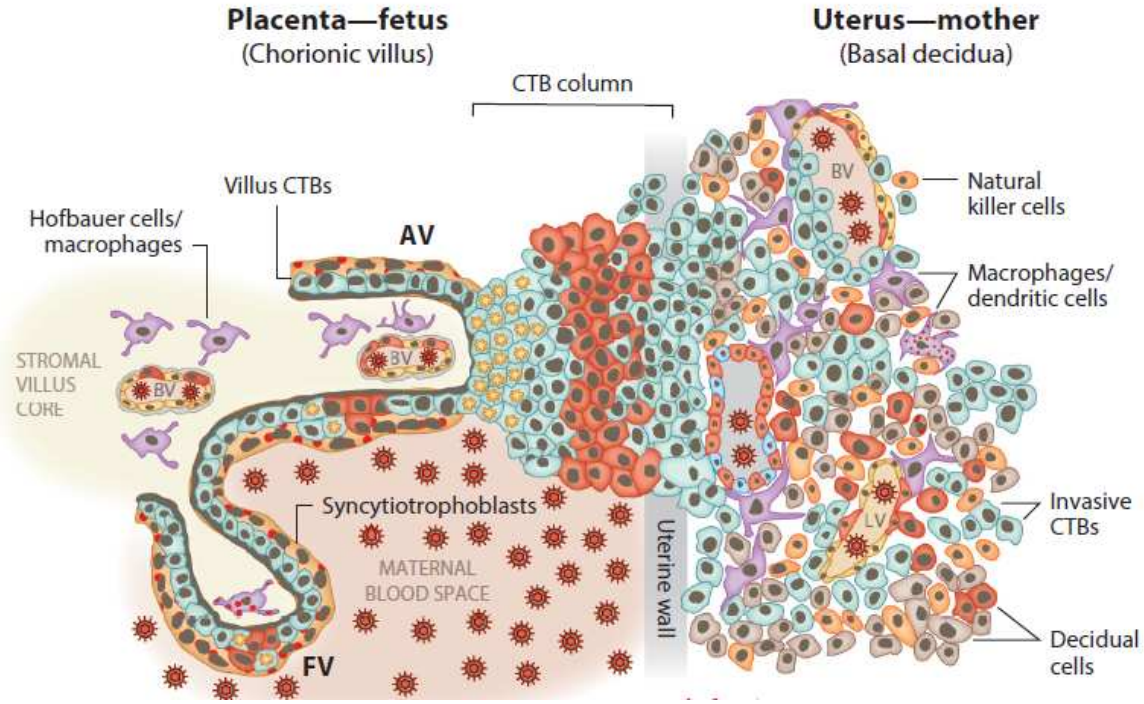


Figure 5. Uterine-placental interface. Cell types that support HCMV replication are indicated by red cytoplasm. The cytotrophoblast (CTB) layer of anchoring villi (AV) and floating villi (FV) plays a key role in the fetal transmission of HCMV infection. BV: blood vessel (adapted from Pereira L. Annu Rev Virol. 2018, 5(1):273-99 [55])

In the case of non-primary infection, it is possible that HCMV is reactivated in macrophages of the uterine wall, causing infection of cytotrophoblast cells with the consequent possibility of fetal infection [57].

HCMV infects the amniotic membranes, alters the development of the cytotrophoblast layer causing consequently structural and functional abnormalities in the placenta [58,59]. Histopathological analysis of placentas from HCMV-infected fetuses showed pronounced villous maldevelopment, diffuse villitis, cytomegalic cells, and areas of necrosis and calcification [52,55,60]. In previous studies, these findings have been associated with functional damage of placenta and brain hypoxia in the fetus [52,60].

Finally, it was proven that HCMV infection impairs the HLA-G expression. This molecule is a component of immune tolerance that protects the fetal cells from removal by maternal immune cells, particularly abundant in the decidua during the first trimester of pregnancy. Consequently, to downregulation of HLA-G, the maternal immune response could be activating against fetal cells [56,59].

2.3 Immune responses in cCMV infection

The importance of adaptive immunity in the control of HCMV infection is well documented in the immunocompromised individuals [61]. In the setting of cCMV infection, it is known that women with primary infection transmit HCMV to fetus at a higher frequency than those with preconceptual virus specific-immunity, suggesting that low avidity and poor neutralizing activity, of maternal antibody against HCMV, increase the probability of viral transmission [44-46,49,62]. However, the maternal antibody response seems not protective against disease once the fetus is infected [48,47]. In the humoral response, gB (involved in viral attachment and penetration) represents the major target for HCMV neutralizing antibodies, followed by gH (implicated in the fusion between host cell membrane and viral envelope). Some studies have evaluated the potential role of the anti-pentamer antibodies (HCMV IgG against gH/gL/UL128-131) in prevention of intrauterine transmission. The authors have demonstrated individual differences in the quality and kinetics of immunity responses and suggest that these findings could explain the variability in intrauterine transmission of HCMV [62-64].

The role of cell-mediated immunity was investigated in more limited number of studies, that prove conflicting data on the importance of early HCMV specific T-lymphocyte responses, to protect from intrauterine transmission [62,65-67]. In contrast with the

extensive literature proving the key role of CD8+ T lymphocyte responses in the outcome of HCMV-infected transplant recipients, some studies on cCMV infection setting found a stronger correlation between HCMV specific CD4+ T-lymphocyte responses and protection from intrauterine viral transmission. In particular, in women who transmit the virus to their fetuses, as compared to women who do not transmit, a delay in the development of HCMV-specific CD4+ T lymphocyte responses, was observed [61,65,66].

In addition to adaptive immune responses, the maternal innate immunity contributes to contrast HCMV replication, acting through effectors as natural killer cells that kill virus infected cells. Nevertheless, throughout the course of its co-evolution with the human host, HCMV has developed mechanisms of interaction that interfere with adaptive and innate immune response to promote immune evasion or inflammation and viral dissemination [5]. The main viral evasion mechanism is based on inhibition of antigen presentation by major histocompatibility complex (MHC) class I and class II. This system, known in humans as HLA (human leukocyte antigen), can be modulate in its expression and/or function by HCMV, that encodes proteins to destroy and detain the expression of HLA molecules on the infected cell surface or to upregulate specific HLA class I molecules binding to immune cell inhibitory receptors [68]. Moreover, HCMV encodes a homolog of the immunomodulatory cytokine human interleukin 10 (hIL-10), cmvIL-10 [69,70]. This molecule preserves the ability to bind and signal through the hIL-10 receptor and it exhibits immunomodulatory functions, including suppression of proinflammatory cytokine in addition to MHC activity inhibition. These strategies contribute to immune evasion during virus infection [3,69].

Finally, recent studies showed that the fetal immune system, long perceived as immature, could contribute to shape the outcome of cCMV infection [71].

2.4 HCMV infection: diagnosis in the mother, fetus and newborn

2.4.1 Diagnosis of maternal HCMV infection

Since cCMV infection is strongly dependent on maternal serological status, diagnostic testing (using serological and virological methods) to detect maternal HCMV infection, can

assist in determining the risk of transmission to fetus [35,36,72]. The diagnosis of primary HCMV infection is straightforward if seroconversion is documented. When HCMV immune status before pregnancy is unknown, the detection of HCMV IgG and IgM should be performed and the presence of HCMV IgM may be used as a marker of active or recent HCMV infection. Of note, Lazzarotto et al., show that the immunoblot is the gold standard test to confirm the presence of IgM antibodies in serum and suggest to perform HCMV IgG avidity measurements generally only when HCMV IgM antibodies are positive [36,72,73]. The detection of positive HCMV IgM together with low-moderate HCMV IgG avidity are good indicators of recent primary infection [4,35,74]. It is important to take into account that low avidity results are found 18-20 weeks after the onset of symptoms [35,36,73].
Virus isolation and/or HCMV-DNA detection in body fluids can only support serological diagnosis and positive results are not associated with a greater risk of infection and damage in fetus or neonate [72,73].

2.4.2 Prenatal diagnosis of fetal HCMV infection

A clinical suspicion or a serologic evidence of maternal HCMV infection and/or abnormal fetal sonographic findings should prompt a detailed examination of the fetal brain [75]. The fetal compartment can be studied by non-invasive method, ultrasonography (US) and invasive prenatal diagnostic investigation (amniocentesis). This latter should be performed after 20-21 weeks of gestation, in mother with documented primary HCMV infection or in cases of fetal abnormalities (compatible with HCMV infection) detected by ultrasound [36]. The presence of HCMV in amniotic fluid may be detected using viral isolation in cell culture or polymerase chain reaction (PCR). However, the major limitation of the invasive and non-invasive techniques is that ultrasound identifies prenatally no more than 15% of infected cases and positive results of amniotic fluids tests do not discriminate between infants who will have symptoms at birth and those who will not, although quantitative PCR might partially enable such a prediction. In some studies, high viral load in amniotic fluid has been correlated with major risk of symptomatic cCMV infection [52,73]. Of note, combination of abnormal fetal lesions with the high viral load in amniotic fluid is currently associated with a poor neurodevelopmental outcome [75-77].

2.4.3 Diagnosis of cCMV infection in newborn

When cCMV infection in newborn is clinically suspected on maternal infection documented, it is necessary to perform a correct diagnosis of infection. Infants congenitally HCMV-infected shed large amounts of virus in saliva and urine [78,79]. Therefore, urine and/or saliva samples, collected within the first 3 weeks of life, should be used for the detection of HCMV-DNA by PCR. However, it is important to consider that the saliva sample must be collected at least 1 hour later the breastfeeding to avoid potential viral contamination of breast milk [36]. Neonates with confirmed cCMV infection, should be promptly seen by pediatrics, audiology (ABR testing) and ophthalmology (Fundoscopic exam) and undergo laboratory testing and brain imaging by US, magnetic resonance imaging (MR), and CT [80].

2.5 Treatment of congenitally HCMV-infected neonates

Despite anti-HCMV treatment, the most of neurological manifestations are irreversible. In addition, the use of antiviral therapy in cCMV infection is limited by the potential risks that must be evaluated together with possible benefits. Clinical randomized trials provided evidence of reduction in hearing loss and mild improvement of manifestations in symptomatic newborns treated at birth [43,81]. Based on these results, currently the antiviral treatment is recommended for newborns with moderately to severely symptomatic cCMV infection. The therapy should be administered within the first month of life for 24 weeks, during which a defined follow up, including ophthalmological and audiological examination should be performed. The therapy consists on administration of valganciclovir 16 mg/kg per dose orally/twice a day [36]. The antiviral treatment was not recommended for asymptomatic cCMV infected newborns in order to prevent the later developing of sequelae.

It is well documented that infants with early diagnosis of NSHL improve cognitive function respect to those with later diagnosis. For this reason, the neonatal screening for cCMV infection has become the focus of many studies in recent years [36].

2.6 Prevention of maternal infection and transmission *in utero*

A lot progress has been made in recent years for develop an efficacy vaccine to prevent HCMV infection. Clinical trials evaluating a recombinant HCMV gB vaccine, showed some efficacy in prevention of infection in young women. However, currently there is no licensed vaccine and only the HCMV educational and hygienic measures have the potential to prevent exposure to HCMV in pregnant women [36,82,83]. Research could be useful to identify education content and methods effective in preventing HCMV maternal infection [36,83].

Finally, passive immunization with HCMV hyperimmunoglobulin, as a potential means to prevent HCMV transmission to the fetus in pregnant women with primary cytomegalovirus infection, has been investigated. Data from a randomized, placebo-controlled study, suggest that the treatment with hyperimmunoglobulin did not significantly modify the course of primary HCMV infection during pregnancy [33,36,84].

CHAPTER 3

Brain damage in congenital HCMV infection

3.0 Brain damage in congenital HCMV infection

3.1 Development of the human central nervous system

The development of the CNS is briefly described below in order to understand the potential pathological role of HCMV in congenital infection.

The organogenesis of the CNS begins with a process called neurulation that starts at 18 days of gestation and results in the development of the neural tube at 4 weeks. Within 4-7 weeks, this neural tube enlarges in its cranial part in three primary vesicles called prosencephalon, mesencephalon, and rhombencephalon (Figure 6) [85,86]. In the fifth week, the prosencephalon develops into the telencephalon and diencephalon, while the rhombencephalon becomes the metencephalon and myelencephalon. The telencephalon evaginates into two later vesicles, the future hemispheres, and basal nuclei. The diencephalon will give rise to the thalamus, hypothalamus, and retinas; the myelencephalon will become the medulla, the metencephalon will form the pons and cerebellum and mesencephalon will develop into the midbrain, including superior and inferior colliculi (Figure 7 and 8) [87]. During the 8-16 weeks, the already formed structures increase in volume and corpus callosum develops. In this period there is the emergence of cortical plate (CP) with synapse formation, biochemical maturation and glial cell differentiation. The CP includes distinct layers of neurons. In fact, at the end of the fourth week, radial glia cells, originating from precursor in the ventricular zone, form a gap junction between their basal processes and ventricular surface. Contrarily, their apical processes enlarge and contact the basal lamina at the pial surface, building the external boundary of CNS. These glial fibers provide a scaffold for neuron migration to the cortical layer (Figure 9). In fact, the young neurons, originating from precursor cells, migrate and during the 8th-16th weeks bypass the early formed layers building a more external layer in the cortex. When neurons arrive in CP, they lose their glial attachment. Adhesion molecules allow the migratory movements of the neurons through glial fibers. This is the radial migration mode, however the neurons could also migrate departing from ganglionic eminence, in the subventricular region, to move in tangential direction. In this same period, other cells differentiate in astrocytes and oligodendroglial cells. In a small period, the neurons migration defines

different layers in encephalic hemispheres that are from external to internal: external marginal layer, CP, subcortical layer, the intermediate zone (with the subplate, this represents the future white matter that will develop) and the ventricular zone with cells in mitotic activity [85]. After 16 weeks, there is an increasing thickness of the cortex and the growing of hemisphere surface due to an increasing number of synapses. The limiting intracranial surface leads to multiple gyri in the cortex. Finally, during brain development the angiogenesis starts to form at 5 weeks, while, the myelinization starts to form in the second half of pregnancy [85,87]. The oligodendrocyte is the CNS cell type responsible for production of the myelin sheath. The stem cells into CNS generate both neurons and glial cells that include oligodendrocytes and astrocytes. These latter provide structural and trophic support for neurons, induce formation of the blood brain barrier (BBB), and regulate CNS synaptogenesis. In addition, in the CNS there are the resident immune cells, located within the brain parenchyma behind the BBB that constitute the microglial cells [88,89]**.**

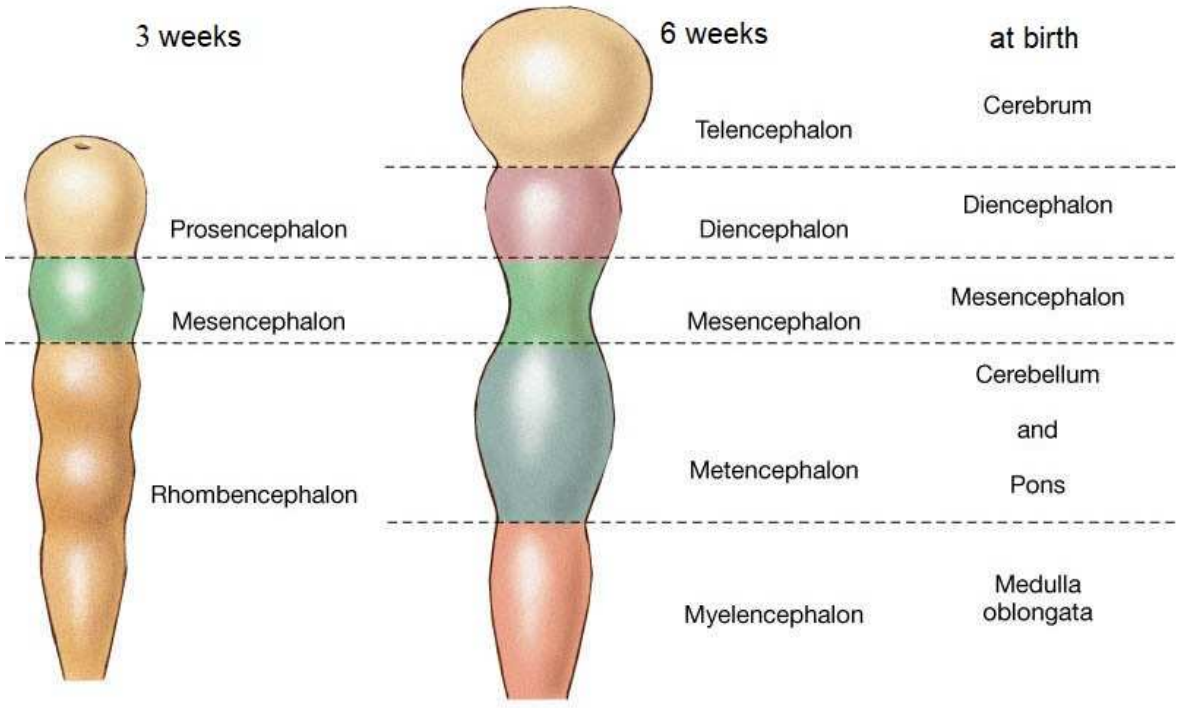


Figure 6. Human CNS: neural tube and derived structures (adapted from Cocco L et al, Anatomia Umana. Edises 2004, 334-505 [90]).

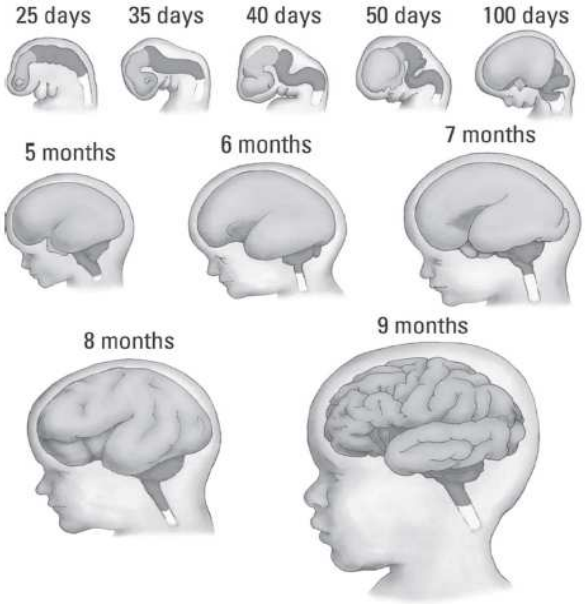


Figure 7. Development of human brain. (from Potter's. Mosby Elsevier. Second Edition. 2007, 1:1959-76 [85]).

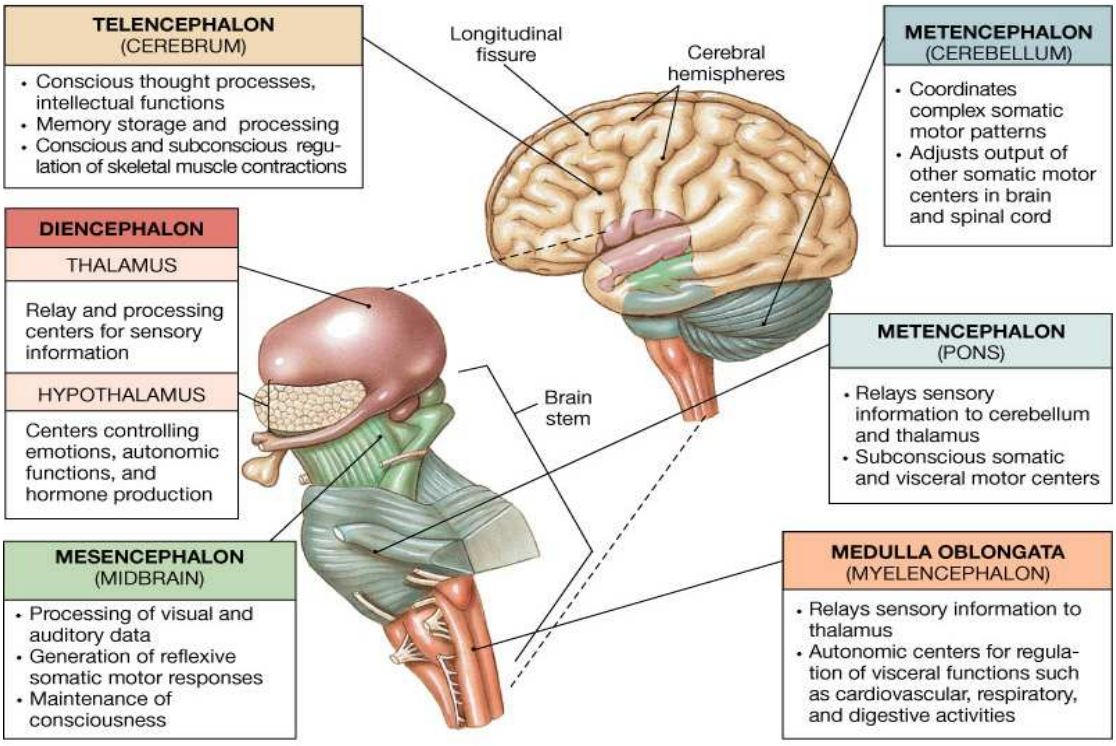
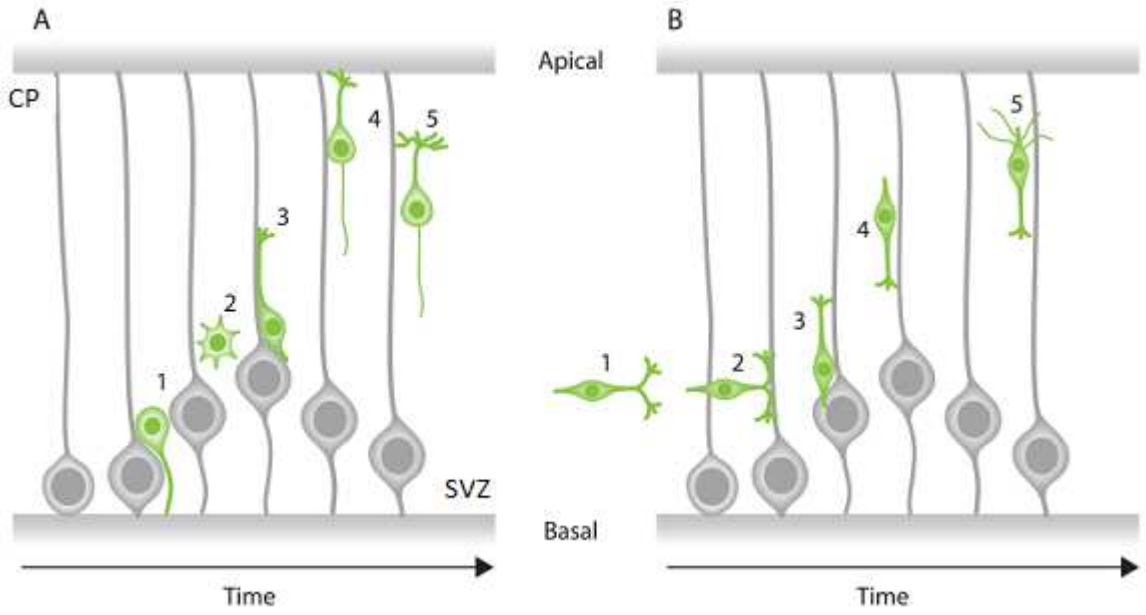


Figure 8. Human CNS at birth (adapted from Cocco L et al, Anatomia Umana. Edises 2004, 334-505 [90]).



CP: cortical plate; SVZ: subventricular zone

Figure 9. Schematic representation of cortical plate (CP) development. Modes of neuronal migration in the CNS: A) Radial migration and (B) tangential migration (adapted from Amini R et al. Neuronal migration and lamination in the vertebrate retina. Front Neurosci. 2017, 11: 742 [91]).

3.2 Neuroimaging findings in cCMV infection

The neurological manifestation of newborns with cCMV infection, including lethargy, hypotonia, seizures, mental retardation and encephalic palsy, are the results of injury in CNS, such as: intracranial calcification, lissencephaly, polymicrogyria, ventriculomegaly, periventricular calcifications, white matter alterations and microcephaly. Currently, these pathological findings are evaluated and characterized by US, MR imaging, and CT. The intracranial calcifications are the most frequent lesion observed in cCMV infection occurring in 34-70% of cases and may be localized in different sites: brain parenchyma, basal ganglia and periventricular regions. This latter is the most common area were the calcification are present (Figure 10). The intracranial calcification is the imaging findings more associated with developmental delays than other abnormalities [92,93].

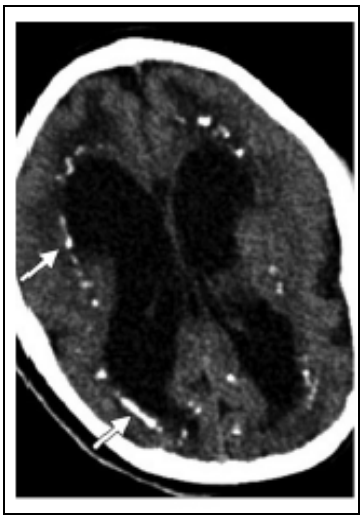


Figure 10. Intracranial calcifications. Unenhanced CT image shows periventricular calcifications in patients with cCMV infection (from Fink KR et al. Radiographics. 2010, 30(7):1779-96 [92]).

The ventriculomegaly is detected in the 45% of cCMV cases. This is associated often with encephalic volume loss, as well as the microcephaly (caused by encephalic or cerebellar hypoplasia) that occurs in 27% of patients with cCMV infection. In these cases, the loss of glial and neuronal volume lead to poor neurologic outcome.

Others common brain lesions detected in cCMV infected newborns are the white matter abnormalities (Figure 11) that are present in 22% of patients. These findings could represent a delayed myelinisation and often are localized in parietal or temporal lobe.

In the 10% of patients with cCMV infection migrational abnormalities are detected, such as lissencephaly and diffuse or focal polymicrogyria (Figure 12) [92,94]. Specifically, lissencephaly is the absence of sulcation in cortical areas that appears as smooth brain surface. This condition is associated to thickened cortical layer that reflect the neuronal loss. The lissencephaly is indicative of fetal infection during early age of gestation and it is associated to worse outcome. On the contrary to lissencephaly, polymicrogyria is the presence of multiple small abnormal gyri that seem to be presented by an area of thickened cortex. This abnormality is correlated to a HCMV infection occurring later in the fetal life period than lissencephaly.

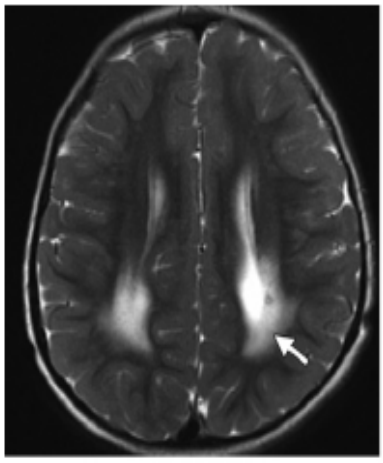


Figure 11. The axial T2 – weighted MR image displays hyperintense posterior white matter lesion (arrow) in patient with cCMV infection (from Fink KR et al. Radiographics. 2010, 30(7):1779-96 [92]).

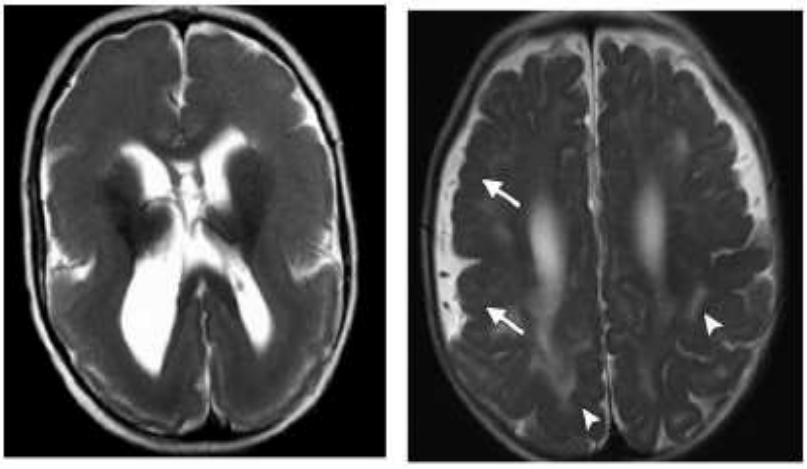


Figure 12. Axial T2 weighted MR image of migrational abnormalities in patients with cCMV infection: lissencephaly (left figure) and polymicrogyria (right figure) (from Fink KR et al. Radiographics. 2010, 30(7):1779-96 [92]).

Other imaging findings may be found in newborns with cCMV infection, such as periventricular cysts, ventricular adhesions and lenticulostriate. The periventricular cysts in temporal lobe with abnormalities of white matter is often considerate specific for HCMV

infection (Figure 13) [92,94]. However, while white matter abnormalities can occur throughout fetal life, each lesion, detected by imaging methods, reflects the timing of fetal infection. Specifically, lissencephaly, intracranial calcifications, encephalic or cerebellar hypoplasia, are more frequently present in fetuses that acquire infection before 18 weeks. Polymicrogyria, migrational abnormalities and cerebellar hypoplasia are more frequent in fetal infection acquired within 18-24 weeks. The fetal infection acquire after these periods have less severe neurological manifestations [92,95].

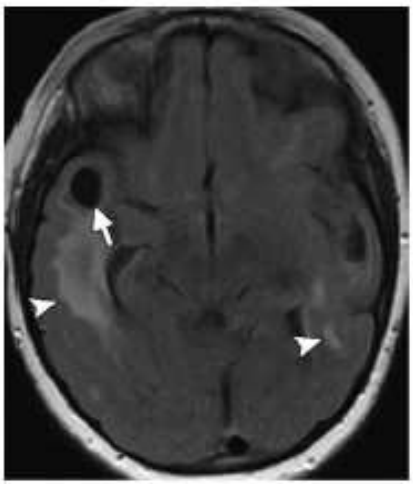


Figure 13. T2-weighted MR image show anterior temporal cyst (arrow) and adjacent white matter disease (arrowheads) in patient with cCMV infection (from Fink KR et al. Radiographics. 2010, 30(7):1779-96 [92]).

3.3 Potential pathogenic mechanisms of brain damage in cCMV infection

In contrast to clinical signs that are transitive (such as anemia, hepatosplenomegaly, and jaundice), the neurodevelopmental sequelae caused by cCMV infection are almost always persistent for life, as a result of irreversible damage in the CNS [49,96]. To date, the pathogenesis of brain injury HCMV-related is unknown [49]. Several studies have tried to explain the route by which HCMV reach the developing fetal CNS, because it is still insufficiently defined. However, species-specificity of HCMV has limited the study in animal models. Nevertheless, since the cCMV infection in humans is most frequently acquired during the early periods of the second trimester of pregnancy and the neonatal

mouse has a CNS embryologically equivalent to human fetus at 15 weeks of gestation, some studies were performed in this animal model [96]. The obtained results showed that, during hematogenous spread, murine CMV (MCMV) disseminates to the CNS and replicates in the brain parenchyma [96,97]. However, the exact mechanism by which HCMV cross the BBB is still unknown [96,97]. It has been hypothesized that the entry of the virus into the CNS could be due to loss of integrity of BBB or mediated by the ECs constituting the BBB, through which the virus spreads to the astrocytic processes [98-100]. Alternatively, as hypothesized for HCMV in cases of encephalitis, the virus could reach the CNS through the cerebrospinal fluid following viral replication in infected epithelial cells of the choroid plexus [98,101,102]. Finally, HCMV infection of ECs induces monocyte extravasations. This could be an additional viral mechanism to reach the CNS and disseminate into the brain; in fact HCMV-infected blood monocyte-derived macrophages could serve as carrier of the virus entry into developing CNS (Figure 14) [49, 98,103].

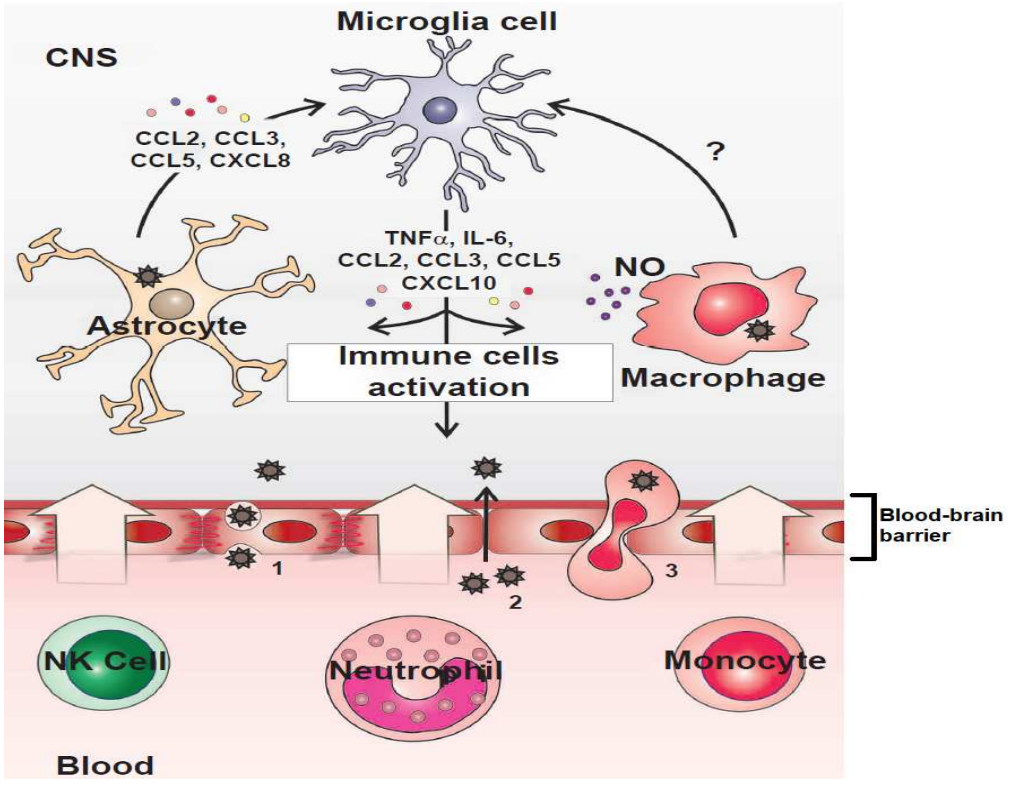


Figure 14. Possible mechanisms by which HCMV could reach CNS: *i)* infection of ECs, *ii)* loss of BBB integrity and *iii)* transport within infected monocytes (adapted from Slavuljica I et al. Cell Mol Immunol. 2015, 12(2):180-91 [96]).

How the virus plays its direct pathogenic role once in the brain has been evaluated by different hypothesis, including that HCMV is teratogen. Studies *in vitro* using human fibroblast have found that these cells infected with HCMV during the S phase of the cell cycle showed two specific breaks at position 1q42 and 1q21 into the chromosome 1. This effect could be inhibited through an incubation of the virus with neutralizing antibodies. In addition, the two break positions are located near the loci DFNA7 and USH2A. The DFNA7 seems to be involved in an inheritance autosomal dominant form of progressive hearing loss [49,104,105], while the USH2A gene encodes a protein involved in an autosomal recessive disorder responsible for both blindness and SNHL [49,106,107]. This evidence could be potentially correlated with the onset of SNHL and visual impairment caused by HCMV [49]. However, in animal model and *in vitro* studies, other mechanisms were investigated to elucidate the direct effect of HCMV replication in brain parenchyma. In particular, an interference of HCMV infection with the apoptotic signalling pathway was observed [49]. The apoptotic process is a critical defense mechanism to eliminate, during organogenesis, the damaged or poorly developed cells as well as the virus infected cells from the host [49]. Viral replication in astrocytes, induces the expression of proapoptotic cell protein p53, which is sequestered by HCMV-IE2 inhibiting or delaying the late apoptotic event [49,108,109]. Two molecules are also encoded by antiapoptotic viral genes, UL36 and UL37, to prevent cell death. The homologs of these genes were also identified in rodent and macaque CMVs [49,110-113]. The inhibition of cell death can be necessary to complete the slow HCMV replication cycle. Post-mortem examination of brain tissue from patients with neurological symptoms caused by cCMV infection, also showed a rare or absent apoptosis of infected neuronal or glial cells [49,114]. In addition, viral antigens were absent in areas with neuronal apoptosis. This suggest an indirect role of HCMV in the neuronal loss through apoptosis process, that could be caused by virus induced neuroinflammatory responses [49]. In fact, in presence of inflammation or tissue injury, the expression of extrinsic apoptosis signals is upregulated playing an important role in normal

brain development. HCMV can have developed an immune evasion strategy, that in turn may lead to apoptotic damage of bystander cells [49,115,116]. In addition, brain injury related to cCMV infection, could be also mediated by cytokines. It is known that immune responses in CNS are due to both, resident brain cells and immune effectors able to infiltrate brain tissue during infection or damage. The resident glial cells (astroglial and microglial cells) produce chemokines and cytokines that have neuroprotective function, controlling viral spread. However, their overexpression may result in neurodegeneration and CNS injury. In particular, chemokines also recruit peripheral immune cells in the brain, such as T-lymphocytes producing additional proinflammatory cytokines that potentially could contribute to cause neurotoxicity during HCMV brain infection [49]. In a previous study, involving human fetuses with cCMV infection, the presence of inflammatory response and immune-mediate damage was showed. Specifically, in HCMV infected brain tissues, diffuse microglial activation, astrocytosis, vascular changes and microglial nodules (mainly composed by activated CD8+ T-lymphocytes) were found. In the same study, placental diffuse villitis and necrosis were detected in fetuses with severe brain damage suggesting that placental insufficiency and hypoperfusion could contribute to the pathogenesis of brain abnormalities [60].

Finally, HCMV directly interacts with regulatory protein altering the cell cycle progression of infected cells. With this strategy the virus may use the cellular DNA replication machinery [49]. Some *in vitro* studies showed that HCMV productively infects cells that are able to undergo mitotic division in vivo, such as neural precursor cells (NPCs) and astrocytes. On the contrary, differentiated cell types, as neurons, do not support HCMV replication, while differentiated glial cells retain their susceptibility to viral infection. [49, 117,118]. In murine model, the MCMV infection inhibits the proliferation of neural stem cells and reduce their capacity to differentiate into neurons. These could be due to the alteration of cellular cycle virus-induced [117]. Finally, in the same animal model, as for differentiated neurons, primordial embryonic stem cells are not susceptible to MCMV infection, suggesting that the susceptibility to viral infection depends on the cellular differentiation state. During fetal life, the proportion of cells susceptible to viral infection in the brain varies at different gestational age and this could be responsible for the variability

of clinical manifestations related to cCMV infection [49]. Studies on distribution of HCMV susceptible cells in fetal brain could help to explain the neuropathogenesis related to cCMV infection. To date, the neuronal development was evaluated mainly in animal model and *in vitro* experiments, while few studies were performed in human adult and fetal brain and these are mostly focused on some encephalic areas [119-121]. However, it is generally acknowledged that neurogenesis involved differential kinds of cells identified mainly by immunohistochemical studies using specific markers (Figure 15) [119-121]. In particular, nestin was used frequently as a marker to identify neural stem/precursor cells. This is an intermediate filament protein expressed in undifferentiated CNS cells and downregulated in mature cells. Despite the fact that stem/precursor cells can express also other markers, such as GFAP (glial fibrillary acidic protein), nestin is widely used to their identification [120,122]. The doublecortin (DCX), is a brain specific microtubule associated protein expressed by migrating, differentiating neuroblasts and it is used to identify these cells [122,123]. Finally, NeuN (neuronal nuclei, or neuronal nuclear antigen) localized in nuclei and perinuclear cytoplasm of mature neurons is detectable exclusively in post-mitotic neurons and is absent in neural progenitor, glia, oligodendrocytes and astrocytes. Other markers of mature neurons are calretinin and calbindin, but the mostly used is NeuN [120,121,124].

The distribution of NeuN expression in the human fetal nervous system was reported by Sarnat HB et al. showing the presence of few mature neurons in the early stage of gestation (Table 3) [125].

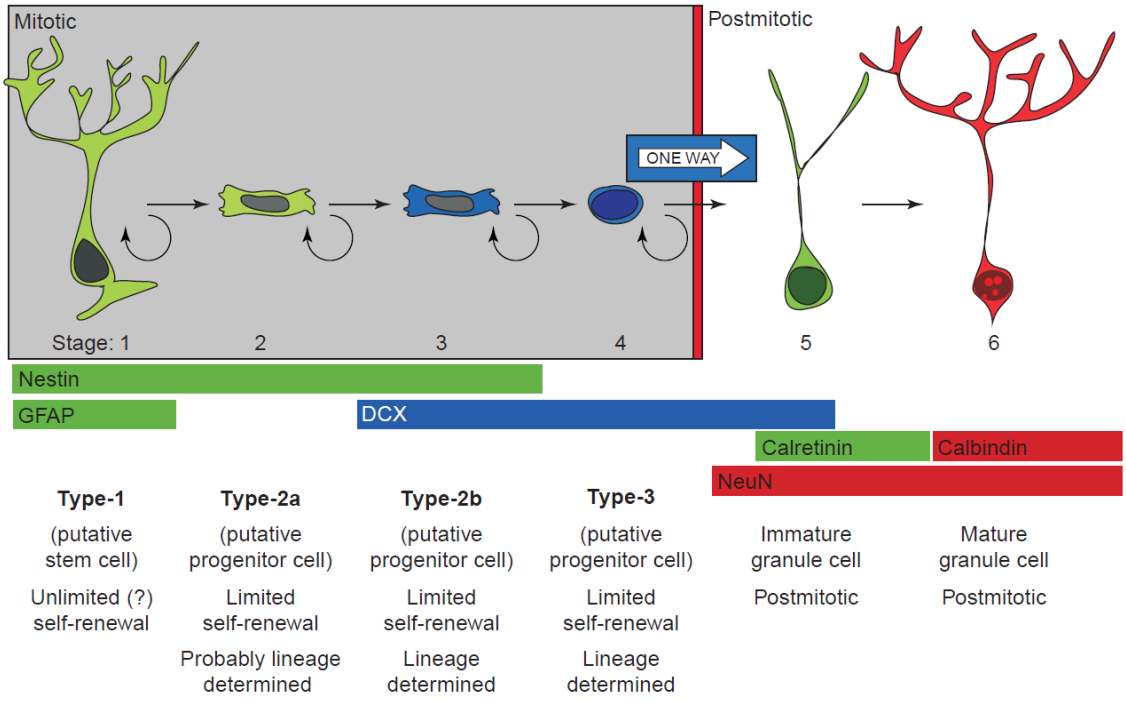


Figure 15. Schematic representation of the neurogenesis process. Stage 1: stem cell, stage 2-4: progenitor cells that differ through their proliferative potential and increasing neuronal differentiation, stage 5: postmitotic stage, stage 6: mature cell (adapted from Kempermann G et al. Trends Neurosci. 2004, 27(8):447-52. [121]).

Gleeson JG demonstrate that DCX is widely expressed in migrating neurons (Table 4) [126,127]. Finally, Yin X et al. evaluated the nestin expression analyzing by immunohistochemistry tissues from different areas of human brain in fetuses at various weeks of gestation. The authors found that the overall proportion of nestin positive cells reduced in all examined sites with the increase in gestational age. However, among the different regions, the higher proportion of nestin positive cells were detected in hippocampus, followed by subventricular zone and striatum (Figure 16, and Table 5). In this study, similar results were obtained for the detection of nestin mRNA by in situ hybridization in the same tissues [128].

Region	Gestational age (weeks)						
	8	10	14	20	22	24	40
Spinal cord							
Ventral horn motor neurons	+	++	++	+++	+++	+++	+++
Dorsal horn neurons	+	+	+	++	+++	+++	+++
Dorsal root ganglion cells	++	++	+++	+++	+++	+++	+++
Brainstem							
Hypoglossal nucleus	0	0	+	+++	+++	+++	+++
Nucleus ambiguus	0	0	+	+++	+++	+++	+++
Nucleus solitarius	0	0	+	++	++	+++	+++
Dorsal motor vagal nucleus	0	0	+	++	+++	+++	+++
Descending trigeminal nucleus	0	0	+	++	+++	+++	+++
Basal pontine nuclei	–	0	+	++	++	+++	+++
Vestibular nuclei	0	0	+	+++	+++	+++	+++
Inferior olivary nuclei	–	0	0	0	0	0	0
Arcuate nucleus	–	0	0	+	++	++	+++
Cerebellum							
Purkinje cells	0	0	0	0	0	0	0
External granule cells, outer layer	–	0	++	++	+++	++	+
External granule cells, inner layer	–	0	++	+++	++	+	0
Internal granule cells	–	0	0	0	0	++	+++
Dentate nucleus	–	0	0	0	0	0	0
Thalamic nuclei	0	0	+	++	+++	+++	+++
Caudate nucleus	0	0	0	+	++	+++	+++
Cerebral cortical plate							
Cajal–Retzius neurons, layer 1	0	0	0	0	0	0	0
Layers 2–3	–	0	0	0	0	++	+++
Layers 4–6	–	0	0	++	+++	+++	+++
Periventricular germinal matrix	–	0	+	+	+	+	0

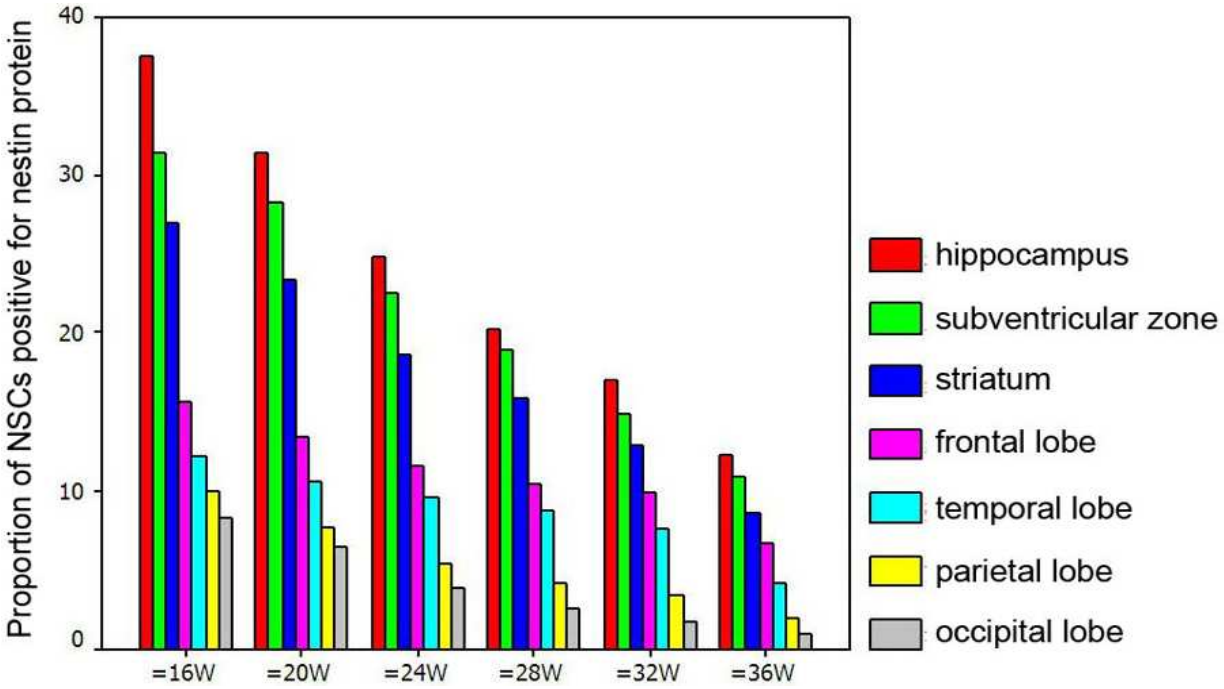
-, not yet formed; 0, no cells; +, 1–25% of cells; ++, 26–50% of cells; +++50–100% of cells.

Table 3. NeuN expression in various regions of the human fetal nervous system (from Sarnat HB et al. Brain Dev.1998, 20(2):88-94. [125])

Age	DCX				
	MZ	CP	WM		VZ
			glial cell	axon	
9–10 GW	–	+/–	–	–	–
11–15 GW	1+	2+	–	1+	2+
16–20 GW	+/–	2+	+/–	2+	1+
21–30 GW	–	2+	1+	1+	1+
31–40 GW	–	1+	2+	+/–	1+
1–24 months	–	1+	2+	–	+/–
2–10 years	–	1+	1+	–	+/–
11–29 years	–	1+	–	–	+/–

MZ: marginal zone, CP: cortical plate, WM: white matter, VZ: ventricular zone, GW: gestational weeks

Table 4. DCX, immunoreactivity in the developing human brains (adapted from Qin J et al. Brain Res. 2000, 863(1-2):225-32. [127])



W= week of gestation

Figure 16. Proportion of nestin positive cells (neural stem cells, NSCs) at different sites of fetal brain (from Yin X et al. Int J Clin Exp Pathol. 2013; 6(12): 2757–64 [119])

Group/ site	hippocampus	subventricular zone	striatum	frontal lobe	temporal lobe	parietal lobe	occipital lobe	χ^2	*P*-value
16 W	46.47	41.42	28.51	14.95	12.12	10.1	9.0	23644.08	<0.01
20 W	36.5	35.5	24.83	13.76	12.57	9.68	7.97	15712.73	<0.01
24 W	30.4	27.36	20.32	12.58	11.03	7.33	6.31	10811.92	<0.01
28 W	22.35	20.57	17.68	11.31	9.28	6.12	4.82	7108.268	<0.01
32 W	19.36	17.38	15.31	9.97	8.22	4.98	2.88	6928.515	<0.01
36 W	15.76	13.21	10.48	7.86	5.38	4.45	1.48	6150.322	<0.01
χ^2	9855.902	8848.345	3697.819	4336.394	1170.125	1435.392	2156.375		
P-value	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01		

W= WG: week of gestation

Table 5. Proportion of nestin positive cells at different sites of fetal brain (from Yin X et al. Int J Clin Exp Pathol. 2013; 6(12): 2757–64 [119])

The predominant localization of the neural stem/precursor cells in the hippocampus and in subventricular zone could be correlated with the ability of HCMV to replicate effectively in

these cells; explaining the neuronal damage in brain of patients with cCMV infection often located around the periventricular zone [49]. However, in order to confirm these hypotheses and to understand the mechanism of neuropathogenesis of HCMV-related brain damage, further studies will be needed to evaluate the differentiation stage of HCMV-infected neural/neuronal cells during cCMV infection.

CHAPTER 4

Aim of the study

4.0 Aim of the study

cCMV infection is the major cause of abnormalities in the CNS, that are irreversible and persist for life [43,52]. In fact, although HCMV can affect multiple organs, the most important sequelae of intrauterine infection are those related to lesions in the CNS, likely because fetal brain is not able to fully regenerate its parenchyma [52]. Neurological outcomes in cCMV infection may include encephalic palsy, mental retardation, SNHL, and visual impairments [53]. These clinical conditions are the result of brain lesions mainly studied by imaging methods, which showed: intracranial calcifications, microcephaly, abnormal periventricular signals, white matter lesions, intraventricular adhesions, ventriculomegaly, altered sulcation/gyral patterns and cerebellar abnormalities [92].

To date, the pathogenesis of injury related to cCMV infection in the developing fetal brain is not yet very well known [5,49]. Recent studies, mainly performed in animal and *in vitro* models, suggested that encephalic damage in cCMV infection could be due to a multifactorial process, that include the effects of direct viral replication, and the indirect effects occurring at two different levels: in the brain, where the infection can induce immune-mediated damage, and in the placenta, where the infection may cause severe placental necrosis with placental insufficiency and, consequently, hypoxic brain damage [49,52,60,96]. However, the direct viral replication in CNS seems to play a key role in the pathogenic mechanism of brain injury related to cCMV infection. Infected cells inside the CNS have altered intercellular communication and deficient response to neurotransmitters. During fetal development, this condition can affect the normal proliferation and migration patterns of neurons, leading to altered brain architecture and function [49,60,128]. In addition, few studies performed on cultured brain cells, showed a preferential tropism of CMVs for some type of cells, such as the immature and proliferating neural/neuronal cells [49,129,130]. These evidences could also suggest a specific brain localization of viral infection, as well as those described for other herpes viruses, such as the herpes simplex virus -1 (HSV-1), for which temporal lobe abnormalities are commonly reported in patients with herpes simplex encephalitis [131,132]. All these findings were poorly investigated in studies involving human cases of cCMV infection.

The aim of this project was to study the brain damage in human fetuses with cCMV infection in order to implement the knowledge on both, histological characteristics of encephalic injury and the mechanism of viral spread in the CNS with potential clinical consequences. In particular, brain tissues from HCMV-infected fetuses were analyzed in order to evaluate, in different encephalic areas: *i)* the distribution of the HCMV-infected cells, *ii)* the tissue viral load and *iii)* the potential viral tropism.

CHAPTER 5

Material and methods

5.0 Material and methods

5.1 Study design

HCMV-infected human fetuses at 21 weeks of gestation, with or without ultrasound abnormalities, from women who opted to terminate their pregnancies were examined. The study was approved by the Ethical Committee of St. Orsola Polyclinic, University Hospital, Bologna, Italy (*approval numbers: 14/2017/U/tess of 14/03/2017; 8/2010/O/Sper of 13/02/2010*). The fetal tissues were analyzed after receiving informed consent from the parents, according to the policies of the Ethical Committee and to the regulation of the Italian Ministry of Health.

For each fetus, tissues from different brain regions were analyzed: the 4 cortical areas (frontal, temporal, occipital, parietal) and underlying white matter, subventricular zone, thalamus, hypothalamus, hippocampus, basal ganglia and cerebellum.

All brain regions were analysed by:

- immunohistochemical staining for the detection of infected brain cells expressing HCMV-antigens;
- haematoxylin-eosin staining to evaluate the histological damage;
- real-time PCR to quantify the tissue viral load.

In addition, the ability of HCMV to infect neural/neuronal fetal brain cells was evaluated in correlation with their differentiation stages for 6/8 fetuses with HCMV-positive cells and HCM-DNA in the brain. This analysis was performed using double immunohistochemical staining for simultaneous detection of HCMV-antigens with markers of neural/neuronal cells.

All the brain tissue analyses described above were also performed in a case control that was a fetus at 21 weeks of gestation from a HCMV seronegative woman who opt to terminate the pregnancy due to fetal cardiac malformation.

The study was carried out in the Laboratory of Virology, Operative Unit of Clinical Microbiology - St. Orsola Polyclinic, University Hospital, Bologna, Italy, in collaboration with:

- the Unit of Obstetrics and Prenatal Medicine, St. Orsola Polyclinic, University Hospital, Bologna, Italy;
- the Operative Unit of Pathology, St. Orsola Polyclinic, University Hospital, Bologna, Italy;
- the Operative Unit of Pathology, St. Maria Nuova Hospital, Reggio-Emilia, Italy.

In this study the terms “brain” or “encephalon” are referred to the current definition of cerebrum, the brainstem and the cerebellum.

5.2 Study population

Ten fetuses at 21 weeks of gestation, with documented HCMV infection were analysed. Specifically, 2 and 8 cases were prospectively and retrospectively enrolled, respectively. All fetuses were from pregnant women with primary HCMV infection arising before the twelfth week of gestation and documented by the detection of anti-HCMV IgM with anti-HCMV low IgG avidity. The diagnosis of fetal infection was based on positive results for the detection of HCMV-DNA in amniotic fluid at 21 weeks of gestation, obtained by real-time PCR. In addition, at the time of amniocentesis, ultrasound examination was performed in all pregnant women, in order to detect potential fetal abnormalities. The results obtained by invasive and non-invasive prenatal diagnosis are reported in Table 6.

An additional fetus at 21 weeks of gestation from HCMV seronegative woman who opt to terminate the pregnancy due to fetal cardiac malformation was analysed as control case.

For each fetus, sections obtained from paraffin-embedded blocks of tissue, previously fixed in 4% formaldehyde, were analyzed.

Case No.	HCMV-DNA in AF (copies/mL)	Ultrasound findings at 21 weeks of gestation
1	>1,250,000	Encephalic periventricular hyperechogenicity, hyperechogenic bowel
2	>1,250,000	Normal
3	182,000	Normal
4	>1,250,000	Normal
5	948,473	Normal
6	323,300	Normal
7	>1,250,000	Normal
8	489,000	Normal
9	>1,250,000	Encephalic periventricular hyperechogenicity, hyperechogenic bowel
10	270,000	Normal

AF: amniotic fluid

Table 6. Results obtained by invasive and non-invasive prenatal diagnosis

5.3 Histological brain damage evaluation

To study the histological abnormalities correlated to HCMV infection in the brain, one section of 3 microns from each encephalic area was analyzed by hematoxylin-eosin staining, performed by standard method. In particular, the presence of the following pathological findings, was evaluated in each brain regions:

- necrosis, characterized by focal or diffuse death of cells in the tissue;
- microglial nodules (occasional or multiple), consisting in clusters of activated microglial cells involved in immune response to HCMV-infected cells;

- microglial activation, proved by microglial body cells with various morphologies: small rod-shape, amoeboid-like and spherical cells**;**
- astrocytosis, characterized by cell body expansion (hypertrophy) and cells with clear nuclei (named Alzheimer type II cells**)**;
- vascular changes, consisting in increased number of vessels with hypertrophy of endothelial cells (that showed plump, cuboidal form and protruded into the lumen).

In addition to the presence, the frequency and severity of the above described pathological finding were considered in order to assess brain damage. However, taking into account that the microglial activation, astrocytosis and the vascular changes are often observed in viral encephalitis, brain damage was classified as severe, moderate or mild, according the following criteria analyzed:

- *severe brain damage*: presence of tissue necrosis and multiple microglial nodules (≥3/brain region);
- *moderate brain damage*: no tissue necrosis, but presence of multiple microglial nodules (≥3/brain region);
- *mild brain damage*: no tissue necrosis, but presence of occasional microglial nodules (<3/brain region).

In all brain regions, 5 fields at 20 high-power field (HPF) were evaluated.

5.4 Detection of infected brain cells expressing HCMV-antigens

To identify the HCMV-positive cells, one section of 3 microns from each encephalic area was analyzed by immunohistochemical staining, performed using anti-CMV [8B1.2, 1G5.2, 2D4.2] mouse monoclonal primary antibody (Cell Marque, USA), that reacts with immediate early, delayed early, and late HCMV-antigen preparation. The HCMV-infected cells distribution in the brain was assessed analysing 5 fields at 10 HPF for each encephalic area. The results were expressed as mean of HCMV-positive cells detected in the 5 fields.

5.5 Quantification of brain tissue viral load

To quantify the brain tissue viral load, 2 sections of 8 microns were analysed, as follows.. After the sections were mounted on glass slides, the selected brain areas were anatomically identified and dissected by scraping it off with a sterile single-use scalpel. Each selected tissue area was placed in a 1.5 mL tube, where the deparaffinization procedure was performed using 160 µL of the Deparaffinization Solution (Qiagen, Germany) with 200 µL of tissue lysis buffer (buffer ATL, Qiagen, Germany) and 20 µL of protease (Proteinase K, Qiagen, Germany). After one hour of incubation at 56°C followed by one hour of incubation at 90°C, DNA extraction was performed using the QIAsymphony®SP instrument with the QIAsymphony DSP DNA Mini Kit (Qiagen, Germany). Purified DNA was eluted in 50 µL and the contained human DNA (hDNA) was quantified using a real-time PCR assays, Quantifiler® Human DNA Quantification Kit (Life Technologies, USA), which amplifies a region of the housekeeping gene hTERT. Five nanograms of hDNA were processed for HCMV-DNA quantification, carried out using a real-time PCR assay, CMV ELITe MGB™ kit (ELITech Group, Italy). Amplification, detection and analysis were performed using the 7500 real-time PCR System (Applied Biosystems, USA). The tissue viral load was reported as number of copies/5ng of hDNA. The lower limit of detection was 10 copies/5ng of hDNA, while the lower limit of quantification (LLoQ) was equal to 13 copies/5 ng of hDNA. Positive results below the LLoQ were censored with a value equal to 10 copies/5ng hDNA.

Although all brain regions were anatomically identified, the tissue viral load in the subventricular zone was not evaluated due to the difficulties to dissect and scrape the extremely thin layer of the periventricular region.

5.6 Differentiation stage of the HCMV-infected neural/neuronal cells

To evaluate the differentiation stage of the HCMV-infected neural/neuronal cells, double immunohistochemical stainings for simultaneously detection of HCMV-antigens with markers of neural/neuronal cells were performed. In particular, nestin was used as a marker to recognize cells in the early stage of neural development, DCX was used as a marker to

identify neural cells with lineage determined and NeuN for postmitotic neural cells detection (see Chapter 3, Figure 15).

Briefly, serial sections of 3 microns from each paraffin block containing the selected brain areas were cut and quickly processed for automated double immunohistochemical staining in a Benchmark Ultra immunostainer (Ventana Medical Systems, USA). The first immunohistochemical reaction was always the anti-CMV staining (pre-diluted mouse monoclonal anti-CMV, clone 8B1.2 1G5.2&2D4.2, Cell Marque USA), visualized using the OptiView DAB detection kit (brown color), followed by the second antibody represented by monoclonal anti-nestin clone 10c2 (Santa Cruz Biotech, USA) diluted 1:400, polyclonal anti-NeuN (Abcam Ltd, UK) diluted 1:300 or monoclonal anti-DCX clone 2G5 (Millipore USA) diluted 1:600. The second immunoreaction was visualized using the Alkaline phosphatase UltraView detection Kit (red color). Sections were counterstained using hematoxylin and Bluing reagent following instrument's manufacturer instructions. All reagents other than the above reported antibodies were from Ventana Medical Systems, USA. Staining protocols are summarized in Table 7.

Test	AR	1st Antibody (CMV)	2nd Antibody	
CMV	UltraCC1 x 32 min at 95°C	16 min - 36°C	-	
HCMV-Nestin	UltraCC1 x 40 min at 95°C	8 min - 36°C	Nestin	36 min - RT
HCMV-NeuN	UltraCC1 x 32 min at95°C	8 min - 36°C	NeuN	40 min - RT
HCMV-DCX	UltraCC1 x 32 min at95°C	8 min - 36°C	DCX	28 min – 36°C

AR: Antigen Retrieval; UltraCC1: Tris-HCl pH 8.2; RT: Room Temperature

Table 7. Double immunohistochemical staining protocols

5.7 Statistical analysis

Statistical analysis was not performed due to the small sample size (10 fetuses) evaluated in this study.

CHAPTER 6

Results

6.0 Results

6.1 Detection of HCMV in the different brain regions

The immunohistochemical staining for HCMV-antigens expression, performed in brain tissues of all 10 fetuses with cCMV infection, revealed HCMV presence in the brain of 8 cases (80%), confirmed also by the detection of HCMV-DNA (Figure 17, Table 8). In the remaining 2 cases (20%), no HCMV-positive cells and no viral DNA in all brain regions were observed. On the basis of these findings, for the subsequent examinations, the attention was focused on the cases that presented a encephalic involvement of HCMV infection (cases 1,2,3,4,5,7,8,9). Among the 8 fetuses with HCMV-positive brain cells, a viral load more than 10^6 copies/mL was detected in amniotic fluid of 5 cases (62.5%) and in 2/8 cases (25%) ultrasound abnormalities were found at 21 weeks of gestation (Table 8). In both these fetuses the pathological findings involved the brain, where a encephalic periventricular hyperechogenicity was observed.

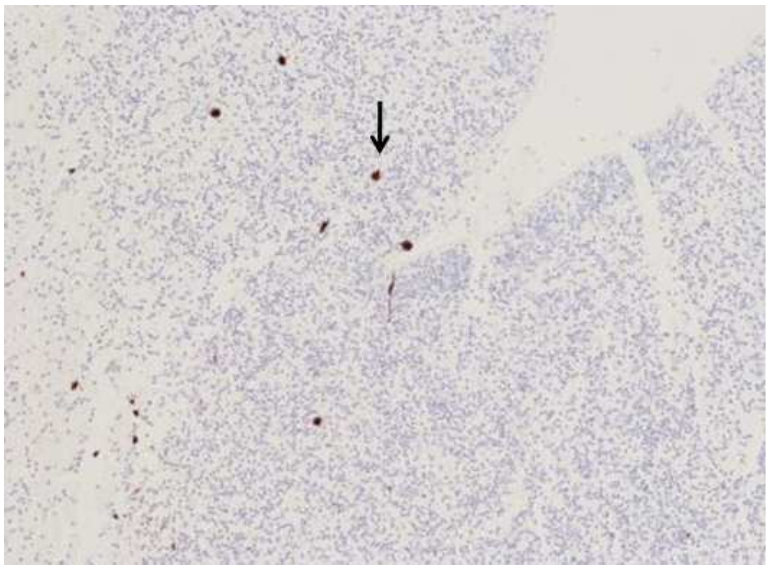


Figure 17. HCMV-positive cells detected by immunohistochemical staining. Brain cells expressing HCMV-antigens are identified by brown staining (arrow). Frontal lobe (10 HPF).

Case No.	HCMV-IHC in brain tissues	HCMV-DNA in brain tissues	HCMV-DNA in AF (copies/mL)	Ultrasound findings at 21 WG
1	**Positive**	**Positive**	>1,250,000	Encephalic periventricular hyperechogenicity, hyperechogenic bowel
2	**Positive**	**Positive**	>1,250,000	Normal
3	**Positive**	**Positive**	182,000	Normal
4	**Positive**	**Positive**	>1,250,000	Normal
5	**Positive**	**Positive**	948,473	Normal
7	**Positive**	**Positive**	>1,250,000	Normal
8	**Positive**	**Positive**	489,000	Normal
9	**Positive**	**Positive**	>1,250,000	Encephalic periventricular hyperechogenicity, hyperechogenic bowel
6	Negative	Negative	323,300	Normal
10	Negative	Negative	270,000	Normal

IHC: Immunohistochemistry, AF: amniotic fluid, WG: weeks of gestation

Table 8. HCMV-positive cells and HCM-DNA detected in brain tissues and findings detected by invasive and non-invasive prenatal diagnosis.

6.2 Detection of pathological findings and evaluation of brain injury

In the 8 fetuses with encephalic HCMV-infected cells and HCMV-DNA in the brain, various pathological findings were observed in the examined encephalic regions. In particular, microglial activation (proved by increased rod-shaped cells as showed in Figure 18) diffuse astrocytosis (represented in Figure 19 as cells with clear nuclei), and vascular changes (as plump endothelial cells reported in Figure 20) were detected in all cases, without differences in brain region distribution. These evidences showed a diffuse encephalic inflammatory reaction. However, as reported in the previous chapter (section 5.3), considering the severity and the frequency of the remaining histological encephalic abnormalities, such as necrosis and microglial nodules, a different degree of brain injury in

the 8 fetuses was identified and classified as severe (1 cases, 12.5%), moderate (3 cases, 37.5%) and mild (4 cases, 50%) (Table 9). In particular, microglial nodules, showed in Figure 21 as clusters of activated microglial cells, were detected in all fetuses. Nevertheless, in 4 cases the frequency of microglial nodules was occasional (< 3/brain region), showing a mild encephalic injury. Among the remaining fetuses, 3 presented multiple microglial nodules (≥ 3/brain region) defining a moderate brain damage and 1 case showed the presence of multiple microglial nodules with cortical necrosis (Figure 22). The latter case was classified as fetus with severe brain damage. As described for astrocytosis, microglial activation and vascular changes, also the microglial nodules were not localized in some encephalic regions, but uniformly distributed into the brain. On the contrary, cortical necrosis was mainly detected in layer III. The brain with severe injury also showed the following lesions: diffuse macrophage infiltration of the leptomeninges, polymicrogyria and periventricular leukomalacia (white matter necrosis). The cerebellum showed extensive parenchymal hemorrhage with scarce residual tissue identified.

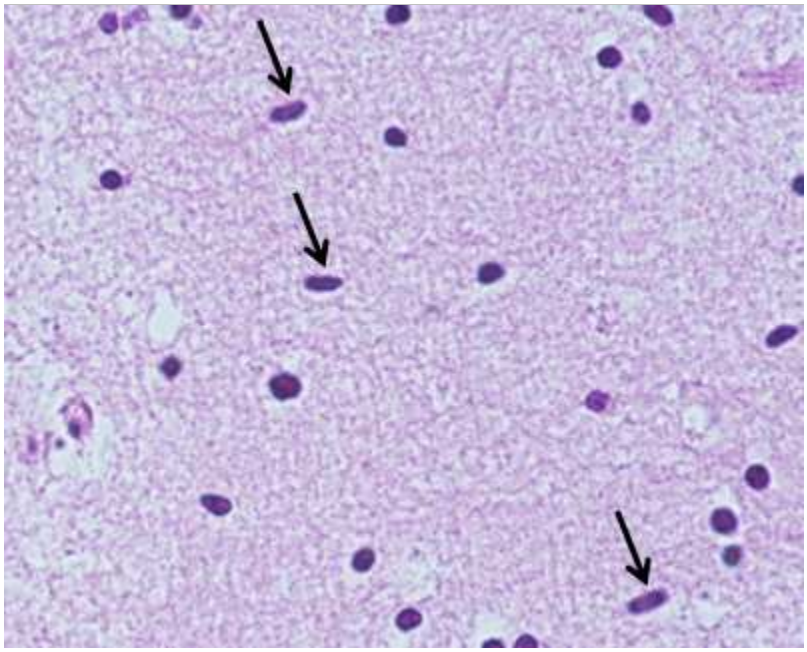


Figure 18. Diffuse microglial activation: increased number of rod-shaped cells (arrows). Hippocampus, hematoxylin-eosyn staining (40 HPF).

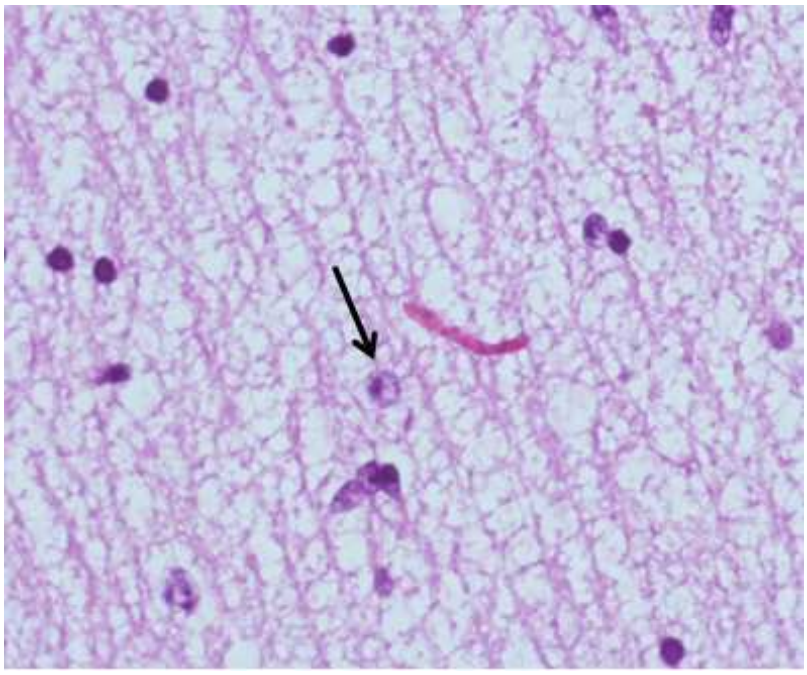


Figure 19. Astrocytosis: cell with clear nuclei, named Alzheimer type II cell (arrow). Temporal lobe, white matter, hematoxylin-eosyn staining (40 HPF).

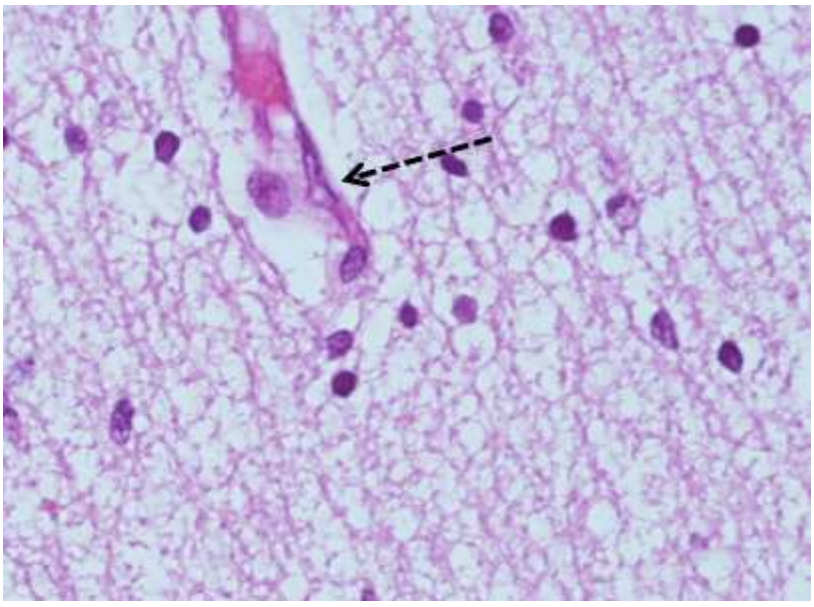


Figure 20. Vascular changes: plump endothelial cell protruding into the vassal lumen (dashed arrow). Temporal lobe, white matter, hematoxylin-eosyn staining (40 HPF)

Case No.	Brain damage	HCMV-DNA in AF (copies/mL)	Ultrasound findings
9	**Severe**	>1,250,000	Encephalic periventricular hyperechogenicity, hyperechogenic bowel
1	**Moderate**	>1,250,000	Encephalic periventricular hyperechogenicity, hyperechogenic bowel
2	**Moderate**	>1,250,000	Normal
7	**Moderate**	>1,250,000	Normal
3	**Mild**	182,000	Normal
4	**Mild**	>1,250,000	Normal
5	**Mild**	948,473	Normal
8	**Mild**	489,000	Normal

AF: amniotic fluid

Table 9. Different degree of brain damage detected in 8 fetuses with HCMV-positive cells and HCMV-DNA in the brain

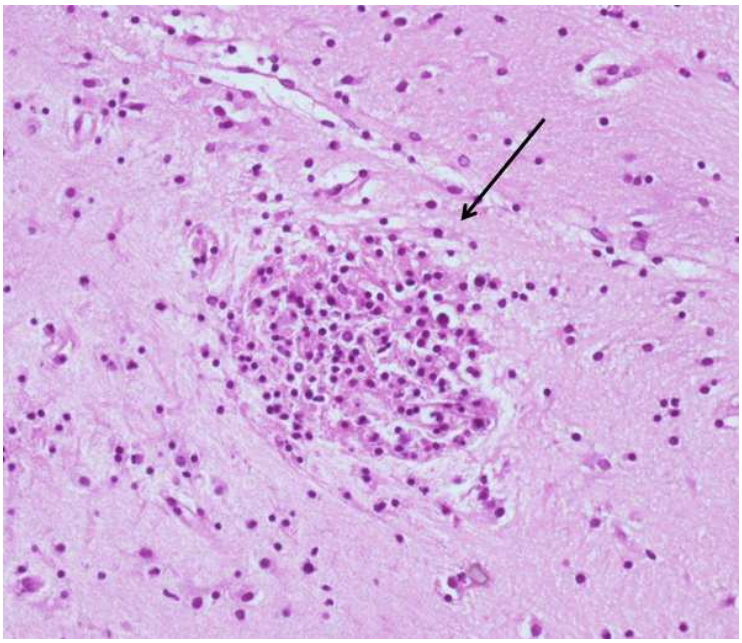


Figure 21. Microglial nodule: cluster of activated microglial cells (arrow). Hypotalamus, hematoxylin-eosin staining (20 HPF)

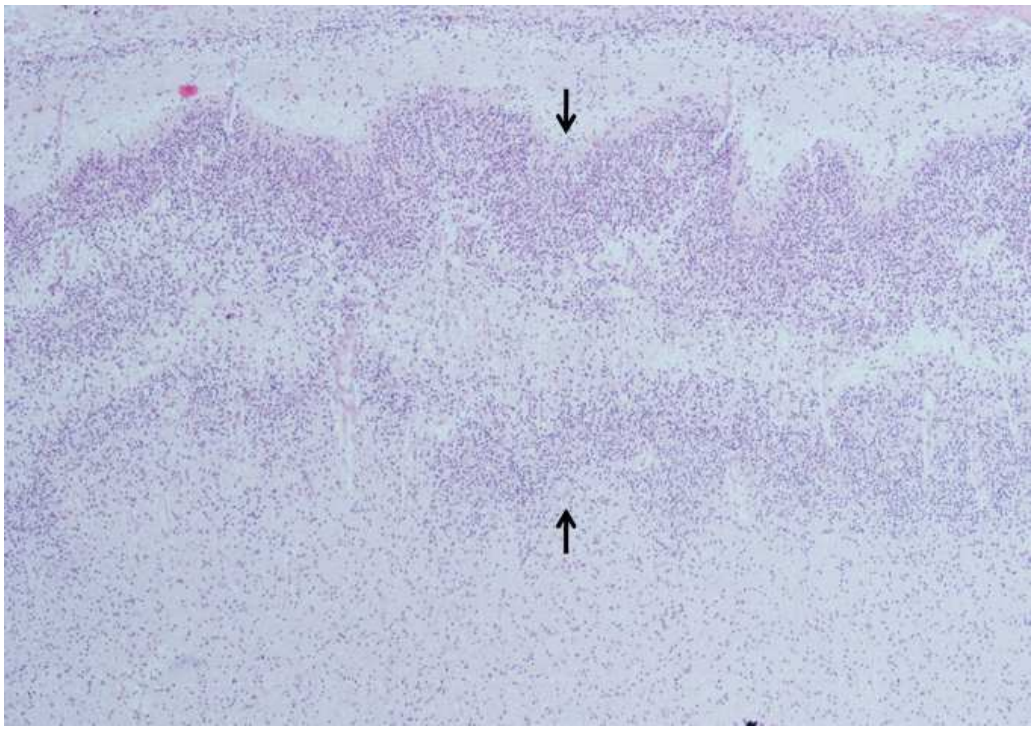


Figure 22. Necrosis in cortical layer III (between the arrows) and polymicrogyria. Temporal lobe, hematoxylin-eosin staining (4 HPF)

In all cases with severe/moderate encephalic damage, high viral load in amniotic fluid (>10^6 copies/ml) were found, and 50% of these cases showed ultrasound pathological findings involving the brain (Table 9).

6.3 Quantification and distribution of tissue viral load into the brain

Brain tissue viral load was quantified in all 8 fetuses with HCMV-positive cells and HCMV-DNA in the encephalon, analyzing different brain areas, except the subventricular zone that was not evaluated due to the difficulties to dissect and scrape the very thin layer of the periventricular region.

The median HCMV-DNA levels detected in the case with severe and moderate brain damage were higher than those found in the fetuses with mild encephalic injury: 92 copies/5ng hDNA (range: 20-380 copies/5ng hDNA) and 87 copies/5ng hDNA (range:

100-7’505 copies/5ng hDNA) versus 10 copies (range: 10-248 copies/5ng hDNA). The median levels of tissue viral load detected in each brain area were reported in Figure 23.

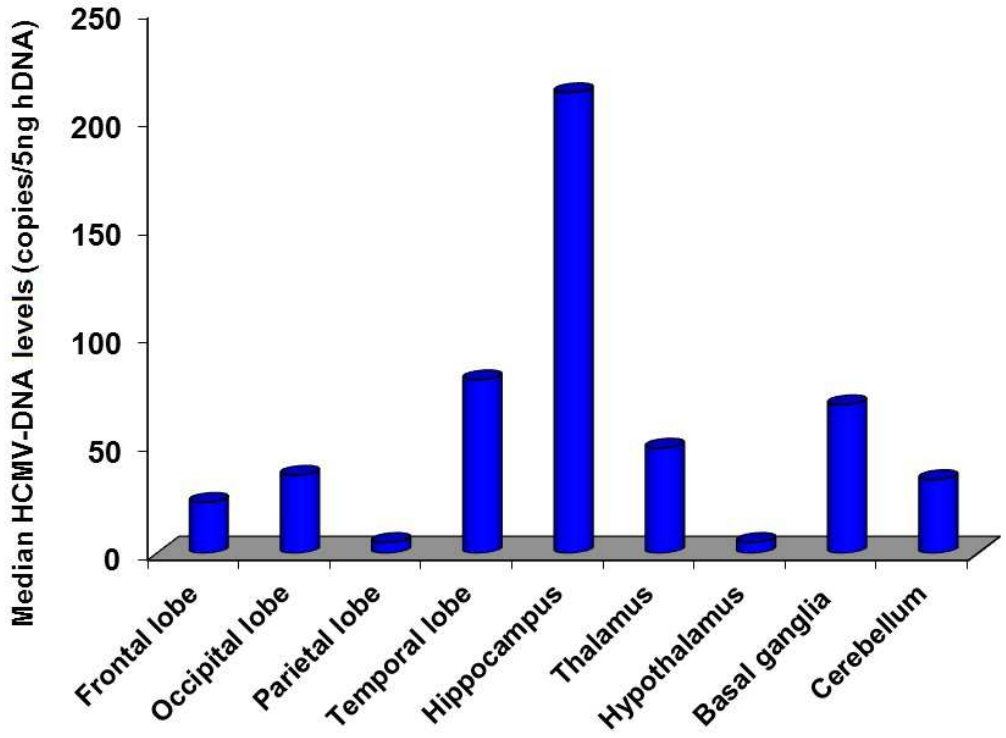
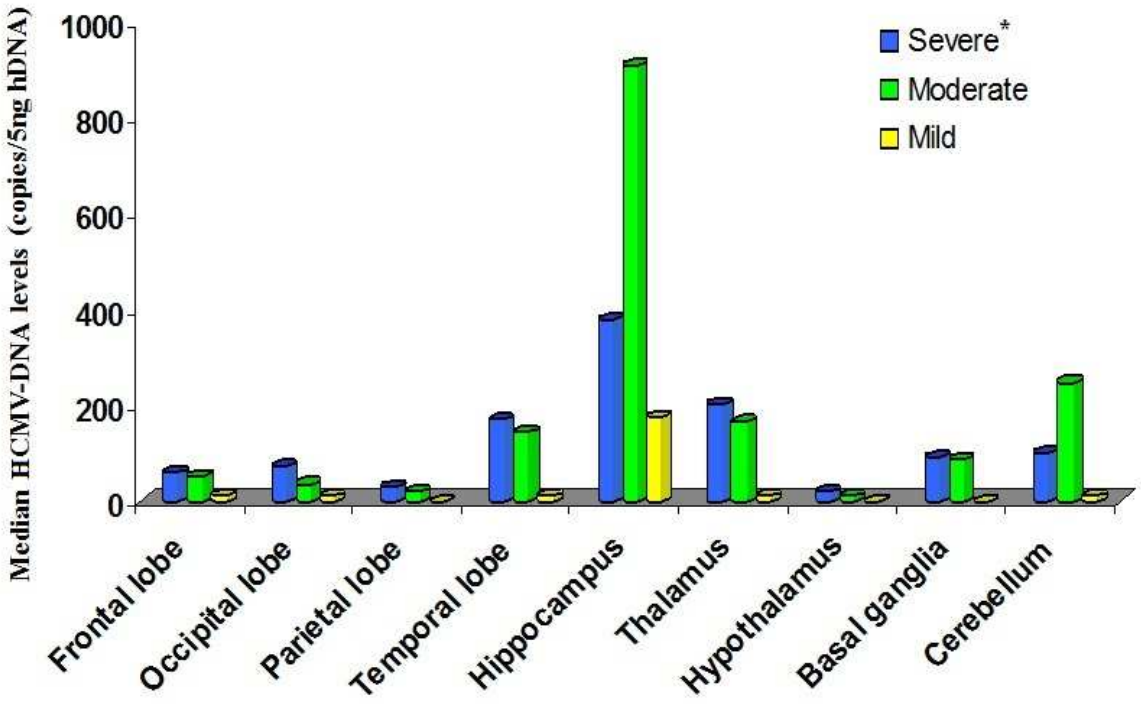


Figure 23. Distribution of tissue viral load in different brain regions. The levels of HCMV-DNA were reported in relation to 5 ng of human genomic DNA (hDNA).

Different distribution of HCMV-DNA levels, in the brain, was found. In particular, the highest median viral load, equal to 212 copies/5ng hDNA (range 10-7’505 copies/5ng hDNA), was detected in the hippocampus; followed by the median HCMV-DNA levels present in the temporal lobe (80 copies/5ng hDNA, range 0-238 copies/5ng hDNA), basal ganglia (68.5 copies/5ng hDNA, range 0-114 copies/5ng hDNA), and thalamus (48.5 copies/5ng hDNA, range 0-744 copies/5ng hDNA). Median values found in other brain regions were: 36 copies/5ng hDNA (range 13-280 copies/5ng hDNA) in occipital lobe, 34 copies/5ng hDNA (range 0-899 copies/5ng hDNA) in cerebellum, 23.5 copies/5ng hDNA (range 10-300 copies/5ng hDNA) in frontal lobe, 5 copies/5ng hDNA (range 0-197

copies/5ng hDNA) in hypothalamus and 5 copies/5ng hDNA (range 0-107 copies/5ng hDNA) in parietal lobe.

Similar results were obtained stratifying the median levels of tissue viral load in correlation with the degree of brain damage (Figure 24). In fact, the highest median HCMV-DNA levels were again identified in the hippocampus, where values equal to 380 copies/5ng hDNA, 910 copies/5ng hDNA (range 105-7'505 copies/5ng hDNA) and 93 copies/5ng hDNA (range 10-248 copies/5ng hDNA) were detected in the cases with severe, moderate and mild brain damage, respectively. However, for severe brain damage, the tissue viral load detected was referred to the only case available. In addition, in this case, the HCMV-DNA values found in the cerebellum may be biased because it was severely hemorrhagic.



The values reported for severe brain damage were referred to single case

Figure 24. Median of HCMV-DNA levels stratifying in correlation with the degree of brain damage.

6.4 Quantification and distribution of HCMV-positive cells into the brain

In all brain regions HCMV-positive cells including neuronal, glial and endothelial cells were found (Figure 25, 26, 27). In the analyzed encephalic regions HCMV-positive cells were present as both solitary and grouped together in clusters. In particular, in some cases, the clusters of HCMV-infected cells were detected as distributed along the migration pathway defined by radial glial fibers (Figure 28).

Considering the number of HCMV-infected cells counted in each fold over the different brain regions, the mean values of HCMV-positive cells detected in the case with severe and moderate encephalic injury were higher than that found in the fetuses with mild brain damage: 2.49 cells (range: 0-9 cells) and 1.57 cells (range: 0-23 cells) versus 0.22 cells (range: 0-11 cells).

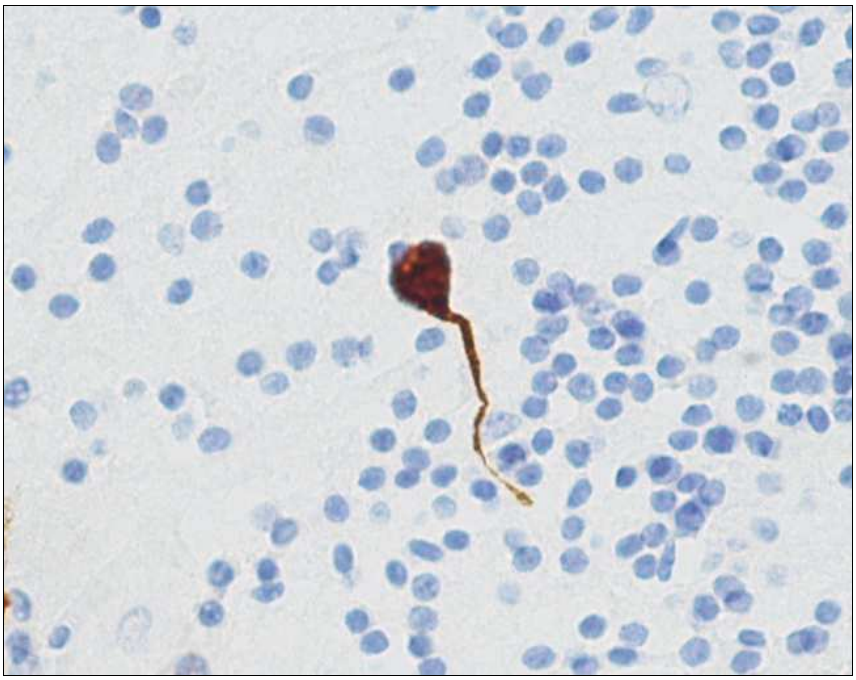


Figure 25. HCMV-infected neuronal cell (brown-stained pyrenophora and axon) detected by IHC in hippocampus (40 HPF).

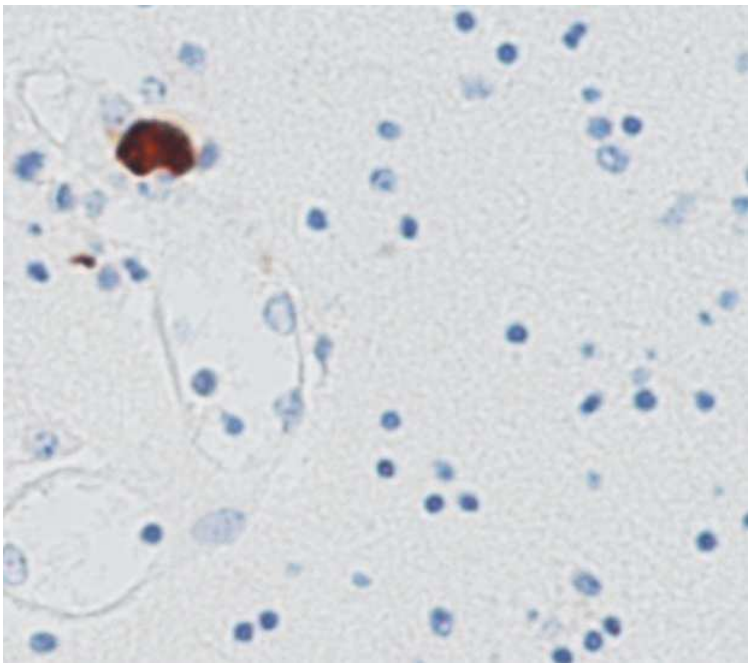


Figure 26. HCMV-infected endothelial cell (brown-stained cell in the wall of vassal) detected by IHC in white matter of temporal lobe (40 HPF).

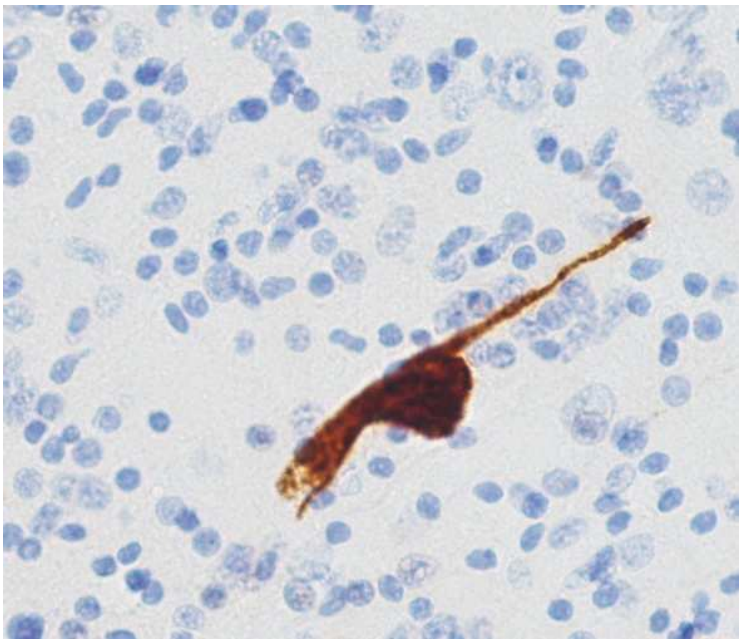


Figure 27. HCMV-infected radial glial cell (brown-stained cell with apical and basal processes) detected by IHC in white matter of temporal lobe (40 HPF).

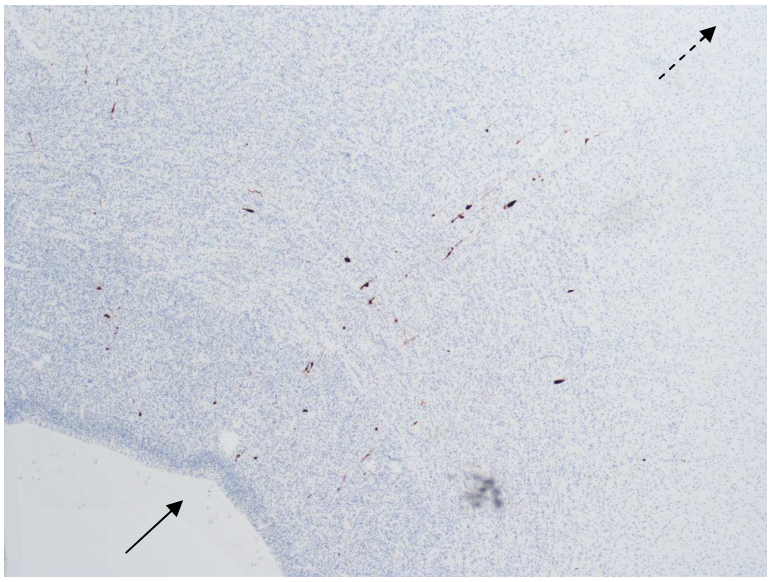


Figure 28. HCMV-infected cells (brown-stained cells) along the migration pathway defined by radial glial fibers from subventricular zone (filled arrow) to cortex (dashed arrow) in temporal lobe (4 HPF).

Analyzing the mean values of HCMV-positive cells in the different brain regions, results similar to those obtained for the quantification of tissue viral load were observed. In fact, the highest mean value of HCMV-infected cells was found in the hippocampus with a value equal to 2.9 cells (range: 0-23 cells) (Figure 29). However, in the analysis of HCMV-positive cell distribution in the brain, also the subventricular zone was included. This area as well as the hippocampus, during fetal life contains high proportion of immature and proliferating brain cells. After the hippocampus, the highest mean value of HCMV-positive cells was detected in subventricular zone, including the periventricular zone in each lobe and the ganglionic eminence, with value equal to 1.8 cells (range: 0-19 cells). In addition, the number of HCMV-infected cells observed in the periventricular region (germinal matrix) were evaluated in each brain lobe and compared with those detected in the cortical area and in white matter (Figure 30).

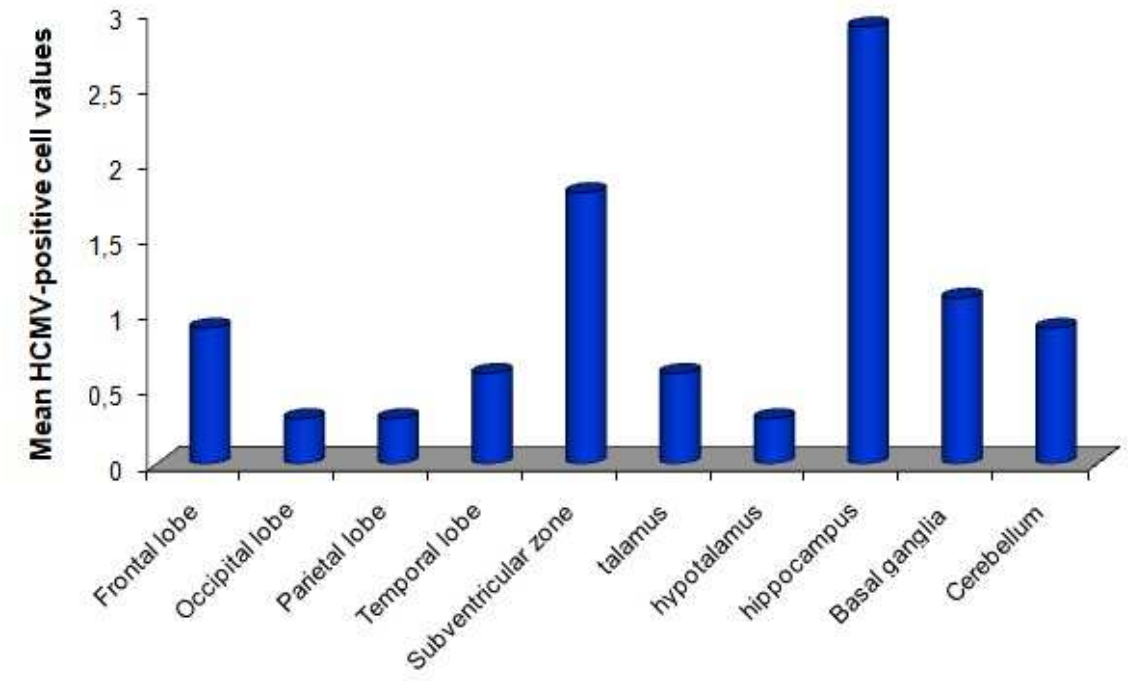


Figure 29. Distribution of mean HCMV-infected cell values in different brain areas.

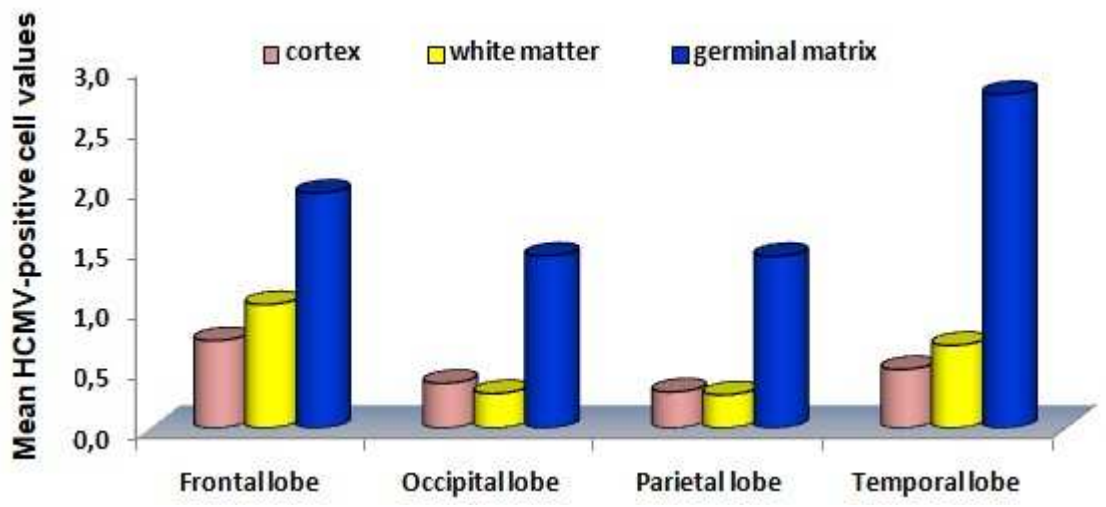


Figure 30. Mean value of HCMV-positive cells in the germinal matrix, white matter and cortical area of each brain lobe.

In each brain lobe, the mean value of HCMV-positive cells detected in the germinal matrix were higher than those observed in the cortical area and in white matter (3.5 cells in germinal matrix [range 0-19 cells], 0.8 cells in white matter [range: 0-7 cells] and 0.5 cells in cortex [range: 0-4 cells]). In particular, the highest mean value of HCMV-positive cells was observed in the periventricular region of temporal lobe. Finally, analyzing the distribution of HCMV-infected cells in the cerebellum, the highest mean value of HCMV-positive cells was found in the white matter (Figure 31).

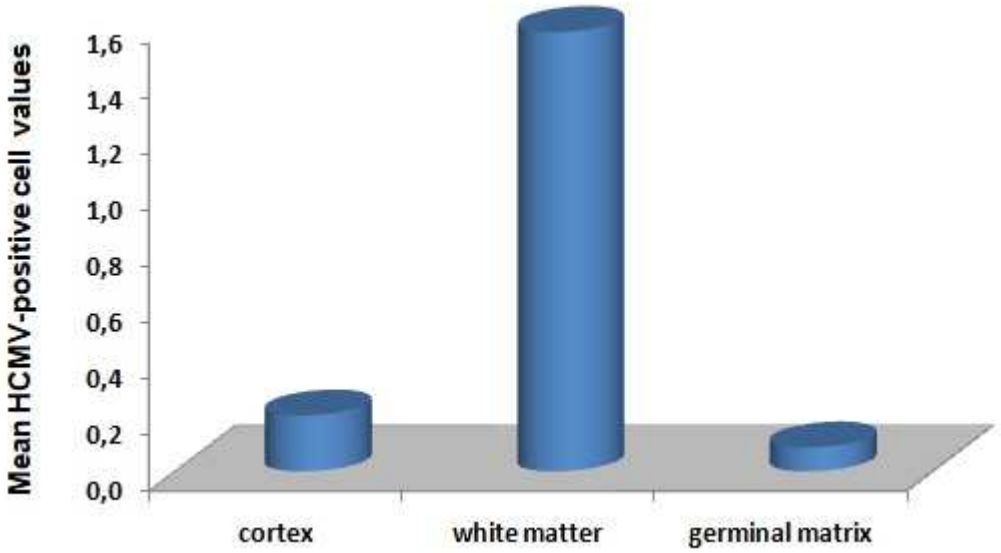


Figure 31. Distribution of mean values of HCMV-positive cells in the cerebellum

6.5 Developmental stage of the HCMV-infected neural/neuronal cells

The differentiation stage of HCMV-infected neural/neuronal cells was evaluated in 6 out of the 8 fetuses with HCMV-positive cells in the brain, cases: 1,2,5,7,8,9. The results, obtained by double immunohistochemical staining for simultaneous detection of HCMV-antigens and nestin as marker identifying neural cells in early stage of differentiation, showed that the 63.3% (441/646) of HCMV-positive cells expressed nestin (Figure 32).

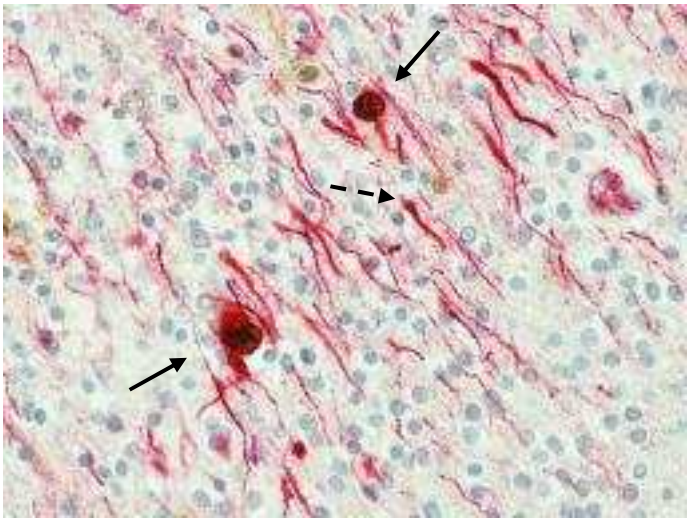


Figure 32. HCMV-positive cells expressing nestin and HCMV-antigens are identified by both red and brown staining (filled arrow); uninfected cells expressing only nestin are identified by red staining (dashed arrow). Subventricular zone (40 HPF)

In the brain of studied cases, nestin was mainly expressed in the subventricular zone and hippocampus, while in the white matter and cortex was weakly detected. In thalamus, hypothalamus and basal ganglia, this marker was not expressed. However, the positive cells for both HCMV-antigens and nestin were found in all brain regions, including the area where nestin was not expressed by non infected neural cells (Figure 33 and 34).

Considering the results obtained using DCX as marker of neuronal precursor cells, almost all HCMV-positive cells showed to express this marker: 94% (Figure 35).

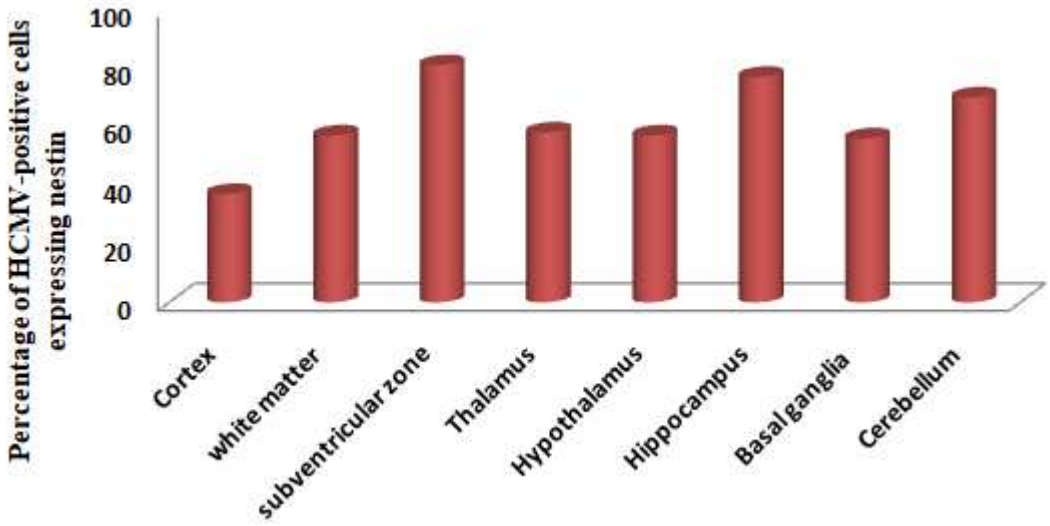


Figure 33. Percentage of HCMV-positive cells expressing nestin in different brain regions. The HCMV-positive cells in the different lobes were analyzed and reported all together as mean values of HCMV-positive cells in cortex and white matter.

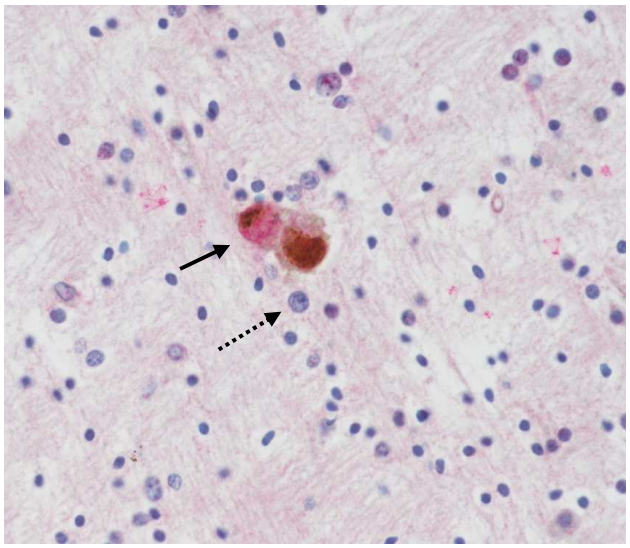


Figure 34. HCMV-positive cell expressing nestin and HCMV-antigens (brown and red stained, filled arrow). The cell is surrounded by uninfected cells that do not express nestin (dashed arrow). White matter, neuronal migration zone (40 HPF).

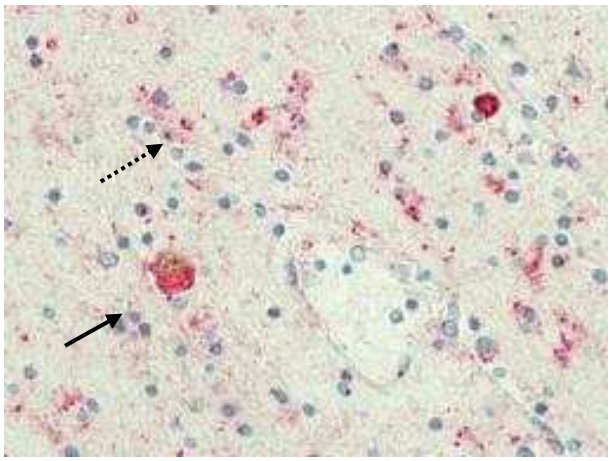


Figure 35. HCMV-positive cell expressing DCX and HCMV-antigens (red and brown stained, filled arrow) surrounded by uninfected cells expressing only DCX (red stained, dashed arrow). Hippocampus (40 HPF).

In the brain of studied fetuses, DCX was diffusely expressed in each region, moreover, cells positive for both HCMV-antigens and DCX were detected in all encephalic areas.

Finally, no HCMV-positive cells expressing NeuN was found in the brain of studied fetuses (Figure 36). This marker, used to identify mature neurons, were mainly detected in the cortex, it was less express in white matter and was not found in subventricular zone and hippocampus of studied cases.

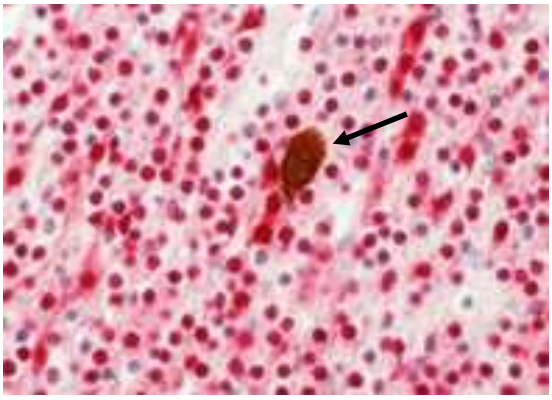


Figure 36. HCMV-positive cell not expressing NeuN (brown cell, arrow) surrounded by uninfected cells positive for NeuN (red cells). Cortex, temporal lobe (40 HPF)

When comparing the same brain region for fetuses with different degree of brain damage, in the case with severe encephalic injury, the expression of NeuN resulted almost absent compared to fetuses with moderate or mild brain damage (Figure 37).

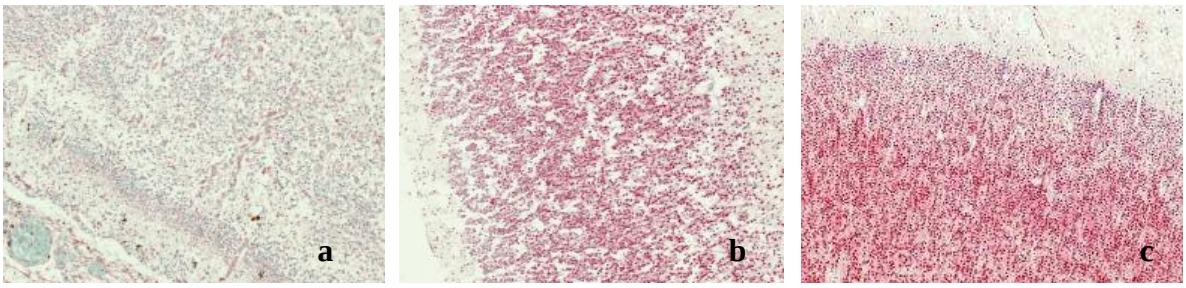


Figure 37. Expression of NeuN (red cells) in cortical area of temporal lobe in case with severe (a) moderate (b) and mild (c) encephalic damage. (4 HPF)

6.6 Control case

In the brain of control case, no pathological findings observed in the encephalon of cases with cCMV infection were found.

The nestin resulted expressed mainly in hippocampus and subventricular zone, it was weakly detected in the white matter and cortical area and it was absent in the thalamus, hipotalamus and basal ganglia.

DCX was diffusely found in all brain regions, while NeuN was mainly detected in the cortical area, it was less present in white matter, thalamus and hypothalamus and absent in subventricular zone and in the hippocampus.

CHAPTER 7

Discussion and conclusion

7.0 Discussion and conclusion

Although cCMV infection is the leading cause of significant damage in brain development, little is known about the neuropathogenic mechanisms by which viral infection lead to human fetal cerebral injury [49]. The knowledge on the neuropathogenesis behind cCMV-related neurological disabilities mainly derives from studies performed on animal models of cerebral infection and cultured human brain cells [49,133].

In the present study, brain tissues from 10 human fetuses at 21 weeks of gestation with cCMV infection were analyzed in order to provide information on encephalic viral replication and on pathogenesis of HCMV-induced brain injuries.

The results show encephalic viral infection in 80% (8/10) of studied fetuses, proved by the detection of HCMV-positive cells and HCMV-DNA in the brain. In the remaining two cases (20%), no evidence of encephalic infection was found, suggesting that not all HCMV-infected fetuses show cerebral involvement of viral replication, as reported in previous studies [52,54,60]. In the subsequent examination, the attention was focused on those 8 cases with HCMV-positive cells and HCMV-DNA detected in the brain. In these cases, different encephalic regions (cortical area and underlying white matter of frontal, occipital, parietal and temporal lobes, subventricular zone, thalamus, hypothalamus, hippocampus, basal ganglia and cerebellum) were analyzed. Taking into consideration the frequency and severity of brain pathological findings in these fetuses, a different degree of encephalic injury was found and classified as severe, moderate and mild in 1 (1/8, 12.5%), 3 (3/8, 37.5%) and 4 (4/5, 50%) cases, respectively. In particular, astrocytosis, microglial activation and vascular changes were diffusely found in the encephalon of all 8 fetuses, showing a disseminate inflammatory response [134,135,136]. Microglial nodules were also observed in all analyzed brains, however their distributions were occasional in cases with mild injury and multiple in those with severe/moderate damage. This histological lesion is a neuropathological characteristic of viral CNS infection [137-142]. In the encephalitis caused by HSV-1 and HCMV, the microglial cells, in the nodules, are involved in phagocytosis of degenerating infected brain cells which are killed by CD8+ cytotoxic T-

lymphocytes [137,140,142]. The presence of activated CD8+ T-cells in microglial nodules, containing HCMV-positive cells, were also demonstrated in a previous study, where the encephalic inflammatory infiltrate, in human fetuses with cCMV infection, were characterized [60]. In addition to the above described pathological findings, in the studied case with severe brain damage, an extensive cortical necrosis with diffuse macrophage infiltration, polymicrogyria and periventricular leukomalacia, were also found. The necrosis was mainly localized in cortical layer III. This lesion in the cortex of HCMV-infected fetuses was detected in a previous study and characterized by inflammatory and apoptotic cells with and without viral inclusion; it could be due not only to direct viral replication, but also to hypoxic condition caused by placental injury HCMV-induced [60].

Except for cortical necrosis and periventricular leukomalacia detected in fetuses with severe encephalic injury, the remaining histological findings (microglial nodules, astrocytosis, microglial activation and vascular changes) were not localised in specific brain areas, but uniformly distributed, probably representing a diffuse inflammatory reaction into the encephalon.

Among the 4 cases with severe/moderate brain damage, the invasive prenatal diagnosis showed a high viral load in amniotic fluid, with values more than 10^6 copies/mL in all cases (100%). In the 50% of cases, pathological findings were detected by non-invasive prenatal diagnosis (ultrasound examination). The data is in agreement with literature reporting that the presence of high viral loads in the amniotic fluid, sampled at the appropriate time, combined with ultrasound evidence of abnormalities in the CNS, are highly suggestive of fetal cCMV infection with poor outcome [72,143,144].

Analyzing the brain tissue viral load, higher median HCMV-DNA values were found in cases with severe/moderate encephalic damage compared to the value detected in case with mild brain injury. Overlapping results were found evaluating the mean values of HCMV-positive cells. This confirms a correlation between the degree of damage and the level of viral replication, as already reported [60]. In addition, in the brain, a different distribution of tissue viral load through the various studied regions was observed and the highest median level of viral genome were identified in the hippocampus (212 copies/5ng hDNA, range: 10-7’505 copies/5ng hDNA). This area showed the highest median HCMV-DNA

values also stratifying the tissue viral load distribution in correlation with the encephalic damage. Moreover, evaluating the localization of HCMV-positive cells in the brain, the highest mean value of HCMV-infected cells was detected again in the hippocampus. This cerebral region resulted to be mainly involved also when brain infection was caused by other neurotropic viruses, such as HSV-1. In fact, several studies, focused on acute encephalitis due to HSV-1, reported a pathological involvement of select brain regions, such as the limbic structure, where the hippocampus is a preferential and highly susceptible target of infection. To date, the tropism of HSV-1 for this cerebral area remains unexplained [145-149]. A preferential replication of CMVs in the cells of dentate gyrus (in the hippocampus) and cerebral subventricular zone, were observed mainly in animal models and in culture cell experiments [100,150]. In human subjects, the main localization of HCMV infection in these cerebral regions was proven in few studies, some of which involving adult immunocompromised patients and premature infants with lethal cCMV infection [150,151].

In the 8 studied fetuses, the tissue viral load in subventricular zone was not evaluated due to difficulties in the dissection of the very thin periventricular region from 8 micron sections. However, the analysis of HCMV-positive cells, in this cerebral area, showed the highest mean value of HCMV-infected cells (1.8 cells, range: 0-19 cells) after those detected in hippocampus (2.9 cells, range: 0-23 cells). In addition, considering the part of subventricular zone (germinal matrix) limited to four cerebral lobes, the highest mean value of HCMV-positive cells detected in each lobe was found in the germinal matrix with respect to cortex and with matter, suggesting a main viral replication in germinal matrix.

Yin X et al., studying human fetuses at different weeks of gestation, observed that during fetal life, the highest proportion of neural/neuronal precursor cells reside in the hippocampus, followed by the amount of these cells detected in subventricular zone [119]. Therefore, taking into account these findings, the highest viral load detected in hippocampus and the higher HCMV-infected cell values found in both hippocampus and in subventricular zone, could indicate a preferential HCMV replication in the cerebral areas where neural/neuronal precursor cells reside. Murine models of MCMV infection, showed that the immature neural cells were the main target of viral replication [152,153].

Moreover, studies performed on human neural precursor cell cultures, demonstrated the greatest susceptibility to HCMV of these cells, in which the effects of viral replication depend on their differentiation state [129,130,154].

In order to confirm these evidences, in 6 out of 8 studied fetuses, that showed different degree of brain damage, the differentiation stage of HCMV-infected neural/neuronal cells was investigated by double immunohistochemical staining for the detection of HCMV-antigens with markers of mitotic and postmitotic neural/neuronal cells. The results showed that the main neural/neuronal marker expressed by HCMV-positive cells was DCX (94%), the antigen identifying the immature neuronal cells with a determined lineage (as neuroblasts). Nestin, that is the marker detecting the neural cells in an early stage of differentiation, was found in the 63.3% of HCMV-positive cells, while no HCMV-infected cell resulted positive for NeuN, the antigen expressed by mature neurons. These findings showed that the HCMV-infected cells were mainly neural and precursor neuronal cells, confirming the preferential tropism of HCMV for immature and proliferating cells of neuronal lineage. On the contrary, the total absence of HCMV-infected cells expressing NeuN, could be explain by the evidence obtained in human cell culture studies, showing that the mature neurons are refractory to HCMV replication [49].

The HCMV-positive cells that did not express neural/neuronal markers, probably were cells different from those of neuronal lineage. Since the HCMV-infection cause cytomegalic effect in the cells, these cells are not morphologically identifiable and specific antigens expression could be detected to identify the type of cell HCMV-infected. However, in this study, only a few infected glial and endothelial cells were identified by their localization and by the presence of peculiar cell structure. This is in agreement with literature, which reports the ability of HCMV to infect different type of cells in the brain [60].

The high proportion of HCMV-positive cells expressing DCX could reflect the diffuse expression of this marker in all brain regions of fetuses with cCMV infection. The same distribution of neural precursor cells was found in both control case and literature data regarding fetuses at 21 weeks of gestation. However, it could also speculate that the massive DCX expression among the HCMV-positive cells may be due to a preferential viral replication in neuronal precursor cells rather than in neural stem cells; however, to

confirm this hypothesis other analysis should be performed (for example, using immunohistochemical staining for the simultaneous detection of HCMV-positive cells with multiple neural markers). Finally, the very high number of HCMV-positive cells expressing DCX could be also explained by an altered ability of infected neuronal precursor cells to differentiate into mature neurons. The total absence of HCMV-positive cells expressing NeuN, in the studied cases, may support this hypothesis. In addition, human cell culture studies demonstrated that the HCMV infection in immature neural/neuronal cells lead to the inhibition or delaying of differentiation process in these cells [49,129,155]. Analyzing the HCMV-infected cells expressing nestin, in correlation with the expression of this marker in the brain regions, interesting data was found on the neural/neuronal stage of differentiation. In particular, as expected for fetuses at 21 weeks of gestation, in fetuses with cCMV infection as well as in control case, nestin antigen was mainly expressed in the subventricular zone and in hippocampus, it was weakly detected in white matter and cortex and it resulted absent in the remaining areas. Despite this, HCMV-infected cells expressing nestin was found in all brain regions, also in areas where this marker was not present, confirming that HCMV infection interfere with neuronal differentiation. In addition, evaluating the NeuN expression in control case and in literature data, this marker resulted mostly expressed in the cortex and weakly present in the white matter. Nevertheless, comparing the NeuN expression in the brain with severe damage with that found in cases with moderate/mild injury, the expression of NeuN resulted almost absent in the cortex area of encephalon with severe damage. Excluding the areas affected by necrosis, in the remaining cortex and white matter areas this could be a further proof that HCMV infection interfere with neuronal differentiation.

Finally, the presence of HCMV-infected cells in the pathways of neuronal migration, confirm that these cells retain their ability to migrate, although this can occur aberrantly, as reported by Cheeran et al [49]. Moreover, because HCMV-infected cells were found in all subventricular zone, including periventricular region and ganglionic eminence, where immature cells depart for radially and tangentially neuronal migration, respectively, both migration modes could be affected. For the cerebellum, a different migration pathway is described. In fact, the neurons that reside in this brain region derived from two distinct

germinal zones: the ventricular zone and the rostral rhombic lip [155]. Therefore, the main localization of HCMV-positive cells detected in the white matter of cerebellum could be explained by the peculiar migration mode in this brain area.

In conclusion, this study demonstrated in human fetuses with cCMV infection, a preferential viral tropism for neural/neuronal precursor cells, that lose or delay their capacity to differentiate, while retain their ability to migrate. All these findings may result in reduced proliferation of immature neural cells, cellular signalling alterations and cell death and aberrant neuronal migration that lead to significant effects in the architecture and function of the fetal brain in development, such as intracranial calcification, polymicrogyria, lissencephaly and other consequences frequently detected in cCMV infection. The pathological findings detected in this study show the brain condition at 21 weeks of gestation and it is difficult to establish the later evolution of the injury. However, the fetus with severe pathological findings could have had a poor outcome, since necrosis, periventicular leukomalacia and polymicrogyria are parenchymal brain lesions associated with serious, permanent neurological manifestations.

In the light of these results that show the direct role of HCMV in the pathogenesis of brain damage cCMV-related, additional features of cerebral injury will have to be investigated in all encephalic regions, such as the effects of the immune responses and the development degree of additional infected cells type involved in the neurogenesis, as the glial cells.

List of abbreviations

BBB: blood brain barrier

cCMV: congenital human cytomegalovirus

CMVs: cytomegaloviruses (human and non-human)

CNS: central nervous system

CP: cortical plate

CT: computed tomography

DCX: doublecortin

ECs: endothelial cells

HCMV: human cytomegalovirus

hDNA: human DNA

HLA: human leukocyte antigen

HPF: high-power field

HSV-1: herpes simplex virus -1

MCMV: murine cytomegalovirus

NeuN: neuronal nuclei

PMNL: polymorpho-nuclear leukocytes

PCR: polymerase chain reaction

SNHL: sensorineural hearing loss

US: ultrasonography

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