UNIVERSITAT DE BARCELONA

Assessment of prenatal imaging, fetal blood parameters, and new pharmacological interventions, in congenital cytomegalovirus infection

Ameth Hawkins Villarreal

Doctoral Thesis

Assessment of prenatal imaging, fetal blood parameters, and new pharmacological interventions, in congenital cytomegalovirus infection

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Declaration

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declare that **Ameth Hawkins-Villarreal** has performed under my supervision the studies presented in the thesis **"Assessment of prenatal imaging, fetal blood parameters, and new pharmacological interventions, in congenital cytomegalovirus infection"**. This thesis has been structured following the normative for PhD thesis as a compendium of publications, to obtain the degree of **International Doctor in Medicine** and the mentioned studies are ready to be presented to a Tribunal.

Anna Goncé Mellgren

Thesis Director

Presentation

The present thesis has been structured following the normative for PhD thesis, as a compendium of publications, to obtain the degree of International Doctor in Medicine. Projects included in this thesis belong to the same research line, leading to seven articles published or submitted for publication in international journals:

1. **Hawkins-Villarreal A**, Moreno-Espinosa AL, Eixarch E, Marcos MA, Martinez-Portilla RJ, Salazar L, Garcia-Otero L, Lopez M, Borrell A, Figueras F, Goncé A. Blood parameters in fetuses infected with cytomegalovirus according to the severity of brain damage and trimester of pregnancy at cordocentesis. *Journal of Clinical Virology* (2019) ***119,*** 37-43: doi:10.1016/j.jcv.2019.08.008.
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4. **Hawkins-Villarreal A**, Moreno-Espinosa AL, Castillo K, Hahner N, Martinez-Portilla RJ, Picone O, Mandelbrot L, Simon I, Gratacós E, Goncé A, Eixarch E. Cortical maturation assessed by magnetic resonance imaging in unaffected/mildly affected fetuses with cytomegalovirus infection.
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5. **Hawkins-Villarreal A#**, Castillo K#, Nadal A, Planas S, Moreno-Espinosa AL, Alarcón A, Figueras F, Rebollo M, Eixarch E, Goncé A. What does the "halo" sign mean in fetal cytomegalovirus infection? Cerebral imaging abnormalities and postmortem histopathology in a cohort of 35 infected fetuses.
   Status: draft completed.

6. Goncé A, **Hawkins-Villarreal A**, Salazar L, Guirado L, Marcos MA, Pascual-Mancho J, Prats P, Lopez M, Eixarch E, Salvia M-D, Fortuny C, Figueras F. Maternal high-dose valacyclovir and its correlation with newborn blood viral load and outcome in congenital cytomegalovirus infection. *J Matern Fetal Neonatal Med*. 2020 Nov 3;1-5. doi: 10.1080/14767058.2020.1843016.
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   Impact Factor: 8.678, 1st quartile

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LIST OF ABBREVIATIONS

AC	Abdominal circumference
ACV	Acyclovir
AF	Amniotic fluid
ANOVA	Analysis of variance and covariance
AUC	Area under the curve
BPD	Biparietal diameter
CCW	Crania cortical width
CNS	Central nervous system
CM	Cisterna magna
cCMV	Congenital cytomegalovirus
CI	Confidence intervals
CC	Corpus callosum
CMV	Cytomegalovirus
CTD	Cerebellar transverse diameter
CTI	Cardiothoracic index
DNA	Deoxyribonucleic acid
DR	Detection rate
DVP	Deepest vertical pocket
EFW	Estimated fetal weight
ELISA	Enzyme-Linked ImmunoSorbent Assay
ET	Echo time
FBS	Fetal blood sampling
FBV	Fetal body volume
FVL	Fetal viral load
FLV	Fetal liver volume
FLV/AC	Fetal liver volume to abdominal circumference ratio
FLV/FBV	Fetal liver volume to abdominal fetal body volume ratio
FOV	Field of view
GCV	Gancyclovir
GA	Gestational age
GGT	Gamma-glutamyl transpeptidase
GPR	Gamma-glutamyl transpeptidase to platelets ratio
HASTE	Half Fourier acquisition single-shot turbo spin-echo
HC	Head circumference

HCMV	Human cytomegalovirus
HIG	Hyperimmunoglobulin
IDIBAPS	Institut d'Ínvestigacions Biomèdiques August Pi ì Sunyer
ICC	Intraclass correlation
IgM	Immunoglobulin M
IgG	Immunoglobulin G
IQR	Interquartile range
IU	International unit
IUGR	Intrauterine growth restriction
LHR	Likelihood ratio
MGA	Mean gestational age
MPI	Maternal primary infection
MRI	Magnetic resonance imaging
NPV	Negative predictive value
NSG	Neurosonography
OR	Odds ratio
PCR	Polymerase chain reaction
PPV	Positive predictive value
RCT	Randomized Control Trial
ROC	Receiver-Operator curve
RT	Repetition time
SBD	Severe brain damage
SD	Standard deviation
Se	Sensitivity
SF	Sylvian fissure
SFA	Sylvian fissure angle
SGA	Small for gestational age
SNHL	Sensorineural hearing loss
Sp	Specificity
TF-US	Transfontanellar ultrasound
TOP	Termination of pregnancy
US	Ultrasound
VCV	Valacyclovir
VMG	Ventriculomegaly
VL	Viral load
WMHS	White matter hyperintensity

SUMMARY IN SPANISH

Antecedente: El citomegalovirus humano (HCMV) es una de las principales causas de infeccione congénita en todo el mundo. La infección congénita por citomegalovirus (CMV) es una causa importante de pérdida auditiva neurosensorial, retraso en el desarrollo neurológico, epilepsia y pérdida de visión.

Hipótesis principal: la precisión diagnóstica/pronóstica prenatal para predecir qué embarazos resultarán en un feto/recién nacido sintomático en la infección congénita por CMV puede mejorar mediante una combinación de pruebas biológicas en sangre fetal, marcadores ecográficos y/o por resonancia magnética (RM).

Objetivos: Evaluar el valor pronóstico de los hallazgos por ecografía y/o por RM fetal junto con el análisis de parámetros en sangre fetal en una serie de fetos con o sin infección comprobadas por CMV. Evaluar los efectos de la terapia antiviral materna con valaciclovir (VCV) tanto para disminuir la transmisión vertical del virus (prevención primaria) como la carga viral en el recién nacido y la evolución de las lesiones ecográficas prenatales.

Metodología: estudio ambispectivo donde incluimos fetos con infección por CMV confirmada en muestra de líquido amniótico y fetos sin infección CMV. La severidad de la infección/afectación fetal se determinó de acuerdo a los hallazgos, en estudio de imagen, ya sea por ultrasonido obstétrico detallado y/o por RM, del sistema nervioso central (SNC). Los fetos fueron categorizados como afectación leve o severa.

Resultados:

1) En el primer artículo incluimos a un grupo de 28 fetos infectados por CMV diagnosticados ante la presencia de anomalías ecográficas observadas en las ecografías de rutina de segundo y tercer trimestre y en los cuales pudimos obtener una muestra de sangre fetal. Encontramos que aquellos fetos que mostraban lesiones severas/graves en el SNC presentaron niveles significativamente más elevados de gamma-glutamyl transpeptidasa (GGT) que aquellos fetos infectados con lesiones leves o sin lesión alguna en el SNC, independientemente de la edad gestacional (EG). También encontramos que aquellos fetos con lesiones graves diagnosticados en el segundo trimestre presentaron niveles significativamente más bajos de plaquetas, niveles más elevados de beta2microglobulina, nieveles más elevados de carga viral,

y presencia de IgM especifica para CMV que aquellos fetos diagnosticados en el tercer trimestre. Para la predicción de daño cerebral, los niveles de GGT ≥ 183 UI/l alcanzaron 71% de sensibilidad, 83% de especificidad (AUC: 0,78), con OR de 2,05 (IC 95%: 1,22-3,43) por 100 UI/l de incremento, ajustado por EG.

2) En el segundo estudio incluimos 26 fetos infectados (20 con daño severo del SNC y 6 con lesiones leves o sin lesión alguna) y una cohorte de 35 fetos no infectados con anomalías cardíacas y de SNC u otras anomalías (14 con daño severo del SNC, 21 con lesiones leves o sin lesión alguna) y encontramos que aquellos fetos con daño neurológico severo/grave independientemente de estar infectados o no presentaron niveles de GGT significativamentes más elevados que aquellos fetos sin lesión neurológica o con lesión leve del SNC.

2.1) En los fetos infectados, tal como era esperable, encontramos niveles de plaquetas significativamente más bajos. Esta diferencia permaneció significativa cuando se compararon los fetos con daño neurológico severo.

2.2) Los niveles de GGT ≥ 183 UI/l, ajustados por EG en cardio/cordocentesis, se asociaron con daño neurológico severo con un OR de 17 (IC 95%: 3,9-72) en ambos grupos

2.3) El ratio GGT/plaquetas (GPR) ajustado por la EG en el momento de la cardio/cordocentesis se asoció con daño neurológico severo con un OR de 3,10 (IC 95 %: 1,30-7,45) por cada 10 UI de aumento de GPR en el grupo infectado por CMV. En comparación con los niveles de GGT por si solos, encontramos una tendencia hacia un mayor rendimiento para la predicción de daño neurológico severo en el grupo infectado por CMV con un GPR≥12 logrando: sensibilidad del 84%, especificidad de 83%, razón de verosimilitud positiva de 5.1 y negativa de 0.19 (AUC: 0.92), p=0.07.

2.4) Al analizar los resultados de acuerdo a una probable plausibilidad biológica encontramos que aquellos fetos infectados por CMV y con daño severo del SNC presentaban los niveles más elevados de GGT y GPR, p<0.001.

3) En el tercer artículo incluimos un total de 32 fetos infectados (uno no mostró hallazgos anormales en ecografía/RM, 16 fetos tenían hallazgos de infección no graves y 15 tenían anomalías cerebrales graves). Como grupo control incluimos 33 fetos sanos. La mediana del volumen hepático fetal medido por RM en los fetos infectados no fue significativamente diferente de el volumen hepático en fetos sanos.

El hallazgo fue similar cuando los fetos infectados fueron comparados de acuerdo a la severidad del daño neurológico. Al ajustar el volumen hepático por la circumferencia abdominal y el volumen corporal total encontramos que los fetos infectados mostraban volumenes significativamente mayores que el grupo control.

4) En el cuarto estudio se incluyeron 24 fetos infectados por CMV y 24 fetos sanos. En los fetos infectados, observamos una medición ecográfica del ventrículo lateral cerebral significativamente mayor, un surco parietooccipital y un surco calcarino significativamente menos profundos, un ángulo de fisura de Sylvio significativamente mayor (superior/inferior); y una puntuación subjetiva de maduración cortical significativamente más baja en el área temporal, el área parietal, el surco parietooccipital y el surco calcarino ($p<0,05$).

5) En el quinto estudio, el signo ecográfico del halo, o hiperecogenicidad periventricular, se detectó en 32 de los 35 fetos analizados (91%). El signo del halo fue el único hallazgo ecográfico en 6 fetos, todos del segundo trimestre. Todos los fetos con halo aislado mostraron un grado leve/moderado de ventriculitis, pero la ventriculitis extensa se observó en mayor proporción en el grupo sin halo y con halo aislado ($p= 0,032$). Todos los fetos con un halo aislado se clasificaron como estadio histológico I sin signos de calcificación cerebral, necrosis de la sustancia blanca o lesión cortical. Por otro lado, todos los fetos con halo no aislado, excepto en un caso, y aquellos con halo ausente, presentaron lesiones cerebrales severas (Estadio II). El 62% de los fetos con halo no aislado y el 100% de los fetos con halo ausente presentaron lesiones cerebrales graves macroscópicas.

6) En el sexto estudio, se evaluó la nueva intervención farmacológica con VCV a dosis elevadas (8 g/24 horas) en fetos infectados: en 8 pacientes embarazadas con infección fetal confirmada se administró el fármaco a una EG mediana de 26,5 semanas (23,8-33,1) en 3 casos sin anomalías ecográficas fetales del SNC y en 5 con anomalías leves/moderadas. El VCV se administró durante una mediana de 10 semanas y fue bien tolerado. Las anomalías ecográficas fetales no progresaron. Al nacer, 3 recién nacidos eran asintomáticos y uno estaba gravemente afectado (coriorretinitis bilateral). La mediana de la carga viral neonatal fue de 502 UI/ml (231-191781) con niveles más bajos cuando se administró tratamiento materno ≥10 semanas, y en casos sin anomalías ecográficas fetales, siendo estas diferencias no significativas.

7) En el último estudio se valoró la utilidad del VCV a dosis elevadas (8 g/24 horas) como tratamiento de prevención secundaria de la infección fetal por CMV. Se incluyeron 143 gestantes con infección primaria, 59 en el grupo VCV y 84 en el grupo control. Después del análisis ajustado por coincidencia de puntuación de propensión "propensity score matching", el tratamiento con VCV se asoció significativamente con una reducción general en la tasa de transmisión materno-fetal (OR = 0,40; IC del 95 %: 0,18-0,90, p= 0,03). Después de la infección primaria periconcepcional, la tasa de transmisión materno-fetal al nacer fue del 7 % en el grupo VCV frente al 10 % en el grupo control (p=1,00); 22 % frente a 41 % después de la infección primaria materna en el primer trimestre (p=0,07) y 25 % frente a 52 % después de la infección primaria materna en el segundo trimestre (p =0,24). Al analizar la eficacia del tratamiento según la viremia materna al inicio del tratamiento, hubo una tendencia hacia una mayor eficacia cuando la viremia fue positiva (21% vs 43%, p=0,07) en comparación a los casos con viremia negativa.

Conclusiones:

1) En los fetos infectados por CMV, la trombocitopenia y los niveles elevados de GGT se asociaron con anomalías cerebrales graves en ecografía/RM. Sin embargo, entre los fetos gravemente afectados, los parámetros sanguíneos, con excepción de la GGT, cambian según la edad gestacional. La sangre fetal podría ser menos predictiva de daño cerebral en el tercer trimestre.

2) Los niveles elevados de GGT se asociaron con daño cerebral severo en fetos infectados y no infectados por CMV. Sin embargo, la infección puede desempeñar un papel, ya que el aumento fue significativamente mayor entre los infectados. Debido al recuento de plaquetas más bajo en los fetos infectados, el ratio GPR puede mejorar la tasa de detección de daño neurológico severo en este grupo.

3) Aunque el aumento de volumen del hígado fetal no se correlacionó con la gravedad de la infección, estos parámetros (volumen hepático corregido por la circumferencia abdominal y el volumen corporal total) podrían usarse como un nuevo marcador del agrandamiento del hígado. Se necesitan más estudios para comprender mejor el valor pronóstico de estos hallazgos.

4) Los fetos infectados por CMV (sin afectación ecográfica y con afección leve) mostraron una maduración cortical retardada en comparación con los controles sanos

5) En fetos infectados por CMV, se observó un signo de halo aislado sólo en el segundo trimestre y se asoció con ventriculitis leve y nódulos microgliales sin signos de necrosis de la sustancia blanca. Aunque observamos una baja probabilidad de daño cerebral severo por histopatología, su valor para la evaluación pronóstica debe confirmarse con un seguimiento ecográfico fetal y una RM cerebral fetal al inicio del tercer trimestre.

6) Las lesiones fetales por CMV permanecieron estables con altas dosis de VCV materno. La carga viral del recién nacido se mantuvo sin cambios significativos independientemente de la duración del tratamiento y de las anomalías fetales/neonatales.

7) El tratamiento con VCV a altas dosis en mujeres embarazadas con infección primaria por CMV en el primer y segundo trimestre reduce el riesgo de transmisión al feto.

1. INTRODUCTION

1. INTRODUCTION

Cytomegalovirus (CMV) remains a major cause of congenital infection and disease during pregnancy with around 0.2-2% newborns being infected at birth.[1,2] Congenital infection with Human CMV (cHCMV) is the first cause of non-genetic sensorineural hearing loss (SNHL), and an important cause of neurological impairments, vision loss and neurodevelopmental delay.[2–4] The prevalence of cCMV varies according to the population, with an estimated 40 to 100% of individuals being infected.[3] The seroprevalence tends to be higher in lower socio-economic groups, racial and ethnic minority populations, and women of higher parity and advanced maternal age.[2,5] CMV infection is acquired through close contact with saliva and urine mostly from young children and through sexual intercourse. After CMV primary infection, the virus can be continuously or intermittently excreted for months or years.[6] Fetal infection may occur after primary as well as after non-primary maternal infection (reactivation or reinfection by a different strain).[6,7] As a result of the high seroprevalence, there is a continuously high reservoir of CMV in the population. Non-immune pregnant women with young children in daycare are at higher risk of infection due to high rates of horizontal transmission through saliva among young children. Since clinical symptoms are usually absent, the only reliable way to estimate the prevalence of infection in a population is through laboratory testing.

CMV can be transmitted from mother to the fetus anytime during gestation (in-utero, intrapartum, or during breast-feeding) but is most likely to cause serious permanent damage to the fetus when the mother develops infection during the first trimester of pregnancy.[4,7] Intrauterine transmission is thought to be the result of trans-placental crossing of the virus, which then replicates in multiple embryonic or fetal tissues.[8] After primary infection the risk of vertical transmission is around 32%[9–12], being much lower (≤3.5%) after non-primary infection[13]. According to Chatzakis et al. the pooled rates of vertical HCMV transmission with maternal primary infection (MPI) are 5.5%, 21%, 37%, 40% and 66% in the preconception period and periconception periods, and the first, second and third trimester, respectively. The rate of fetal insult after MPI was higher in the preconception period and first trimester of pregnancy with 29% and 19%, respectively; whereas in the second and third trimester the pooled rate of fetal insult was not greater than 1%.[12] In MPI with transmission to the fetus during the first trimester of pregnancy[12], the percentage of congenitally infected

symptomatic children with permanent sequelae is estimated to be 28%, whereas the percentage of children without symptoms at birth who later develop hearing loss is estimated to be 13.5%.[4,5,14]

Both, the diagnosis of primary infection early in pregnancy and the presence of compatible fetal signs on ultrasound (US) allow the diagnosis of CMV infection in the fetus. Viral DNA detection by polymerase chain reaction (PCR) in the amniotic fluid has been recognized as the reference method for the diagnosis of fetal infection.[15] To achieve an accurate diagnosis the procedure must be performed after 17 weeks of pregnancy and at least 8 weeks after maternal infection.[16,17] The sensitivity of PCR in amniotic fluid has been reported to be between 90 to 95% with a 5 to 10% false negative rate explained by late trans-placental passage of the virus.[14]

The morbidity of the cCMV in infancy, including hearing loss and neurodevelopmental delay, has been associated with abnormal prenatal US and magnetic resonance imaging (MRI) findings. However, direct extrapolation of the neonatal syndrome to the fetus is difficult.

1.1 Prognostic markers of fetal infection

Once the diagnosis of fetal infection is achieved, the prognosis is established based on a combination of fetal US and MRI findings *(Table 1)* although fetal laboratory tests can also play a role. Prenatal imaging in cCMV is primarily focused on the brain, given the neurotropism of the virus.[18,19] Imaging in cCMV has two main objectives: detection of fetal structural anomalies for correct diagnosis and provision of prognostic information.

1.1.1 *Prognostic value of US findings*

Fetal imaging with US has been the method of choice for antenatal detection of fetal anomalies. The role of US examination in predicting the presence of symptoms at birth has been highlighted by some authors. However, this has been questioned by other groups who found the sensitivity of US in predicting symptomatic newborns to be only 21%.[25] Cerebral anomalies observed with US can be assumed to carry a poor prognosis,[21] but this assumption does not necessarily hold for ascites, hyperechogenic bowel, and hepatomegaly.[22]

The prevalence of central nervous system (CNS)-US findings in fetal CMV after a MPI in the preconception period and first trimester is around 40%[12]. Several fetal cerebral abnormalities have been associated with a poor prognosis, with an odds ratio (OR) for a poor outcome as high as 41.[23] Brain involvement might be delayed up until late in pregnancy and the prognostic value of US at diagnosis is expected to be lower than the prognostic value of imaging obtained later in pregnancy. The negative predictive value (NPV) of US features noted at the time of diagnosis was as high as 93%.[24] The predictive value of non-severe US abnormalities has been evaluated in only 2 studies, with odds ratios (ORs) of 7.4 and 18.4 for poor outcome when showing extra-cerebral symptoms, with a positive predictive value (PPV) of 54% to 60%.[23,24] Faure-Bardon *et al*. also reported that the NPV in CMV-infected fetuses following MPI in the first trimester with a normal second trimester assessment for the prediction of symptoms at birth and for the prediction of moderate to severe sequela is 73% and 82%, respectively. When extracerebral findings were observed they found a PPV of almost 50% for the prediction of symptoms at birth and 30% for the prediction of moderate to severe sequela.[25]

The identification of anomalies in the context of fetal CMV infection should prompt a thorough targeted US examination of the brain in search of features that can predict poor outcomes.[19,26] A normal neurosonographic examination performed by expert sonographers, especially at around 32 weeks, is a good predictor of normal neurodevelopmental outcomes.[25,27–29]

Table 1. Classification of prenatal US and MRI abnormalities in congenital CMV infection

Severe US/MRI brain abnormalities*	Mild US/MRI brain abnormalities**	Extra-cerebral US abnormalities**
Severe ventriculomegaly (≥ 15 mm)	Intraventricular adhesions	^ Hyperechogenic bowel
Microcephaly (HC ≤ -3 SD)	Mild ventriculomegaly (10-14.9 mm)	^^ Intrauterine growth restriction
Periventricular hyperechogenicity	Isolated calcifications	† Hepatomegaly (right lobe ≥ p95)
Enlarged sub-arachnoid space (CCW>95th centile)$^{\int}$ (Microencephaly)	Calcifications of lenticulostriate vessels in basal ganglia	Intra-hepatic calcifications
Porencephaly (porencephalic cysts)	Sub-ependymal cysts	Pleural effusion
Corpus callosum agenesis/dysgenesis	White matter hyperintensity (MRI)	Ascites
Corpus callosum hypoplasia (CC <5th centile)		Pericardial effusion

Cerebellar/vermian hypoplasia (CTD <5th centile) or cerebellar hemorrhage	Fetal hydrops
Abnormal gyration / Cortical dysplasia (MRI)	† Cardiomegaly (CTI ≥ 3.4 cm)
	Oligohydramnios (DVP ≤ 2 cm)
	Polyhydramnios (DVP ≥ 10 cm)
	Placentomegaly ≥ 40 mm

** Lesions with Poor prognosis* *** Lesions with Uncertain prognosis* **** Lesions with Good prognosis*

DVP: Deepest vertical pocket

^ Considered when the echogenicity of the bowel is equal or more intense than that of the fetal bones

^^ Considered when the estimated fetal weight is below the 10th centile according to specific population tables with or without Doppler ultrasound alterations.

*† Hepatomegaly is considered when the right hepatic lobe length is ≥p95 * Vol. 39, N0. 2, February 2011*

† CTI: considered abnormal if the heart is more than one-third of the thoracic diameter

∫ Malinger et al. Prenat Diagn 2000 Nov;20(11):890-3

US: ultrasound, MRI: magnetic resonance imaging, HC: head circumference, SD: standard deviation, CCW: cranio-cortical width, CC: corpus callosum, CTD: cerebellar transverse diameter, CTI: Cardiothoracic Index

Adapted from: 1- *Leruez-Ville et al. Prognosis evaluation of fetal CMV infection. Am J Obstet Gynecol 2016;215:342.e1-9.*

2- *Goncé et al. TORCH and B19 Parvovirus infections during Pregnancy.* www.fetalmedbarcelona.org

Although a targeted scan when the operator is aware of fetal CMV has a high sensitivity, and PPV and NPV values[9,30], it should be stressed that a scan performed as part of routine antenatal care has shown to have a poor sensitivity for fetal infection (15%) and a poor PPV for long-term sequela (28-35%).[9,20]

1.1.2 *Prognostic value of MRI findings*

MRI can provide important additional information especially of the cortical structure, cerebellar growth and white matter signaling. Moreover, it can also clarify the spectrum of neurological findings related to the time of fetal infection during pregnancy. It is certainly useful in the assessment of fetuses with extracerebral features without brain abnormalities detected with US. MRI can also be a useful contribution for prenatal brain imaging, particularly in the late second and third trimester of pregnancy, especially when US suggests a potential brain anomaly or lesion[31]. Previous studies have proposed fetal MRI as a supplementary and/or complementary tool in the detection of brain anomalies in several circumstances and has been utilized for the evaluation of structural and functional characteristics including metabolic profile of the brain[19,31–36]

The prediction of neurological impairment and SNHL in CMV infection by MRI is feasible between 27 and 33 weeks of gestation and has been reported to have a high NPV (96.8%-99%).[37,38] CMV can infect a wide variety of brain cells, including

neurons, astrocytes, microglia and endothelial cells, and may be involved in different mechanisms of injury resulting in altered neuronal proliferation, migration, and cortical cell organization.[18,39–41] MRI findings in cCMV are often unspecific, with ventriculomegaly and white matter hyperintensity being the most commonly described CNS anomalies.[19]

Other authors have described an MRI pattern predictive of congenital CMV infection consisting of multifocal lesions predominantly involving deep parietal white matter, with or without gyral abnormalities. When gyral abnormalities are present, leukoencephalopathy may also be diffuse. The presence of abnormalities in the anterior part of the temporal lobe increases the likelihood of the presence of CMV infection.[42]

Cannie *et al*.[37] developed an MRI grading score in a series of CMV infected fetuses ranging from normal findings (grade 1), the presence of white matter hyperintensity (WMHS) (grade 2 and 3), and cysts in the temporal and/or occipital lobe (grade 4) to the presence of migration disorders, cerebellar hypoplasia, and microcephaly (grade 5). According to regression analysis the time of MPI and MRI grading were independent predictors of SNHL. Furthermore, MRI grading was the only predictor of neurological impairment. Regarding the importance of WMHS, they observed that SNHL was associated with isolated hyperintensity in the temporal lobes in 14% of fetuses. No association was found between neurological impairment and isolated hyperintensity, and was, thus, considered a good postnatal prognosis. The presence of cysts in the MRI (grade 4) was associated with SNHL in 55% of the cases and with neurological impairment in 25%. Since SHNL can affect the development of language and communication and therefore the overall development in childhood[43,44], the ability to predict SHNL is of great importance. Given the high NPV of the MRI in the prediction of SNHL and neurological impairment following a fetal infection after a first trimester MPI, parents should wait until the late second trimester (27 weeks) for a better prognosis of outcome by MRI. However, normal imaging does not rule out the development of SHNL and minor neurodevelopmental abnormalities.[45] Moreover, persistent infection can cause a combination of lesions that warrant a strict neuroimaging follow-up.[46,47]

1.1.3 *Prognostic value of laboratory parameters in fetal blood*

Fetal blood sampling (FBS) by cordocentesis for diagnostic purposes, especially to determine the karyotype, has fallen in disuse and has been replaced by amniocentesis. However, blood analysis in cCMV infection has been useful for the assessment of infected neonates.[48] Although the reported sensitivities for CMV-DNA in fetal blood range from 41% to 92%,[49–52] FBS does not increase the detection rate of fetal infection. Nonetheless, investigation of fetal hematological and biochemical parameters might be beneficial in assessing the clinical condition of the fetus.[20] Three studies have demonstrated the utility of FBS, with both thrombocytopenia ($< 50{,}000/mm^3$ in one study and $< 100{,}000/mm^3$ in two studies); and a high viral load (≥ 4.93 $\log_{10}$ IU/ml and 4.5 $\log_{10}$ copies/ml) being associated with a greater risk of been symptomatic at birth or severe brain abnormalities.[5,23,24] High levels of β_2 microglobulin (> 11.5 mg/L) in fetal blood have also been associated with poor outcomes.[5]

High gamma-glutamyl-transpeptidase (GGT) levels have been proposed as a parameter of fetal CMV infection and can be significantly related to fetal organ damage[5,17,23,53]. GGT is an enzyme with considerable redox activity in biliary epithelial cells, the myocardium, kidney, and pancreas, and is most expressed in the brain particularly in microglia and endothelial cells[53–55]. However, the exact cell localization, expression, function, and regulation of GGT in the brain has yet to be elucidated. Rivera *et al.*, reported that an increase in GGT levels could be related to fetal stress although the exact origin is unknown.[54]

As a result of these discrepancies, new approaches have been proposed based on HCMV DNA quantification in amniotic fluid[15,56] or combined assessment of US findings and low platelet count in fetal blood samples.[23] However, it seems unlikely that a single test or examination can predict the collective events that might lead to permanent damage.[5]

1.2 Treatment of fetal infection

Treatment options for fetal CMV infection during pregnancy to prevent maternal fetal transmission or reduce the severity of fetal CMV-associated symptoms are limited[57]. Gancyclovir (GCV)/valgancyclovir and valacyclovir (VCV) are active against CMV and are being used in the treatment of immunocompromised

individuals. Moreover, intravenous GCV and its oral prodrug valganciclovir have proven effectiveness in preventing hearing loss in symptomatically infected infants.[58,59] However, these antivirals are contraindicated during pregnancy due to concerns related to the teratogenic effects in fetal germ cells.[46,47] Even if there are reports of successful treatment with GCV[48,49] and valganciclovir[49,50] in infected fetuses, their use during pregnancy is currently not recommended. VCV is an ester derivative of acyclovir which is active against CMV DNA polymerase, when used at high doses (i.e. 8 g per day). High-dose VCV is a drug used for universal prophylaxis in liver and kidney transplant recipients.[60] At a dose of 8 g/day, VCV has shown to be more effective against herpes viruses[61] in individuals with immunosuppressive conditions[62]. Exposure to acyclovir and VCV has shown to be safe during pregnancy and its administration in the first trimester of pregnancy has not shown to be associated with an increased risk of major birth defects.[63]

Regarding the prevention of fetal sequela, in a first study Jacquemard *et al*. showed that VCV crosses the placental barrier and decreases the CMV viral load in 80% of infected fetuses with higher concentration in amniotic fluid than fetal and maternal blood. Moreover, a reduction in viral load in fetal blood was correlated with a good outcome, even in cases with US abnormalities already present prior to the beginning of treatment.[28] After these preliminary results, a second study by the same group showed that compared to a historical cohort, maternal oral administration of high-dose VCV to the mother was safe and may improve outcomes in infected fetuses diagnosed at >21 weeks and considered to be moderately symptomatic, showing a 50% reduction in asymptomatic newborns among treated fetuses ($p<0.05$).[55]

On the other hand, regarding the prevention of fetal infection (secondary prevention), a recent prospective double-blinded, placebo-control randomized controlled trial by Nissan *et al*. showed that the administration of high-dose VCV (4 g twice/day) is effective in reducing fetal CMV infection after MPI acquired early in pregnancy. Pregnant women with serological evidence of MPI either periconceptionally or during the first trimester were randomly assigned to receive high-dose VCV or placebo from enrolment until amniocentesis at 21-22 weeks of gestation. There was a significant reduction in the positive amniocenteses for CMV in the VCV group compared to the placebo group with an OR:0.29 (95% confidence interval [CI]: 0.09-0.90) for vertical CMV transmission. Additionally, Faure-Bardon *et al*. reported a

decrease in maternal-fetal transmission in a retrospective study using VCV (4g twice/day) in patients with MPI diagnosed before 14 weeks of gestation. Treatment was started as soon as MPI was biologically proven and continued at least until amniocentesis was performed at 17 – 22 weeks of gestation.[59] Both studies showed a reduction of over 60% of the maternal-fetal transmission rate. These recent data have shown promising results with the administration of VCV soon after maternal infection, although this can only be implemented under systematic maternal screening.

Nigro *et al*. conducted a first study that showed a significantly 60% reduction in the risk of cCMV with monthly intravenous hyperimmunoglobulin (HIG) therapy.[65] However, in a randomized trial, HIG did not significantly modify the course of primary CMV infection to prevent fetal infection in 123 pregnant women who presented a MPI between 5 and 26 weeks of gestation.[66] Similarly, HIG did not significantly modify maternal CMV-DNA blood levels, and nor did it significantly modify CMV-DNA levels in the placentas.[56] However, a recent observational study by Kagan *et al*. using a repeated dose (biweekly) of HIG, in patients with a confirmed first trimester MPI, showed a reduction of over 70% in the maternal-fetal transmission rate compared to a matched historical cohort. Nonetheless, the use of intravenous HIG is much more costly than VCV[67,68] and requires to be applied in a tertiary level hospital.

Given these results and considering the potential for progression of fetal CMV infection in utero, intrauterine treatment may be justified in MPI in the first trimester of pregnancy/periconceptional period to prevent vertical transmission. Moreover, although there is less evidence, high-dose VCV administration is probably justified in fetuses with mild CMV infection (CNS or extra CNS/moderate findings).[22] until results of new studies with better treatment options are available.

Finally, updated estimates of the impact of congenital CMV are also needed for increased awareness of the true burden of the infection and disease in every country. This would help allocation of public health resources, and determination of the cost-effectiveness or cost-benefit of potential interventions.[2]

2. HYPOTHESES

HYPOTHESES

MAIN HYPOTHESIS

The accuracy to predict the outcome of a CMV infected fetus may be improved by a combination of fetal blood parameters, fetal US, and MRI features.

SPECIFIC HYPOTHESES

1. In CMV-infected fetuses, selective virological and biological blood parameters correlate with the severity of brain damage.
2. GGT levels in fetal blood may correlate with the severity of CNS damage in both CMV-infected and uninfected fetuses.
3. A combination of GGT levels and platelet count in fetal blood sampling may improve the prediction of brain damage in CMV-infected and uninfected fetuses.
4. Hepatomegaly may be a marker of symptomatic fetuses in congenital CMV infection.
5. Fetal liver volume may be a surrogate marker of liver enlargement and can be depicted by MRI and correlated with the fetal liver weight at birth.
6. Unaffected and mildly affected CMV-infected fetuses with mild sonographic involvement may have underdeveloped cortical maturation as compared with healthy controls.
7. In CMV-infected fetuses, the periventricular hyperechogenicity, the “halo sign”, may be a sign of poor prognosis related to severe ventriculitis and white matter injury.
8. The use of high dose valacyclovir (VCV) may prevent the appearance of fetal anomalies or reduce the progression of fetal abnormalities in those CMV-infected fetuses with mild/moderate sonographic findings
9. VCV as a secondary prevention measure may reduce the risk of maternal-fetal transmission of CMV.

3. OBJECTIVES

3. OBJECTIVES

MAIN OBJECTIVE

To evaluate the prognostic value of fetal US/MRI features together with that of fetal blood parameters at the time of diagnosis in a series of fetal CMV infection.

SECONDARY AIMS

1. To assess the value of hematological, biochemical, and virological blood parameters in CMV infected fetuses to predict the severity of fetal brain damage, and to evaluate these parameters according to the gestational age at cordocentesis.
2. To evaluate the prognostic value of fetal US imaging together with platelet count in fetal blood at the time of the diagnosis in a series of confirmed fetal CMV infections.
3. To evaluate the prognostic value of GGT levels in fetal blood for the severity of brain damage in a series of CMV-infected and uninfected fetuses.
4. To assess if the performance of the GGT/platelets ratio in fetal blood better predicts the severity of brain damage in CMV-infected and uninfected fetuses
5. To assess the FLVs obtained by MRI in CMV-infected fetuses and compare them with those of a group of healthy fetuses.
6. To evaluate if the FLV of CMV-infected fetuses differs according to the severity of brain damage determined by US/MRI.
7. To obtain information about the halo sign, one of the most frequent sonographic abnormalities observed in fetuses infected with CMV and generally considered a feature of poor prognosis. To describe its association with other brain imaging abnormalities and analyze its correlation with histopathological findings.
8. To evaluate maternal tolerance, fetal outcome, and newborn viral load in a cohort of pregnancies with fetal CMV infection with absent/mild US/MRI abnormalities treated with high-dose VCV.
9. To evaluate the efficacy and safety of VCV treatment for secondary prevention of congenital CMV infection after maternal primary infection in the periconceptional period, and the first and second trimester of pregnancy.

5. MATERIAL, METHODS & RESULTS

STUDY 1

Blood parameters in fetuses infected with cytomegalovirus according to the severity of brain damage and trimester of pregnancy at cordocentesis.

Hawkins-Villarreal A, Moreno-Espinosa AL, Eixarch E, Marcos MA, Martinez-Portilla RJ, Salazar L, Garcia-Otero L, Lopez M, Borrell A, Figueras F, Goncé A. *Journal of Clinical Virology* (2019) ***119,*** 37-43.
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Blood parameters in fetuses infected with cytomegalovirus according to the severity of brain damage and trimester of pregnancy at cordocentesis

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ABSTRACT

Background and objective: Cytomegalovirus (CMV) remains a major cause of congenital infection and disease. During pregnancy, symptomatic cases can be detected through ultrasound (US) features, nevertheless, prognostic assessment is difficult. The aim of this study was to assess the predictive value of specific blood parameters in CMV infected fetuses.

Study design: Twenty-eight CMV-infected fetuses in which a cordocentesis had been performed were included. Fetuses were considered severely or mildly affected according to prenatal US/MRI brain damage. Fetal blood parameters were assessed for the prediction of severe brain abnormalities, and compared according to the trimester of pregnancy. Logistic regression and receiver operating curve analysis were performed.

Results: Thrombocytopenia ($\leq$100,000/mm^3; *p:0.03*) and high levels of gamma-glutamyl transpeptidase (GGT) ($\geq$151 IU/L; *p:0.02*) signaled severity. For the prediction of brain damage, GGT levels $\geq$ 183 UI/l achieved 71% sensitivity, 83% specificity (*AUC: 0.78*), and OR of 2.05 (95% CI: 1.22–3.43) per 100 IU/l increase, adjusted for gestational age. However, thrombocytopenia (91% vs 50%; *p: 0.04*), β_2 microglobulin > 10.4 mg/l (60% vs 0% *p: 0.03),* CMV-DNA > 50,000 copies/ml (80% vs 25%; *p: 0.02)*, and positive IgM (70% vs 17%; *p: 0.04)* were observed significantly more often in severely damaged fetuses sampled $\leq$28 weeks than thereafter.

Conclusion: In CMV infected fetuses, thrombocytopenia and high levels of GGT are associated with severe US/MRI brain abnormalities. Nevertheless, among severely affected fetuses, blood parameters, with exception of GGT, change according to gestational age. Fetal blood could be less predictive of brain damage in the third trimester.

1. Background

Cytomegalovirus (CMV) is the most common congenital infection, and remains worldwide a major cause of sensorineural hearing loss and neurodevelopmental abnormalities [1]. Since routine maternal screening is currently not recommended, congenital CMV is underdiagnosed. Detection during pregnancy is usually achieved when suggestive sonographic signs are found during routine scans, although their value in the diagnosis of fetal infection is limited [2]. Once fetal infection is confirmed, serial targeted ultrasound (US) examinations, and magnetic resonance imaging (MRI) as a complementary tool, have shown a good sensitivity in the identification of symptomatic newborns. [3–7]. Severe fetal brain lesions have been associated with dismal prognosis, with odds ratio as high as 41 [8]. However, although the brain is a major target of end-organ damage, a precise cellular marker of brain damage remains uncharacterized [9]. The need for a more accurate evaluation has led to investigating postnatal blood parameters in symptomatic neonates, to be applied in infected fetuses, although their predictive value to identify symptomatic newborns is conflicting [8,10–13]. Moreover, the diagnostic performance according to the trimester at cordocentesis has not been yet compared. The aim of this study was to assess the value of haemathological, biochemical, and

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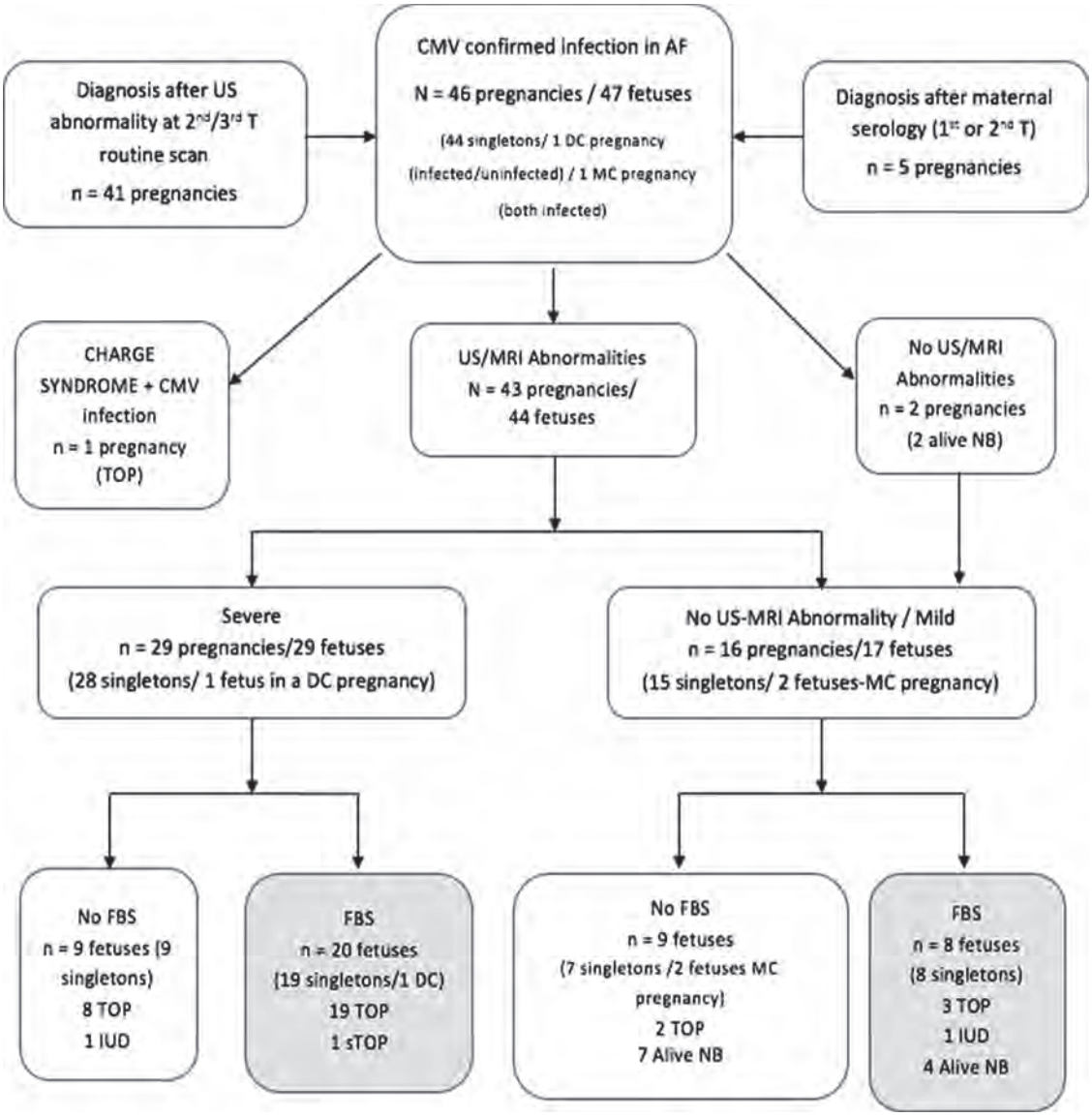

Fig. 1. *Flow chart:* Pregnancies with confirmed fetal CMV infection followed-up at our center between 2006 and 2018. Description of the outcome according to the severity of infection, and cordocentesis performed for fetal blood analysis.
DC: dichorionic, MC: monochorionic, AF: amniotic fluid, US: ultrasound, MRI: magnetic resonance imaging, FBS: fetal blood sampling, TOP: termination of pregnancy, sTOP: selective termination of pregnancy, IUD: intrauterine demise. NB: newborn.

virological blood parameters in CMV infected fetuses to predict severity of fetal brain damage, and to evaluate them according to the gestational age at cordocentesis.

2. Materials and methods

A series of consecutive pregnancies with CMV fetal infection confirmed in amniotic fluid by a positive polymerase chain reaction (PCR), attended at Hospital Clinic, Barcelona, in which fetal blood sampling (FBS) was performed as a complementary investigational tool over a 13-year period (January 2006-December 2018) (Fig. 1). In the absence of routine CMV screening in pregnancy, most of the cases were diagnosed on the evidence of US abnormalities detected during the second or third trimester routine scans. Other causes of fetal defects such as chromosomal abnormalities and toxoplasmosis infection were ruled out at the time of amniotic fluid study.

Fetal examination and follow-up consisted of serial US including a detailed neurosonography. All scans were carried out by experienced examiners using high-resolution US equipment (Voluson 730 Expert and E6 or E8, GE Healthcare, Kretz, Zipf, Austria). In pregnancies reaching the third trimester a fetal MRI was performed at 30–32 weeks (1.5 T GE Sigma Horizon, Echo speed, LX MRI scanner, Milwaukee, WI, USA), or earlier if US examination revealed brain lesions. Fetal infection was classified as severe (fetuses with severe US/MRI brain findings) or mild (fetuses with mild/absent US/MRI brain findings or with extra-cerebral ones) according to Table 1 classification [14].

After fetal imaging, women were counseled about newborn prognosis, and termination of pregnancy (TOP) was discussed according to Spanish laws. Cordocentesis for further studies in fetal blood was offered after evaluating its risks and diagnostic limitations. Written informed consent was obtained, and in most cases, the cordocentesis was undertaken at the time of TOP. Second trimester cordocentesis was defined when performed up to 28.0 gestational weeks, and third trimester when carried out thereafter. Fetal blood was analyzed for platelet count, β_2-microglobulin, gamma-glutamyl transpeptidase (GGT), CMV-DNAemia, and CMV specific-IgM antibodies. Platelet count was determined using ABX Pentra 60 −HORIBA Ltd; Japan. Thrombocytopenia was defined as a platelet-count threshold of

Table 1
Classification of prenatal US and MRI abnormalities in congenital CMV infection.

Severe US/MRI brain abnormalities*	Mild US/MRI brain abnormalities**	Extra-cerebral US abnormalities**
Severe ventriculomegaly (≥ 15 mm)	Intraventricular adhesions	ˆHyperechogenic bowel
Microcephaly (HC ≤ -3 SD)	Mild ventriculomegaly (10-14.9 mm)	ˆˆIntrauterine growth restriction
Periventricular hyperechogenicity	Isolated Calcifications	Hepatomegaly (right lobe ≥ 40 mm)
Enlarged sub-arachnoid space (CCW > 95th centile) (Micrencephaly)	Calcifications of lenticulostriate vessels in basal ganglia	Intra-hepatic calcifications
Porencephaly (porencephalic cysts)	Sub-ependymal cysts	Pleural effusion
	White matter hyperintensity (MRI)	Ascites
Agenesis/dysgenesis of corpus callosum		Pericardial effusion
Cerebellar/vermian hypoplasia (CTD < 5th centile) or cerebellar hemorrhage		Fetal hydrops
Abnormal gyration / Cortical dysplasia (MRI)		† Cardiomegaly
		Oligohydramnios (DVP ≤ 2 cm)
		Polyhydramnios (DVP ≥ 10 cm)
		Placentomegaly ≥ 40 mm

*Lesions of poor prognosis: fetuses with at least one severe brain US or MRI abnormality **Lesions of uncertain prognosis: fetuses with mild brain US or MRI abnormalities or extra-cerebral US abnormalities exclusively.
US: Ultrasound. MRI: Magnetic Resonance Imaging. HC: head circumference; CCW: crania-cortical width; CTD: cerebellar transverse diameter;
DVP: deepest vertical pocket.
ˆ Considered when the echogenicity of the bowel is equal or more intense than that of the fetal bones.
ˆˆ Considered when the estimated fetal weight is below the 10th centile according to specific population tables with or without Doppler ultrasound anomaly.
† Considered if the heart is more than one-third of the thoracic diameter.
Adapted from: 1- Leruez-Ville et al. Prognosis evaluation of fetal CMV infection. Am JObstet Gynecol 2016;215:342.e1-9.
2- Goncé et al. TORCH and B19 Parvovirus infections during Pregnancy. www.fetalmedbarcelona.org.

100,000/mm^3. β_2-microglobulin concentration was measured using Siemens Dimension Vista® N-Latex® β_2-microglobulin reagent cartridge (Siemens Healthineers, Inc., Newark, Del., USA); high-risk levels were considered establishing two different cut-offs: ≥10.4 mg/l and ≥11.5 mg/l as defined by Fabbri et al. [11]. Quantitative determination of GGT was performed using a *in-vitro* diagnostic test [Siemens Dimension® EXL clinical chemistry system Flex® reagent cartridge (Siemens Healthineers, Inc., Newark, Del., USA)]; levels ≥151 UI/L were considered abnormal [15]. Extraction of CMV DNA for all the maternal, fetal, and neonatal samples was performed using QIAsymphony system (Qiagen, Hilden, Germany), and viral load by PCR CMV Real Time (Nanogen Advanced Diagnostics, Italy) with a threshold of 20 copies/ml to define positivity. High viral load in FBS was defined using two thresholds: 30,000 copies/ml (≥ 448 $\log_{10}$ IU/ml) and 50,000 copies/ml (≥ 493 $\log_{10}$ IU/ml), as both have been associated with symptomatic status at birth. [11,14]. CMV-IgM specific antibodies were determined using commercially available enzyme immunoassay [CMV IgM ELISA VIDAS (Biomerieux S.A., Spain)].

In cases of fetal demise and TOP, routine postmortem macroscopic and microscopic examination of the fetus and the placenta was performed, after informed consent obtained from the parents. In cases with an alive newborn, congenital CMV was confirmed by a positive PCR from urine sampled within 2 weeks of birth. Postnataly, the infection was classified as either symptomatic (mild, or moderate to severe), or asymptomatic according to the recent consensus document by Rawlinson *et a*l. [16].

2.1. Ethical approval

This study was approved by the Hospital Clinic ethics committee: **Reg. HCB/2017/0564**.

2.2. Statistical analysis

Results of non-viral and viral assays were compared according to the severity of the congenital CMV infection. Variables were analyzed according to the severity of brain damage in US/MRI imaging. Quantitative variables were assessed using Shapiro-Wilk's test for normality, and normally distributed variables were compared using *t*-test and expressed as mean and standard deviation (SD). Non-normally distributed quantitative variables were compared using U-Mann-Whitney test and expressed as median and interquartile range (IQR: p25-75). Qualitative variables were compared using X^2 and Fisher's exact test. A sub-analysis of fetal cord blood parameters was assessed according to trimester of gestation. Receiver-Operator Curve (ROC) analysis was performed in order to establish the best cut-off point for variables that were significantly different between severely and mildly affected fetuses. Univariate logistic regression was carried out for each independent factor for an outcome of severe brain damage. A robust bias-corrected estimation was used to calculate 95% confidence intervals and p-values. Predictive performance for GGT as a continuous variable was calculated using ROC analysis. Sensitivity, specificity, positive and negative likelihood ratios and as well as area under the ROC curve were calculated. P-value < 0.05 was considered significant. Data were analyzed using STATA, v.15.0 (College Station, Texas)

3. Results

A total of 46 pregnancies and 47 fetuses with CMV infection were included in the study (Fig. 1). With the exception of five pregnancies in which the diagnosis of fetal infection was established after maternal CMV screening in the first or second trimester decided by the patient's practitioner, all other pregnancies were diagnosed on the evidence of US abnormalities, 31 detected during second and 10 during third trimester routine scans. Cordocentesis for further evaluation was accepted by 28 (61%), 20 with severe fetal US/MRI abnormalities, and 8 with non-severe (n = 7) or absent (n = 1) findings. Among the one with absent findings, maternal primary infection was diagnosed after first trimester screening **(Supplementary Tables 1a & 1b).** Baseline characteristics of pregnancies comparing those with and without FBS, and according to the severity of brain-damage are summarized in **Supplementary Table-2 and** Table 2, respectively. Regarding the time of cordocentesis, 18 were performed in the second trimester (12 severely and 6 mildly/non-affected fetuses) at a mean gestational age (MGA) and SD of 23.4 (2.1) weeks, and 10 in the third (8 severely and 2 mildly affected fetuses) at a MGA of 33.2 (2.6) weeks. Twenty-two fetuses (78%) were sampled at the time of TOP. The mean time elapsed between US diagnosis and FBS was 3.4 weeks.

The most frequent fetal brain US abnormality was periventricular hyperechogenicity involving the whole periventricular area (Fig. 2) found in 54% of fetuses, and the most frequent extra-cerebral US abnormality was hyperechogenic bowel (39%). Cerebral MRI was performed in 14 fetuses (50%), between 23.0 and 37.0 weeks, and provided relevant additional information regarding an abnormal cortical

Table 2
Baseline characteristics according to the severity of brain damage in pregnancies with CMV infected fetuses with FBS.

Characteristic	Mild/No US Ab n = 8	Severe US Ab n = 20	*p** value
Maternal Age, years, mean (SD)	30.5 (5.3)	31.4 (5.9)	0.71
Caucasian Ethnicity (%)	100	100	1.00
Low educational level, n (%)**	0 (0)	8/19 (42)	0.08
Multiparity (%)	62	60	0.90
Child at nursery (< 3y child) (%)	50	60	0.27
Gestational age at diagnosis of fetal infection, mean (SD)	22.5 (2.7)	25.5 (4.8)	0.12
≤ 28.0 weeks, n (%)	7 (87)	14 (70)	0.33
Fetal gender (female), n (%)	5 (26)	14 (74)	0.70
AF-CMV million copies/ml, median (IQR)	12.9 (7.2-12.9)	10 (1.14-14)	0.31
Gestational age at cordocentesis, mean (SD)	24.4 (1.8)	27.9 (1.1)	0.11
≤ 28.0 weeks	6 (75)	12 (60)	0.45

Data are presented as mean and standard deviation (SD), frequencies or percentage (%), medians (IQR: interquartile range: p25-75). * *p* value as determined with the *t*-test, Mann-Whitney U, X^2 or Fisher's exact test.
** Low educational level was defined as primary school studies only. FBS: fetal blood sampling. US Ab: ultrasound abnormality. AF: amniotic fluid.

development in 3 (Fig. 3). The US and MRI findings are summarized in **Supplementary-Table 3**.

Concerning FBS results, the only two parameters significantly associated with the degree of brain damage were thrombocytopenia and GGT levels, with a median value significantly higher in the severely affected fetuses **(**Table 3 / Fig. 4**)**. The best performance in the prediction of severe brain damage was achieved with a GGT cutoff-of ≥183 UI/l with 71% sensitivity, 83% specificity, 4.3 positive and 0.34 negative likelihood ratios (LHR) (*AUC: 0.78*), and an OR of 2.05 (95% CI: 1.22–3.43) per 100 IU/l increase. (Fig. 5).

When the distribution of fetal blood parameters was compared among all fetuses according to gestational age at cordocentesis, we observed that in the second trimester, the mean platelet count was lower (69.4/mm^3 vs 117.9/mm^3, *p=0.04),* the median β_2-microglobulin was higher (11.9 mg/l vs 7.5, p < 0.01), and CMV IgM and viral load > 50 000 copies were significantly more frequent (71% vs 13%, p < 0.01 and 79% vs 33%, p = 0.03, respectively) **(Supplementary Table-4)**.

Subsequently, we analyzed the validity of blood parameters according to gestational age among the severely damaged fetuses, and observed that thrombocytopenia, IgM antibodies, and DNAemia > 50 000 copies/ml were obtained significantly more often ≤28 weeks (*p = 0.04, p = 0.04 and p = 0.02, respectively*). Moreover, high levels of β_2 microglobulin appeared exclusively in the second trimester (*p* = 0.03). GGT was the only parameter that did not vary according to the trimester of pregnancy **(**Table 4**).**

4. Discussion

In this series, we observed that among fetuses diagnosed with CMV infection in the second or third trimester of pregnancy, GGT and thrombocytopenia were the only blood parameters significantly associated with severe US/MRI brain lesions. However, while GGT levels showed stable values along the pregnancy, thrombocytopenia, high levels of β_2-microglobuline and DNAemia, and positive IgM antibodies were observed significantly more often in the severely damaged fetuses diagnosed before 28 weeks.

Thrombocytopenia has been reported as an independent factor responsible for a poor perinatal outcome. Fabbri et al and Leurez-Ville et al. found an association between fetal thrombocytopenia in the second trimester and being symptomatic at birth. [11,14]. Leurez-Ville et al. showed that with platelet count ≤114,000/mm3 there was a 62.5% risk of a symptomatic status at birth or at TOP. They concluded that the prognostic assessment for being symptomatic at birth is possible as early as in the second trimester by combining a targeted US-examination, viral-load, and platelet count in fetal blood [14]. In contrast, Enders *et a*l [13] who included second and third trimester FBS, did not observe differences in non-virological markers between fetuses with normal and abnormal US findings. In our series, thrombocytopenia was also observed more often in the severely damaged fetuses though more frequently in the second trimester.

Dreux et al. [17] identified β_2-microglobulin as a reliable marker of fetal CMV infection, and Fabbri et al. reported that β_2-microglobulin was the most reliable non-viral marker for prediction of fetal damage in the second trimester [11]. We did not observe any differences in β_2-microglobulin levels when comparing based on the severity of damage. However, sub-analysis according to the time of cordocentesis in these fetuses, high levels of β_2-microglobulin were found exclusively in those fetuses diagnosed with severe damage in the second trimester.

Regarding virological parameters, higher levels of DNAaemia in symptomatic fetuses have been described [11–14] but with a wide overlap, leading to a poor prognostic value [11,13] as also described in newborns [12]. Different cut-off values have been proposed for fetal prognostic assessment in the second trimester that vary from 4.48 log10 IU/mL to 4.93 log10 IU/mL [11,14], but a cut-off value has not been established in the third trimester. Among our fetuses, there were higher levels in the severely damaged group ≤28 weeks. However, when comparing according to the severity of damage no differences were observed. A few studies have reported that fetuses with US abnormalities have significantly higher values of CMV specific-IgM antibodies

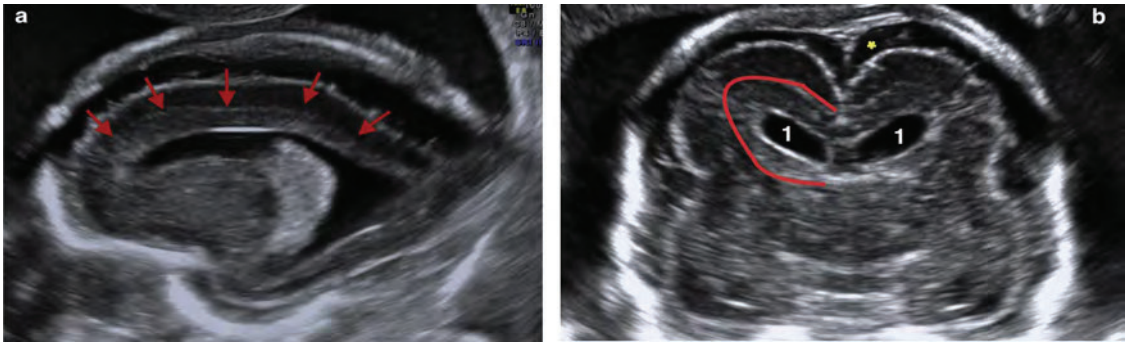

Fig. 2. Fetal neurosonography: transvaginal approach: 24.0 weeks (Case 5 – supplementary table 1a) **(a)** Parasagittal plane of the fetal head in the three horns view showing periventricular hyperechogenicity also known as ∴ periventricular halo ∴ (red arrow heads). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).
(b) Coronal transcaudate plane showing dilation of both anterior horns (1) of the lateral ventricles. We delineated the periventricular hyperechogenicity **("halo sign")** in one of the anterior horns. Noted the augmented sinus-cortical space (yellow asterisk) as a sign of micrencephaly.

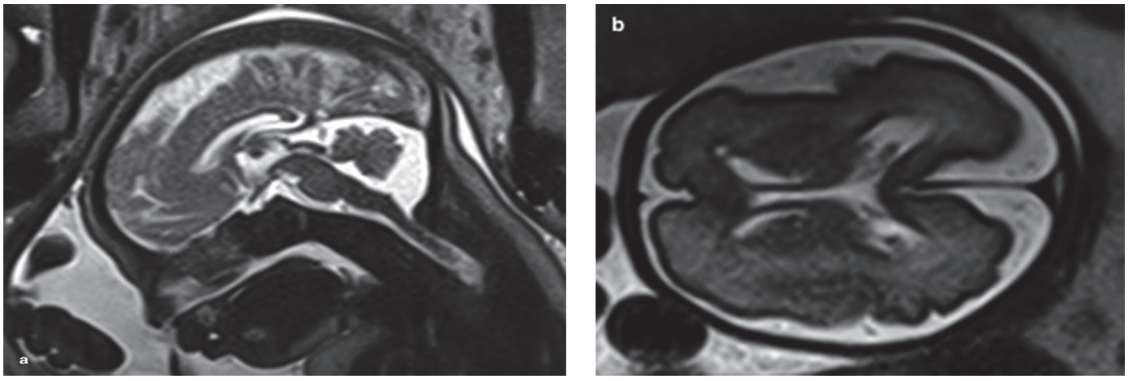

Fig. 3. Fetal MRI image. **(a)** 36.0 weeks. (case 15, supplementary table 1a (3)) - Sagittal plane of the fetal head showing hypoplastic corpus callosum and cortical dysplasia **(b)** 29.0 weeks (case 9, supplementary table 1a (2)) - Axial view showing mild ventriculomegaly, delay in cortical maturation, enlarged subarachnoid space, and white matter hyperintensity.

compared to asymptomatically infected ones, though their usefulness to distinguish symptomatic from asymptomatic newborns is poor [11,13]. We did not observe differences in the presence of CMV-IgM when comparing the severity of damage, but found a significant decrease in IgM positivity in FBS obtained in the third trimester.

According to our results, it could be hypothesized that after an acute phase of the infection, fetal thrombocytopenia recovers, and β_2-microglobulin and DNAemia decrease in the third trimester. Moreover, a longer period elapsed from fetal infection would allow time for IgM to reach its peak earlier in pregnancy, and already be negative in the third trimester.

CMV infection can induce several inflammatory mediators that may induce cytotoxicity [9]. GGT is an enzyme involved in the transfer of amino acids and considered to participate in glutathione-coupled detoxification processes with great activity in biliary epithelial cells, as well as in other tissues, such as the brain [18–20]. GGT does not cross the placenta and cord levels derive entirely from the fetus [15]. There is little information on abnormal fetal values, although high levels of GGT have been reported as a reliable parameter of CMV infection. [11,13,21]. Enders et al. [13] did not observe significant differences in GGT levels according to the presence of US findings in CMV-infected fetuses, although levels above 350 IU/l were found exclusively in those with severe abnormalities. A series of more than 3000 unselected fetal blood samplings showed that the mean GGT value during second and third trimester was 97.5 IU/l with little variation between 20 and 40 weeks [15]. This finding may explain why we observed significant differences according to the severity of damage, but not to the trimester

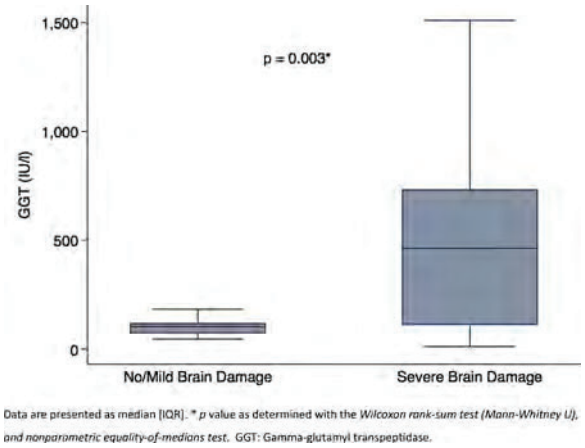

Fig. 4. Gamma-glutamyl transpeptidase levels according to severity of brain damage.
Data are presented as median [IQR]. * *p* value as determined with the *Wilcoxon rank-sum test (Mann-Whitney U), and nonparametric equality-of-medians test.* GGT: Gamma-glutamyl transpeptidase.

at cordocentesis. To the best of our knowledge, an association of the severity of brain lesions with high levels of GGT has never been previously reported. Although it could be interpreted that among the severely damaged fetuses the source of GGT elevated levels is derived from biliary obstruction, our hypothesis is that CMV cerebral-infection

Table 3
Fetal blood sampling results according to the severity of brain damage in CMV infected fetuses.

Characteristic	Mild/No US Ab n = 8		Severe US Ab n = 20		*p** value
Platelet count /mm^3, mean (SD)	125.5 (28.9)		71.3 (15.4)		0.08
Low platelet count (≤ 100,000), n (%)	2/8 (25)		14/19 (74)		0.03*
GGT IU/l, median (p25-75)	103.5 (72-120)		463 (111-734)		0.003*
GGT ≥ 183 IU/l, n (%)	0/6 (0)		10/14 (71.4)		0.011*
GGT ≥ 151 IU/l, n (%)	1/6 (16.7)		10/14 (71.4)		0.024*
GGT ≥ 120 IU/l, n (%)	2/6 (33)		10/14 (71.4)		0.11
β_2 microglobulin (mg/l), median (IQR)	11 (8.7 - 12.7)		9.5 (7.2 - 12.3)		0.41
β_2 microglobulin ≥ 11.5 mg/l, n (%)	2/5 (40)		6/16 (37.5)		0.92
β_2 microglobulin ≥ 10.4 mg/l, n (%)	3/5 (60)		6/16 (37.5)		0.38
CMV-DNA thousands copies/ml, median (IQR)	118 (56-205)		96 (16-428)		0.54
CMV-DNA > 30,000 copies/ml, n (%)	3/5 (60)		11/18 (61)		0.60
CMV-DNA > 50,000 copies/ml, n (%)		3/5 (60)	10/18 (55)		0.47
Positive Fetal IgM n, (%)	3/6 (50)		8/16 (50)	1.00	

Data are presented as mean and standard deviation (SD), percentage (%), medians (IQR: interquartile range: p25-75).
* p value as determined with the *t*-test, Mann-Whitney U, X2 or Fisher's exact test. AF: amniotic fluid GGT: Gamma-glutamyl transpeptidase. US Ab: ultrasound abnormality.

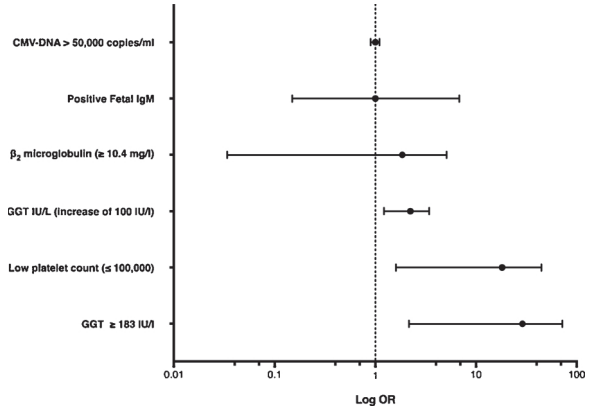

Fig. 5. Individual risk for severe outcome according to fetal blood parameters. All *p* values and confidence intervals were calculated with robust bias-corrected logistic regression

increases GGT expression and activity in the brain, and that it could help discriminate severe cases. Recently, we have obtained similar results when comparing GGT levels at the time of TOP in a group of uninfected fetuses, with (N = 14) and without (N = 20) brain damage [50% sensitivity, 95% specificity, 9.5 positive LHR, and 0.53 negative LHR (*AUC: 0.82*) for the same GGT cutoff-(≥187 UI/l)] [Unpublished data]. These results are still hypothetical, and more research is required.

The strengths of our study are: it is the first one to compare blood parameters in untreated infected fetuses according to the trimester at diagnosis. In addition, it is the first study to establish a cut-off value for GGT levels in the prediction of severe brain lesions. Blood parameters, except for GGT, were nearer normal ranges in the third trimester, also in the severely damaged fetuses. This could be valuable information at the time of considering a cordocentesis for further prognosis assessment as it is an invasive procedure that entails considerable risk.

Our study has several limitations. First, the retrospective nature of the analysis. Second, the small sample size, especially among pregnancies with mildly affected fetuses that are underdiagnosed in the absence of maternal routine screening, and the fact that not all laboratory parameters were available for all fetuses. Third, there could have been an overlap in the grading of severity because it is not known what would have been the evolution of cases classified as mildly damaged in the second trimester if they had survived. This, however, is inherent to a classification of risk in the second trimester. In addition, there was a lack of standardized necropsy protocol for congenital CMV infection.

In conclusion, in CMV-infected fetuses, platelet count and GGT levels were significantly associated with severe US/MRI brain damage. However, thrombocytopenia, high levels of β_2-microglobulin, high CMV-DNAemia, and specific-IgM antibodies were less frequently observed in the third trimester. Our results suggest that with the exception of GGT, blood parameters could be predictive of fetal damage only in the second trimester.

Summary

A series of pregnancies with confirmed fetal CMV infection which underwent cordocentesis. Fetuses were considered severely or mildly affected based on US/MRI findings. Fetal blood parameters were compared according to the severity of brain damage and trimester of pregnancy at cordocentesis.

Ethical approval

This study was approved by the Hospital Clinic ethic committee: **Reg. HCB/2017/0564**.

Credit author statement

All authors fulfill all conditions required for authorship, have seen and approved the manuscript, and all have significantly contributed to the work as follows: Anna Goncé and Ameth Hawkins-Villarreal (conception and design of the study); Ameth Hawkins-Villarreal, Ana L. Moreno-Espinosa, Elisenda Eixarch, Laura Garcia-Otero, Laura Salazar (acquisition of data); Ameth Hawkins-Villarreal, Raigam J. Portilla-Martinez, Anna Goncé, Francesc Figueras (analysis and interpretation of data); M. Angeles Marcos (analysis and interpretation of virological essays). Anna Goncé (supervision). Anna Goncé, Ameth Hawkins-Villarreal, Ana L. Moreno-Espinosa (writing-original draft). Anna Goncé, Ameth Hawkins-Villarreal, Antoni Borrell, Francesc Figueras, Marta Lopez (writing, revision and editing of the submitted article).

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Table 4
Fetal blood sampling results according to trimester at cordocentesis in CMV infected fetuses with severe brain damage.

Characteristic	≤ 28.0 weeks n = 12	> 28.0 weeks n = 8	*p** value
Platelet count 10^3 /mm^3, mean (SD)	49.8 (46)	100.9 (83)	0.11
Low platelet count (≤ 100,000), n (%)	**10/11 (91)**	**4/8 (50)**	**0.04***
GGT IU/l, median (IQR)	463 (111-898)	495 (170-698)	0.55
GGT ≥ 183 IU/l, n (%)	7/10 (70)	3/4 (75)	0.85
GGT ≥ 151 IU/l, n (%)	7/10 (70)	3/4 (75)	0.85
GGT ≥ 120 IU/l, n (%)	7/10 (70)	3/4 (75)	0.85
β_2 microglobulin (mg/L), median (IQR)	**11.9 (9.7-13.0)**	**7.5 (6.4-7.7)**	**0.03***
β_2 microglobulin ≥ 11.5 mg/L, n (%)	**6/10 (60)**	**0/6 (0)**	**0.03***
β_2 microglobulin ≥ 10.4 mg/L, n (%)	**6/10 (60)**	**0/6 (0)**	**0.03***
CMV-DNA thousand copies/ml, median (IQR)	147 (89,4-456)	108 (3,2-228)	0.05
CMV-DNA > 30,000 copies/ml, n (%)	8/10 (80)	3/8 (38)	0.07
CMV-DNA > 50,000 copies/ml, n (%)	**8/10 (80)**	**2/8 (25)**	**0.02***
Positive fetal IgM n, (%)	**7/10 (70)**	**1/6 (17)**	**0.04***

Data are presented as mean and standard deviation, frequencies or percentage (%), medians [IQR: interquartile range: p25-75].
* p value as determined with the *t*-test, Mann-Whitney U, X2 or Fisher's exact test.
2nd Trimester: ≤ 28 weeks of gestation, 3rd Trimester: > 28 weeks of gestation. GGT: Gamma-glutamyl transpeptidase.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jcv.2019.08.008.

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STUDY 2

Increased Gamma-Glutamyl Transpeptidase and Gamma-Glutamyl Transpeptidase/Platelet Ratio: Biological Markers of Severe Brain Damage in Cytomegalovirus Infected and Uninfected Fetuses

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Oral presentation:

1) ISUOG Virtual Congress 2021
2) FMF 19th World Congress in Fetal Medicine 2022

Status: draft in preparation.

Abstract

Objective: The main objective of this study was to evaluate the association of gamma-glutamyl-transpeptidase (GGT) with severe brain damage (SBD) in cytomegalovirus (CMV)-infected and uninfected fetuses and examine whether this biomarker differs between groups. Our secondary aim was to assess whether implementing the use of the GGT/platelet ratio (GPR) would increase the detection rate for SBD in CMV-infected and uninfected fetuses.

Materials & methods: GGT levels and platelet count were analyzed in fetal blood samples in consecutive cases of CMV-infection and in a cohort of uninfected fetuses with neurological, cardiac, or other abnormalities. GPR was calculated with the following formula: GGT (international units [IU]/L)/platelet count (10^9/L) x 10. The severity of neurological findings was determined in all fetuses by prenatal neuroimaging in alive newborns, and by postmortem in cases of termination of pregnancy (TOP). GGT levels, platelet count, and the GPR levels were compared according to brain-damage severity and CMV-infection status. Logistic regression, Receiver-Operator Curve (ROC) analysis, and test of equality of ROC areas were performed.

Results: Twenty-six CMV-infected (20 SBD and 6 mild/no brain lesions) and 35 uninfected-fetuses (18 brain and 17 no brain abnormalities) were included. The median (interquartile [IQR]) gestational age (GA) at cardio/cordocentesis was 25.1(22.6-29.6) weeks with no differences between groups. The median (IQR) GGT levels were significantly higher among fetuses with SBD than those with mild/no brain-damage: 227 IU/l (73-662) vs 98 IU/l (46-109), p=0.001. GGT levels ≥183 IU/l, adjusted for GA at cardio/cordocentesis, were associated with SBD with an odds ratio (OR) of 11 (95% confidence interval [CI]: 2.8-43) in both groups. When adjusted for GA at cardio/cordocentesis GGT level values were significantly higher in SBD-CMV-infected compared to SBD-uninfected fetuses, median (IQR): 423 IU/l (110-816) vs.118 IU/l (59-245), p=0.023. For SBD prediction in the uninfected group GGT levels ≥187 IU/l achieved 39%

sensitivity (Se), 95 specificity (Sp), 6.61 positive and 0.65 negative likelihood ratios (LHR). As previously reported by our group, the prediction was higher for CMV-infected fetuses, with a detection rate of 70% and 83% Sp for a GGT cut-off ≥183 IU/L.

The median (IQR) platelet count was significantly lower among CMV-infected vs. uninfected fetuses: 100 (27-155) $x10^3$ /mm^3 vs. 204 (137-245) $x10^3$ /mm^3, p<0.001. This difference remained when SBD fetuses were compared according to infection status. There were no variations in platelet levels regardless of the severity of brain damage in the uninfected group, p=0.53.

The median (IQR) GPR was significantly higher among fetuses with SBD than those with mild/no brain-damage: 21.5 IU (7.1-51.6) vs 4.4 IU (3.0-9.4), p<0.001. As expected, however, we observed significantly higher median (IQR) GPR levels among SBD CMV-infected than SBD uninfected fetuses: 49.1 IU (23.1-256.5) vs 9.0 IU (2.6-13.6), p<0.001. GPR adjusted for GA at cardio/cordocentesis was associated with SBD with an OR of 7.31 (95% CI: 1.91-28.0) per every 10 IU increase of GPR in the CMV-infected group. When compared to GGT levels alone, we found a tendency towards an increased performance for SBD prediction in the CMV-infected group with a GPR≥12 achieving 84% Se, 83% Sp, 5.1 positive and 0.19 negative LHR (*AUC: 0.92*), p=0.07. In the uninfected group we observe no improvement in the performance for the prediction of SBD with the GPR compared to the GGT levels, p=0.43.

Conclusions: Increased GGT levels were associated with SBD in CMV-infected and uninfected fetuses. Nevertheless, infection may play a role since the increase was significantly higher among the infected fetuses. Due to the lower platelet count in CMV-infected fetuses, the GPR may improve the detection rate of SBD in this group.

1-Background

Gamma-glutamyl-transpeptidase (GGT) is an enzyme with considerable redox activity in biliary epithelial cells, the kidney, pancreas, and brain (microglial/pericytes/endothelial cells).[1–4] It is the enzyme catalyzing reduced glutathione, and its metabolism pathway has been implied in various pathophysiological conditions and is now considered as a risk factor in cardiac and cerebrovascular diseases[5–9]. In cerebral micro-vessels, GGT is expressed at high levels and appears to be involved in the maintenance of blood-brain barrier integrity.[10] In systemic inflammation, the disruption of endothelial integrity promotes an increase of reactive oxygen species in the brain, platelet adhesion and aggregation, resulting in decreased platelet count and increased of GGT serum levels[10–12]. Cytomegalovirus (CMV) infection can induce direct cytopathic effects and several inflammatory mediators that may induce cytotoxicity[13,14]. In a previous study we observed that increased GGT levels were related to severe-brain-damage (SBD) in CMV-infected fetuses irrespective of gestational age (GA)[15]. Moreover, thrombocytopenia in CMV-infected fetuses, especially in the second trimester, has been associated with brain damage[16–19]. The GGT/platelet ratio (GPR) was first proposed in 2014 as an inflammatory biomarker influencing liver fibrosis and cirrhosis, and further studies on GPR indicated its remarkable predictive ability for hepatocellular carcinoma[20]. Recently, this biomarker has been associated with neurological deficits due to hypoxic-anoxic ischemic brain injury in coronary artery disease and other comorbidities[21]. The clinical significance of these biomarkers in CMV-infected and uninfected fetuses is unknown. Since systemic inflammation is a milestone in the physiopathology of fetal CMV infection that could trigger an increase in GGT levels and cause thrombocytopenia due to increased platelet aggregation, we speculate that the GPR could be associated with poor prognosis in congenital CMV.

The main objective of this study was to evaluate the association of GGT with SBD in CMV-infected and uninfected fetuses and examine whether this biomarker differs between groups. Our secondary aim was to assess whether implementing the use of the GPR would increase the detection rate for SBD in CMV-infected and uninfected fetuses.

2-Materials and Methods

This was an ambispective study in which a series of consecutive pregnancies with fetal CMV infection confirmed in amniotic fluid (AF) by a positive polymerase chain reaction (PCR) from 2008 to 2020 (part of previously published data [15]) was compared to a cohort of uninfected fetuses with neurological, cardiac, or other abnormalities diagnosed in the same single center from 2017 to 2019. In all cases fetal blood sampling (FBS) was performed for complementary fetal diagnostic assessment or as an investigational tool at the time of termination of pregnancy (TOP) (**Figure 1)**. Fetal blood was analyzed for hemoglobin, platelet count, β_2-microglobulin and GGT levels. Fetal blood parameters were determined as described previously[15]. GPR was calculated with the following formula: GGT (international units [IU]/l)/platelet count (10^9/L) x 10.

CMV infection group: In the absence of routine CMV screening in pregnancy, most of the CMV cases were diagnosed on the evidence of abnormal ultrasound (US) findings during routine second or third trimester scans. Extraction of CMV DNA for all the maternal, fetal, and neonatal samples was performed using the QIAsymphony system (Qiagen, Hilden, Germany), and viral load by CMV Real Time PCR (Nanogen Advanced Diagnostics, Italy) with a threshold of 20 copies/ml to define positivity. Other causes of fetal defects such as chromosomal abnormalities and fetal toxoplasmosis were ruled out at the time of the AF study. Follow-up consisted of serial US including a detailed neurosonography. Brain abnormalities were classified as severe /mild or absent according to the classification of Leruez-Ville *et al.*[16],. and the severity of brain lesions was confirmed at postmortem evaluation in cases ending in TOP.

Uninfected fetuses cohort: this cohort consisted of fetuses that underwent TOP due to severe abnormalities, and one alive fetus (born at term) (**Figure 1)**. In cases with central nervous system (CNS) findings compatible with CMV, the infection was ruled out in AF. Regarding genetic studies, quantitative fluorescent-PCR/chromosomal microarray or exome analysis included in the workshop for structural abnormalities was performed to ruled out associated chromosomal or genetic anomalies. The severity of brain abnormalities was confirmed at postmortem.

In both groups, scans were carried out by experienced examiners using high-resolution US equipment (Voluson 730 Expert and E6 or E8, GE Healthcare, Kretz, Zipf, Austria); and in some cases, magnetic resonance imaging (MRI) (1.5T Magnetom Aera syngo MR D13; Siemens, Erlangen, Germany), to better establish prognosis if US examination revealed brain abnormalities.

Ethical approval: This study was approved by the Ethics Committee of the Hospital Clinic of Barcelona: **Reg. HCB/2017/0564**

Statistical Analysis

GGT levels, platelet count, and the GPR were compared according to brain damage severity and CMV infection status. Quantitative variables were assessed using the Shapiro-Wilk test for normality, and normally distributed variables were compared using the t-test and expressed as mean and standard deviation (SD). Non-normally distributed quantitative variables were compared using the Mann-Whitney U test or the Kruskal-Wallis equality-of-populations rank test and expressed as median and interquartile range (IQR: p25-75). Qualitative variables were compared using the Chi squared (X^2) and Fisher exact tests. Receiver-Operator Curve (ROC) analysis was performed to establish the best cut-off point for variables that were significantly different between severely and mildly affected fetuses. Univariate logistic regression was carried out for each independent factor for an outcome of SBD. A robust bias-corrected estimation was used to calculate 95% confidence intervals (CI) and p-values. Predictive performance for GGT and the GPR as a continuous variable was calculated using ROC analysis. Sensitivity (Se), specificity (Sp), and positive and negative likelihood ratios (LHR) and as well as area under the ROC curve were calculated. A test of equality of ROC areas was performed. A p-value <0.05 was considered significant. Data were analyzed using STATA, v.15.0 (College Station, Texas).

3-Results

Twenty-six CMV-infected (20 SBD and 6 mild/no brain lesions) and 35 uninfected fetuses (18 SBD and 17 no brain abnormalities) were included (**Figure 1**). The description of abnormalities in uninfected fetuses is shown in **Table 1**. Regarding CMV-infected fetuses, brain abnormalities

diagnosed by US or MRI are shown in **Supplementary table 1**. Six cases were considered mildly affected, showing only mild brain (n = 2), no brain abnormalities (n = 2), and no brain abnormalities with extra-CNS findings (n = 2) at the time of cordocentesis. The median (IQR) gestational age (GA) at cardio/cordocentesis was 25.1(22.6-29.6) weeks, with no differences between groups (**Table 2**). GA at birth/TOP and birthweight were similar between groups (**Table 2)**.

In CMV-infected fetuses the median (IQR) for GGT levels, the GPR and β_2-microglobulin was significantly higher compared to uninfected fetuses ($p<0.05$) and the median (IQR) platelet count was significantly lower: 100 (27-155) $x10^3$ /mm^3 vs. 204 (137-245) $x10^3$ /mm^3, $p<0.001$ (**Table 3**). Moreover, in uninfected fetuses, GGT levels were the only blood parameter significantly higher in SBD fetuses compared to those with no brain damage (**Supplementary table 2**). Blood parameter results among infected fetuses compared according to brain damage are shown in **Supplementary table 3**.

Regardless of their infection status, in fetuses with SBD, the median (IQR) GGT levels and the GPR were significantly higher while hemoglobin levels and platelet count were significantly lower, $p<0.05$ (**Table 4**). The median (IQR) GGT levels among fetuses with SBD were: 227 IU/l (173-662) vs 98 IU/l (46-109), p= 0.001 (**Table 4**). When adjusted for GA at FBS, significantly higher GGT levels values were observed in SBD CMV-infected compared to uninfected SBD fetuses [median (IQR): 423 IU/l (110-816) vs. 118 IU/l (59-245), p=0.023] (**Figure 2).** As expected, the median (IQR) platelet count was significantly lower among SBD CMV-infected vs. uninfected SBD fetuses: 49 (19-122) $x10^3$ /mm^3 vs. 200 (158-214) $x10^3$ /mm^3, $p<0.001$. **Table 5**. There were no variations in platelet levels regardless of the severity of brain damage in the uninfected group, p=0.53 (**Supplementary table 2).**

The median (IQR) GPR was significantly higher among fetuses with SBD than those with mild/no brain-damage: 21.5 IU (7.1-51.6) vs. 4.4 IU (3.0-9.4); $p<0.001$ (**Table 4**). However, we observed significantly higher median (IQR) GPR levels among SBD CMV-infected than SBD uninfected fetuses: 49.1 IU (23.1-256.5) vs. 9.0 IU (2.6-13.6), p=0.002 (**Table 5).**

GGT levels ≥183 IU/l, adjusted for GA at cardio/cordocentesis, were associated with SBD with an odds ratio (OR) of 11 (95%CI: 2.8-43) in both groups. For SBD prediction in the uninfected group GGT ≥187 IU/l achieved a Se of 39%, Sp of 94%, and a 6.61 positive LHR and 0.65 negative LHR (*AUC: 0.70*). As previously published by our group, the prediction regarding CMV-infected fetuses was higher, with a detection rate of 70% and 83% Sp for a GGT cut-off ≥183 IU/L (*AUC: 0.78*).

The GPR adjusted for GA at FBS was associated with SBD with an OR of 7.31 (95% CI: 1.91-28.0) per every 10 IU increase of GPR in the CMV-infected group**.** When compared to GGT levels alone, we found a tendency towards an increased performance for SBD prediction in the CMV-infected group with a GPR≥12 achieving 84% Se, 83% Sp, and a 5.1 positive and 0.19 negative LHR (*AUC: 0.92*), p=0.07 (**Figure 3**). In the uninfected group there was no increase or improvement in the performance of the prediction of SBD with the GPR compared to the GGT levels, p=0.43 (**Figure 3**). The GGT and GPR levels remained higher when assuming a possible biological plausibility adjusted for GA at FBS, p<0.001 (**Figure 4**).

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Tables

Table 1. Description of US/MRI and genetic abnormalities in uninfected fetuses.

	Diagnosis
I: CNS abnormality (n = 18) *	Corpus callosum dysgenesis (n = 2)
	Partial corpus callosum agenesis (n = 1)
	Parenchymal hemorrhage (n = 6)
	Porencephaly (n = 1)
	Cerebellar or vermian hypoplasia (n = 7)
	Severe ventriculomegaly (n = 2)
	Abnormal cortical gyration (n = 6)
II: Complex congenital cardiopathy (n = 9)	Double outlet (hypoplastic) RV, with outflow tracts in TGA with pulmonary hypoplasia.
	Poly-valvular pathology with pulmonary atresia and critical aortic stenosis.
	AV canal ostium secundum type, (47 XX +21)
	Persistence of LSVC with drainage in the coronary sinus with probable dysplastic tricuspid valve (arr: 8p23.2p22 (183250-18157968) x1)
	Pulmonary atresia type 1
	Right isomerism
	Critical coarctation of the aorta with non-hypoplastic LV and reduced aortic isthmus size
	Tricuspid atresia with hypoplastic RV
	Hypoplastic left heart syndrome
III: Other abnormalities (n = 8)	Early severe intrauterine growth restriction
	Osteogenesis imperfecta [mutation COL1A1 p.(G254R)]
	Osteochondrodysplasia
	Suspicion of fetal anemia
	Cleft lip-palate
	Malposition of hands and feet, LV foci, choroid plexus cysts (deletion 22q11.21. arr: 22q11.21(17274835_19835417) x1)
	Arthrogryposis
	Fetal hydrops of unknown cause

CNS: central nervous system. RV: right ventricle. TGA: transposition of great arteries. AV: atrio-ventricular. LSVC: left superior vena cava. LV: left ventricle. Arr: array CGH. * Fetuses could have more than one CNS abnormality.

Table 2. Baseline characteristics of the study population.

Characteristic	CMV n = 26	No-CMV n = 35	*p** value
Maternal age, years, mean (SD)	32.0 (5.2)	33.8 (5.0)	0.17
Ethnicity (Caucasian), (%)	96	86	0.18
Multiparity, (%)	73	31	0.001*
Low educational level, (%)	30	19	0.31
Fetal gender (female), (%)	54	56	0.88
GA at amniocentesis, median (IQR)	22.9 (21.1 – 26.6)	23.7 (21.3 – 28.4)	0.57
GA at cordocentesis, median (IQR)	25.1 (22.6 – 29.6)	25.7 (22.6 – 30.6)	0.80
Fetal ultrasound/MRI abnormality, n (%)			
None	2/26 (8)	0/35 (0)	0.18
Extra CNS abnormality‡	16/26 (62)	21/35 (60)	0.90
CNS abnormality	22/26 (85)	18/35 (51)	0.007*
Severe CNS abnormality	20/26 (77)	18/35 (51)	0.04*
GA at birth/TOP weeks, median (IQR)	25.6 (23.3 – 31.3)	24.0 (22.9 – 29.0)	0.41
Birthweight g, median (IQR)	749 (506 - 1686)	604 (522 - 1218)	0.56

Data are presented as mean, standard deviation (SD), frequencies or percentage (%), median (IQR: interquartile range: p25-75). *p value determined with the t-test, Mann-Whitney U, X^2 or Fisher's exact test. CMV: cytomegalovirus. GA: gestational age. MRI: magnetic resonance imaging. CNS: central nervous system. TOP: termination of pregnancy. NB: newborn. ‡ Fetuses could have both CNS and extra-CNS abnormalities at the same time.

Table 3. Fetal blood sampling results in fetuses with and without cytomegalovirus infection

Characteristic	CMV n = 26	No-CMV n = 35	*p** value
Hemoglobin (g/dL)	10.9 (8.9 - 12.6)	12.1 (11 - 13.7)	0.037*
Platelet count 10^3 /mm^3	100 (27 - 155)	204 (137 - 245)	< 0.001*
GGT IU/l	274 (98 - 667)	99 (57 - 152)	0.006*
GPR IU	36.7 (7.4 - 110.4)	5.2 (2.7 - 10.6)	< 0.001*
β_2 microglobulin (mg/L)	10.5 (7.5 - 12.4)	3.7 (3.2 - 4.2)	< 0.001*

Data presented as median (IQR: interquartile range: p25-75). *p value as determined with the Mann-Whitney U, X^2 or Fisher's exact test. CMV: cytomegalovirus. GGT: gamma-glutamyl transpeptidase. GPR: GGT/platelets ratio. IU: international units.

Table 4. Fetal blood sampling results according to the severity of brain damage.

Characteristic	Severe BD n = 38	Mild**/no BD n = 23	*p** value
Hemoglobin (g/dL)	11.0 (9.3 - 12.6)	12.2 (11.4 - 13.6)	0.008*
Platelet count 10^3 /mm^3	137 (48 - 198)	205 (116 - 245)	0.019*
GGT IU/l	227 (73 - 662)	98 (46 - 109)	0.001*
GPR IU	21.5 (7.1 - 51.6)	4.4 (3.0 - 9.4)	<0.001*
β_2 microglobulin (mg/L)	5.2 (3.7 - 11.3)	4.1 (3.6 - 5.0)	0.101

Data are presented as median (IQR: interquartile range: p25-75), frequencies or percentage (%). **p* value as determined with the Mann-Whitney U test, X^2, Fisher's exact test, or nonparametric equality-of-medians test. BD: brain damage. GGT: gamma-glutamyl transpeptidase. GPR: GGT/Platelets ratio. IU: international units. ** Mild brain damage applies only to cytomegalovirus-infected fetuses.

Table 5. Fetal blood sampling results in fetuses with severe brain damage according to cytomegalovirus infection status.

Characteristic	SBD_CMV n = 20	SBD_No-CMV n = 18	*p** value
Hemoglobin (g/dL), median (IQR)	10.6 (7.9 - 12.2)	11.8 (10.3 - 13.4)	0.11
Platelet count 10^3 /mm^3, median (IQR)	49 (19 - 122)	200 (158 - 214)	<0.001*
GGT IU/l, median (IQR)	423 (110 - 816)	118 (59 - 245)	0.023*
GGT ≥ 183 IU/l, n (%)	14/20 (70)	7/18 (39)	0.054*
GGT ≥ 151 IU/l, n (%)	14/20 (70)	7/18 (39)	0.054*
GPR IU, median (IQR)	49.1 (23.1 - 256.5)	9.0 (2.6 - 13.6)	<0.001*
GPR ≥ 12	16/20 (80)	6/18 (33)	0.002*
β_2 microglobulin (mg/L), median (IQR)	10.6 (7.2 - 12.4)	3.6 (3.0 - 4.0)	<0.001*

Data are presented as medians (IQR: interquartile range: p25-75), frequencies or percentage (%). **p* value as determined with the Mann-Whitney U, X^2 or Fisher's exact test. SBD: severe brain damage. CMV: cytomegalovirus. GGT: gamma-glutamyl transpeptidase. GPR: GGT/Platelets ratio. IU: international units.

Figures

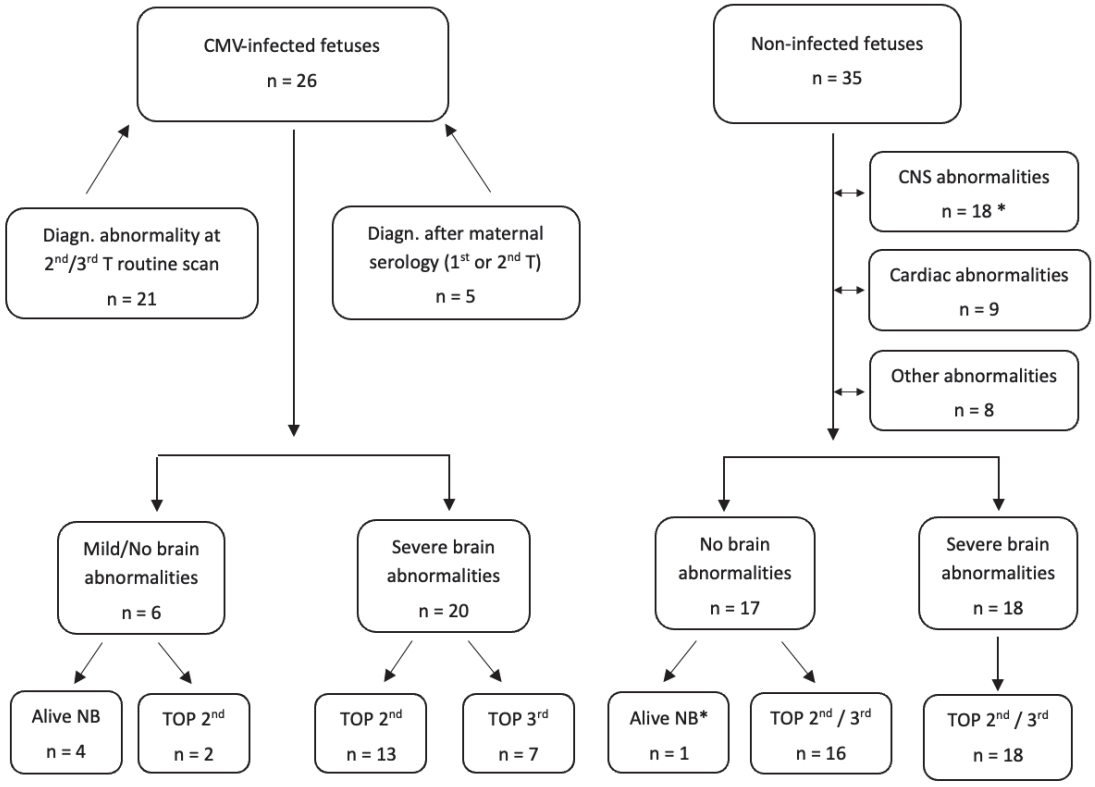

Figure 1: *Flow chart:* CMV-infected and non-infected fetuses with cardio/cordocentesis performed for fetal blood analysis. Description of the outcome according to the severity of brain abnormalities. CMV: cytomegalovirus, Diagn: diagnosis, CNS: central nervous system, TOP: termination of pregnancy, NB: newborn at term. TOP 2nd/TOP 3rd: Termination of pregnancy in 2nd or 3rd trimester. * Fetus under surveillance for suspicion of anemia.

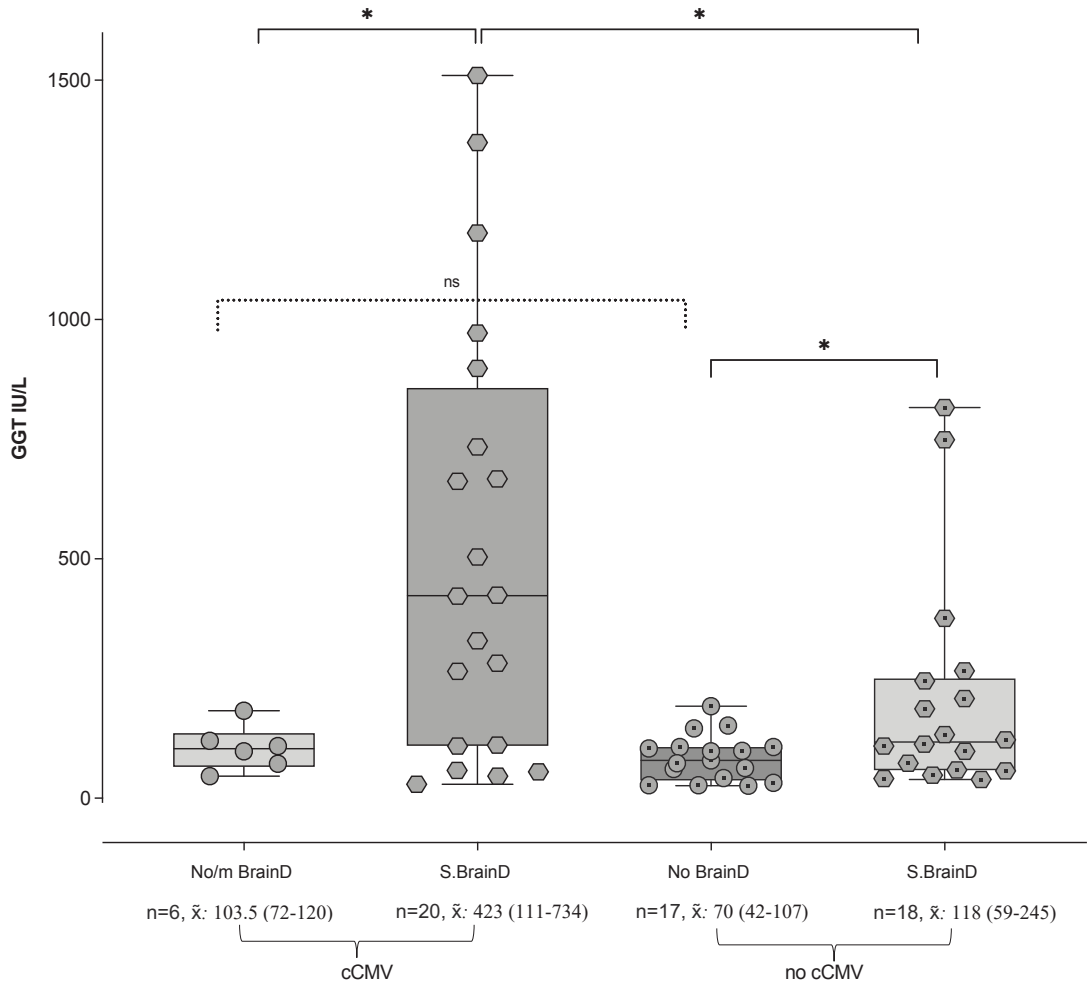

Figure 2. Box plots of GGT levels among study groups. Data presented as median(x̃) [IQR]. Horizontal bars at the top of each panel indicate differences between data pairs. *p value (adjusted for GA at FBS) as determined with Kruskal-Wallis equality-of-populations rank test and non-parametric pairwise comparisons. GGT: Gamma-glutamyl transpeptidase. cCMV: infected fetuses. no cCMV: uninfected fetuses. No/m BrainD: infected fetuses with mild or no brain damage. S. BrainD: severe brain damage. No BrainD: uninfected fetuses with no brain damage. * p= <0.05, ns= >0.05.

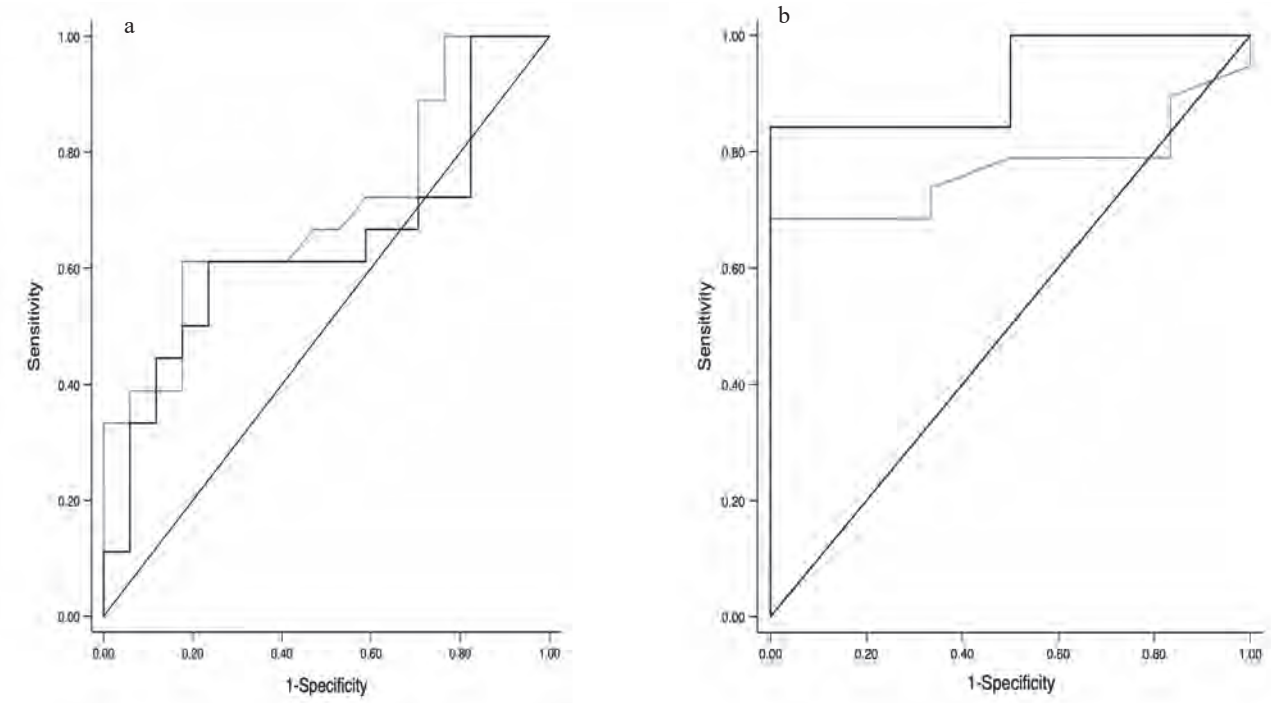

Figure 3. Receiver operating characteristic (ROC) curves for the prediction of severe brain damage comparing values of GGT (gray line) and GPR (black line) in **a.** uninfected and **b.** infected fetuses.

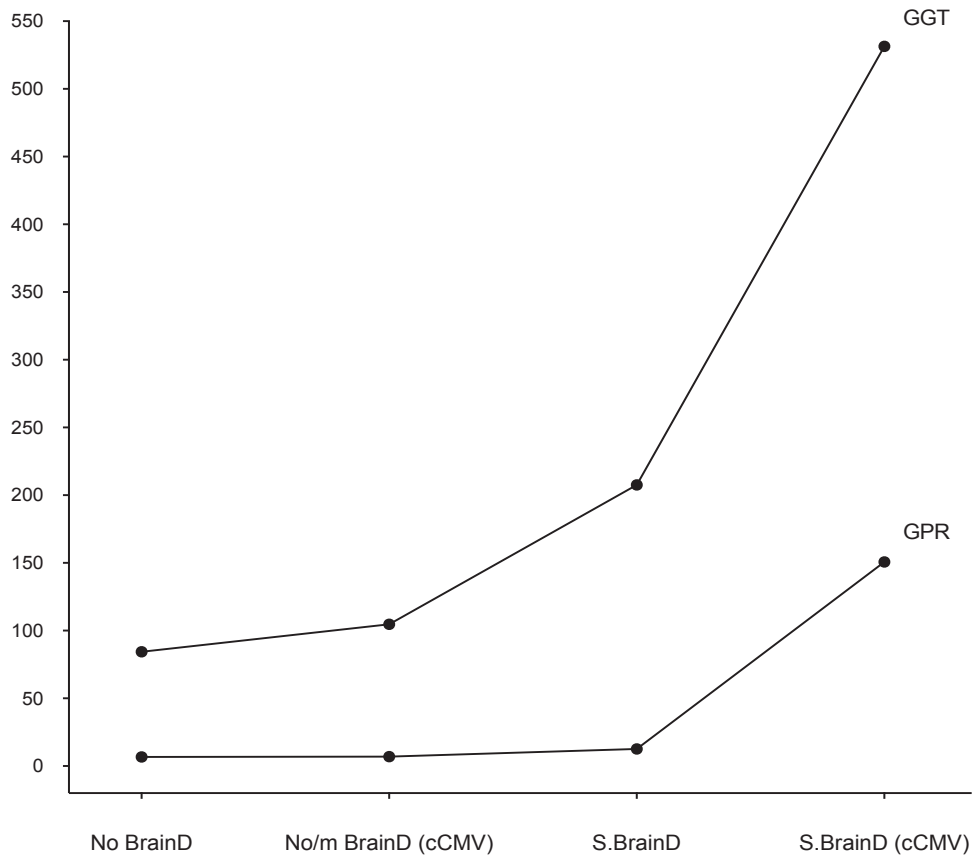

Figure 4: Linear trend analysis: GPR (**A**), GGT (**B**). Data presented as mean (SEM). **p* value as determined with parametric linear regression tendency test (Jonckheere-Terpstra Test); ***p*<0.001**. GGT: gamma-glutamyl transpeptidase. GPR/Platelet ratio. No BrainD: uninfected fetuses with no brain damage. No/m BrainD (cCMV): infected fetuses with mild or no brain damage. S. BrainD: uninfected fetuses with severe brain damage. S. BrainD (cCMV): infected fetuses with severe brain damage.

Supplementary material

Supplementary table 1. Description of central nervous system abnormalities in cytomegalovirus infected fetuses.

	CMV n = 26
Severe CNS features	
Severe VMG, n (%)	1 (4)
Microcephaly, n (%)	2 (8)
Microencephaly (Enlarged SAS), n (%)	5 (19)
Porencephaly, n (%)	0 (0)
Periventricular hyperechogenicity, n (%)	13 (50)
Corpus callosum hypoplasia, n (%)	7 (27)
Cerebellar or vermian hypoplasia, n (%)	7 (27)
Cerebellar hemorrhagic cyst, n (%)	1 (4)
Parenchymal hemorrhage, n (%)	0 (0)
Abnormal sulcus gyration, n (%)	6 (23)
Mild CNS features	
Mild VMG, n (%)	1 (4)
Intraventricular adhesions, n (%)	2 (8)
Isolated calcifications, n (%)	1 (4)
Sub-ependymal cysts, n (%)	2 (8)

Data are presented as frequencies or percentage (%). Fetuses could have more than one central nervous system abnormality. VMG: ventriculomegaly. SAS: Sub-arachnoid space.

Supplementary table 2. Fetal blood sampling results in fetuses without cytomegalovirus infection according to the severity of brain damage.

Characteristic	Severe BD n = 18	no BD n = 17	*p** value
Hemoglobin, median (IQR)	11.8 (10.3 - 13.4)	12.2 (11.5 - 13.9)	0.30
Platelet count 10^3 /mm^3, median (IQR)	200 (158 - 214)	234 (118 - 260)	0.53
GGT IU/l, median (IQR)	118 (59 - 245)	79 (42 - 107)	0.04*
GGT ≥ 183 IU/l, n (%)	7/18 (39)	1/17 (6)	0.02*
GGT ≥ 151 IU/l, n (%)	7/18 (39)	2/17 (12)	0.07
GPR IU, median (IQR)	9.0 (2.6 - 13.6)	4.4 (2.7 - 7.4)	0.17
β_2 microglobulin (mg/L), median (IQR)	3.6 (3.0 - 4.0)	3.7 (3.5 - 4.3)	0.32

Data are presented as median (IQR: interquartile range: p25-75), frequencies or percentage (%). *p value as determined with the Mann-Whitney U test, X^2, Fisher's exact test. CMV: cytomegalovirus. BD: brain-damage. GGT: gamma-glutamyl transpeptidase. GPR: GGT/platelet ratio. IU: international units.

Supplementary table 3. Fetal blood sampling results in cytomegalovirus-infected fetuses according to the severity of brain damage.

Characteristic	Severe BD n = 20	Mild/no BD n = 6	*p** value
Hemoglobin, median (IQR)	10.6 (7.9 - 12.2)	12.0 (11.1 - 12.6)	0.06
Platelet count 10^3 /mm^3, median (IQR)	49 (19 - 122)	151 (116 - 212)	0.01*
GGT IU/l, median (IQR)	423 (110 - 816)	104 (72 - 120)	0.04*
GGT ≥ 183 IU/l, n (%)	14/20 (70)	1/6 (17)	0.02*
GGT ≥ 151 IU/l, n (%)	14/20 (70)	1/6 (17)	0.02*
GPR IU, median (IQR)	49.1 (23.1 - 256.5)	6.3 (4.0 - 10.8)	0.002*
β_2 microglobulin (mg/L), median (IQR)	10.6 (7.2 - 12.4)	9.9 (8.2 - 11.9)	0.91

Data are presented as median (IQR: interquartile range: p25-75), frequencies or percentage (%). *p value as determined with the Mann-Whitney U, X^2, Fisher's exact test. BD: brain-damage. GGT: gamma-glutamyl transpeptidase. GPR: GGT/platelet ratio. IU: international units.

STUDY 3

Fetal Liver Volume Assessment Using Magnetic Resonance Imaging in Fetuses with Cytomegalovirus Infection

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Fetal Liver Volume Assessment Using Magnetic Resonance Imaging in Fetuses With Cytomegalovirus Infection†

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Objective: To assess fetal liver volume (FLV) by magnetic resonance imaging (MRI) in cytomegalovirus (CMV)-infected fetuses compared to a group of healthy fetuses.

Method: Most infected cases were diagnosed by the evidence of ultrasound abnormalities during routine scans and in some after maternal CMV screening. CMV-infected fetuses were considered severely or mildly affected according to prenatal brain lesions identified by ultrasound (US)/MRI. We assessed FLV, the FLV to abdominal circumference (AC) ratio (FLV/AC-ratio), and the FLV to fetal body volume (FBV) ratio (FLV/FBV-ratio). As controls, we included 33 healthy fetuses. Hepatomegaly was evaluated post-mortem in 11 cases of congenital CMV infection. Parametric trend and intraclass correlation analyses were performed.

Results: There were no significant differences in FLV between infected (n = 32) and healthy fetuses. On correcting the FLV for AC and FBV, we observed a significantly higher FLV in CMV-infected fetuses. There were no significant differences in the FLV, or the FLV/AC or FLV/FBV-ratios according to the severity of brain abnormalities. There was excellent concordance between the fetal liver weight estimated by MRI and liver weight obtained post-mortem. Hepatomegaly was not detected in any CMV-infected fetus.

Conclusion: In CMV-infected fetuses, FLV corrected for AC and FBV was higher compared to healthy controls, indicating relative hepatomegaly. These parameters could potentially be used as surrogate markers of liver enlargement.

Keywords: magnetic resonance imaging, fetal liver, pregnancy, fetal cytomegalovirus infection, fetal brain abnormalities

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INTRODUCTION

Cytomegalovirus (CMV) is the most common congenital infection and is a major cause of sensorineural hearing loss and neurodevelopmental abnormalities worldwide (1). As maternal screening of infection is not recommended, its detection during pregnancy is usually achieved when sonographic signs suggestive of infection are found during routine scans (2). After confirmation of fetal infection, the combination of serial targeted ultrasound (US) examinations and magnetic resonance imaging (MRI) as a complementary tool for brain assessment have shown good diagnostic performance for determining symptomatic status at birth (3–7). In infants with symptomatic disease, the manifestations can range from unspecific-mild to multi-systemic involvement, with a particular predilection toward the reticuloendothelial system, especially the liver (8, 9). Although the brain is a major target of congenital CMV-infection (10), there is a lack of information on hepatic involvement in these fetuses. Traditionally, estimation of fetal liver size has been based on liver length measurement performed by US (11–13), however, there is no standardized imaging methodology for the assessment of hepatomegaly.

The aim of this study was to compare fetal liver volume (FLV) in CMV-infected with that of healthy fetuses. In addition, we compared FLV according to the severity of fetal brain abnormalities in infected fetuses and correlated the liver weight estimated by MRI with that found in cases with termination of pregnancy (TOP).

MATERIALS AND METHODS

This was a retrospective case-control study including consecutive pregnancies with a CMV-infected fetus in which prenatal MRI was performed for prognostic assessment over a 13-year period (July 2006-December 2019) in BCNatal (Hospital Clínic and Hospital Sant Joan de Déu, Barcelona, Spain) and during a 4-year period (January 2015-September 2019) in Hôpital Louis-Mourier, Paris, France. The study was approved by the Institutional Review Board of the Hospital Clinic **(HCB/2017/0564)** and Hôpital Louis-Mourier **(CEERB-Paris Nord/2020-012)**.

Cytomegalovirus-infected fetuses were considered severely or mildly affected according to prenatal brain US/MRI findings observed in US/MRI, as described previously (14, 15) (**Supplementary Table 1**). Most cases were diagnosed with the presence of US abnormalities found during routine second or third trimester scans. The remaining cases were diagnosed after maternal CMV screening by patients' physicians. Fetal CMV infection was confirmed with extraction of CMV DNA from amniotic fluid samples using the QIAsymphony system (Qiagen, Hilden, Germany). Chromosomal abnormalities and toxoplasmosis infection were ruled out at the time of the amniotic fluid study. The estimated fetal weight (EFW) at the time of the MRI was defined as that obtained by US no more than 2 weeks before or after the MRI. Small for gestational age (SGA) was defined as EFW by US below the 10th percentile. After fetal US/MRI, women were counseled about the prognosis of the newborn. TOP was discussed according to Spanish/French laws. In these case of TOP, routine post-mortem examination (macroscopic and microscopic) of the fetus and the placenta was performed after obtaining informed consent from the parents. The maceration status of the fetus was established using the Langley criteria (16). Organs, including the liver, were weighed on an electronic scale as part of the standard autopsy procedure. The time from delivery to autopsy was also recorded. In cases with a live newborn, congenital CMV was confirmed by a positive polymerase chain reaction (PCR) of a urine or saliva sample taken within the first 48 h of birth. The control group was made up of low-risk singleton pregnancies with fetuses of normal growth without structural abnormalities attended at BCNatal resulting in healthy newborns that were also a control group in a previous prospective cohort study on prenatal MRI (17, 18). All the pregnant women included agreed to participate and provided signed informed consent. Controls did not undergo any additional genetic or infection testing apart from routine blood exams during pregnancy.

Magnetic Resonance Imaging Acquisition

Magnetic resonance imaging was performed in a clinical MRI system (1.5T Magnetom Aera syngo MR D13; Siemens, Erlangen, Germany in Hospital Clinic and 1.5T GE SIGNA Artist, Echo speed, LX MRI scanner, Milwaukee, WI, United States, in Hôpital Louis-Mourier) with a 5-channel cardiac coil in both centers. No maternal or fetal sedation was used. T2-weighted images were obtained using half Fourier single-shot turbo spin-echo (HASTE) sequences (Hospital Clinic: time of repetition [RT] = 1000 ms, echo time [ET] = 137 ms, slice thickness = 3.5 mm, gap = 3.5 mm, voxel size = 0.5859375 mm × 0.5859375 mm × 3.5 mm, field of view [FOV] = 225 mm × 300 mm, matrix = 256 mm × 192 mm, flip angle = 135°, acquisition time = 165 s. Hôpital Louis-Mourier: RT = 1120 ms, ET = 92 ms, slice thickness = 3.5 mm, gap = 4.0 mm, voxel size = 0.5469 mm × 0.5469 mm × 3.5 mm, FOV = 225 × 300, matrix = 256 mm × 320 mm, flip angle = 90°, acquisition time = 93 s) in axial, coronal, and sagittal planes according to fetal orientation. The total fetal body and liver area of the fetuses was assessed by manually tracing the region of interest on each image slice with tissue present using Fiji Image-J2 software. **Figures 1A,B**. Volumes were calculated using the sequence that allowed complete visualization of the fetus and the fetal liver without motion-induced artifacts. For the estimation of liver volume, the main portal vein, and gallbladder were excluded. The abdominal circumference (AC) was measured in the MRI using the same landmarks used for US measurement (19, 20). FLV was calculated in cubic centimeters (cm^3) as follows: [(total liver area × pixel spacing^2) × (slice thickness + GAP)]/1000. The total fetal body volume (FBV) in cubic centimeters (cm^3) was calculated as: [(total body area × pixel spacing^2) × (slice thickness + GAP)]/1000. We corrected the FLV for AC and for FBV (adapted from previously published methodology) (21, 22). To obtain the fetal liver weight estimated by MRI we used a literature-derived density of 1.05 g/cm^3 from pediatric standards (23). We considered the GAP as a part of the formula to calculate

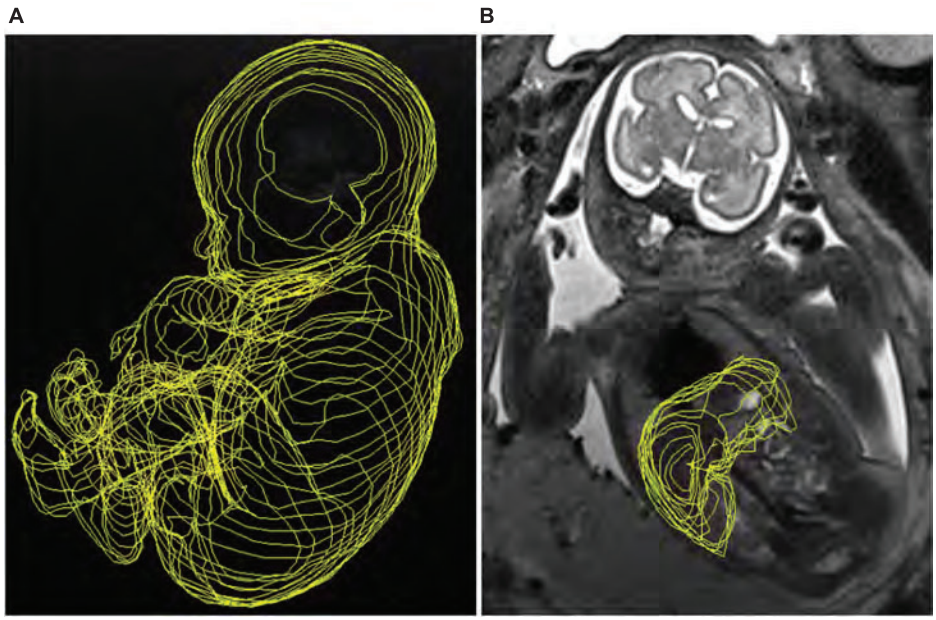

FIGURE 1 | (A) Manual tracing of the total fetal body obtained from a T2-weighted imaging sequence in sagittal view. **(B)** Manual tracing image of the fetal liver volume obtained from a T2-weighted imaging sequence in coronal view.

the liver volume because this methodology was standard prior to 2020. The intensity of the liver signal in MRI analysis was not evaluated. MRI measurements were performed only at one center (BCNatal). Two of the physicians in charge of the MRI measurements were blinded to the diagnosis of fetal CMV-infection (KC, and LT) and the third knew the fetal CMV status (AH-V). The FLV was not assessed by US.

Statistical Analysis

We compared the FLV, the FLV/FBV-ratio and the FLV/AC-ratio between CMV-infected and healthy fetuses and according to the severity of CMV infection. The variables were analyzed according to the severity of brain abnormalities in US/MRI imaging. Quantitative variables were assessed using the Shapiro–Wilk test for normality, and normally distributed variables were compared using the *t*-test and expressed as mean and standard deviation (SD). Non-normally distributed quantitative variables were compared using the Mann-Whitney *U*-test and expressed as median and interquartile range (IQR: p25–75). Qualitative variables were compared using the Chi squared (X^2) and Fisher exact tests. A sub-analysis of variance and covariance (one-way ANOVA with Bonferroni *post-hoc* test) of the FLV, FLV/FBV-ratio and FLV/AC-ratio was performed according to a possible biological trend (healthy vs. mild vs. severe CMV-infected fetuses). Interobserver reliability analysis of FLV was performed comparing the measurements of AH-V and LT by a two-way random effect model to assess the intraclass correlation coefficient (ICC) estimates and their 95% confident intervals (95% CI) in 16 randomly chosen healthy fetuses. Concordance analysis between the fetal liver weight estimated by MRI and liver weight obtained post-mortem was made by a two-way random effect model to assess the ICC estimates and their 95% confidence intervals (CI) in those fetuses with anatomopathological examination.

Power estimation for an alpha error of 0.05 was performed using the two-sample means Satterthwaite's *t*-test. A robust bias-corrected estimation was used to calculate the 95%CI and *p*-values. A p-value < 0.05 was considered significant. Data were analyzed using STATA, v.15.0 (College Station, TX, United States).

RESULTS

Population Characteristics

A total of 31 pregnancies and 32 fetuses with CMV infection were included in the study (one monochorionic diamniotic pregnancy with both fetuses infected; one dichorionic diamniotic pregnancy with one fetus infected). The control group consisted of 33 singleton pregnancies. Twenty-two CMV-infected pregnancies were followed in the Fetal Infection Unit at the Hospital Clinic of Barcelona and 9 were followed in the Maternal-Fetal Medicine Unit at the Hôpital Louis-Mourier, Paris. Infection was confirmed by a positive PCR in amniotic fluid in 27 fetuses. Five patients at Hôpital Louis-Mourier declined amniocentesis, and infection was confirmed in urine of the newborns at birth. In 11 pregnancies fetal infection was suspected after maternal CMV screening in the first or second trimester. In 20 pregnancies, infection was suspected in the presence of fetal US anomalies: 13 during routine scans in the second trimester and 7 during the third trimester. Eighteen patients (58%) had a confirmed first trimester primary CMV infection, one had a second-trimester

seroconversion, and in 12 (39%) the type of maternal infection was unknown. The mean gestational age (GA) at diagnosis was 26.4 (4.5) weeks. Of the total sample of CMV-infected fetuses, one showed no abnormal US/MRI findings, 16 fetuses had non-severe features of infection, and 15 had severe brain abnormalities. The most frequent US/MRI findings were periventricular hyperechogenicity ("halo sign"), ventriculomegaly, abnormal gyration and white matter hyperintensity in 71, 29, 43, and 67% of the fetuses, respectively. The most frequent extra-CNS findings were SGA and hyperechogenic bowel in 70 and 35% of the cases. The prenatal US/MRI findings are shown in **Supplementary Table 3**.

Among the severely affected pregnancies, 13 women opted for TOP and post-mortem study was accepted by 11 (**Figure 2**: **Flowchart**). The median time from delivery to anatomopathological examination was 48 h. The median (IQR) of GA post-mortem was 30.3 (28.0–34.5) weeks. The median time (IQR) from MRI to post-mortem examination in cases with TOP was 1.0 (0.81–1.4) weeks. Eighty-two percent of the TOP-fetuses had none to mild maceration status. The median (IQR) of liver weight at post-mortem was 88.6 g (59.8–139).

The median GA (IQR) at MRI in the study population was 31.6 (28.6–33.4) weeks. The characteristics of pregnancies with and without CMV infection and according to the severity of brain abnormalities are summarized in **Tables 1**, **2**. We observed a significantly higher proportion of pregnant women with a child less than 3 years old of age, SGA at US and at birth among CMV-infected fetuses **Table 1**. We also found a tendency to low educational level among mothers with pregnancies involving severe CMV-infected fetuses, $p = 0.05$, **Table 2**.

Results of Magnetic Resonance Imaging Analysis:

We found no significant differences in FLV between CMV-infected fetuses and healthy-fetuses, (FLV [IQR]: 139.7 [112–161] vs. 126.4 [98–151], $\boldsymbol{p = 0.22}$), **Figure 3A**. When the FLV was corrected for AC and FBV we observed significantly

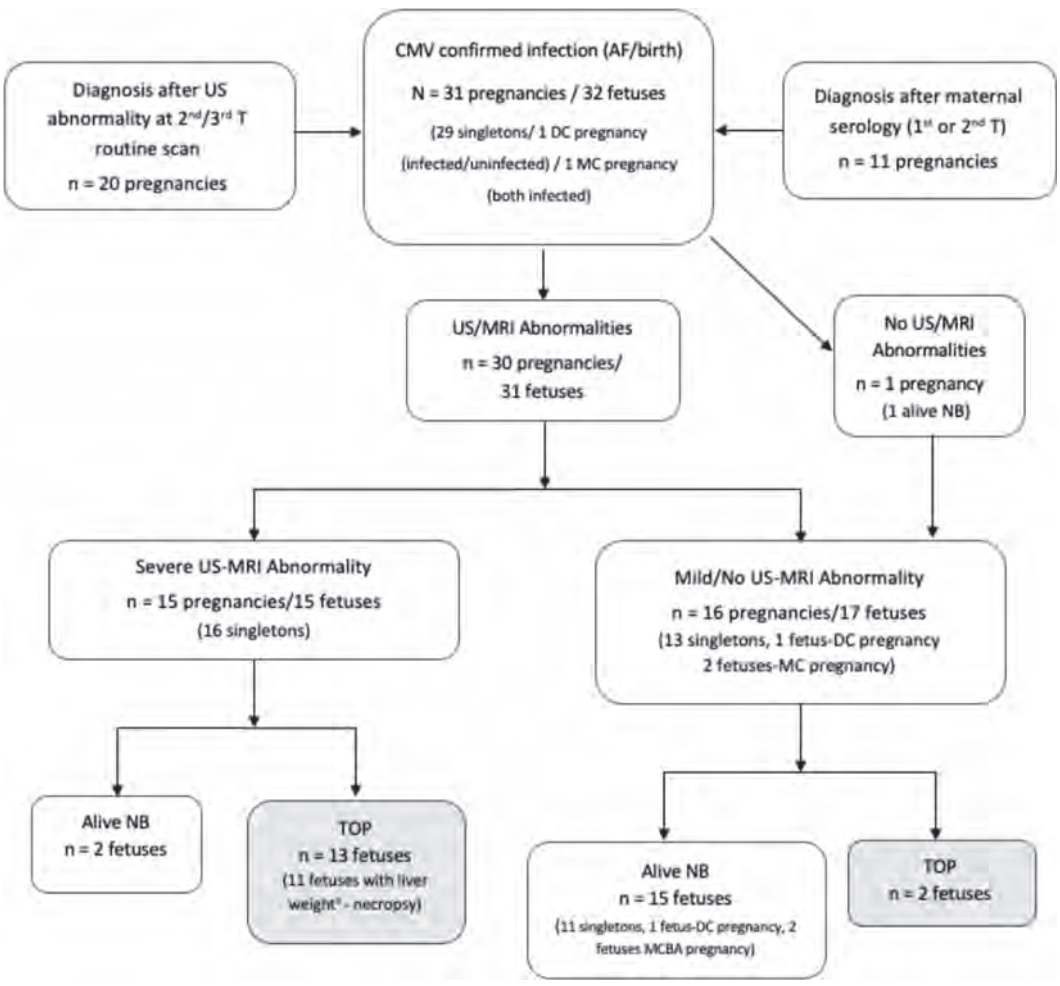

FIGURE 2 | Flowchart: Follow-up of pregnancies with confirmed fetal CMV infection. Description of outcomes according to the severity of infection, anatomopathological examination performed and liver weight at necropsy‡. DC, dichorionic; MC, monochorionic; AF, amniotic fluid; US, ultrasound; MRI, magnetic resonance imaging; TOP, termination of pregnancy; NB, newborn.

TABLE 1 | Baseline characteristics in pregnancies with and without congenital CMV infection.

Characteristic	cCMV *n* = 31 preg/32 fetuses	Controls** *n* = 33 preg/fetuses	*p** value
Maternal age, years, mean (SD)	32.4 (5.1)	32.8 (5.6)	0.86
Ethnicity (Caucasian), (%)	81	73	0.46
Multiparity, (%)	58	42	0.13
Low educational level, (%)	30	27	0.81
Child at nursery (<3 year child), (%)	**62**	**33**	**0.01***
Fetal gender (female), (%)	62	51	0.37
Gestational age at MRI, mean (SD)	31.2 (2.7)	30.7 (3.0)	0.46
EFW (g) at the time of MRI, mean (SD)	1570 (649)	1845 (519)	0.11
Small for gestational age, (%)	**34**	**5**	**0.012***
AC at the time of MRI (mm), mean (SD)	259.3 (38.1)	271.1 (42.2)	0.24
Fetal body volume (cm^3), mean (SD)	2371.2 (889)	2588.2 (897)	0.34
Gestational age at birth, mean (SD)	**37.6 (3.16)**	**39.3 (1.25)**	**0.025***
Birthweight (grams)†, mean (SD)	**2706 (822)**	**3326 (369)**	**0.002***

Data are presented as mean and standard deviation (SD), standard error of the mean (SEM), frequencies or percentage (%).
**p-value as determined with the t-test, Mann-Whitney U, X^2 or Fisher's exact test.*
cCMV, congenital cytomegalovirus; Preg, pregnancies; MRI, magnetic resonance imaging; EFW, estimated fetal weight.
***Controls: healthy fetuses.*
AC, abdominal circumference.
MCDA twin pregnancy in cCMV cases.
†*Seventeen cCMV cases with a live newborn included. The bold values indicate significant differences.*

TABLE 2 | Baseline characteristics of pregnancies with congenital CMV infection according to the severity of brain abnormalities.

Characteristic	Mild cCMV *n* = 16 preg/17 fetuses	Severe cCMV *n* = 15 preg/fetuses	*p** value
Maternal age, years, mean (SD)	32.8 (4.9)	32.4 (5.4)	0.83
Ethnicity (Caucasian) (%)	80	81	0.93
Multiparity, (%)	63	73	0.52
Low educational level, (%)	**13**	**47**	**0.05**
Child at nursery (<3 year child), (%)	56	73	0.32
Fetal gender (female), (%)	53	69	0.61
Gestational age at diagnosis in AF, mean (SD)	25.2 (4.9)	27.6 (3.8)	0.18
Gestational age at MRI, mean (SD)	31.9 (1.9)	30.4 (3.0)	0.13
EFW (g) at the time of MRI, mean (SD)	1758 (694)	1402 (539)	0.10
AC[á] at the time of MRI (mm), mean (SD)	270 (36.0)	246 (37.1)	0.10
Fetal body volume (cm^3), mean (SD)	2482 (900)	2260 (896)	0.50

Data are presented as mean and standard deviation (SD), frequencies or percentage (%).
**p-value as determined with the t-test, Mann-Whitney U, X^2 or Fisher's exact test.*
cCMV, congenital cytomegalovirus; Preg, pregnancies; AF, amniotic fluid.
[á]*AC, abdominal circumference.*
One MCDA twin pregnancy in mild cases. The bold values indicate significant differences.

higher liver volumes in CMV-infected fetuses compared to the healthy controls [FLV/AC-ratio (SEM): 5.18 (0.14) vs. 4.41 (0.14), $\boldsymbol{p = < 0.001}$; FLV/FBV-ratio (SEM): 6.11 (0.26) vs. 4.81 (0.13), $\boldsymbol{p = < 0.001}$], **Figure 3B1,B2**.

When the three study groups were compared, we also observed significantly higher ratios in the mild and in the severely infected cases compared to the healthy controls, **Figure 4A**. The FLV/AC-ratio and FLV/FBV-ratio remained higher when assuming a biological trend adjusted for GA at MRI, ($\boldsymbol{p = < 0.001}$) (parametric trend analysis), **Figure 4B**. However, we did not observe significant differences in the median liver volume or in the FLV/AC and FLV/FBV ratios according to the severity of CMV fetal brain abnormalities, **Supplementary Figures 1A,B1,B2**. The US/MRI findings, FLV, FLV/AC-ratio, FLV/FBV-ratio and liver weight at necropsy in severely affected fetuses with TOP and post-mortem examination are summarized in **Supplementary Table 2**. FLV showed a good correlation with the AC, GA at MRI, and FBV (r = 0.86, 0.82, 0.80, respectively; $\boldsymbol{p = < 0.001}$). There was excellent agreement between examiners [ICC = 0.96 (95% CI: 0.90–0.98); $\boldsymbol{p = 0.01}$; n = 16]. We also observed excellent concordance between fetal liver weight estimated by MRI and that obtained at anatomopathological examination [ICC = 0.95 (95% CI: 0.75–0.98): $\boldsymbol{p = < 0.001}$]. There was no case of hepatomegaly in the post-mortem examination.

DISCUSSION

Our results show that hepatomegaly assessed by MRI is probably an uncommon finding in congenital CMV. Although there were no significant differences in FLV, when adjusted for fetal size (i.e.,

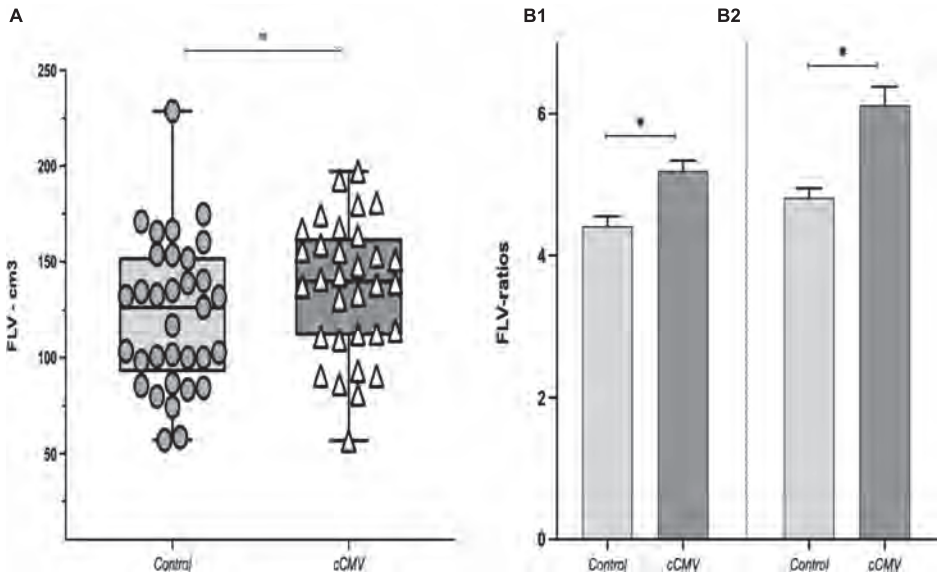

FIGURE 3 | (A) Fetal liver volume (FLV) between study groups, data presented as median (IQR: interquartile range: p25–p75). [*p-value as determined with the Wilcoxon rank-sum test (Mann-Whitney U); $\boldsymbol{p = 0.22}$]. **(B1)** FLV/AC-ratio (cm^3/cm), and **(B2)** FLV/FBV-ratio between the study groups. Data presented as mean (SD). *p-value as determined with the t-test; $\boldsymbol{p < 0.001}$ in both. cCMV: congenital cytomegalovirus fetuses (n = 32). Controls: (n = 33). AC, abdominal circumference; FBV, fetal body volume.

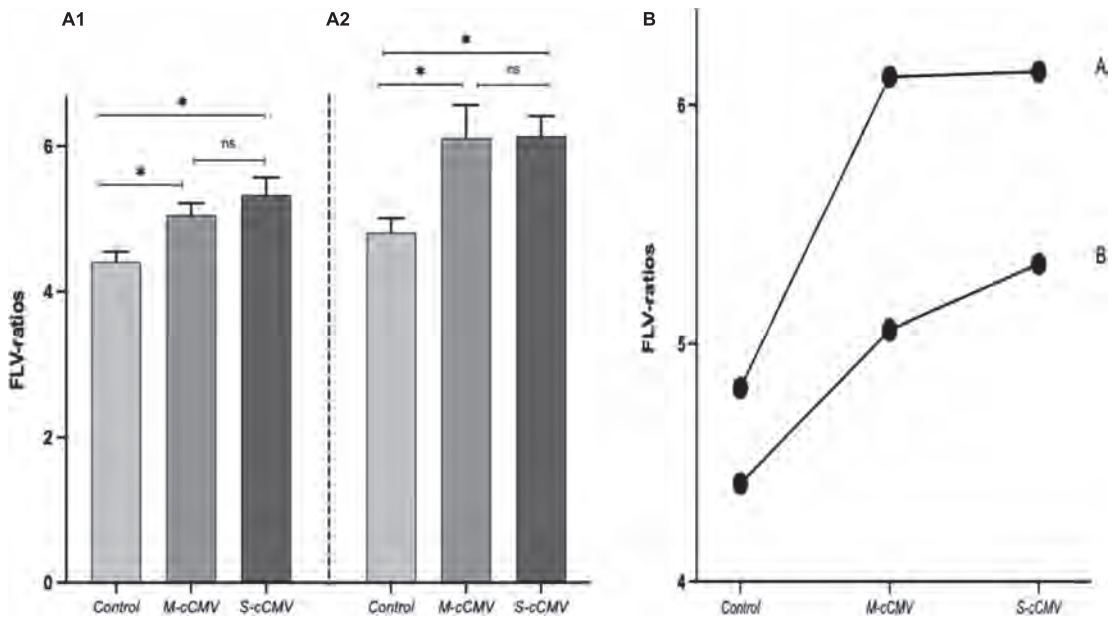

FIGURE 4 | Comparison between the study sub-groups. Fetal liver volume (FLV) ratios. **(A1)** FLV/AC-ratio, **(A2)** FLV/FBV-ratio. Data presented as mean (SD). *p-value as determined with analysis of variance and covariance (one-way ANOVA); $\boldsymbol{p < 0.001}$. **(B)** Linear trend analysis: FLV/FBV-ratio **(A)**, FLV/AC-ratio **(B)**. Data presented as mean (SD). *p-value as determined with parametric linear regression tendency test; $\boldsymbol{p < 0.001}$. Control, healthy fetuses; M-cCMV, mild congenital cytomegalovirus fetuses; S-cCMV, severe congenital cytomegalovirus fetuses; ns, not statistically significant.

AC or FV-B) the FLV was increased in CMV-infected fetuses compared to healthy controls. We could hypothesize that this is due to liver enlargement in relation to body composition.

Although adjustment for AC and FBV is not a standard procedure and the diagnostic accuracy is not established, other authors have used ratios to better understand the pathophysiology of different fetal conditions. Cannie et al. demonstrated that the observed/expected fetal lung volume based on FBV could predict fetuses at high risk of pulmonary hypoplasia (21, 22). It can be argued that using the head

circumference or cephalic diameter might have been a better normalization method in our study; however, microcephaly, a common feature of severe CMV infection, makes it less ideal.

As expected, one-third of the CMV-infected fetuses were SGA at the time of MRI and at birth (24, 25). The liver volume of these fetuses at MRI was similar to that of the control group. In this regard, Duncan et al. found that 63% of fetuses with an individualized birthweight <10th centile had a normal liver volume estimated by MRI (26). Interestingly, despite the larger hepatic ratios and greater number of SGA fetuses in the CMV-infected group in our sample, there were no statistically significant differences regarding AC measured at MRI. Despite the liver sizes, a lower AC would have been expected. There is controversy regarding the AC and liver size in intrauterine growth restriction (IUGR) fetuses. In this regard, Roberts et al. enrolled 98 fetuses with an AC below the 10th centile and found a liver length within normal limits in 82% (27). This may be because the measurement of AC may reflect not only liver size but also that of other intra-abdominal organs and the amount of fetal subcutaneous fat (28). Another possible explanation for the lack of significant differences in the AC is that compensatory arterial mechanisms may protect the liver from a reduction in blood flow in IUGR fetuses and thereby preserve the liver size in relation to the body composition (29, 30). In CMV infection the physiopathology of growth restriction and liver size could be related to mechanisms different from those in restricted fetuses due to placental dysfunction that could explain the finding of a relative liver enlargement in our cases.

Although we found a significantly higher FLV/AC-ratio and FLV/FBV-ratio in CMV cases compared to heathy controls, there were no significant differences in these ratios between mild and severely affected CMV-infected fetuses. We can speculate that perhaps mildly infected fetuses may already have hepatic involvement. Furthermore, hepatic involvement is not exclusively associated with severe fetal infection (31). Hepatic tropism and organ dysfunction in symptomatic CMV-infected newborns (9) is common. However, liver dysfunction as an isolated feature could be related to an acute phase of the infection in the second or third trimester and may not necessarily be related to adverse outcomes.

The mean liver weight in CMV-infected fetuses undergoing TOP at 30 weeks of gestation was 91.6 g. This was similar to the liver weights (53.4 to 98 g) of non-infected fetuses described by Maroun et al. at the same gestational age (32). This emphasizes our finding of no difference in the FLV estimated by MRI between CMV-infected and non-infected fetuses. As part of the internal validity of this study, we obtained excellent concordance between the FLV/weight estimated by MRI and the liver weight at post-mortem assessment. Liver weight is directly affected by the maceration status of the fetus (32, 33), being more affected in type-III maceration. However, 82% of the TOP-fetuses in our series had type-I maceration. Shelmerdine et al. found an excellent overall correlation between the liver volume estimated by MRI prior to autopsy and liver weight during autopsy ($r = 0.98$) in 45 fetuses (60% with none to mild maceration) in a mean time of 10 days from delivery to autopsy (34). Other authors have also found an excellent correlation between the volume estimated by fetal MRI and post-mortem in different organs such as the brain (99% for cerebrum, 89% for cerebellum) supporting the reliability of MRI for weight assessment (35).

This study has some strengths and limitations that should be mentioned. One of the main strengths is the comparison of CMV-infected fetuses with healthy-controls, and between infected fetuses according to severity of infection. Moreover, the measurements were manually performed by the same single examiner in both centers, thereby reducing the variability. In addition, this is one of the first studies describing not only liver volume but also comparing the weight of the liver examined post-mortem with the liver weight estimated by MRI in CMV-infected fetuses undergoing TOP, providing information about the reliability of this measurement. Among the limitations, we first acknowledge the retrospective nature of the analysis. Second, the different MRI protocol between centers. Third, the small sample size in the infected group according to the severity of central nervous system abnormalities may preclude the finding of differences between these two groups and the overlapping of the results. Fourth, asymptomatic CMV infection was not discarded among healthy controls: however, this probability is very low since congenital infection occurs in 0.7% of all fetuses/newborns (1). Moreover, *in vivo* measurement of liver weight was obtained only in pregnancies undergoing TOP in the severely affected CMV group, limiting the interpretation of our results. Nevertheless, we considered these data as part of the internal validity of the study and despite only being available in a small proportion of fetuses, the data demonstrate the excellent correlation between the two measurements. Finally, although assessment of fetal volumes performed in this study provided additional data on the extent of the pathophysiology of congenital CMV-infection, its usefulness in clinical practice could be questioned. Moreover, measurement of FBV by MRI is time consuming (almost twice the time required for determining FLV) (23); nonetheless, as technology improves and semi-automatic delineation of fetal structures advances (36), the time to calculate volumes will likely be reduced.

CONCLUSION

The liver volume obtained by MRI in CMV-infected fetuses was relatively greater than that of healthy-controls after adjustment for AC and FBV. Although increased FLV was not correlated with the severity of infection, these parameters could potentially be used as a surrogate marker of liver enlargement. Further studies are warranted to better understand the prognostic value of these findings.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are not publicly available due to restrictions according to patient privacy regulations but are available from the corresponding author on reasonable request.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of the Hospital Clinic (HCB/2017/0564) and Hôpital Louis-Mourier (CEERB-Paris Nord/2020-012). Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

EE, AH-V, and AG: conceptualization and design of the study. AH-V, ALM-E, EE, NH, ANak, OP, and NS: acquisition of data. ANad: anatomopathological examination. AH-V, RJM-P, AG, and EE: analysis and interpretation of data. AH-V, KC, and LT: MRI measurements. AG: supervision. AG, AH-V, and ALM-E: writing—original draft. AG, AH-V, EE, and FF: writing, revision, and editing of the submitted article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.889976/full#supplementary-material

Supplementary Figure 1 | Fetal liver volume (FLV) and FLV-ratios according to the severity of brain abnormalities in CMV-infected fetuses (mild: $n = 17$, severe: $n = 15$). **(A)** FLV (cm^3), data presented as median (IQR: interquartile range: p25–p75). *p-value determined with the Wilcoxon rank-sum test (Mann-Whitney U); $\boldsymbol{p = 0.68}$. **(B1)** FLV/AC-ratio. *p-value as determined with the t-test; $\boldsymbol{p = 0.39}$. **(B2)** FLV/FBV-ratio, data presented as mean (SEM, standard error of the mean).

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STUDY 4

Cortical maturation assessed by magnetic resonance imaging in unaffected/mildly affected fetuses with cytomegalovirus infection.

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Title: Cortical maturation assessed by magnetic resonance imaging in unaffected/mildly affected fetuses with cytomegalovirus infection*

Short title: MRI cortical development in fetal CMV infection.

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* Presented as oral communication at: European Congenital Cytomegalovirus Initiative (ECCI) Congress, November 13–14, 2020.

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Contribution:

What are the novel findings of this work?

This is the first study on congenital CMV-infection demonstrating delayed cortical maturation (underdeveloped calcarine, parietooccipital sulci and larger Sylvian fissure angle in addition to a lower cortical-grading in parietal and temporal areas) in sonographically unaffected or mildly affected fetuses assessed by magnetic resonance imaging (MRI).

What are the clinical implications of this work?

These data suggest that fetal CMV infection, even when considered of good prognosis, seems to be associated with an altered pattern of fetal brain cortical maturation compared to healthy, noninfected fetuses. Our results reinforce the use of MRI as complementary tool for assessing brain structure at 32 weeks of gestation in CMV-infected fetuses even without US abnormalities

Key words: fetal brain, cortical development, pregnancy, fetal cytomegalovirus infection, fetal magnetic resonance imaging.

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What are the clinical implications of this work?

Fetal CMV infection, even considered of good prognosis, seems to be associated with an altered pattern of fetal brain cortical maturation compared to healthy, noninfected fetuses. Our results reinforce the use of MRI as complementary tool for assessing brain structure at 32 weeks of gestation in CMV-infected fetuses even without US abnormalities.

Key words: fetal brain, cortical development, pregnancy, fetal cytomegalovirus infection, fetal magnetic resonance imaging.

ABSTRACT

Objective: This study aimed to comprehensively assess the pattern of cortical maturation by magnetic resonance imaging (MRI) in fetuses with unaffected and mildly affected CMV infection determined by ultrasound (US) and establish possible differences compared to healthy controls.

Methods: Twenty-four CMV-infected fetuses (7 US unaffected, 17 US mildly affected) and 24 healthy controls between 27 and 36 weeks of gestation undergoing fetal MRI were included. Fetuses were considered unaffected or mildly affected according to prenatal US/MRI neuroimaging findings. We compared fetal sulci depth, Sylvian fissure depth, Sylvian fissure, and angle and cortical maturation grading of specific sulci and areas among study groups. Regression, parametric trend and intraclass correlation analysis were performed.

Results: Compared to controls, in the CMV-infected fetuses showed a significantly larger median (interquartile range: IQR) width of the lateral ventricles [right (mm): 3.9 (2.6-5.3) vs. 7.8 (5.9-9.9); left:

4.2 (3.2-5.3) vs. 7.5 (6.0-10.9)], significantly decreased parietooccipital sulcus [right (mm): 15.9 (14.7-17.3) vs. 12.6 (11.3-13.5); left: 16.0 (13.3-17.5) vs. 12.3 (10.6-13.5)] and calcarine sulcus depth [right (mm): 17.5 (16.1-18.7) vs. 15.4 (14.4-16.3); left: 16.7 (15.6-18.9) vs. 14.6 (14.1-15.6)], $p<0.001$; and a significantly larger upper and lower Sylvian fissure angle [upper, right (°): 42.8 (35.8-45.8) vs. 48.9 (38.4-64.7); left: 40.9 (34.2-45.8) vs. 48.2 (41.9-60.7)], [lower, right: 41.6 (34.4-49.2) vs. 48.9 (40.6-60.9); left: 42.2 (38.8-46.9) vs. 48.9 (39.5-57.5)]; $p<0.05$. In addition, the infected fetuses had a significantly lower cortical-grading in the temporal and parietal areas, and the parietooccipital and calcarine sulcus compared to healthy fetuses ($p<0.05$). These differences persisted when adjusting for gestational age, ipsilateral atrium width, fetal gender and considering being small for gestational age as a confounding/interacting factor.

Conclusion: Unaffected and mildly affected CMV-infected fetuses with mild sonographic involvement showed underdeveloped cortical maturation compared to healthy controls. These results suggest that congenital CMV infection, even in non-severely affected fetuses, which are typically considered of good prognosis, could be related to altered brain cortical structure. Further research is warranted to better elucidate its correlation with neurodevelopmental outcomes.

1-Background

Cytomegalovirus (CMV) is the most common congenital infection and remains a major cause of sensorineural hearing loss and neurodevelopmental abnormalities worldwide[1]. Detection during pregnancy is usually done when sonographic signs suggestive of infection are found during routine scans[2]. Maternal screening of infection has recently been recommended since there is treatment to prevent vertical CMV transmission[3]. After confirmation of fetal infection, the addition of magnetic resonance imaging (MRI) as a complementary tool to targeted ultrasound (US) for examination of the fetal brain increases the positive predictive value for the diagnosis of brain abnormalities in CMV-infected fetuses[4–7]. The use of these two imaging modalities is helpful for appropriate counseling since current prognosis assessment in fetal CMV-infection is mainly based on cerebral findings[4].

The developing brain is vulnerable to inflammation secondary to the fetal CMV-infection causing cell injury. Neuronal injury early in pregnancy can lead to significant life-long neurocognitive impairment[8,9]. Long-term sequelae are expected in 40-60% of symptomatic survivors, and in 10-20% of asymptomatic children[5]. A significant percentage of fetuses with normal third-trimester US become symptomatic at birth or develop delayed congenital (cCMV)-associated symptoms[10,11] and MRI is recommended to better visualize temporal lobes and better depict gross cortical abnormalities. The cerebral cortex develops in three overlapping stages: cell proliferation, neuronal migration, and cortical organization[12,13]. Cortical development appears as gyri and sulci late in pregnancy due to neuronal migration[14–16]. Sulcal development is a markers of cortical maturation and is used as an indicator of cortical development[12,16,17] and may also be related to the cytoarchitectural organization of the brain[18]. Although the central nervous system (CNS) is a major target of cCMV-infection[19], there is scant information on cortical maturation in infected fetuses without US/MRI-findings or in those with US/MRI features considered of good/uncertain prognosis. Comprehensive characterization of the brain cortical development in-utero by MRI is a critically important reference point that can provide more information about underlying structural changes related to the mild fetal infection[16,18,20].

The aim of this study was to comprehensively compare cortical maturation in terms of sulci depth, sulci grading and area grading in CMV-infected fetuses and healthy controls.

2-Materials and methods

We performed a retrospective case-control study that included consecutive pregnancies with a CMV-infected fetus undergoing a prenatal MRI as a complementary clinical tool to aid in the prognostic assessment of these patients over an 11-year period (March 2009-December 2020) in BCNatal (Hospital Clínic and Hospital Sant Joan de Déu) and over a 3-year period (February 2016-September 2019) in Hôpital Louis-Mourier. The study was approved by the Institutional Review Board of the Hospital Clinic **(HCB/2017/0564)** and Hôpital Louis-Mourier **(CEERB-Paris Nord/2020-012).**

Infected fetuses were considered unaffected or mildly affected according to prenatal US/MRI findings[21,22]. Sixty-one percent were diagnosed after maternal CMV screening according to local hospital policy (France) or by attending physicians. The remaining 39% of the cases were diagnosed by

evidence of US abnormalities during the routine second or third trimester scans. CMV infection was confirmed with extraction of CMV DNA for fetal and neonatal samples using the QIAsymphony system (Qiagen, Hilden, Germany). Chromosomal abnormalities and toxoplasmosis infection were ruled out at the time of amniotic fluid study. After fetal US/MRI, women were counseled about the prognosis of the newborn. Termination of pregnancy (TOP) was discussed according to Spanish/French laws. In cases of TOP, after obtaining informed consent from the parents, routine postmortem examination of the fetus and the placenta was performed. In cases with a live newborn, cCMV was confirmed by a positive polymerase chain reaction (PCR) of a urine or saliva sample taken within the first 48 hrs of birth. Administraton of valacyclovir treatment for the prevention of progression of fetal brain lesions was recorded. The control group was made up of singleton low-risk pregnancies with normal growth fetuses without structural abnormalities and healthy newborns attended at BCNatal from a larger prospective cohort[23,24]. Controls did not undergo any additional genetic or infection testing aside from routine blood tests during pregnancy.

MRI Acquisition:

Magnetic resonance imaging was performed in both centers with a clinical MRI system (1.5T Magnetom Aera syngo MR D13; Siemens, Erlangen, Germany in Hospital Clinic and 1.5T GE Signa Horizon, Echo speed, LX MRI scanner, Milwaukee, WI, USA in Hôpital Louis-Mourier) with a 5-channel cardiac coil. No maternal or fetal sedation was used[25]. The 3 orthogonal planes of the fetal head were used to measure sulci depth and grading by T2-weighted sequences obtained using a single-shot, fast spin-echo (Hospital Clinic: repetition time [RT]= 1000 ms, echo time [ET] = 137 ms, slice thickness = 3.5 mm, gap = 3.5 mm, field of view [FOV] = 225 x 300 mm, voxel size = 0.59 x 0.59 x 3.5 mm, matrix = 256 x 192 mm, flip angle = 135°, acquisition time = 32 s; Hôpital Louis-Mourier: RT= 1120 ms, ET = 92.7 ms, slice thickness = 3.5 mm, gap = 3.5 mm, FOV= 300 x 300 mm, voxel size = 0.55 x 0.55 x 3.5 mm, matrix = 320 x 256 mm, flip angle = 90°, acquisition time = 32 s).

MRI Analysis

Biparietal diameter (BPD), head circumference, posterior lateral ventricular width and all normative biometry of the fetal brain were determined according to Kyriakopoulou et al[26]. MRI measurements were performed by A.H-V (blinded to CMV-infection status).

Sulci Depth: sulcal measurments were performed off-line using HOROS™ Project medical image viewer v3.3.6 software (LGPL-3.0) for T2- weighted sequences with a 3.5-mm slice thickness. Laterality was assessed by determining the fetus position in utero. Anatomic planes for measurements were assessed using the International Society of Ultrasound in Obstetrics and Gynecology guidelines for sonographic examination of the fetal nervous system[27,28]. Sulci and fissure measurements in millimeters (mm) of both hemispheres was assessed by tracing a perpendicular line from the midline towards the border of the specific sulcus as previously described by Hahner et al[23]. To assure a 90° degree angle, lines were traced using the HOROS™ Software perpendicular lines tool. A straight line was traced from the frontal bone to the occipital bone in axial planes and vertically from the vault to the skull base in coronal planes. All measurements were normalized by BPD and multiplied by 100.

Sylvian fissure angles (SFA): SFA were measured in a coronal transthalamic plane (landmarks: anterior horns of lateral ventricles, cavum septi pellucidi, third ventricle at the point of maximum development, parietal, and temporal lobes) adapting previously methodology[14]. From the midline, a perpendicular horizontal line should be drawn on the third ventricle until the extracerebral fluid compartment on both sides. Four lines were drawn: bilaterally along the upper external and internal side of the Sylvian fissure (SF). The upper and lower SFA formed by these lines were measured, using the horizontal line as the reference (0°). **Figure 1a**.

Cortical Grading: cortical areas and cortical sulci/fissure grading were performed according to Pistorius et al., with a scoring methodology ranging from Grade 0 (no maturation) to Grade 5 (maximum maturation)[29].

Statistical analysis

Data between fetuses with CMV infection (unaffected/mildly affected) and healthy fetuses were compared, according to the US/MRI abnormalities (CNS and/or extra-CNS). Quantitative variables

were assessed using Shapiro-Wilk's test for normality, and normally distributed variables were compared using the t-test and expressed as mean and standard deviation (SD). Non-normally distributed quantitative variables were compared using the Mann-Whitney U test and expressed as median and interquartile range (IQR: p25-75). Qualitative variables were compared using the Chi squared test and Fisher's exact tests. To correct for fetal head size, we adjusted sulcal depth/SFA by biparietal diameter. For brain quantitative variables a linear regression analysis adjusted for ipsilateral ventricular width and gestational age at MRI was performed. The variable small-for-gestational-age (SGA) was analyzed as a probable confounding/interaction factor. A sub-analysis of variance and covariance using a non-parametric test (Kruskal-Wallis equality of population rank test) was performed. Non-parametric tendency analysis (Jonckheere-Terpstra Test) was performed according to a possible biological trend (healthy vs. unaffected CMV fetuses vs. mild affected CMV-infected fetuses). To assess reproducibility of SFA measurement using MRI, interobserver reliability analysis was done comparing the measurements of A.H-V & K.C (blinded to each other) applying the intraclass correlation (ICC) estimates and their 95% confidence interval (95% CI) in 13 randomly chosen healthy fetuses by a two-way random effect model with absolute agreement. A robust estimation was used to calculate 95% CIs and p-values. P-value <0.05 was considered significant. Data were analyzed using STATA, v.15.0 (College Station, Texas).

3-Results

Population characteristics:

MRI was performed in 45 pregnancies and 46 fetuses between 27 and 36 weeks of gestation: 24 CMV-infected fetuses [23 pregnancies: 21 singletons, one dichorionic-pregnancy (one infected), and one monochorionic-diamniotic (MCDA) pregnancy (both infected), **Figure 2**] and 24 healthy fetuses (all singleton pregnancies) as the control group. Infection was confirmed by a positive PCR in amniotic fluid in 21 fetuses. Three patients at Hôpital Louis-Mourier declined amniocentesis, and infection was confirmed in the urine of the newborns at birth. Thirteen patients (57%) had a confirmed first trimester confirmed primary CMV infection; two presented second-trimester seroconversion; two had a non-primary infection, and in six (26%) the type of maternal infection was unknown (one MCDA

pregnancy). The median (IQR) gestational age (GA) at diagnosis of fetal infection was 22.5 (21.4-30.0) weeks. High-dose valacyclovir (8g/24h) was administered to 12 patients (52%) as tertiary prevention for prevention of fetal sequelae at a median (IQR) GA of 26.0 (23.7-32.6) weeks, with a median treatment duration of 13 (7-15) weeks. The median gestational age at MRI was 32.6 weeks (IQR: 31.6-33.6), with no significant differences between study groups, **Table 1**. We observed a significantly higher proportion of multiparity, 3-year-old children, SGA, and a significantly lower birthweight among CMV-infected pregnancies, **Table 1**.

The most frequent cerebral US findings were subependymal cysts (21%), hyperechogenic caudate nucleus and lenticulostriate vasculopathy in 17% of the fetuses. The most frequent extra-cerebral findings were SGA and hyperechogenic bowel in 21% of the cases. Among the MRI cerebral findings, in 37% of cases we observed white matter hyperintensity (WMHS) and temporal lobe cyst in 25%. The prenatal US/MRI findings of the 17 mildly affected fetuses are shown in **Supplementary table 1.**

MRI analysis results:

In the CMV-infected fetuses we observed significantly larger lateral ventricles width compared to healthy controls [right (mm): 7.8 (5.9-9.9) vs. 3.9 (2.6-5.3); left: 7.5 (6.0-10.9) vs. 4.2 (3.2-5.3)], $p<0.001$, with no differences in other brain structures (**Supplementary Table 2).** When compared according to the degree of involvement, mildly affected CMV-infected fetuses showed larger ventricular widths compared to the unaffected (**Figure 3)** with a significant trend according to severity, $p<0.001$.

Cortical development measurements in the study groups are shown in **Table 2.** We observed a significantly decreased depth in the parietooccipital sulcus and calcarine sulcus in CMV-infected fetuses (unaffected and mildly affected) compared to controls; $p<0.001$. This difference remained when assuming a biological trend, $p<0.001$. We observed significantly larger SFAs (upper/lower) bilaterally in mildly affected CMV-infected fetuses compared to healthy controls; $p<0.001$. There was a significant linear tendency when assuming a biological trend in the upper/lower right SFA and in the upper left SFA, $p<0.05$. In addition, significantly lower cortical-grading in the temporal and parietal areas, parietooccipital sulcus, and calcarine sulcus was observed in mildly affected CMV-infected fetuses vs. healthy controls; $p<0.05$, **Figure 4.** No significant differences were found in the SF, cingulate sulcus,

superior temporal sulcus, central sulcus, frontal area, and mesial area between CMV-infected fetuses compared to healthy controls. (**Supplementary figure 1**)

Finally, there was excellent agreement of the four SFAs between examiners. Upper right: ICC= 0.95 (95%CI: 0.83–0.98); **p<0.001**. Lower right: ICC= 0.90 (95%CI: 0.50–0.97); **p<0.001**. Upper left: ICC= 0.95 (95%CI: 0.82–0.98); **p<0.001**. Lower left: ICC= 0.96 (95%CI: 0.87–0.98); **p<0.001**.

4-Discussion

This study provides evidence that in cCMV-infection, sonographically unaffected or mildly affected fetuses, present a significant delayed pattern of cortical brain maturation compared to healthy fetuses. Parietooccipital and calcarine sulci depth was significantly reduced, with a significantly larger SFA and lower cortical-grading in the parietooccipital and calcarine sulci, temporal and parietal areas. To our knowledge, this is the first study to characterize cortical brain development according to sulci depth and cortical-grading assessed by MRI-analysis in CMV-infected fetuses with normal and mild US/MRI findings compared to healthy-controls.

Descriptions of structural brain findings in CMV-infected fetuses with mild or no brain involvement are scarce. Hoffman et al. described a significantly smaller temporal lobe volume normalized to whole brain volume between 24 to 38 weeks of gestation in CMV-infected fetuses without MRI findings or with white matter hyperintensity (WMHS) compared to controls, with marked changes in those with WMHS[30]. There are contradictory findings regarding the apparent diffusion coefficient (ADC), which allows a quantitative measurement of brain maturation. Katorza et al. described higher ADC-values in the parietal and temporal lobes of CMV-infected fetuses compared to fetuses with WMHS[31] of unkown etiology, suggesting lesser brain maturation. Contrarily, Kotovich et al.[32] found lower ADC-values in CMV-fetuses without WMHS and unremarkable fetal MRI results compared to matched GA-uninfected controls. These differences could be related to different phases of cellular injury. In our series, 37% of the cases had WMHS, and almost a third had unremarkable US/MRI. Likewise, we found significantly lower cortical-grading in temporal and parietal lobes and a linear tendency to higher upper/lower SFA regardless of the presence or absence of WMHS.

Being an SGA fetus could confound our findings, since 21% of CMV-infected fetuses were SGA. Based on our regression analysis, being SGA did not confound our results. Moreover, previous data in small fetuses without cCMV showed an increased insular depth and reduced SF depth[33–35], changes not observed in our CMV-infected fetuses.

Our data showed that in CMV-infected fetuses the upper SFA was significantly larger than in controls, likely suggesting underdeveloped cortical maturation of the superior operculization process of the SF[36,37]. Our findings agree with Pooh et al. who described that the SFA may be an indicator for the subsequent development of cortical malformation.

CMV-infection at different GAs may have a distinct pattern of cellular and developmental effects on the brain that may ultimately determine the neurological outcomes[10,38–44]. More than half our cases were first trimester infections except for two seroconversions at the beginning of the 2nd trimester, and in a quarter of pregnancies the time of infection was unknown. Although the impact of latent CMV-infection is unclear[9,43], we hypothesized that the larger ventricular width in our CMV-infected fetuses could reflect persisting inflammation due to latent infection, possibly resulting in an underdeveloped cortical maturation. Cellular proliferation occurs in the germinal matrix on the ventricular zone of ventricular walls from 8 to 16 weeks of gestation, followed by neuronal migration, and finally synaptogenesis characterized by the appearance of sulci and gyri[12,18,45], being critical steps for the sulcation process.

We hypothesized that the decreased parietooccipital and calcarine sulci depth, and lower cortical-grading in the temporal and parietal-areas and parietooccipital and calcarine sulci in the mildly affected CMV-infected fetuses is related to the cell injury caused by infection during brain embryonic development and strongly related to the ventricular system. Sarnat et al. described that the depth of a fissure/sulcus may be influenced by the adjacent ventricular system[46]. The occipital horn is the most recent recess of the lateral ventricle, being, hence, most vulnerable between weeks 6 and 9. Although we could not determine the timing of infection in slightly over a quarter of our sample, these fetuses may have acquired the infection in the first trimester or early second trimester of pregnancy, potentially explaining our findings.

Our results reinforce the suggestion that MRI be considered a complementary tool for assessing brain structure at 32-weeks of gestation in CMV-infected fetuses even without US abnormalities.

Identification of unaffected/mildly affected CMV-infected fetuses at risk of altered neurodevelopment due to subtle prenatal changes in cortical development has potential importance in clinical practice. Evaluation of sulci depth, grading and SFA is feasible and can provide an overview of brain cortex maturation to detect subtle alterations in specific regions that could explain neurodevelopmental outcomes in infected fetuses. It should be stressed that due to the paucity of data on normal sulci depth, we could not provide a cut-off value that could be useful in clinical practice. Moreover, we acknowledge that these findings may need correlation with neurological outcomes. Nonetheless, in fetuses with isolated non-severe ventriculomegaly, similar changes in parietooccipital and calcarine sulci have been reported. These neonates showed weaker, albeit non-significant, performance in the motor and range of state clusters in the Brazelton test.[23]

The main strengths of this study are MRI assessment in a well characterized group of CMV-infected fetuses and manual MRI measurements by a single examiner blinded to the CMV-infection status. We applied the SFA, a recently described neurosonography measurement with excellent interobserver agreement, that can serve as a screening tool for malformations of cortical development[14]. Lastly, we described the use of the SFA assessed by MRI in cCMV-infection that can improve our understanding of cortical brain architecture and the pathophysiology of fetal CMV-infection. The main limitations are the retrospective nature of the analysis and the small sample size mainly of unaffected CMV-infected fetuses. Neither could we establish the exact time of infection in almost a third of the cases and 52% of the patients were treated with valacyclovir as preventive treatment but we were unable to determine possible potential effects on our findings. Finally, asymptomatic CMV-infection was not excluded among controls, although this probability is very low considering a 0.7% incidence of cCMV.

In conclusion, CMV-infected fetuses with unremarkable US/MRI or with mild involvement showed underdeveloped cortical maturation assessed by MRI compared to healthy controls. Postnatal follow-up studies are warranted to understand the consequences of delayed cortical maturation in CMV-infected fetuses without US/MRI findings and in those with mild involvement.

Conflict of interest: None

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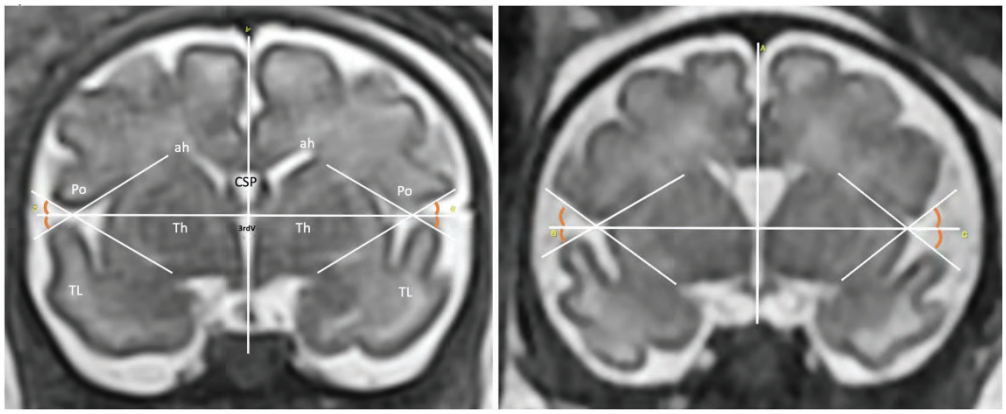

Figure 1. Illustrative coronal transthalamic plane magnetic resonance images showing the measurement of the upper/lower Sylvian fissure angles (SFA) in both hemispheres. (a) 33-week healthy control fetus. (b) 33-week CMV-infected fetus. Note that the CMV-infected fetus has a wider upper and lower SFA.
ah: anterior horns of lateral ventricles, CSP: cavum septi pellucidi, 3rdV: third ventricle, Th: thalamus, Po: parietal operculum, TL: temporal lobe.

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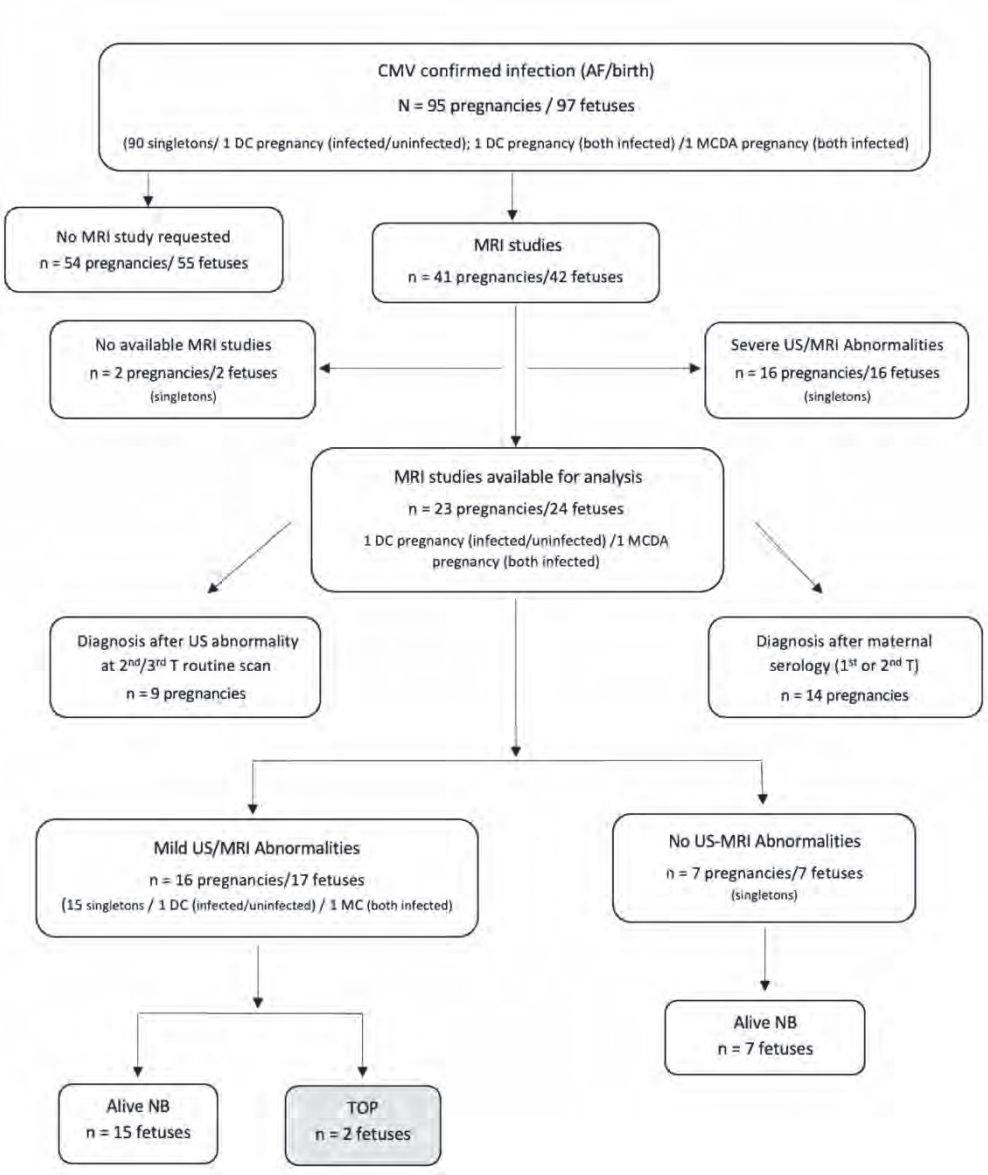

Figure 2: Flow chart: Pregnancies followed with confirmed fetal CMV infection. Description of the outcome according to the US/MRI features.
DC: dichorionic, MCDA: monochorionic diamniotic, AF: amniotic fluid, US: ultrasound, MRI: magnetic resonance imaging, TOP: termination of pregnancy, NB: newborn.

202x242mm (300 x 300 DPI)

Table 1. Baseline characteristics of the study population.

Characteristic	Controls n = 24 preg/24 f	cCMV n = 23 preg/24 f	*p** value
Maternal Age (years), mean (SD)	33.1 (6.4)	32.9 (4.4)	0.93
Ethnicity (Caucasian), n (%)	19/24 (79)	16/23 (70)	0.45
Multiparity, n (%)	**9/24 (38)**	**19/23 (83)**	**0.003**
Low Educational level, n (%)	5/24 (21)	6/23 (26)	0.81
3-year-old child, n (%)	**6/24 (25)**	**16/23 (70)**	**0.003**
Gestational age at MRI, median (IQR)	32.8 (29.5-33.8)	32.4 (31.7-33.4)	0.91
EFW (g) at the time of MRI, mean (SD)	1979 (416)	1884 (621)	0.58
Small for gestational age, n (%)	**0/24 (0)**	**5/24 (21)**	**0.05**
Fetal gender (female), n (%)	13/24 (54)	11/24 (46)	0.56
Gestational age at birth, median (IQR)	39.4 (37.8 - 40.1)	38.8 (37.6 - 39.4)	0.19
Birthweight (g), median (IQR)	**3320 (3006 - 3610)**	**2955 (2652 - 3298)**	**0.027**

Data are presented as mean and standard deviation (SD), standard error (SE), frequencies or percentage (%), median (IQR: interquartile range: p25-p75). **p* value as determined with the *t*-test, Mann-Whitney U, X^2 or Fisher's exact test.

cCMV: congenital cytomegalovirus. preg: pregnancies, f: fetuses. MRI: magnetic resonance imaging. EFW: estimated fetal weight. BPD: biparietal diameter. HC: head circumference. cCMV unaffected, n=7; cCMV mildly affected, n=17.

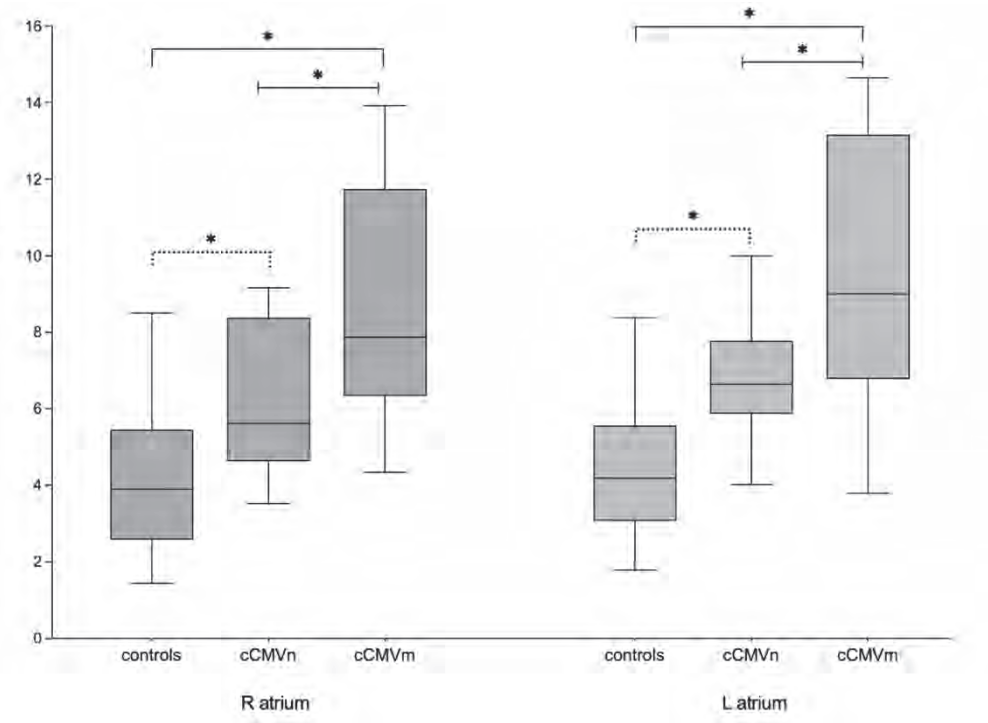

Figure 3. Boxplot graph of lateral ventricular width in right and left brain hemispheres in study groups. p value estimated with the Kruskal-Wallis equality of population rank test, adjusted for gestational age at MRI. *p <0.05, (controls: healthy fetuses, cCMVn: unaffected CMV-infected fetuses, cCMVm: mildly affected CMV-infected fetuses). R: right, L: left.

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Table 2. Magnetic resonance imaging cortical development parameters among study groups.

Characteristic	Controls n = 24	cCMVn n = 7	cCMVm n = 17	*p** value	*p*† value
Right hemisphere ^					
Insula (mm)	29.7 (28.3 - 30.9)	29.4 (28.2 - 31.4)	29.9 (28.8 - 31.5)	0.67	0.69
Sylvian fissure (mm)	15.9 (14.7 - 17.1)	15.4 (13.7 - 16.2)	15.8 (15.1 - 17.1)	0.46	0.92
Parietooccipital sulcus (mm)	15.9 (13.5 - 17.3)	12.7 (11.9 - 13.3) *	12.5 (11.3 - 13.8) †	**0.002**	**< 0.001**
Cingulate sulcus (mm)	4.76 (4.15 - 6.52)	4.32 (4.04 - 5.17)	4.77 (4.03 - 5.61)	0.44	0.61
Calcarine sulcus (mm)	17.5 (16.1 - 18.7)	15.6 (14.7 - 16.9) *	15.4 (13.9 - 16.0) †	**0.015**	**< 0.001**
Upper Sylvian fissure angle (°)	42.8 (35.8 - 45.8)	41.6 (33.5 - 58.6)	50.1 (40.4 - 65.5) †	0.71	**0.012**
Lower Sylvian fissure angle (°)	41.6 (34.4 - 49.2)	42.6 (38.6 - 49.1)	50.7 (45.8 - 64.6) †	0.79	**0.009**
Left hemisphere ^					
Insula (mm)	29.5 (28.5 - 30.7)	29.4 (28.2 - 31.9)	29.4 (29.0 - 31.2)	0.90	0.94
Sylvian fissure (mm)	16.5 (15.6 - 17.8)	16.1 (14.7 - 17.4)	16.1 (15.2 - 17.6)	0.77	0.95
Parietooccipital sulcus (mm)	16.0 (13.3 - 17.5)	13.1 (10.1 - 14.4) *	11.6 (10.8 - 12.4) †	**0.004**	**< 0.001**
Cingulate sulcus (mm)	4.82 (4.02 - 6.57)	4.59 (3.90 - 5.40)	4.79 (3.84 - 5.51)	0.61	0.53
Calcarine sulcus (mm)	16.7 (15.6 - 18.9)	15.4 (14.2 - 15.9) *	14.6 (14.1 - 15.5) †	**0.018**	**< 0.001**
Upper Sylvian fissure angle (°)	40.9 (34.2 - 45.8)	45.3 (40.1 - 59.5)	51.0 (43.1 - 61.3) †	0.11	**< 0.001**
Lower Sylvian fissure angle (°)	42.2 (38.8 - 46.9)	40.7 (34.8 - 48.9)	54.6 (46.6 - 61.9) †	0.54	**< 0.001**

Data are presented as median (IQR: interquartile range: p25-p75). *p* value (<0.05) determined with the Kruskal-Wallis equality of populations rank test and adjusted by ipsilateral atrium and gestational age at MRI, and SGA as a confounding factor compared to controls. *p value between healthy controls vs. cCMVn. †p value between healthy controls vs. cCMVm. ^ Variables normalized by BPD: biparietal diameter and multiplied by 100. cCMVn: unaffected cytomegalovirus infected fetuses. cCMVm: mildly affected cytomegalovirus infected fetuses.

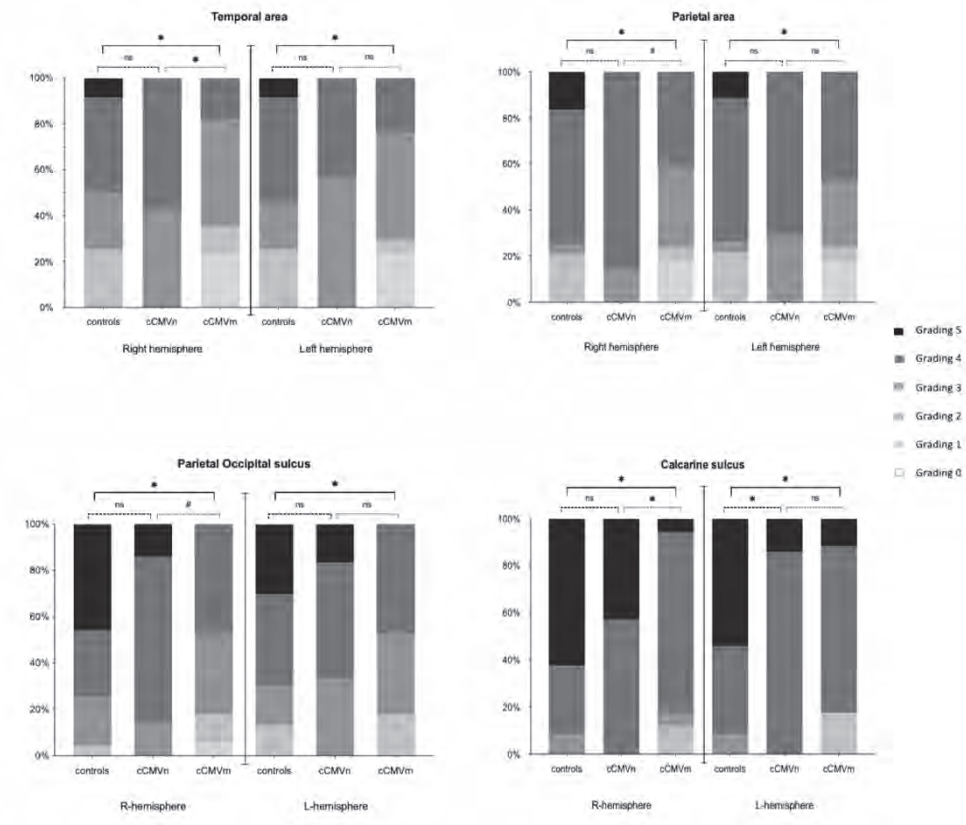

Figure 4. Cortical grading of areas and sulci among study groups. Distribution of cortical grading scores of significantly different cortical areas and sulci of right and left hemispheres between controls: healthy fetuses, vs. cCMVn: unaffected cytomegalovirus-infected fetuses, vs. cCMVm: mildly affected cytomegalovirus-infected fetuses. p value by X2 or Fisher's exact test. *p<0.05 adjusted for ipsilateral atrium and gestational age at MRI.

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STUDY 5

What does the "halo" sign mean in fetal cytomegalovirus infection? Cerebral imaging abnormalities and postmortem histopathology in a cohort of 35 infected fetuses

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Status: draft completed

Contribution:

What are the novel findings of this work?

This is the largest cohort of periventricular echogenic halo in cytomegalovirus infected fetuses with a targeted histopathological examination. The halo sign, when observed isolated, was detected exclusively during second trimester, and was correlated with mild ventriculitis and mild histopathological staging.

What are the clinical implications of this work?

In fetuses with isolated halo sign in the second trimester there is a low probability of severe brain damage. However, its prognostic value for counseling parents, requires an accurate brain imaging follow-up including neurosonographies and magnetic resonance imaging.

Key words: pregnancy, fetal cytomegalovirus infection, fetal ultrasound, fetal histopathology, fetal MRI, periventricular halo, prognosis.

ABSTRACT

Objectives: To analyze the histopathological correlation of the halo sign and its association with other brain imaging abnormalities in fetuses infected with cytomegalovirus (CMV)**.**

Methods: Retrospective evaluation of all fetuses with severe CMV infection based on central nervous system (CNS) abnormalities that ended in termination of pregnancy (TOP) or fetal demise, diagnosed at a single center. A Maternal-Fetal Medicine expert reanalyzed the images from the transabdominal and transvaginal neurosonography scans. The halo sign was defined as homogeneous periventricular echogenicity observed in the three fetal brain orthogonal planes (axial, parasagittal and coronal).

Isolated halo was defined as the only CNS finding, and non-isolated halo as observed concomitant with other CNS anomalies. An expert fetal radiologist reanalyzed magnetic resonance imaging

(MRI) in cases in which it had been performed. Both experts were blinded to histological results. Hematoxylin-eosin-stained histologic slides were independently reviewed by two experienced pathologists blinded to neuroimaging results. Ventriculitis was classified in four grades and severity, or progression of the lesions was categorized into two stages.

Results: Thirty-five CMV-infected fetuses were included, 25 with ultrasound (US) diagnosis in the second trimester, and 10 in the third; one fetal demise and 34 TOP. The halo sign was detected in 91% fetuses (23 in the second, and 9 in the third trimester). The halo sign was the only US finding in 6 fetuses, all from the second trimester. Median gestational age at US scan among the isolated-halo and non-isolated halo cases was similar. The 3 fetuses with absent halo sign were diagnosed at 21, 26, and 35 weeks. Among the non-isolated halo cases, imaging severity was not associated with trimester at diagnosis, except for microencephaly which was more frequent in the second trimester 10/18 (56%) vs. 1/8 (13%), p=0.04. When analyzing histopathological findings, ventriculitis was observed in all fetuses with an isolated halo but most of them, 4/6 (66%) showed a mild grade. Extensive ventriculitis was more frequent in fetuses with non-isolated halo, 21/26 (81%) and non-halo ones: 2/3 (66%), p=0.032. All fetuses with an isolated halo were classified as histological Stage I with no signs of brain calcifications, white-matter necrosis, or cortical injury. On the other hand, all fetuses with non-isolated halo and those with absent halo, showed severe brain lesions (Stage II). Among fetuses with non-isolated halo, histological lesions did not progress with gestational age, and we even observed a tendency to more frequent white-matter necrosis in the second trimester [10/15 (67%) vs 3/11 (27%), (p=0.06)].

Conclusions: In CMV-infected fetuses, isolated halo sign was observed only in the second trimester and was associated with mild/moderate ventriculitis without signs of white-matter calcifications or necrosis. Although we observed a low probability of severe brain damage by histopathology, detailed sonographic follow-up and MRI in the early third trimester are required to counsel parents.

INTRODUCTION:

Congenital cytomegalovirus (CMV) is the most common congenital infection worldwide, the leading cause of non-genetic sensorineural hearing loss, and an important cause of neurodevelopmental disabilities in children. Prenatal counseling on the prognosis of congenital CMV is challenging, and it is largely based on fetal imaging. Ultrasound (US) abnormalities are seen in only a small proportion of CMV-infected fetuses, and subtle or nonspecific US features are likely to remain undetected[1,2]. While severe abnormal fetal cerebral US and magnetic resonance imaging (MRI) findings have been well defined and are the most significant predictive markers for adverse outcomes, less severe abnormalities carry a more uncertain prognosis[3,4].

Periventricular hyperechogenicity (halo sign), defined as homogeneous periventricular echogenicity observed in the parasagittal plane and confirmed in the axial and coronal planes[5,6], is one of the most common abnormalities in fetuses infected with CMV. Although the method of acquisition of US images was not transvaginal, in 1991 Tassin et al. described a pattern of bilateral periventricular ringlike zones that seemed specific for intrauterine CMV disease and could represent the earliest abnormality prior to the development of subependymal calcifications[7]. Halo was first described by Malinger in 2003[5], though it was first named "halo sign" by Simonazzi et al. in 2010[2]. The halo, which is better depicted by transvaginal route and not visible by MRI, has been described as a sign of ventriculitis[2,6], germinal matrix injury and as a severe sign of white matter lesion[2]. Thus, the halo sign is generally considered a marker of poor prognosis, but the specific underlying brain histopathology is unknown[8–10].

Only a few studies have examined postmortem CNS tissue of fetuses with periventricular halo histologically, and this study would provide understanding of the pattern and the severity of brain damage in such cases. The objective of this study was to analyze the correlation of the halo sign with histopathological findings to define its prognostic value.

METHODS:

Fetuses with severe CMV infection based on CNS abnormalities, ending in fetal demise or termination of pregnancy (TOP) in full compliance with Spanish law, diagnosed at a single center from 2006 to 2021, were retrospectively evaluated. **Figure 1.** Initial fetal examination and follow-up consisted of serial scanning including detailed neurosonography (NSG). Fetal infection had been confirmed in all cases in amniotic fluid. Extraction of CMV DNA and viral load by real time-PCR CMV were performed as described previously[11]. Fetal infection had been classified as severe (fetuses with severe brain US/MRI findings) according to Leruez-Ville et al[4,11], and isolated halo was considered of poor prognosis. All US scans were carried out by experienced examiners using high-resolution US equipment (Voluson 730 Expert and E6 or E8, GE Healthcare, Kretz, Zipf, Austria). All NSGs were obtained by transabdominal approach, and in cases with vertex presentation followed by transvaginal approach. In pregnancies reaching the third trimester, fetal MRI was performed at 30-32 weeks (1.5T GE Sigma Horizon, Echo speed, LX MRI scanner, Milwaukee, WI, USA). In some cases, with inconclusive NSG in the late second trimester, MRI was performed earlier, at 26-28 weeks.

For the present study, a Maternal-Fetal Medicine expert who was blinded to histological results reanalyzed the transabdominal and transvaginal NSGs obtained in these fetuses. The halo sign was defined as homogeneous bilateral periventricular echogenicity, observed in the parasagittal plane, and confirmed in axial and coronal planes. **Figure 2**. Isolated halo was defined as the only CNS finding, and non-isolated halo as observed concomitant with other CNS anomalies. The corpus callosum, cerebellum, cerebellar vermis, and gyral pattern were also evaluated[12]. Extra-CNS findings were evaluated, yet not considered for the analysis. Moreover, in cases in which MRI had been performed, an expert fetal radiologist, also blinded to NSG and histological results, reanalyzed the images. The cut-off for the US diagnosis of microcephaly was defined as a fetal head circumference ≥ 3 standard deviations (SD) below the mean for gestational age[12]. Microencephaly by NSG was defined as an enlarged subarachnoid space with a cranial-cortical width >95th percentile, and by MRI as brain biparietal diameter <1st percentile[12,13].

All cases included, had been submitted to pathology for conventional complete fetal autopsy. Afterwards, histologic slides, gross pictures, and pathology reports were retrieved from the institutional files. Hematoxylin-eosin-stained histologic slides were reviewed independently by two pathologists blinded to US/MRI results, and they achieved a final diagnosis by consensus. Histologic cerebral lesions (microglial nodules, perivascular infiltrates, necrosis or calcification in cortex or white matter, polymicrogyria, and CMV-infected diagnostic cells) were classified according to presence and extent and were defined as absent, mild: only in one slide, and severe: extensive in a single slide or present in multiple slides. **Figure 3**. Ventriculitis was classified in 4 grades: 0 (absent), 1 (identified only by immunohistochemistry), 2 (focal), 3 (extensive or seen in multiple preparations). Cases with histologically normal periventricular tissue were further investigated for the presence of cytotoxic T-cells through immunohistochemistry with specific antibodies against CD-8. Microencephaly was defined as encephalic weight lesser than two standard deviations below the expected for gestational age (GA) according to Bartosch et al[14].

To develop a classification of the severity or progression of the lesions, pathologic findings were tabulated, and cases were sorted from those with the least number of different lesions through those with macroscopic anomalies. The combination of lesions found among the cases with the least number of lesions was classified as mild or less severe (Stage I) whereas all the other combinations were classified as severe (Stage II). **Table 1.**

This study was approved by the Hospital Clinic Ethics Committee: **Reg. HCB/2020/0322**.

Statistical analysis

Histological and NSG imaging findings were compared between CMV-infected fetuses according to the presence or absence of the halo sing, and whether this was isolated or concomitant with other neuroimaging abnormalities. Quantitative variables were assessed using Shapiro-Wilk's test for normality, and normally distributed variables were compared using the t-test and expressed as mean and SD. Non-normally distributed quantitative variables were compared using the Mann-Whitney U test and expressed as median and interquartile range (IQR: p25-75). Qualitative variables were expressed as numbers and percentages and compared using the Chi squared test

(X^2) and Fisher's exact test. P-value <0.05 was considered significant. Data were analyzed using STATA, v.15.0 (College Station, Texas).

RESULTS:

Population characteristics

Thirty-five CMV-infected fetuses that ended in TOP in the second (n=23) and third trimester (n=11) and one intrauterine fetal demise in the third trimester were included. **Figure 1.** Diagnosis of fetal infection was performed on the evidence of CNS-US abnormalities at routine second (n=22) or third trimester scans (n=7), and in the remaining 6 cases (with isolated halo sign) congenital CMV was diagnosed after extra-CNS abnormalities during second trimester routine US scan (n=2), maternal symptomatology in the first trimester (n=2), close contact with a CMV infected individual in the first trimester (n=1) and first-trimester CMV screening decided by the patient's practitioner (n=1). Regarding type of maternal infection, eighteen patients (51%) had a confirmed first trimester primary CMV infection, one patient had a first trimester non-primary infection, and in 16 (46%) the type of maternal infection was unknown. The baseline characteristics of the study group are listed in **Table 2**. Median (IQR) GA at diagnosis of congenital CMV infection was 22.1 (21.0 - 26.5) weeks. The halo sign had been diagnosed in 32 fetuses (91%) and confirmed at retrospective evaluation in all cases. The median (IQR) GA at the diagnosis of the halo sign was 24.0 (22.6 – 28.9) weeks. Twenty-six fetuses had a non-isolated halo sign with a median (IQR) GA at diagnosis of periventricular halo of 24.4 (22.8-30.1) weeks and 6 fetuses had an isolated halo sign with a median (IQR) GA at diagnosis of 22.6 (22-23.4) weeks, p=0.10. The three fetuses with absent halo sign were diagnosed at 21, 26, and 35 weeks. The median time (IQR) from NSG to TOP was 5 (2-7) days. The median (IQR) of GA at postmortem was 24.4 (22.8-30.4) weeks.

Ultrasonographic findings:

Main associated NSG findings are listed in **Table 3**. In the non-isolated halo fetuses, the sign was

associated with other severe CNS abnormalities [microcephaly (35%), microencephaly (42%), cerebellar and/or vermian hypoplasia (73%), corpus callosum agenesis/dysgenesis (58%), abnormal gyration (58%)] in all except one fetus with mild ventriculomegaly. The severity of associated US findings was not related to trimester at diagnosis, excluding microencephaly which was more frequent at second trimester: 56% vs 13%, p=0.04. **Supplementary table 1.** Among the 3 fetuses without the halo sign, severe US brain lesions were observed in 100% and were similar to those with non-isolated halo. The extra-CNS abnormalities are listed in **Supplementary table 2.**

MRI findings:

Fetal MRI was performed in 13 cases (37%) at a median (IQR) GA of 29.3 (27.7-31) weeks, 12 in fetuses with the halo sign, and one in a fetus without halo. The main MRI abnormalities in fetuses with halo sign were abnormal cortical development [75%, mostly polymicrogyria (89% of them)], microcephaly (67%), microencephaly (58%) and cerebellar hypoplasia (50%), **Supplementary table 3.** Fetal MRI confirmed the severity of US brain abnormalities in 11 fetuses (10 with a non-isolated halo and the one without halo). Moreover, in one fetus with halo sign and mild US unilateral ventriculomegaly at 29 weeks, MRI at 30 weeks detected a diffuse abnormal cortical development compatible with polymicrogyria. Finally, a second trimester MRI at 26 weeks in one fetus with isolated halo did not show brain abnormalities.

Histopathological findings:

Histological lesions were diagnosed in all cases with variable extent and morphology. The most prevalent histological lesions were ventriculitis in 31/35 (89%), and microglial nodules in 29/35 (83%), followed in descending order by CMV inclusions 24/35 (69%), polymicrogyria 21/35 (60%), cortical foci of necrosis 16/35 (46%), white matter foci of necrosis 14/35 (40%), perivascular inflammatory infiltrates 6/35 (17%), and calcifications (periventricular and white matter 9% each; cortical 3%). Macroscopic lesions were observed in 19/35 (54%) of cases**, Table**

4. Seven cases showed only ventriculitis and/or microglial nodules and were classified as Stage I. All other cases showed additional lesions, either microscopic or macroscopic and were classified as Stage II.

Ventriculitis Grading

All fetuses with an isolated halo showed signs of ventriculitis, most of them (66%) mild (Grade 1). Regarding fetuses with non-isolated halo and those without the halo sign, 3/26 (11%) and 1/3 (33%) respectively, did not show signs of ventriculitis, although the rest of them showed higher grades of ventriculitis (Grade 2-3), compared to fetuses from the isolated halo group: (81% vs. 67%, vs. 34%), p=0.032**, Table 4.**

Isolated halo group:

All six cases fell in the histologic Stage I. Microglial nodules and CMV inclusions were identified in 83 and 50% of these fetuses, respectively. The fetuses of this group did not show other brain parenchymal lesions, nor calcifications or perivascular infiltrates. **Table 4.** Histopathologic Stage I was associated with the isolated halo sign in the whole series, p<0.001, **Table 5.**

Non-isolated halo group:

Twenty-five out of the 26 cases (96%) already were in histological Stage II, **Table 5**. Microglial nodules were observed in 81%, and CMV-inclusion cells in 73%. White matter necrosis, cortical necrosis and polymicrogyria were found in 48%, 54% and 73%, respectively, **Table 4**. White-matter necrosis was more frequent, albeit non-significant, in the second trimester [10/15 (67%) vs 3/11 (27%), p=0.06]**, Supplementary table 4.** Likewise, there were not differences in the histological severity stage, when compared by trimester at TOP. **Supplementary table 5.** The remaining fetus classified as histological Stage I showed microcephaly and microencephaly both at US and MRI.

Non-halo group:

All three cases in this group were classified as histological Stage II. All the fetuses in this group presented microglial nodules and CMV-inclusions were present in 67%. White matter necrosis was found in 33%, whereas cortical necrosis and polymicrogyria were detected in 67%. **Table 4.**

DISCUSSION

This is the largest study performing a thorough histological examination of the fetal brain obtained from cases of CMV infection with halo sign identified during the second or third trimester scans. Previous research has considered the halo as a sign of severe CNS injury, but this was not confirmed in our study. In this series, isolated halo sign was mostly associated with mild ventriculitis, microglial nodules, and CMV-infected cells, and contrary to previously reported, we did not observe[5] white matter necrosis[2,6].

Cerebral damage was extensively assessed for each brain region, and histopathological severity staging was established. Although all fetuses with isolated halo showed only a low histopathological severity stage, microglial nodules suggesting immune-mediated damage tended to be more diffuse than among fetuses without the halo sign, or those with additional severe imaging abnormalities. Though severe parenchymal lesions were not observed, diffuse microglial nodules in the brain of CMV infected fetuses have been correlated with direct injury[15]. Gabrielli et al., reported a classification of CNS lesions including a score of inflammation in midterm CMV-infected fetuses with/without sonographic findings[16]. They observed that the inflammatory response was associated with the severity of brain damage, however we did not confirm this finding maybe due to methodological differences

Since the halo sign is almost exclusively diagnosed through transvaginal approach and fetal infection was diagnosed principally on the presence of US abnormalities during second/third trimester routine scans, the halo was associated with more severe US findings in over three-quarter of cases. Probably because of it, histopathological staging among non-isolated halo cases, was severe in almost all cases, including polymicrogyria in nearly three-quarters, and white matter/cortical necrosis in one half. This result is similar to that published by Simonazzi et al.

where the halo sign, as a mid-gestation finding, was associated with other severe CNS abnormalities and white matter injury[1,2,5].

The halo-sign was absent in less than 10% of cases in our cohort, confirming that in CMV-infected fetuses with brain involvement this sign is quite prevalent, mainly at midterm as previously described[2]. Two-thirds of fetuses without the halo-sign belonged to the second trimester making it unlikely for the sign to have already disappeared at the time of diagnosis. In fact, 90% of fetuses from the third trimester still showed this sign and they displayed analogous severe brain imaging abnormalities as those without halo. Moreover, the absence of the halo-sign did not preclude severe CNS histological findings, given that all of them had severe macroscopic lesions. However, our sample does not allow to demonstrate a distinctive histological pattern between fetuses without halo and those with a non-isolated one.

Since all cases with isolated halo were exclusively from mid-gestation it could be hypothesized that it is an early sign of immune-mediated damage that would have evolved to more severe brain damage and histological lesions if the fetuses had survived. Nevertheless, among fetuses with non-isolated halo, we did not observe more severe histological damage in the third trimester. Moreover, in theses fetuses, microencephaly detected by NSG was significantly more frequent in the second trimester. Although, these results preclude these hypotheses, we lack data to support either progression to severe brain damage or remission.

In CMV infected fetuses, MRI early in the third trimester has shown to increase detection of CNS abnormalities, especially those involving temporal lobes and cortical development. Fetal MRI is a complementary tool to US and increases prediction of symptoms at birth and long-term sequelae[17–20]. In our series, MRI in the early third trimester helped for the confirmation of severe brain abnormalities in fetuses showing the halo sign together with other US abnormalities, although one case with microencephaly diagnosed both at US and MRI and not confirmed at postmortem, needs further consideration; we hypothesized, that it could be a false positive or might be a result of different diagnostic approaches between the methods.

Although second trimester MRI is not routine practice and it is not well validated, it was performed at 26 weeks in one fetus with an isolated halo and did not show additional findings[18–20]. Since MRI was not performed on the other isolated-halo fetuses, we might have missed brain abnormalities. Nevertheless, this possibility is low since in all these fetuses histopathological damage was mild at the time of postmortem in the second trimester.

This study has some strengths and limitations. The main strength is that this is the largest cohort of halo detected by US in CMV-infected fetuses during second and third trimester with targeted histopathological examination. A larger cohort is unlikely reproducible in subsequent studies. Moreover, all the neuroimaging and pathology specialists were blinded to each other.

Among the limitations, we first acknowledge the retrospective nature of the analysis. Second, the small sample size in the isolated-halo and non-halo groups. Third, halo is a subtle sonographic marker that may be observer-dependent. However, in this study, all images were evaluated by the same observer. A standardized quantitative data (DICOM) would have helped to correlate the intensity of the halo sign and the degree of inflammation. Further studies are warranted to establish different halo types and their relation to prognosis. In addition, our series only included fetuses that ended up in a TOP or fetal demise, that might have biased our findings to the more severe spectrum of the disease. However, it could be argued that it could have been a conservative bias, since in less affected fetuses the correlation with halo sign and ventriculitis is likely to be even weaker than in those severely affected.

Finally, under the hypothesis of the halo as a transient sonographic sign, and since prenatal CMV screening is not recommended, we do not know how many infected fetuses may have been born with a transient halo during the period of the study. Follow-up of fetuses and newborns with isolated halo would be a key element to understand the real significance of this sign. However, this could only be feasible under a universal first trimester screening program; justified after demonstration of over 60% reduction of vertical transmission in mothers treated with valacyclovir[21,22]. The follow up of infected fetuses should include a NSG preferably obtained by transvaginal approach, enabling the detection of halo. Since, in some centers the transvaginal

approach is seldom performed, we encourage to include both parasagittal-coronal planes to the standard axial plane to confirm the halo sign.

In conclusion, our data showed that in CMV-infected fetuses isolated halo sign is observed exclusively in the second trimester and associated with histological findings that suggest immune-mediated damage, but not severe brain lesions. Detailed sonographic follow-up and MRI early in the third trimester are required to counsel parents.

Disclosures of interest: None declared.

Contribution to authorship:

All authors fulfill all the conditions required for authorship, have seen and approved the manuscript, and all have significantly contributed to the work as follows: Anna Goncé, Alfons Nadal and Ameth Hawkins-Villarreal (conception and design of the study); Ameth Hawkins-Villarreal, Karen Castillo, Ana L. Moreno-Espinosa (acquisition of data); Elisenda Eixarch (fetal CNS-US reevaluation) , Mónica Rebollo-Polo (MRI analysis), Alfons Nadal and Silvia Planas (histopathological examination); Ameth Hawkins-Villarreal, Anna Goncé, Karen Castillo (analysis and interpretation of data); Anna Goncé (supervision). Anna Goncé, Ameth Hawkins-Villarreal, Karen Castillo (writing-original draft). Anna Goncé, Ameth Hawkins-Villarreal, Karen Castillo, Ana Alarcón, Alfons Nadal, Eduard Gratacós and Francesc Figueras (writing, revision and editing of the submitted article).

Details of ethics approval:

This study was approved by the Hospital Clinic Ethics Committee: **Reg. HCB/2020/0322.**

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Figures

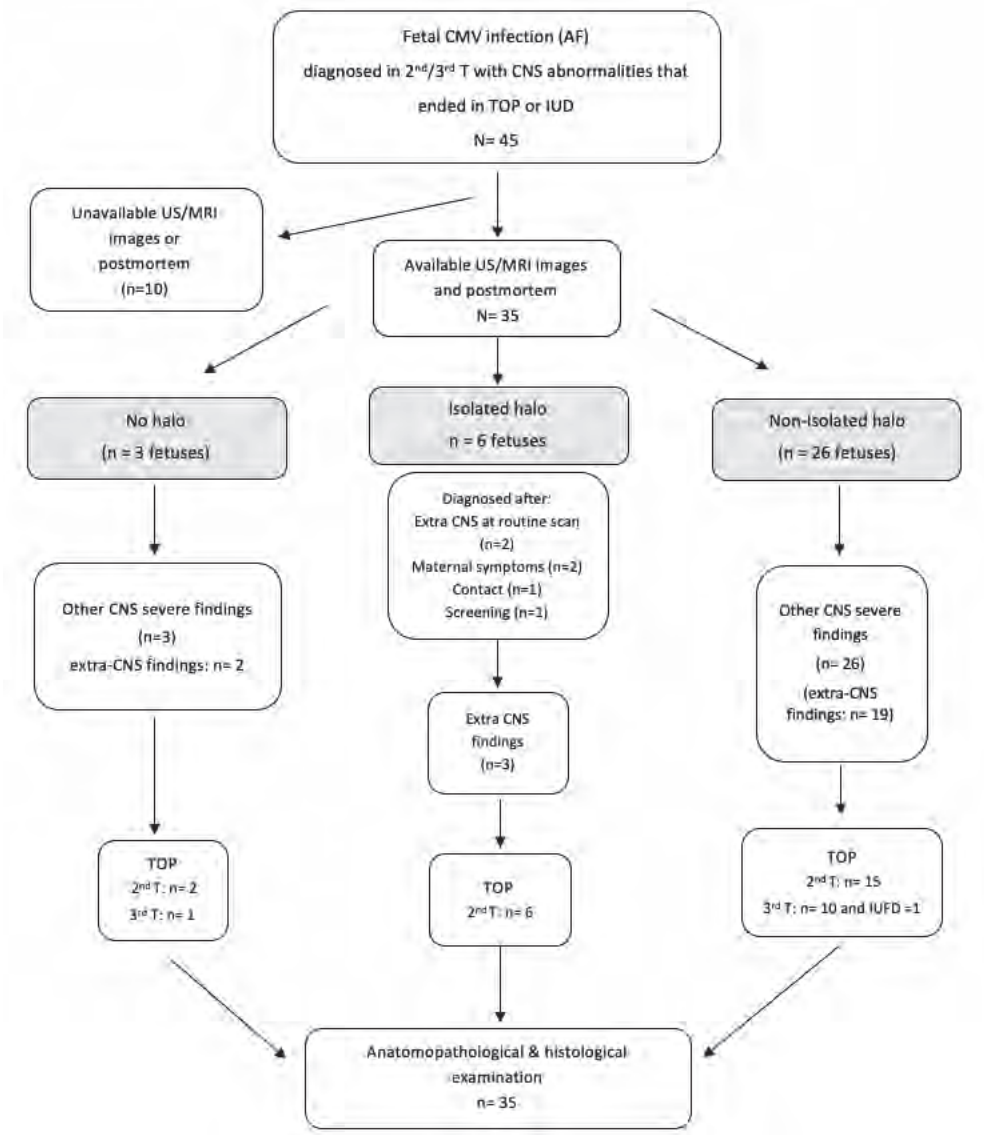

Figure 1: *Flowchart:* Retrospective evaluation of cases with fetal CMV infection and CNS abnormalities that ended in TOP or fetal demise. Description according to the presence or absence of the halo sign. CMV: cytomegalovirus, AF: amniotic fluid, TOP: termination of pregnancy, CNS: central nervous system, US: ultrasound, IUFD: intrauterine fetal demise.

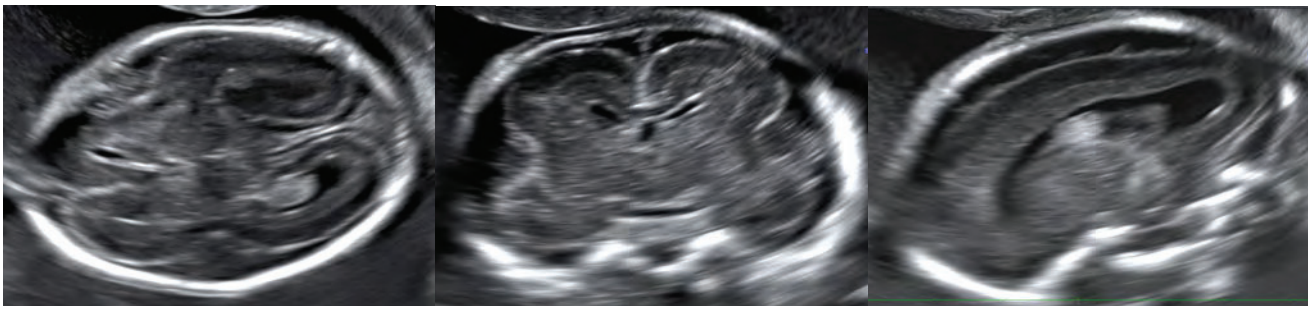

Fig.2. Periventricular hyperechogenicity at 21 weeks of gestation in a fetus with isolated halo sign. (**a**) Transvaginal axial, (**b**) transvaginal coronal, and **c**) transvaginal parasagittal planes. Appreciate the clear delimitation between the periventricular zone and the segment of the cerebral cortex (arrows). The halo sign is visualized in all the orthogonal planes.

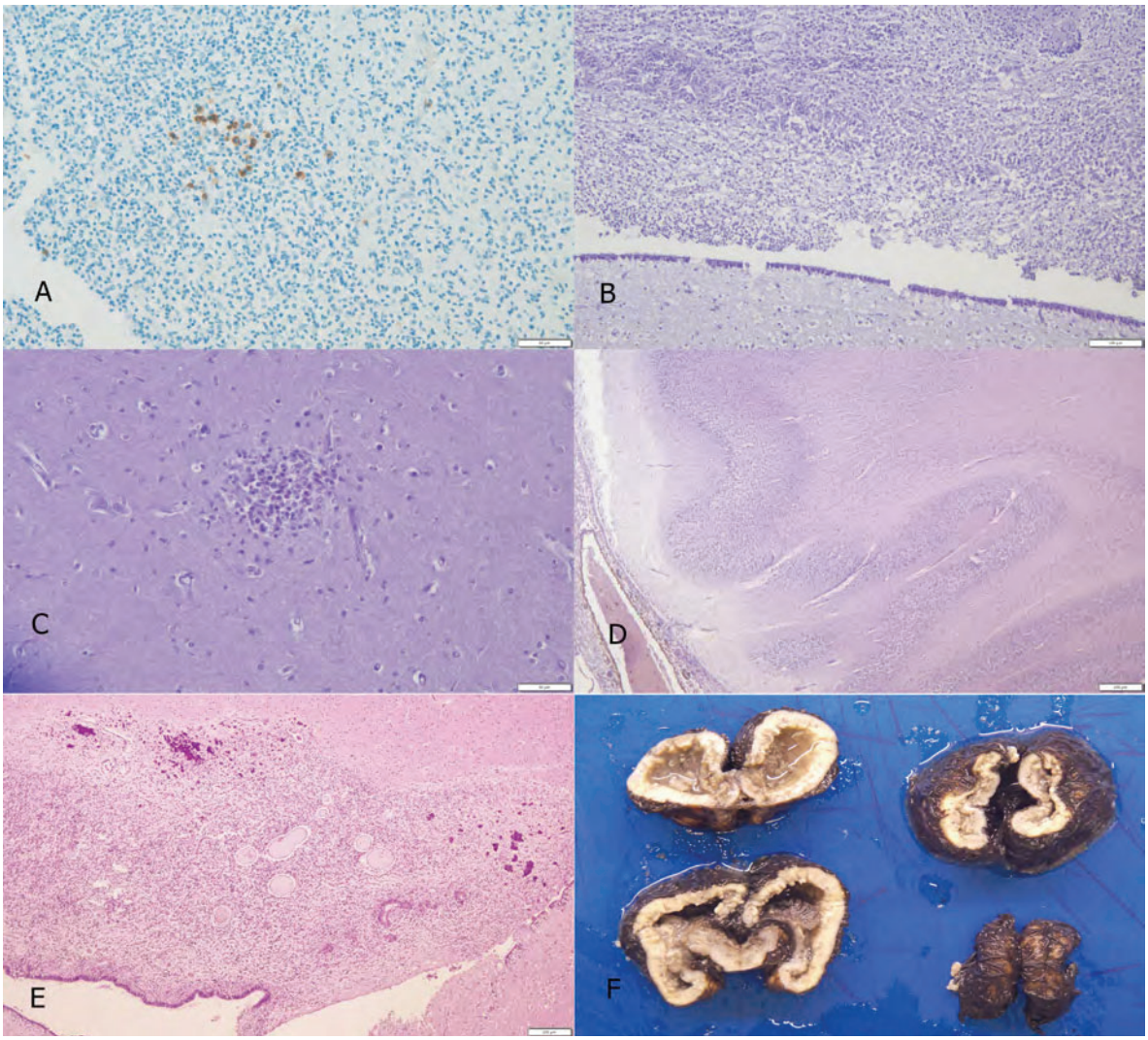

Figure 3. A. The mildest form of ventriculitis is detected immunohistochemically showing clusters of cytotoxic T-lymphocytes in the germinal matrix identified by the brown staining in the cytoplasm. Anti-CD8 immunohistochemistry, hematoxylin counterstain. Original magnification 20x. **B.** Severe ventriculitis (top half of the picture) shows discontinuity of the ependymal layer with cell overgrowth through the gaps in the ependyma. Continuous ependymal layer lining the cerebral tissue in the lower part of the picture. Hematoxylin and eosin (H&E) staining. Original magnification 10x. **C.** Microglial nodule. Round shaped cluster of microglial cells within the white matter. H&E staining. Original magnification 20x. **D.** Polymicrogyria. Cortical cell layers follow a microgyral (festooned) pattern with an excessive number of abnormally small gyri. Meningeal coverings in the left low corner. H&E staining. Original magnification 4x. **E**. A histological example of stage 2 shoes the ventriculitis (with multiple ependymal pseudo ducts, vascular dilatation, and cellular scarcity) and the presence of microcalcifications (irregularly shaped dark and deeply hematoxylin stained) in the border between periventricular germinal matrix and white matter. H&E staining. Original magnification 4x. **F**. Sections obtained from a fixed brain show the macroscopic appearance of severe bilateral ventriculomegaly suggesting the development of hydranencephaly.

Tables

Table 1. Histopathological staging

Stage	
I: Mild	Macroscopic: normal and Microscopic: Only Ventriculitis, microglial nodules and/or viral inclusions
II: Severe	Either Macroscopic lesions including microencephaly, porencephaly, ventriculomegaly, etc. And/or Microscopic: additional lesions (perivascular infiltrates, calcifications, necrosis [white matter, cortical], polymicrogyria)

Table 2. Baseline characteristics of the study group.

Characteristic	(n=35)
Maternal Age, years, median (IQR)	31.6 (29.5 – 34.8)
Caucasian Ethnicity (%)	100
Low educational level, n (%) *	8/31 (26)
Multiparity, n (%)	23 (66)
3-year-old child, n (%)	22 (63)
Type of infection, n (%)	
Primary	18 (50)
Non-primary	1 (3)
Unknown	16 (46)
Rationale for diagnosis of fetal infection	
Anomalies in routine US scan, n (%)	31 (88)
Maternal symptoms, n (%)	2 (6)
Screening, n (%)	1 (3)
Direct contact, n (%)	1 (3)
Gestational age at diagnosis of fetal infection, median (IQR)	22.1 (21.0 – 26.5)
< 28.0 weeks, n (%)	28 (80)
Periventricular hyperechogenicity ("halo"), n (%)	32 (91)
Gestational age at diagnosis of halo, median (IQR)	24.0 (22.6 – 28.9)
< 28.0 weeks, n (%)	24/32 (75)
Fetal gender (female), n (%)	19 (54)
Gestational age at termination of pregnancy, median (IQR)	24.4 (22.8 – 30.4)
< 28.0 weeks, n (%)	23 (65)

Data are presented as medians (IQR: interquartile range: p25-75frequencies or percentage (%). * Low educational level was defined as primary school studies only. NSG: neurosonography. US: ultrasound, AF: amniotic fluid, CNS: central nervous system.

Table 3. Associated neurosonographic findings in CMV infected fetuses with/without the halo sign.

Characteristic	Ni_halo n = 26	No halo n = 3
Severe US features		
Severe VMG, n (%)	4 (15)	0 (0)
Microcephaly, n (%)	9 (35)	3 (100)
Micrencephaly (Enlarged SAS), n (%)	11 (42)	1 (33)
Porencephaly, n (%)	3 (12)	0 (0)
Corpus callosum abnormality *, n (%)	15 (58)	2 (67)
Cerebellar/vermis hypoplasia, n (%)	19 (73)	3 (100)
Abnormal sulcus gyration, n (%)	15 (58)	2 (67)
Mild US features		
Mild VMG, n (%)	7 (27)	1 (33)
Intraventricular adhesions, n (%)	3 (12)	1 (33)
Isolated calcifications, n (%)	7 (27)	0 (0)
Lenticulostriate vasculopathy, n (%)	14 (54)	2 (67)
Sub-ependymal cysts, n (%)	3 (12)	1 (33)
Anterior horns hyperechogenicity, n (%)	2 (8)	0 (25)

Data are presented as frequencies or percentage (%). Fetuses could have a severe CNS finding together with mild CNS abnormalities. ‡ Ni_halo: non-isolated halo sign. US: ultrasound. VMG: ventriculomegaly. SAS: Sub-arachnoid space. * Corpus callosum <p5, dysgenesis or agenesis.

Table 4. Main histological findings in CMV infected fetuses according to the type of halo sign.

Characteristic	IHalo n= 6	nI_Halo n= 26	No Halo n= 3	p* value
Ventriculitis grading				
Grade 0	0 (0)	3 (11)	1 (33)	
Grade 1	4 (66)	2 (8)	0 (0)	**0.032**
Grade 2	1 (17)	7 (27)	1 (33)	
Grade 3	1 (17)	14 (54)	1 (33)	
Microglial nodules	5 (83)	21 (81)	3 (100)	
Focal	1 (20)	10 (48)	3 (100)	0.288
Diffuse	4 (80)	11 (52)	0	
CMV inclusions	3 (50)	19 (73)	2 (67)	
Focal	2 (67)	5 (26)	0	0.436
Diffuse	1 (33)	14 (74)	2 (100)	
Periventricular calcifications	0 (0)	3 (12)	0 (0)	0.987
Perivascular infiltration	0 (0)	6 (23)	0 (0)	0.797
White matter calcifications	0 (0)	3 (12)	0 (0)	0.967
Cortical calcifications	0 (0)	1 (4)	0 (0)	0.859
Withe matter necrosis	0 (0)	13 (48)	1 (33)	
Focal	0	6 (46)	1 (100)	0.223
Diffuse	0	7 (54)	0	
Cortical necrosis	0 (0)	14 (54)	2 (67)	
Focal	0	5 (36)	1 (50)	0.147
Diffuse	0	9 (64)	1 (50)	
Polymicrogyria	0 (0)	19 (73)	2 (67)	
Focal	0	3 (16)	0	
Diffuse	0	16 (84)	2 (100)	**0.017**
Macroscopic lesions	0 (0)	16 (62)	3 (100)	**0.004**

Data are presented as frequencies or percentage (%) * *p* value as determined with X^2 or Fisher's exact test. iHalo: isolated halo sign, Ni_Halo: non-isolated halo sign.

Table 5. Histopathological stage according to the presence of halo sign.

Stage	IHalo n= 6	nI_Halo n= 26	No Halo n= 3
Stage I	6 (100%)	1 (4%)	0 (0%)
Stage II	0 (0%)	25 (96%)	3 (100%)

Data presented as n (%). IHalo: isolated halo sign. nI_Halo: non-isolated halo sign.

***p* value <0.001.**

Supplementary material

Supplementary Table 1. Frequency of severe neurosonographic findings in cytomegalovirus infected fetuses with non-isolated halo according to trimester of pregnancy.

Characteristic	2nd Trimester n = 18	3rd Trimester n = 8	*p** value
Severe US features			
Severe VMG	3 (17)	1 (13)	0.786
Microcephaly	7 (39)	2 (25)	0.492
Microencephaly (Enlarged SAS)	**10 (56)**	**1 (13)**	**0.040**
Porencephaly	2 (11)	1 (13)	0.919
Corpus callosum abnormality **	11 (61)	4 (50)	0.597
Cerebellar and/or vermis hypoplasia	14 (78)	5 (63)	0.418
Abnormal sulcus gyration	10 (56)	5 (62)	0.741

Data are given as n (%) * *p* value as determined with X^2 or Fisher's exact test. US: ultrasound, VMG: ventriculomegaly, SAS: Sub-arachnoid space. ** corpus callosum <p5, dysgenesis or agenesis.

Supplementary Table 2. Extra-central nervous system findings in cytomegalovirus infected fetuses.

Characteristics	n= 35 (%)
Hyperechogenic bowel	10 (28)
IUGR	10 (28)
Oligo/Anhydramnios	4 (11)
Cardiomegaly	3 (9)
Hydrops fetalis	2 (6)
Fetal ascites	2 (6)
Pericardial effusion	2 (6)
Hepatosplenomegaly	1 (3)
Liver calcifications	1 (3)
Fetal anemia	1 (3)
Placentomegaly	1 (3)
None	15 (43)

Data are given as n (%). Fetuses could have more than one anomaly.

Supplementary Table 3. Main magnetic resonance imaging findings in cytomegalovirus infected fetuses with the "halo" sign.

Characteristics	n= 12 (%)
Ventriculomegaly	5 (42)
Mild	5 (42)
Severe	0 (0)
Isolated calcifications	3 (25)
Intraventricular adhesions	3 (25)
Lenticulostriate vasculopathy	7 (58)
Subependymal cysts	2 (17)
Temporal lobe cysts	4 (33)
White matter hyperintensity	8 (67)
Microcephaly	8 (67)
Porencephaly	0 (0)
Corpus callosum dysgenesis	1 (8)
Cerebellar hypoplasia	6 (50)
Vermian hypoplasia	1 (8)
Abnormal cortical development	9 (75)
Microencephaly*	7 (58)

Data are given as n (%). MRI: magnetic resonance imaging. *Enlarged sub-arachnoid space, brain biparietal diameter <p1.[12-13]

Supplementary Table 4. Main histological findings in cytomegalovirus infected fetuses with non-isolated halo comparing second vs. third trimester.

Characteristic	2nd Trimester n = 15	3rd Trimester n = 11	*p** value
Ventriculitis grading			
Grade 0	2 (13)	1 (9)	
Grade 1	1 (7)	1 (9)	0.985
Grade 2	4 (27)	3 (27)	
Grade 3	8 (53)	6 (55)	
Microglial nodules	12 (80)	9 (82)	
Focal	7 (58)	3 (33)	0.520
Diffuse	5 (42)	6 (67)	
CMV Inclusions	11 (73)	8 (73)	
Focal	4 (36)	1 (12)	0.507
Diffuse	7 (64)	7 (88)	
Perivascular infiltration	3 (20)	3 (27)	0.664
Periventricular calcifications	2 (13)	1 (9)	0.738
White matter calcifications	1 (7)	2 (18)	0.364
Cortical calcifications	0 (0)	1 (9)	0.423
White matter necrosis	**10 (67)**	**3 (27)**	**0.056**
Focal	5 (50)	1 (33)	
Diffuse	5 (50)	2 (66)	
Cortical necrosis	10 (60)	5 (45)	
Focal	2 (22)	3 (60)	0.462
Diffuse	7 (78)	2 (40)	
Polymicrogyria	9 (60)	10 (90)	
Focal	2 (22)	1 (10)	0.466
Diffuse	7 (78)	9 (90)	

Data are given as n (%) * *p* value as determined with X^2 or Fisher's exact test.

STUDY 6

Maternal high dose valacyclovir and its correlation with newborn blood viral load and outcome in congenital cytomegalovirus infection.

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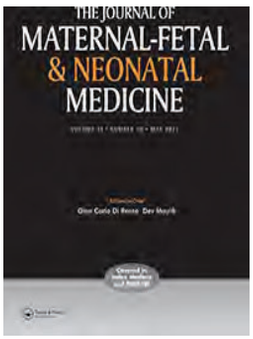

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REPORT

Maternal high-dose valacyclovir and its correlation with newborn blood viral load and outcome in congenital cytomegalovirus infection

Anna Goncé[a,b], Ameth Hawkins-Villarreal[a,c], Laura Salazar[a], Laura Guirado[a], Maria-Angeles Marcos[d], Jara Pascual Mancho[e], Pilar Prats[f], Marta López[a,b], Elisenda Eixarch[a,b], Maria-Dolors Salvia[a], Claudia Fortuny[g] and Francesc Figueras[a,b]

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ABSTRACT

Background/objective: Currently, there is no validated treatment for fetal cytomegalovirus (CMV). Two studies suggest that high-dose maternal valacyclovir decreases fetal viral load and improves outcomes in moderately-symptomatic fetuses. We offered valacyclovir in cases of fetal infection lacking ultrasound abnormalities or with non-severe infection. Maternal tolerability, fetal outcome and newborn blood viral load were evaluated in pregnancies of mothers receiving valacyclovir.

Study design: We performed a case series including 8 pregnancies with fetal CMV classified as unaffected/mildly-moderately affected. Mothers received valacyclovir (8 g/24h) from fetal infection diagnosis to delivery. Standard newborn evaluation was performed, and viremia was determined in the first 48 h of life and compared according to length of maternal treatment and presence/absence of prenatal anomalies.

Results: Valacyclovir was administered at a median gestational age of 26.5 weeks (23.8–33.1) in 3 cases without fetal abnormalities, and 5 with mild/moderate abnormalities. Three were 3 first trimester primary infections, one non-primary infection, and in 4 the type of infection was unknown. Valacyclovir was well-tolerated. Fetal features did not progress. Three newborns were asymptomatic, and one was severely affected (bilateral chorioretinitis). The median newborn viral load (IQR) was 502 IU/mL (231–191781) with lower levels when maternal treatment was administered $\geq$10 weeks, and in cases without fetal abnormalities [median 234 IU/mL (228–711) vs. 4061 (292–510500) $p = .18$; and 234 IU/mL (228–379500) vs. 711 IU/mL (292–4061) $p = .65$, respectively], these differences being non-significant.

Conclusions: Fetal CMV lesions remained stable with high-dose maternal valacyclovir. Newborn viral load was unchanged despite treatment duration and fetal/neonatal abnormalities.

Summary: Fetal cytomegalovirus lesions remained stable with high-dose maternal valacyclovir. Newborn viral load was unchanged despite treatment duration and fetal/newborn abnormalities.

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Fetal cytomegalovirus infection; fetal treatment; high-dose valacyclovir

Introduction

Congenital cytomegalovirus (CMV) is a significant source of neurosensory hearing loss and developmental delay [1]. In the absence of maternal screening, fetal infection is mainly diagnosed based on the evidence of sonographic abnormalities at the time of routine scans. Unfortunately, prenatal treatment has not yet been validated [2]. However, two studies from a single center observed that high-dose maternal valacyclovir is safe, decreases the viral load (VL) in fetal blood, and might improve outcomes in moderately symptomatic fetuses [3,4]. Moreover, recent data have shown promising results with the administration of

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valacyclovir soon after maternal infection to prevent vertical transmission [5,6], although this can only be implemented under systematic maternal screening. According to the current guidelines at our institution, high-dose maternal valacyclovir is administered in cases of fetal CMV infection without ultrasound (US) lesions or with non-severe abnormalities.

The aim of the study was to evaluate maternal tolerance, fetal outcome, and newborn blood-VL in pregnant women treated with high-dose valacyclovir.

Materials and methods

This was a case series of 8 consecutive pregnancies in which fetal CMV-infection was confirmed in amniotic fluid by positive polymerase-chain-reaction (PCR). High-dose valacyclovir was administered from diagnosis of fetal infection to delivery at the Hospital Clínic, Barcelona (January 2017–May 2019). The cases were diagnosed on the presence of US-abnormalities detected during routine scans, or after first-trimester maternal screening by the patient's physician. The pregnancies had been diagnosed at our institution or referred for evaluation and decision-making. In cases diagnosed by ultrasound, maternal CMV serology was obtained. Fetuses were classified as severely/mildly/unaffected according to the presence/absence of US or magnetic resonance imaging (MRI) abnormalities [7,8]. Valacyclovir was offered in cases without fetal abnormalities or with mild/moderate abnormalities (extracerebral or mild cerebral signs). High-dose valacyclovir was prescribed off-label with no need for ethical approval; nevertheless, patients were informed of the limited evidence of its efficacy and written informed consent was obtained. Valacyclovir was given at an oral dose of 8 g/day (2 g/6 h) until delivery [4]. Maternal blood evaluation, including complete blood count and liver enzymes, was undertaken before the first dose, and every 2 weeks during treatment. Fetal follow-up consisted of serial US every 3 weeks conducted by experienced examiners using high-resolution equipment (Voluson 730 Expert and E6 or E8, GE Healthcare, Kretz, Zipf, Austria). Fetal MRI was also performed after 30 weeks (1.5 T GE Sigma Horizon, Echo speed, LX MRI scanner, Milwaukee, WI, USA). After birth, congenital infection was confirmed by a positive PCR in urine, and blood-VL was determined within 2 days of life. Extraction of CMV-DNA was performed using MagNaPure (Roche Diagnostics Germany), and VL by PCR-CMV Real-Time (Nanogen Advanced Diagnostics, Italy) with a threshold of 20 IU/mL to define positivity. Newborn blood-VL was compared according to length of maternal treatment ($\leq$10 weeks/$>$10 weeks), and the presence/absence of fetal abnormalities. Newborns were examined following standard clinical evaluation, including a transfontanellar-US (TF-US) within the first days of life. Infection was classified as either symptomatic or asymptomatic, according to the Rawlinson et al. [2] consensus document.

Statistical analysis

Blood VL at birth was compared according to treatment duration and presence/absence of fetal lesions. Quantitative variables were assessed using the Shapiro-Wilk test for normality. Normally distributed variables were compared with the t-test and expressed as mean and standard deviation (SD). Non-normally distributed quantitative variables were compared using the Mann-Whitney-U test and expressed as median and interquartile range (IQR: p. 25–75). Qualitative variables were compared with the X^2 or Fisher test. Linear regression analysis was performed to establish correlation with fetal outcome and blood-VL. A p-value $<.05$ was considered significant. Data were analyzed using STATA, v.15.0 (College Station, Texas).

Results

Valacyclovir was administered to 8 pregnant women [7 singletons and one dichorionic twin (co-twin uninfected)] at a median gestational age of 26.5 weeks (23.8–33.1) and a mean treatment duration of 10.1 weeks (4–16). The Table 1 shows the time and method of diagnosis of fetal infection (US features or maternal screening), type of maternal infection (primary vs. non-primary), and fetal US and MRI abnormalities. All patients completed the treatment with no reports of adverse events or alterations in blood tests. US fetal lesion progression was absent. Ascites reverted at 31 weeks in the fetus from the twin-pregnancy (**case-5**). After birth, 3 newborns were asymptomatic, with a normal TF-US; in 2, low-birthweight was the only clinical sign; 2 showed the same mild/moderate central nervous system (CNS) brain lesion observed prenatally; and in one, the brain lesions had improved (**case-6**). Auditory brainstem response was normal in all newborns except one with unilateral hearing loss and bilateral chorioretinitis; this infant was considered severely CMV symptomatic. Maternal blood collected during the first trimester confirmed a non-primary maternal infection (**case-8**).

Table 1. Description of cases with maternal valacyclovir treatment.

Id	Maternal CMV serologyMaternal serum CMV-DNA Type of infection	GADiag fetal infection (AF)	VL in AF (copies/mL or IU/mL)	Fetal US findings	Fetal MRI findings	GA start VCV (weeks of pregnancy)	Duration VCV (weeks)	VL newborn (IU/mL)	NB TF-US findings /NB infection definition
1	IgG pos/IgM pos (12w) IgG-avidty low (13 w) CMV-DNA: NA **Primary infection 1st T**	21	380000	No	None	25.4	15	234	None/Asymptomatic
2	IgG pos/IgM pos (10w) IgG-avidty low (14 w) CMV-DNA: pos (14w) **Primary infection 1st T**	21	23529415	No	None	22.3	16	379500	None/Asymptomatic
3	IgG pos/IgM pos (11w) IgG-avidty low (14 w) CMV-DNA: pos (14w) **Primary infection 1st T**	21	2486474	No	None	21.6	11	228	None/Asymptomatic
4	IgG pos/IgM pos (25w) IgG pos/IgM pos (28w) IgG-avidity high (28w) CMV-DNA: low pos (28w) **Primary infection 1st T?**	25		IUGR	None	27.5	11	0	None/LBW (only symptom)
5 DC twin	IgG pos/IgM neg (20w) IgG pos/IgM neg (25w) IgG-avidty high (25 w) CMV-DNA: neg (25w) **Unknown**	21	10000000	Hyperechogenic bowel Ascites	WM hypersignal temporal lobes	24.4	13	711	None/LBW (only symptom)
6	IgG pos/IgM mild pos (35w) IgG-avidty high (35 w) CMV-DNA: negative (35w) **Unknown**	32 (34.5)	10000000	Mild VMG (unilateral) Germinal matrix hemorrhage	Mild VMG Subependymal cysts Diffuse WM hyperintensity	35.3	5	510500	Mild unilateral VMG (mildly symptomatic)
7	IgG pos/IgM neg (32w) IgG-avidity. NA CMV-DNA: NA **Unknown**	32	23529412	IUGR Subependymal cysts	Mild VMG WM hyperintensity temporal lobes	33.2	4	4061	Germinal matrix cysts/ LBW (mildly symptomatic)
8	IgG pos/IgM neg (10w) IgG pos/IgM neg (32 wks) IgG-avidty NA CMV-DNA: neg (32w) **Non-primary infection**	32	13000000	Subependymal cysts IV adhesions Calcifications LSV	Diffuse WM hyperintensity	33.0	6	292	Germinal matrix hemorrhage ABR: unilateral hearing loss Fundoscopy: bilateral chorioretinitis (severely symptomatic)

AF: amniotic fluid; VL: viral load; Pos: positive; Neg: negative; NA: non-available; GA: gestational age; GADiag: gestational age at diagnosis; VCV: valacyclovir; DC: dichorionic; US: ultrasound; MRI: magnetic resonance imaging; IUGR: intrauterine growth restriction; VMG: ventriculomegaly; WM: white matter; LSV: lenticulostriate vessels; IV: intraventricular; NB: newborn; LBW: low birth weight; TF-US: transfontanellar ultrasound; ABR: auditory brainstem response.

The median newborn blood-VL was 502 IU/mL with a wide range of distribution (IQR:231–191781). Analysis according to the length of maternal treatment (>10 weeks vs. ≤10 weeks) showed a trend to a lower VL in longer treatments: 234 IU/mL (IQR 228–711) vs. 4061 (IQR 292–510500), although the difference was non-significant ($p = .18$). Moreover, no differences were observed in newborn VL when comparing the presence/absence of fetal lesions: 711 IU/mL (IQR 292–461) vs. 234 (IQR 228–379500) ($p = .65$). However, these two groups overlapped, as fetuses with lesions were diagnosed later with a shorter maternal treatment time.

Discussion

According to the results of a recent study [4], high-dose oral valacyclovir was offered off-label to pregnant women carrying a CMV-infected fetus without or with mild/moderate sonographic abnormalities. Maternal tolerability and adherence were excellent, although newborn blood-VL was not suppressed even after more than 10 weeks of maternal treatment and showed a wide overlap between fetuses with and without US abnormalities.

A recent study [9] showed that newborn blood VL has no clinically predictive value for long-term outcomes, although other studies have identified an association [10,11]. Nevertheless, one of the aims of prenatal administration of valacyclovir is to decrease fetal VL. In the first study, high-dose maternal valacyclovir was administered in pregnancies with symptomatic fetuses, showing a significant reduction in CMV-VL after 1–12 weeks of maternal treatment [3]. In the present series, high-dose valacyclovir did not correlate with newborn blood VL.

Regarding treatment effectiveness, no case showed an evolution of prenatal CNS or extra-CNS lesions. However, in nearly half, the diagnosis of fetal lesions was performed in the third trimester with limited time for demonstrating impairment progression and treatment benefits.

The strength of our study is the addition of valuable information to the limited data regarding prenatal treatment with high-dose maternal valacyclovir in pregnancies with CMV-infected fetuses administered early after the diagnosis of fetal infection before detectable abnormal US, and after the detection of non-severe lesions. Nevertheless, this study has several limitations. It was not a randomized study comparing results between treated/untreated fetuses. Given the difficulties in matching similar cases, we did not compare newborn VL and outcome with a historical cohort of untreated fetuses. Moreover, the wide variation in the type of CMV infection, timing, and duration of treatment make it difficult to draw conclusions. Finally, long-term follow-up of the infants was beyond the scope of the study.

Our findings encourage the use of high-dose oral maternal valacyclovir in pregnancies with CMV-infected fetuses without sonographic abnormalities or with mild/moderate ones. Although newborn VL remains detectable in most cases, this treatment is safe and might reduce the progression of fetal lesions. Nevertheless, randomized clinical trials and further studies with more potent antiviral drugs are warranted.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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STUDY 7

New data on efficacy of valaciclovir in secondary prevention of maternal-fetal transmission of CMV

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New data on efficacy of valaciclovir in secondary prevention of maternal–fetal transmission of CMV

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Short title: Valaciclovir to prevent fetal transmission of CMV

Keywords: Congenital cytomegalovirus; maternal–fetal transmission; prenatal treatment; secondary prevention; TORCH; valaciclovir; viremia

Contribution

What are the novel findings of this work?

Valaciclovir reduces the risk of maternal-fetal transmission of CMV, with a trend even in the second trimester of pregnancy. Moreover, its efficacy seems to be greater in the case of positive maternal viremia at initiation.

What are the clinical implications of this work?

There are no longer many barriers to a widespread screening during pregnancy. This screening should be early and repeated in order to best date maternal seroconversion and initiate VCV treatment as soon as possible. Continuation of VCV treatment after negative amniocentesis and treatment of 2nd trimester seroconversions are discussed.

Abstract

Objective Congenital cytomegalovirus (CMV) infection is the leading cause of non-genetic hearing and neurological deficits. The aim of our study was to evaluate the efficacy and safety of valaciclovir (VCV) in prevention of CMV transmission to the fetus after maternal primary infection.

Methods Retrospective, multicenter study evaluating the rate of CMV maternal-fetal transmission in patients with a primary CMV infection treated with VCV at a dosage of 8g per day (VCV group) compared to a group of untreated women. Each case was assessed virologically to confirm maternal primary infection and to provide accurate dating. The primary endpoint was the presence of congenital CMV infection diagnosed on urine samples at birth. The efficacy of VCV treatment was assessed using logistic regression analysis adjusted for a propensity score.

Results 143 patients were included in the final analysis, 59 in the VCV group, and 84 in the control group. After propensity score adjusted analysis, VCV treatment was significantly associated with an overall reduction in the rate of maternal-fetal transmission (OR = 0.40; 95% CI 0.18-0.90, p=0.03). After periconceptional primary infection, the rate of maternal-fetal transmission at birth was 7% (1/14) in the VCV group vs 10% (1/10) in the control group (p=1.00); 22% (8/36) vs 41% (19/46) after 1st trimester maternal primary infection (p=0.07) and 25% (2/8) vs 52% (14/27) after 2nd trimester maternal primary infection (p=0.24). When analyzing efficacy of treatment according to maternal viremia at treatment initiation, there was a trend towards greater efficacy when viremia was positive (21% vs 43%, p=0.07) compared to when viremia was negative (22% vs 17%, p=0.66). Maternal treatment side effects are reported.

Conclusion VCV treatment of pregnant women with primary CMV infection in the first and second trimesters reduces the risk of transmission to the fetus.

Introduction

Congenital cytomegalovirus (CMV) infection is the leading worldwide cause of congenital viral infections, with a birth prevalence of approximately 0.5% and is an important cause of congenital neurological deficits and the first non-genetic cause of hearing loss [1,2]. Systematic screening for CMV infection during pregnancy is not currently recommended in France. To date, the main obstacle to screening, apart from the fact that the diagnosis of secondary infections and reactivation is not possible, was the lack of available treatment, particularly for the prevention of mother-to-fetal transmission[3].

Valaciclovir (VCV) is an ester derivative of aciclovir which is active against CMV DNA polymerase, when used at high doses (i.e. 8 g per day). Regarding its use in pregnancy, published data in exposed pregnant women are numerous and reassuring, showing no teratogenic risk, but in other indications and at a lower dosage[4]. Regarding anti-CMV activity, its efficacy was initially documented in several randomized trials evaluating prophylactic treatments against CMV reactivation in renal transplant recipients[5,6].

In the context of CMV infection during pregnancy, VCV was first evaluated as a treatment for documented symptomatic fetal infections[7,8]. Regarding prevention of maternal-fetal transmission, a randomized controlled trial evaluated efficacy of VCV against placebo and showed a reduction in maternal-fetal transmission of up to 70%[9]. Treatment was initiated after periconceptional or first trimester primary infection, and the primary endpoint was the presence or absence of CMV detected by amniocentesis performed at around 21 weeks' gestation (WG). Additionally, Faure-Bardon et al. published a retrospective case control study, which also found a decrease in maternal-fetal transmission, only assessed by CMV PCR results at the time of amniocentesis[10]. However, late transmission of CMV after amniocentesis and late infection after treatment discontinuation after a negative amniocentesis have been described[11]. Thus, data on neonatal outcome are needed[12–14]. Furthermore, the efficacy of VCV after the first trimester of pregnancy has never been evaluated.

The aim of our study was to evaluate the efficacy and safety of VCV treatment for the secondary prevention of congenital CMV infection after primary maternal infection regardless of the trimester of infection, in newborns at birth.

Methods

This was a retrospective, multicenter study (Hôpital Louis Mourier, Colombes, APHP; Hôpital Antoine Béclère, Clamart, APHP; Centre Hospitalier Universitaire Angers) including all patients referred for a primary maternal CMV infection between November 2014 and June 2021 for the Louis Mourier center and between January 2018 and June 2021 for the Béclère and Angers centers.

All women with CMV primary infection during pregnancy or the periconceptional period were included. Patients were excluded if primary infection occurred more than 1 month before the date of onset of pregnancy or if patients were referred for ultrasound abnormalities related to congenital CMV infection. After virological expertise, patients for whom primary infection was not certain were excluded from the study.

After confirmation of the maternal primary infection, patients received multidisciplinary counseling on the risk of transmission, infection at birth, possible symptoms for fetuses, and long-term sequelae as well as on the screening strategy based on ultrasound. Amniocentesis was discussed. Patients were offered VCV treatment based on the knowledge of benefit/risk known at the time of consultation. Patients who refused treatment over the same periods were considered as a control group. Treatment was offered orally, at high dose (8 g/day). Patients who discontinued treatment were not excluded to mimic an intention-to-treat analysis.

After initiation of VCV treatment, amniocentesis was offered to all patients 6 weeks after onset of infection and after 21 WG according to usual management in these centers[15]. After the CMV PCR result in amniotic fluid was known, continuation of VCV until birth could differ between centers and was discussed according to the knowledge at the time of the consultation. During the entire treatment with VCV, patients were monitored clinically and by laboratory tests (blood count, liver function and renal function) on a weekly basis at Antoine Béclère Hospital and monthly at Louis Mourier and Angers

Hospital. Patients were also monitored by fetal ultrasound every month and newborns were tested at birth according to the usual protocol with a salivary and urine and if positive control by blood PCR test.

The primary outcome was diagnosis of congenital CMV infection at birth, defined as a positive saliva, urine and/or blood sample < 24h after birth. The analysis was also performed on the result of CMV PCR in amniotic fluid at the time of amniocentesis. Secondary endpoints were: quantification of virus in blood in infected neonates. In addition, all adverse maternal and fetal events related to VCV treatment were recorded.

Maternal CMV primary infection diagnosis and dating

All cases were assessed virologically and reviewed to confirm and date the maternal primary infection. Diagnosis of maternal primary CMV infection relied on serology:

- Seroconversion identified on two sequential serum samples with negative specific CMV-IgG on the first sample and positive CMV-IgG on the second sample in the same laboratory;
- Positive specific CMV-IgG and IgM, and low IgG avidity (VIDAS bioMérieux, Marcy l'Etoile, France).

The estimated time since the onset of primary infection relied on previously described criteria [16]. Briefly we considered:

- Positive CMV-IgM/negative CMV-IgG: 15 days after onset of infection;
- Positive CMV-IgM/positive CMV-IgG with avidity index < 20%: 2 to 4 weeks after onset of infection;
- Positive CMV-IgM/positive CMV- IgG with avidity index between 20 and 40%: 5 to 7 weeks after onset of infection;
- Positive CMV-IgM/positive CMV-IgG with avidity index between 40 and 60%: 8 to 12 weeks after onset of infection.

In order to classify patients in the two latter categories, additional parameters were considered, including IgG kinetics and serological results on previous serum samples.

Infections were defined as periconceptional when the infection occurred within 4 weeks of the date of onset of pregnancy, first trimester when the infection occurred between 2 and 14 WG, second trimester when the infection occurred between 14 and 28 WG, and third trimester when the infection occurred after 28 WG.

Statistical analysis

To assess the efficacy of VCV and to limit indication bias, we used a propensity score assessing a woman's likelihood of being treated according to the trimester of infection (periconceptional, first trimester, second trimester, or third trimester) and the viremia at the time of treatment (negative, detectable unquantifiable, detectable quantifiable, and not known). A logistic regression analysis adjusted for the propensity score was then used to estimate the treatment effect. The treatment effect was assessed in the overall population, as well as in each subgroup of women by trimester of primary infection, and by category of maternal viremia at the time of treatment (negative, detectable unquantifiable, detectable quantifiable, and not known). Maternal viremia was not used in the propensity score because of the risk of overadjustment.

Continuous data were presented as medians and 25^{th} to 74^{th} percentiles. Categorical data were presented as counts and percentages. Student's t test was performed to compare amniotic and neonatal viral load as appropriate, after log transformation of the variables.

All analyses were performed using Stata software (StataCorp, College Station, Texas).

Ethics statement

This study was approved by the Paris Nord biomedical research ethics review committee (CEERB) under No. 2020-012.

Results

During the study period, 230 patients were referred for maternal CMV primary infection. Of these, 87 were excluded because the primary infection was not certain or anteconceptional according to virological expertise, the CMV infection had not been diagnosed in the framework of a screening, or the patients had received valaciclovir in treatment of a congenital infection. (Figure 1). Finally, 143 patients were included in the final analysis: 59 in the VCV group and 84 in the control group (Figure 1). Patient characteristics were similar between the two groups and are described in Table 1. The time from infection to initiation of treatment was 49.8 days (IQR 36.2-56), and treatment was initiated at a median term of 13.6 weeks (11 - 17) (Table 1). CMV seroconversion characteristics and imaging findings (US and MRI) are detailed in table 2 and 3.

After propensity score adjusted analysis considering the term of seroconversion, VCV treatment was significantly associated with a reduction in the rate of maternal-fetal transmission (OR = 0.40; CI95% 0.18-0.90, p=0.029) (Figure 2). This result was unchanged and maintained significance after exclusion of the 2 cases of third-trimester infections (p=0.028).

Prior to propensity score matching, the overall maternal-fetal transmission rate was significantly lower in the VCV group (19%, 11/59) compared to the control group (40%, 34/84) (p=0.006). After periconceptional primary -infection, the rate of maternal-fetal transmission at birth was 7% (1/14) in the VCV group vs 10% (1/10) in the control group (p=1.00). After first trimester primary infection, the maternal-fetal transmission rate was 22% (8/36) in the VCV group vs 41% (19/46) in the control group (p=0.068). After second trimester maternal primary infection, the maternal-fetal transmission rate was 25% (2/8) in the VCV group vs 52% (14/27) in the control group (p=0.244). In the case of third trimester maternal primary infection, the maternal-fetal transmission rate was 0% (0/1) in the VCV group vs 0% (0/1) in the control group (Figure 2).

Results on second trimester infection are detailed into 2 periods: 14 – 18 weeks (early second trimester) and 19-27 weeks (late second trimester). VCV group transmission rate was 0% (0/3) for the early second trimester and 40% (2/5) for the late second trimester. Control group transmission rate was 28% (2/7) for the early second trimester and 57% (11/19) for the late second trimester.

Among patients with negative CMV PCR in amniotic fluid, one newborn was infected at birth in the treatment group (1/44, 2%) compared to two newborns in the control group (2/33, 6%). All newborns infected after negative amniocentesis were asymptomatic at birth. The patient treated with VCV who gave birth to an infected newborn after negative amniocentesis, had an amniocentesis at 32 weeks' gestation after a second trimester seroconversion (ie 24 WG). VCV was started 6 weeks after seroconversion and continued until the end of the pregnancy.

Concerning the patient treated with VCV to prevent maternal-fetal transmission, amniocentesis was performed 8 weeks after a second trimester seroconversion for which the patient was treated with VCV until the end of the pregnancy.

Among treated patients, 88% (52/59) had blood drawn for CMV viral load at first consultation, ie. at the time of initiation of a possible VCV treatment, of which 56% (29/52) were positive. Among control patients, 26% (52/84) had blood drawn for viral load, of which 54% (28/52) were positive.

Considering treated and untreated patients, maternal viremia at the first consultation appeared to be associated with an increased risk of maternal-fetal transmission. When maternal viremia was detectable, the transmission rate in any trimester was 32% (18/57), compared to a transmission rate of 19% (9/47) when maternal viremia was negative (OR 2.7 [95% CI 0.81-8.86]). This trend was also found after seroconversion in the first trimester, ie 27% (9/33) when maternal viremia was positive and 20% (5/25) when maternal viremia was negative. The median time to initiation of therapy after seroconversion for viremia-positive patients and viremia-negative patients was 46.2 and 49.7 days, respectively. When analyzing efficacy of treatment according to maternal viremia, there was a trend

towards greater efficacy when viremia was positive (21% vs 43%, p=0.072) compared to when viremia was negative (22% vs 17%, p=0.659) (Figure 3).

Viral quantifications after maternal fetal transmission were compared between the VCV and control groups. Patients treated with VCV showed a trend towards lower viral load in amniotic fluid (5.2 vs 5.9 log copies/mL, p=0.44) and in newborn cord blood (2.9 vs 4.1 log copies/mL; p=0.02) among children with positive viral load at birth.

The main maternal side effects described were mild and non-specific, and included back pain (4/59), transit disorders (3/59), nausea (3/59), dizziness (1/59) and macrocytosis (1/59). However, one patient, with no previous history, developed acute renal failure (creatinine level 291 micromol/L) associated with back pain and pruritus 14 days after initiation of VCV at 9.4 days' gestation. Renal function resolved spontaneously within a few days after discontinuation of treatment. Pregnancy continued without incident and the patient delivered an asymptomatic uninfected child at term.

Discussion

Main findings

We found that VCV reduced the risk of maternal-fetal transmission of CMV by nearly 60%, which is consistent with previous studies [9],[17]. The incidence of first trimester transmission with VCV was on the order of 20%, as in the randomized clinical trial by Shahar-Nissan et al. [9] We also studied treatment for primary infection in the second trimester, which has not been described before, and found a two-fold lower transmission rate with VCV, although not reaching significance probably due to lack of power.

Clinical implications

The efficacy of valaciclovir to prevent maternal-fetal transmission is an encouragement to screen for CMV early in pregnancy, with repeat testing if negative in order to detect seroconversions and initiate prompt treatment.

In view of our results, screening for CMV in the second trimester of pregnancy can also be discussed. The value of detecting or even preventing congenital CMV infection in the second trimester is a topic of controversy. Faure-Bardon et al. reported no sequelae following infections in the second or third trimester [17], but Bilavsky et al. [14,18]found hearing loss at birth in 4.3% of children with late maternal-fetal transmission and severely affected fetuses have been described after second-trimester infections [19]. Although neonatal risks are less severe than for first-trimester infections, VCV should be considered for second-trimester infections.

The risk of vertical transmission following a negative amniocentesis is another important clinical issue. In untreated cohorts, 4 to 15% of neonates with a negative amniocentesis have congenital CMV [14,19–23]. The main hypothesis is transmission occurring after the amniocentesis, rather than poor sensitivity of the PCR technique[14]. In our cohort, the majority of neonates who were CMV-infected after a

negative amniocentesis was from the group which did not receive VCV. Also, we did not always stop VCV following a negative amniocentesis, which may contribute to our low rate of infected neonates after negative amniocenteses compared to the study by Faure-Bardon et al. [17]. Shahar-Nissan et al. who stopped treatment after amniocentesis, found similar rates of infected newborns after negative amniocentesis of 10% in the VCV group and 6% in controls[9]. In an Italian series, the rate of transmission was 2/12 at amniocentesis and 5/12 at birth, thus almost 30% of the infected newborns became infected following a negative amniocentesis[11]. Thus, continuing VCV following a negative amniocentesis can be discussed to prevent delayed transmission with its potential impact for the child.

We found an association between maternal viremia at presentation and the risk of maternal-fetal transmission, as previously demonstrated [24]. This is the first study to evaluate the efficacy of VCV according to maternal viremia after CMV seroconversion. VCV seemed to be effective mainly in case of positive viremia, where maternal-fetal transmission of was halved, while there was no significant impact when the viremia was undetectable. The duration of viremia is variable, 1 to 5 weeks in immunocompetent persons[25]. Maternal-fetal transmission probably occurs preferentially at the time of viremia, which explains why treatment is more effective in case of positive maternal viremia. The time from seroconversion to treatment initiation is a major prognostic factor for the effectiveness of VCV to prevent maternal-fetal transmission[9]. This may be related to reducing maternal viremia near its peak. Additionally, in case fetal CMV infection does occur, VCV decreases viral load in the amniotic fluid and blood at birth[8].

The dosage of 8 g/day was adapted from the prevention of CMV infections in transplant patients, used in the pharmacokinetic study of Jaquemard et al. and in the trial of Shahar-Nissan et al. [8,9]. Acute renal failure is a known complication of intravenous VCV therapy, and has also been reported following oral treatment in case reports, and may be associated with neurotoxicity [26–29]. The mechanisms are precipitation and crystallization of the drug in the renal tubules, resulting in obstruction and possible cell necrosis [30–32] and direct damage to the renal tubules by an aldehyde metabolite of VCV [33]. Although

no case was described by Shahar-Nissan et al., Faure-Bardon et al. described a case similar to our case of renal failure on high-dose VCV [9,10]. In the first case, the dosage was 4g bid, whereas our patient received 2g four times daily. Both cases resolved spontaneously within a few days after discontinuation of treatment. No case of fetal renal damage has been reported to date. Repeated clinical and laboratory monitoring is required throughout the duration of treatment. Other non-specific symptoms reported during follow-up were back pain, transit disorders, nausea, dizziness and macrocytosis.

Research implications

The efficacy of VCV for secondary prevention of CMV infection is now established, but the benefit/risk balance remains to be determined for use in the second trimester. The only alternative to VCV studied to date is hyperimmune immunoglobulin, but the results of two randomized controlled trials were negative[34–38].

Other antiviral treatments could be discussed. Ganciclovir has not been used due to concerns for toxicity to fetal germ cells, although it is more effective on CMV[39]. Letermovir, which has specific anti-CMV activity and is well tolerated, is currently under evaluation to treat infected fetuses (NCT04732260).

Weaknesses

The main limitation of our study is its retrospective nature. The indication bias may have been to use VCV in the highest risk situations, possibly underestimating its efficacy. We used a propensity score to limit treatment bias. Another limitation is that CMV screening was not systematic. In addition, practices have evolved within the same center and between centers over time. Symptomatic congenital CMV infections at the time of management was excluded to avoid selection bias. However, if they had been included in the untreated group, these patients would have tended to increase the transmission rate and thus enhance the efficacy of VCV.

Finally, although this is one of the largest cohorts evaluating VCV to prevent maternal-fetal transmission of CMV, power was lacking to confirm efficacy for periconceptional and second-trimester seroconversions.

Conclusion

Our study adds evidence that VCV reduces the risk of maternal-fetal transmission of CMV, including in the second trimester of pregnancy. Moreover, its efficacy seems to be greater in the case of positive viremia. Treatment should be initiated as soon as possible in case of primary infection in the periconceptional period or in the first trimester of pregnancy. Information about primary prevention of maternal CMV infection should be given to all women who are or intend to be pregnant. The efficacy of secondary prevention in case of maternal infection strongly supports a policy of CMV screening during pregnancy.

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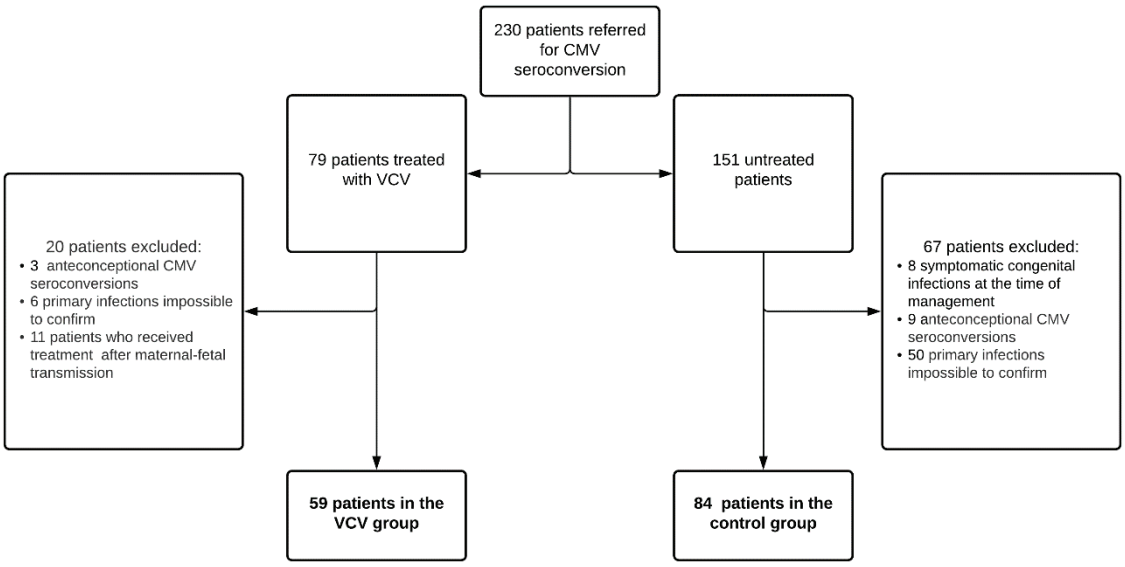
Figure legends

Figure 1. Flow chart.

Figure 2. Maternal-fetal transmission rate at birth. (a) All terms; (b) Transmission by trimester of primary infection. Patients who seroconverted in the 3rd trimester are not represented because of the small number of patients (N=2). *Statistically significant before and after adjustment for the propensity score.

Figure 3. Maternal-fetal transmission at birth according to maternal viremia at the first consultation after maternal CMV primary infection and at time to initiation VCV. If negative viremia: (22% vs 17% (p=0.659) and if positive viremia: 21% vs 43% (p=0.072).



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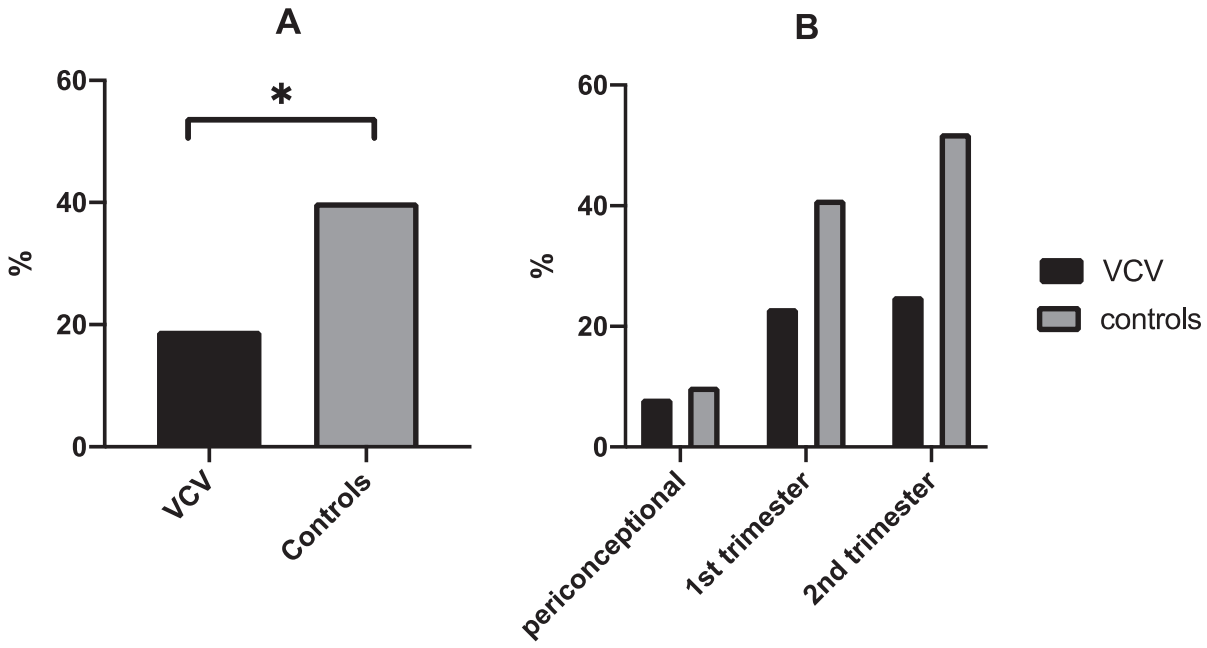

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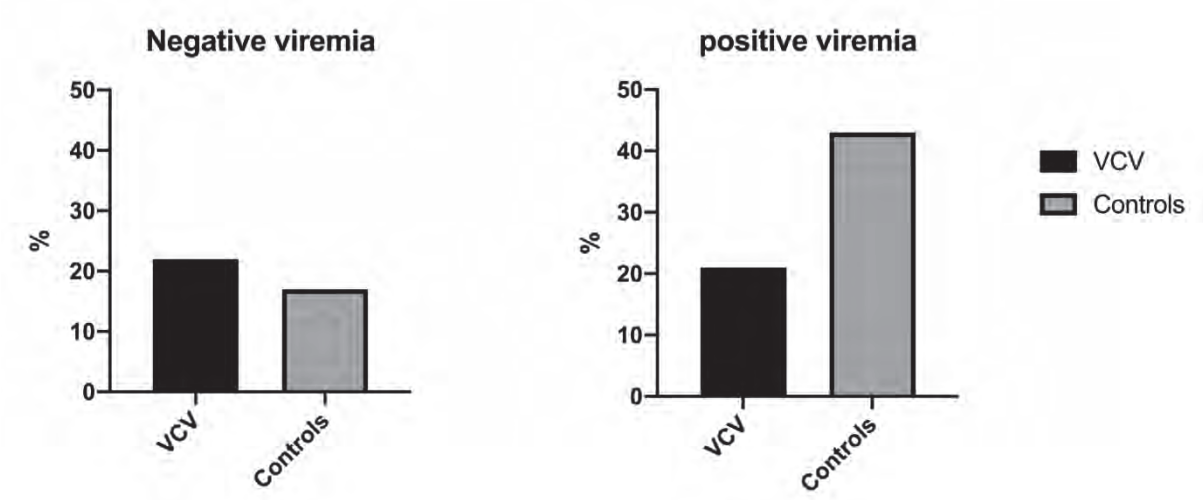

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Table 1 Maternal, obstetrical and CMV seroconversion characteristics

			VCV	Untreated control	Total
Maternal characteristics	**Number of patients**		59	84	143
	Maternal characteristics (median – IQR)	**Age**	32 (29 ; 34)	31.6 (29-34)	32.1 (29-34)
		Parity	2 (2-3)	2 (2-3)	2 (2-3)
		BMI	27 (25-28)	27 (25-30)	28 (26-30)
	Obstetrical characteristics	**Singleton**	58 (98%)	84 (100%)	142 (99%)
		Twin	1 (2%)	0 (0%)	1 (1%)
CMV infection characteristic	**Gestational age at infections**	**periconceptional**	14 (24%)	10 (12%)	24 (17%)
		1st trimester	36 (61%)	46 (55%)	82 (57%)
		2nd trimester	8 (14%)	27 (32%)	35 (24%)
		3nd trimester	1 (2%)	1 (1%)	2 (1%)
	Maternal viremia	**Positive**	29 (49%)	28 (33%)	57 (40%)
		Quantifiable	24 (41%)	15 (18%)	39 (27%)
		Detectable non-quantifiable	5 (8%)	13 (15%)	18 (13%)
		Negative	23 (39%)	24 (29%)	47 (33%)
		Not done	7 (12%)	32 (38%)	39 (27%)
	Gestational age at treatment initiation (weeks) (median, IQR,)		13,6 (11 – 17)		
	Time from infection to therapy initiation, (days) (median, IQR, days)		49.2 (36.2-56)		
	Amniocentesis	**Positive**	10 (17%)	16 (19%)	26 (18%)
		Negative	44 (75%)	33 (39%)	77 (54%)
		Not done	5 (8%)	35 (42%)	40 (28%)
	Gestational age at amniocentesis (weeks) (median, IQR)		21.5 (20.6 – 23.1)	22.2 (21 – 28)	21.6 (20-23)
	Duration of VCV therapy (median, IQR in days)		72.1 (58 – 105.3)		
	Vertical transmission rate at birth		11 (19%)	34 (40%)	45 (31%)

Table 2 CMV seroconversion characteristics and issue for periconceptional and first trimester

	Periconceptional		1st trimester	
	VCV	**Control**	**VCV**	**Control**
Number of patients	14	10	36	46
Gestational age at treatment initiation (weeks) (median, IQR,)	10 (9,5; 13,9)		13,25 (11,9; 15,63)	
Time from infection to therapy initiation (weeks) (median, IQR, days)	8 (7,25; 11,9)		7 (4,4; 8)	
Amniocentesis performed	13 (92%)	3 (3%)	35 (97%)	31 (67%)
positive	1 (8%)	0 (0%)	8 (23%)	10 (32%)
Infection at birth	1 (7%)	1 (10%)	8(22%)	19 (41%)
Imaging				
US abnormalities	1 (7%)	0 (0%)	4 (11%)	7 (13%)
Cerebral calcifications			1	
microcephaly	1		1	1
Echogenic bowel	1		3	3
Fetal growth restrictions				2
Subependymal cysts			1	3
Cerebral ventriculomegaly				2
Hepatomegaly or splenomegaly			2	4
Hepatic calcifications				1
MRI performed	1 (7%)	2 (20%)	6 (16%)	12 (26%)
MRI abnormalities	1 (7%)	0 (0%)	3 (8%)	5 (10%)
Microcephaly	1		1	1
white matter lesions	1		2	5
subependymal cysts			2	3
ventriculomegaly				2
gyral abnormalities			1	1
calcifications			1	
cerebellar anomalies				1

Table 3 CMV seroconversion characteristics and issue for second trimester

	14-18 weeks		19-27 weeks	
	VCV	**Control**	**VCV**	**Control**
Number of patients	3	7	5	20
Gestational age at treatment initiation (weeks) (median, IQR,)	23 (22; 23)		28 (27; 30)	
Time from infection to therapy initiation, (weeks) (median, IQR, days)	8 (7,25; 11,9)		6 (5,3; 6,6)	
Amniocentesis performed	3 (100%)	5 (71%)	3 (60%)	8 (40%)
positive	0 (0%)	1 (20%)	1 (33%)	5 (62%)
Infection at birth	0 (0%)	2 (29%)	2 (40%)	12 (60%)
Imaging				
US abnormalities	0 (0%)	1 (14%)	0 (0%)	1 (8%)
Cerebral calcifications				
microcephaly				
Echogenic bowel		1		1
Fetal growth restrictions		1		
Subependymal cysts				
Cerebral ventriculomegaly				
Hepatomegaly or splenomegaly				
Hepatic calcifications				
MRI performed	0 (0%)	2 (28%)	0 (0%)	4 (20%)
MRI abnormalities	0 (0%)	0 (0%)	0 (0%)	1 (5%)
Microcephaly				
white matter lesions				1
subependymal cysts				
ventriculomegaly				
gyral abnormalities				
calcifications				
cerebellar anomalies				

6. DISCUSSION

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Prenatal prognostic assessment in congenital CMV remains a challenge nowadays. However, although the brain is a major target of end-organ damage, a precise cellular marker of brain damage remains uncharacterized. The need for a more accurate evaluation has led to the investigation of postnatal blood parameters in symptomatic neonates, to be applied in infected fetuses, as well as US and MRI markers, although their predictive value to identify symptomatic newborns may be contradictory. This thesis offers additional data on the extent of the pathophysiology of cCMV-infection. Although it comprises mostly retrospective analyses, our results allowed us to demonstrate the distinctive involvement in CMV-infected fetuses according to the severity of US cerebral findings and trimester at diagnosis of fetal infection. Our work has shown that in CMV-infected fetuses i) the use of fetal blood parameters particularly thrombocytopenia and GGT levels, can predict severe brain damage (SBD), ii) fetal liver volume (FLV) adjusted for fetal body composition can be used as a surrogate marker of liver enlargement though hepatomegaly was an uncommon finding iii) CMV-infected fetuses with mild or normal US/MRI findings show underdeveloped cortical maturation iv) the isolated halo sign at mid-gestation may be related to a low probability of SBD by histopathology. Finally, we report our experience in the use of high-dose VCV in pregnancy.

In our **first study**, we observed that among fetuses diagnosed with CMV infection in the second or third trimester of pregnancy, GGT levels and thrombocytopenia were the only blood parameters significantly associated with severe US/MRI brain lesions. However, while GGT levels showed stable values along the pregnancy, thrombocytopenia, high levels of β_2-microglobulin and DNAemia, and positive IgM antibodies were observed significantly more often in the severely damaged fetuses diagnosed before 28 weeks. Thrombocytopenia has been reported to be an independent factor responsible for poor perinatal outcome. Fabbri *et al* and Leruez-Ville *et al*. found an association between fetal thrombocytopenia in the second trimester and symptomatic status at birth.[5,82]. Leruez-Ville *et al.* showed that the risk of a being symptomatic increases to 63% when combining a targeted US examination, viral-load, and platelet count ≤114,000/mm^3 in fetal blood as early as in mid-trimester[82].

It could be hypothesized that after an acute phase of the infection, fetal thrombocytopenia recovers, and β_2-microglobulin and DNAemia decrease in the third trimester.

CMV infection can induce several inflammatory mediators that may induce cytotoxicity[79]. GGT is an enzyme considered to participate in glutathione-coupled detoxification processes with great redox activity in several organs particularly in the brain[54,55,85]. GGT does not cross the placenta and cord levels derive entirely from the fetus[86]. High GGT levels have been reported as a parameter of fetal CMV infection and probably related to end-organ damage by some authors[5,17,87]. Benoist *et al*. did not observe differences in abnormal GGT levels (levels above the expected for gestational age [GA]) between CMV-infected fetuses with poor and good outcome. Moreover, this group described a similar proportion of fetuses with abnormal cerebral US findings with normal or abnormal GGT levels[23]. On the other hand, significantly higher median GGT values have been reported in CMV-infected (both symptomatic and asymptomatic) compared to non-infected fetuses, while no significant differences were observed when compared according to the severity of the infection[5]. Enders *et al*.[17] showed that CMV-infected fetuses (with and without US abnormalities) had significantly higher GGT median levels (122 international units [IU]/L) as compared to uninfected fetuses. Furthermore, there were no significant differences when compared according to the presence or absence of US abnormalities. Nevertheless, GGT levels above 350 IU/L were exclusively found in symptomatic infected fetuses with severe US abnormalities[17].

In a unselected series of more than 3000 fetal blood samples including infected and uninfected fetuses, the mean GGT value during the second and third trimesters remained below 100 IU/l, with little variation between 20 and 40 weeks[86]. These findings may explain why we observed significant differences according to the severity of brain damage but not to the trimester at cordocentesis. An association of the severity of fetal brain lesions with high levels of GGT has not been reported previously. Although it could be interpreted that among the severely affected fetuses the source of increased GGT levels is likely due to hepatic or multiorgan dysfunction, our hypothesis is that CMV-SNC infection induces a severe inflammatory response that increases GGT expression and activity in the brain.

In our **second study** we aimed to confirm the clinical significance of GGT levels in infected and uninfected fetuses in relation to the severity of cerebral US abnormalities, as well to assess if the detection rate of severe brain involvement improves by the addition of the platelet count as a ratio with GGT. As reported in our first study we corroborated that the GGT levels were significantly higher among fetuses with SBD than those with mild or no brain damage in both infected and uninfected fetuses. However, when compared according to the infection status, higher GGT levels were observed in SBD-CMV-infected compared to SBD-uninfected fetuses with a significantly higher prediction for SBD at the same cutoff value. Similar to a previous study, we demonstrated that the median platelet count was significantly lower among CMV-infected fetuses[88] in both those with SBD and those who were undamaged. Due to this thrombocytopenia in CMV-infected fetuses, the gamma-glutamyl transpeptidase-to-platelet ratio (GPR) was significantly higher among those with SBD than those with mild/no brain-damage. When compared to GGT levels alone, we found a tendency towards an increased performance for SBD prediction in the CMV-infected group with a GPR≥12 achieving a sensitivity of 84%, at a same false positive rate.

The strengths of our first two studies are: i) for the first time we have established a relationship between GGT levels and brain damage, ii) we have established a cut-off value for GGT levels for predicting severe brain lesions in CMV-infected as well as uninfected fetuses; iii) in CMV-infected fetuses, the predictive value of GGT for SBD could be improved using the GPR. In a clinical setting, this could be valuable information at the time of considering cordocentesis for further assessment of prognosis particularly in cases with moderate US-cerebral findings.

In cCMV infants with symptomatic disease, the manifestations can range from unspecific-mild to multisystemic involvement, with a particular proclivity towards the reticuloendothelial system, especially the liver.[89] Hepatic tropism and organ dysfunction in symptomatic CMV-infected newborns is common.[90] However, the medical literature regarding hepatic involvement in CMV-infected fetuses is scant. It could be argued that fetuses with severe damage included could have increased GGT levels due to possible liver dysfunction. However, we analyzed whether GGT levels at the time of fetal cordocentesis correlated with liver volumes and we found no

correlation between GGT levels and liver volumes or fetal liver volume to abdominal circumference (FLV/AC) or fetal liver volume /fetal body volume (FLV/FBV) ratios (adjusted for GA at MRI) with an r = -0.048 and R2: 0.0135 (unpublished data). This finding supports the hypotheses constructed in the **first two studies**.

There is no standardized imaging methodology for the assessment of hepatomegaly since fetal liver size has routinely been evaluated using US-liver length measurement. In **study 3** our results showed that in fetuses infected with CMV hepatomegaly could be assessed by MRI, although it was an uncommon finding. We demonstrated excellent concordance between the liver weight estimated by MRI and the liver weight at postmortem. Likewise, Shelmerdine *et al.*[91] also found an excellent overall correlation between the liver volume estimated by MRI prior to autopsy and liver weight during autopsy in a series of uninfected fetuses.

Although there were no significant differences in FLV among our fetuses, when adjusted for fetal size (i.e., abdominal circumference [AC] or fetal body volume [FBV]) the FLV was increased in CMV-infected fetuses compared to healthy controls. We could hypothesize that this is due to liver enlargement in relation to body composition. Adjustment for AC and FBV is not a standard procedure and the diagnostic accuracy is not established, although other authors have used ratios to better understand the extent of the pathophysiology of different fetal conditions.[92,93]

Despite the significantly higher FLV/AC-ratio and FLV/FBV-ratio in CMV cases compared to heathy controls, there were no significant differences in these ratios between mild and severely CMV-affected fetuses. We hypothesized that mildly infected fetuses may already have hepatic involvement that could be transitory. Liver dysfunction as an isolated feature could be related to an acute phase of the infection and may not necessarily be related to adverse outcomes.

In a previous series of CMV-infected fetuses undergoing termination of pregnancy (TOP) at 30 weeks of gestation, Maroun *et al.* found the liver weight to be similar to that of non-infected fetuses of the same GA.[94] This emphasizes our finding of no difference in the FLV estimated by MRI between CMV-infected and non-infected fetuses.

MRI abnormalities in the cortical maturation in fetal life have been increasingly diagnosed in infants and children with developmental delay[95,96]. However, description of MRI cortical maturation in CMV-infected fetuses with mild or no brain involvement is scarce.

In **study 4** we provide evidence that fetuses with CMV infection, sonographically unaffected or mildly affected, present a significant delayed pattern of cortical brain maturation compared to healthy control fetuses. Infected fetuses showed a significantly reduced parieto-occipital sulcus and calcarine sulcus depth, with a significantly larger Sylvian fissure angle (SFA), and lower cortical grading in the parietooccipital sulcus, calcarine sulcus, temporal area, and the parietal area. To our knowledge, this is the first study to characterize cortical brain development by means of sulci depth and cortical grading assessed by MRI analysis in CMV-infected fetuses with normal or mild US/MRI findings compared to healthy controls.

Although the impact of latent CMV-infection is currently unclear[40,97], we hypothesized that the larger ventricular width observed in our population of CMV-infected fetuses could reflect persisting inflammation due to a latent stage of infection. This latent infection could result in underdeveloped cortical maturation. The walls of the ventricles are critical for normal brain development. These walls are constituted by neural stem progenitor cells, responsible for the susceptibility of the CNS.[98–100]

Sarnat *et al*. described that the depth of a fissure/sulcus may be influenced by the adjacent ventricular system[101]. Although we could not determine the timing of infection in a slightly over a quarter of our sample, we hypothesized that the decreased sulci depth in the CMV-infected population compared to controls, is related to the cell injury caused by viral infection during embryonic brain development and is strongly related to the ventricular system.

Several authors have described volume changes in the temporal lobe of infected fetuses with unremarkable MRI or with isolated WMHS[102–104] at a similar GA as in our cohort suggesting a lower degree of brain maturation related to different stages of cellular injury. In our series, over a third of the cases had unremarkable US/MRI and/or WMHS. Nonetheless, we found significantly lower cortical grading in the temporal and parietal lobes as well as a linear tendency to a higher upper/lower SFA regardless of the presence or absence of WMHS.

Although less than a quarter of CMV infected fetuses were small for gestational age (SGA), we did not find this to interact or confound our results based on our regression analysis. Moreover, the data published by our research group in SGA fetuses showed an increased insular depth and reduced Sylvian fissure (SF) depth[105–107], with these two changes not being observed in our CMV-infected fetuses.

One discussion- worthy finding of our results is that the upper SFA in CMV-infected fetuses, which represents the superior/parietal landmark covering of the insula, was significantly larger compared to controls, likely suggesting underdeveloped cortical maturation of the superior operculization process of the SF[95,108]. Our findings appear to be in line with those of Pooh *et al*. who described that the SFA may potentially be a strong indicator for the subsequent development of cortical malformation.

We acknowledge that our findings may need correlation with neurological outcomes. Nonetheless, in fetuses with isolated non-severe ventriculomegaly (ventricular width of 12 ± 2 mm), similar changes in parietooccipital sulcus and calcarine sulcus have been reported.[109] These neonates showed weaker, albeit non-significant, performance in the motor and range of state clusters in the Brazelton test.

Our results reinforce the use of MRI as a complementary tool for assessing brain structure at 32 weeks of gestation in CMV-infected fetuses even without US abnormalities.

US abnormalities are seen in only a small proportion of CMV-infected fetuses and subtle or nonspecific US features are likely to escape detection. This is the case of the periventricular hyperechogenicity or "the halo sign" which is one of the most common US abnormalities in fetuses infected with CMV. This finding is generally considered a marker of poor prognosis, but the specific brain damage involved is unknown.

Our **fifth study** is the largest and carried out a thorough histological examination of the fetal brain obtained from cases of CMV infection with halo sign identified during the second or third trimester scans. In this series, an isolated halo sign was mainly associated with mild ventriculitis, microglial nodules, and CMV-infected cells, and contrary to previous reports we did not observe white matter necrosis[110]

Cerebral damage was extensively assessed in each brain region, and histopathological severity staging was established. Although all fetuses with isolated halo showed only

a low histopathological severity stage, microglial nodules suggesting immune-mediated damage tended to be more diffuse than among fetuses without the halo sign, or in those with additional severe imaging abnormalities. While severe parenchymal lesions were not observed, diffuse microglial nodules in the brain of CMV-infected fetuses have been correlated with direct injury[15]. Gabrielli *et al.* reported a classification of CNS lesions including an inflammation score in midterm CMV-infected fetuses with/without sonographic findings[16]. They observed that inflammatory response was associated with the severity of brain damage; however, we did not confirm this finding perhaps due to methodological differences

Since in our cohort the halo sign and fetal infection was diagnosed primarily on the presence of US abnormalities during second/third trimester routine scans, the halo was associated with more severe US findings in over three-quarter of cases. This likely explains why histopathological staging in non-isolated halo cases was severe in almost all the cases. This result is similar to that published by Simonazzi *et al.*[110] where the halo sign, as a mid-gestation finding, was associated with other severe CNS abnormalities and white matter injury[1,2,5].

Since all cases with isolated halo were exclusively from mid-gestation it could be hypothesized that it is an early sign of immune-mediated damage that would have evolved to more SBD if the fetuses had survived. Nevertheless, among fetuses with a non-isolated halo, we did not observe more severe histological damage in the third trimester. Moreover, in these fetuses, microencephaly detected by neurosonography (NSG) was significantly more frequent in the second trimester. Although, these results preclude these hypotheses, we lack data to support either progression to SBD or remission.

Follow-up of fetuses and newborns with an isolated halo would be a key element to understand the real significance of this sign. The follow-up of infected fetuses should include a NSG preferably obtained by transvaginal approach, enabling the detection of the halo sign. Since in some centers the transvaginal approach is seldom performed, we encourage the inclusion of both parasagittal-coronal planes to the standard axial plane to confirm the halo sign.

Systematic screening for CMV infection during pregnancy is not currently recommended. The main obstacle to screening, apart from the fact that the diagnosis

of secondary infections and reactivation is not possible, is the lack of maternal/fetal **treatment**. In the context of CMV infection during pregnancy, VCV was first evaluated as a treatment for documented symptomatic fetal infections and recent data have shown promising results with the administration of valacyclovir soon after maternal infection to prevent vertical transmission.[111,112]

In **study 6**, we included 8 pregnant women with confirmed CMV fetal infection in amniotic fluid to whom we administered high-dose VCV in the second/third trimester. In more than half of the cases, fetal infection was diagnosed on the evidence of mild US abnormalities in routine scans.

All patients completed the treatment with a median duration of 10 weeks with no reports of adverse or collateral outcomes. Although one of the aims of prenatal administration of VCV is to decrease fetal viral load we did not find any correlation with newborn blood viral load (VL).

Regarding treatment effectiveness, no case showed an evolution of prenatal CNS or extra-CNS lesions during follow-up. In one fetus ascites was reverted during treatment as described with the use of VGC.[113] After birth, two newborns showed the same mild/moderate CNS brain abnormalities observed prenatally; and in one, brain lesions had improved.

The strength of our study is the addition of valuable information to the limited data regarding prenatal treatment with high-dose maternal VCV in pregnancies with CMV-infected fetuses administered early after the diagnosis of fetal infection, before the detection of abnormal US or after the detection of non-severe lesions.

Our findings encourage together with the findings of Leruez-Ville *et al.*[88] the use of high-dose oral maternal VCV in pregnancies with CMV-infected fetuses with or without mild/moderate sonographic abnormalities. Although newborn VL is detectable in most cases, this treatment is safe and might reduce the progression of fetal lesions.

Regarding the prevention of maternal-fetal transmission, two previous studies have shown promising results with the administration of VCV soon after maternal infection to prevent vertical transmission, reporting a reduction in maternal-fetal transmission of more than 60%.[112,114]

Furthermore, the efficacy of VCV after the first trimester of pregnancy has never been evaluated. Hence, the aim of our last study (**study 7**) was to evaluate the efficacy and safety of VCV treatment for the secondary prevention of cCMV infection after primary maternal infection regardless of the trimester of infection. We found that VCV reduced the risk of maternal-fetal transmission of CMV in almost two-thirds of cases, consistent with previous studies[25,112].

We also reported results from the treatment of MPI in the second trimester and found a two-fold, albeit not significant, decrease in the transmission rate with VCV probably due to a lack of power.

In view of our results, the utility of screening for CMV in the second trimester of pregnancy could be debated. The value of diagnosing and preventing cCMV in the second trimester is controversial. The neonatal risks are less severe than following first-trimester infections, however the risk of sequelae should be of concern[115]. Faure-Bardon *et al.* reported no sequelae following second or third trimester infections[10]. However, Bilavsky *et al*. found a 2.2% risk of hearing loss at birth in children who had a late maternal-fetal transmission. Lipitz *et al*. described a 5.6% of any auditory damage or neurodevelopmental delay in patients with second trimester infection[115]. Although neonatal risk in second trimester infection is less severe than in first trimester infections, VCV should be considered in second trimester infections.

The risk of vertical transmission following a negative amniocentesis is another important clinical issue. In untreated cohorts, 4 to 15% of neonates with a negative amniocentesis have congenital CMV[12,116–120]. The main hypothesis is transmission occurring after amniocentesis, rather than poor sensitivity of the PCR technique[116]. In our cohort, most neonates who were CMV-infected after a negative amniocentesis were from the group which did not receive VCV. In addition, VCV was not always discontinued following a negative amniocentesis, which may contribute to our low rate of infected neonates after negative amniocenteses compared to the study by Faure-Bardon *et al*[10]. Shahar-Nissan *et al*. stopped treatment after amniocentesis and found similar rates of infected newborns after negative amniocentesis of 10% in the VCV group and 6% in controls[112]. In an Italian series, the rate of transmission was 2/12 at amniocentesis and 5/12 at birth; thus almost 30% of the infected newborns became infected following a negative amniocentesis[121]. Taking this into account,

continuing VCV treatment following a negative amniocentesis should be considered to prevent delayed transmission and its potential impact on the child.

Similar to a previous study, we found an association between maternal viremia at presentation and the risk of maternal-fetal transmission[122]. This is the first study to evaluate the efficacy of VCV according to maternal viremia after CMV seroconversion. VCV seemed to mainly be effective in the case of positive viremia, halving maternal-fetal transmission, while there was no significant impact when the viremia was undetectable. The duration of viremia is variable, being from 1 to 5 weeks in immunocompetent persons[123]. Maternal-fetal transmission probably occurs preferentially at the time of viremia, which explains why treatment is more effective in cases of positive maternal viremia. The time from seroconversion to treatment initiation is a major prognostic factor for the effectiveness of VCV to prevent maternal-fetal transmission[112]. This may be related to reducing maternal viremia near its peak.

Relevance, clinical and research implications

Our findings provide relevant clinical implications in terms of the prediction of SBD, assessment of fetuses with mild US-findings or even infected fetuses without US/MRI findings. Nonetheless, our results need confirmation with larger series of infected fetuses especially regarding GGT levels and their association with fetal brain damage, the value of an isolated halo sign, and the cortical development in asymptomatic infected fetuses. It should be stressed that since there is a paucity of data on the normal values of sulci depth, we could not provide a cut-off value that would be useful in clinical practice. Moreover, we acknowledge that all these findings may need correlation with neurological outcomes to fully appreciate their clinical value.

Regarding the recent breakthroughs in prenatal treatment have shown that VCV reduces maternal-fetal transmission by 71% after periconceptional and first trimester MPI and also reduces CMV-related lesions[88,112], study 7 provides additional data along the same line. Since the efficacy of VCV for the prevention of maternal-fetal transmission is now established, the benefit/risk balance, and the perspective of cost-effectiveness of population-based serological screening in the first trimester remains

to be determined. Previous cost-effectiveness studies have concluded that universal serological maternal screening is highly dependent on the incidence of primary CMV infection, and on the effectiveness of in utero treatment[67,68]. Two recent studies using a theoretical cohort compared two strategies: 1) usual care (diagnosis of fetal CMV infections following abnormal mid-trimester US), and 2) universal first-trimester screening with/without treatment of infected mothers. Seror *et al.*[124] showed that moving to a universal first trimester screening approach (serological testing at 7 and 12 weeks) would increase detection rates from 15% to 94% although at significantly higher costs. However, implementing secondary prevention with VCV would significantly improve the outcomes of newborns with a 58% reduction of severely infected alive newborns for a 3.5% increase in total costs related to treatment[124]. On the other hand, Fisher *et al.*[125] reported that universal screening and treatment would be cost-effective only if VCV efficacy achieved a 75.9% reduction in risk of vertical transmission, which represents an additional 5% compared to the results of Sahar-Nissan *et al.*[112]. Given the importance of prevention of societal burden associated with cCMV, future estimates of cost-effectiveness are urgently needed in countries with low maternal seroprevalence (50-60%) and should prompt national health systems to develop consensus guidelines. Unfortunately, maternal screening does not apply to countries with a high maternal seroprevalence. In order to avoid inequities, it is essential to find new diagnostic tools.

Regarding other antiviral treatments to improve the prognosis of newborns once the fetus is infected, maternal valganciclovir has not been used in fetal life due to concerns for toxicity[126–128]. Further investigation is warranted since several case reports have not shown negative effects in fetuses already showing US abnormalities [113,129,130]. Letermovir, a new molecule with specific anti-CMV activity that appears to be well tolerated and safe in pregnancy, is currently being evaluated, in a RCT comparing its efficacy vs. VCV in infected fetuses (NCT04732260)

Strengths and limitations

This project has some strengths and limitations that are worthy of comment. First, it is the first series of data reported that compares blood parameters in untreated infected fetuses according to the trimester at diagnosis. In addition, we established a

cut-off value for GGT levels for the prediction of severe brain lesions. We have shown that other blood parameters were closer to normal ranges in the third trimester, also in severely damaged fetuses. Moreover, we have suggested that increased GGT levels are associated with SBD in CMV-uninfected fetuses. Nevertheless, the CMV-infected fetuses showed significantly higher GGT levels likely due to multiorgan dysfunction and generalized inflammation processes.

We highlighted that despite the lack of differences in the liver volumes of infected fetuses according to the severity of brain damage once corrected by fetal body composition we could identify those fetuses with larger liver volumes compared to the healthy fetuses. In addition, this is one of the first studies describing not only liver volume, but which also compares the weight of the liver examined postmortem with the liver weight estimated by MRI, providing information about the reliability of this measurement.

Another main strength is that, in our **fourth study**, MRI assessment was performed in a well characterized group of CMV-infected fetuses with mild US-findings. MRI measurements were done manually by a single examiner blinded to the CMV infection status. We applied a recently described measurement in NSG, the SFA, which has excellent interobserver agreement and may be useful as a screening tool for malformations of cortical development. Lastly, for the first time we described the use of the SFA assessed by MRI in cCMV infection that can also aid in the understanding of cortical brain architecture and provide additional data on the extent of the pathophysiology in fetal CMV infection. All of the above reinforce the use of MRI in the mid-third trimester to evaluate brain cortical structure in CMV-infected fetuses, even in the absence of US-findings.

Another strong point is that we have reported the largest cohort of halo sign detected by US in CMV-infected fetuses during the second and third trimester with targeted histopathological examination. A larger cohort is unlikely reproducible in subsequent studies. Moreover, all the neuroimaging and pathology specialists were blinded to each other.

Regarding treatment, although we reported a case series, the strength of **study 6** is the addition of valuable information to the limited data regarding prenatal treatment with high-dose maternal VCV in pregnancies with CMV-infected fetuses before detectable abnormal US, or after the detection of non-severe lesions

Lastly, **study 7** is the first to evaluate the efficacy of VCV as secondary prevention, taking into consideration maternal viremia and second trimester infections.

Among the limitations, we first acknowledge the retrospective nature of the analysis. However, it is difficult to perform prospective studies in a single center given the low prevalence of the infection in our setting. Secondly, the small sample size mainly in the mildly affected and unaffected CMV-infected fetuses that are underdiagnosed in the absence of maternal routine screening.

There could have been an overlap in the grading of severity because it is not known what the evolution of cases classified as mildly damaged in the second trimester would have been if they had survived. This, however, is inherent to a classification of risk in the second trimester. In addition, in our first study there was a lack of a standardized necropsy protocol for congenital CMV infection.

Third, *in vivo* measurement of liver weight was obtained only in pregnancies undergoing TOP in the severely affected CMV group, limiting the interpretation of our results

Fourth, we were not able to establish the exact time of maternal infections, nor the type of infection, in almost a half of the cases. Given the absence of routine screening most fetal infections were detected by US abnormalities. However, the nature of the CNS abnormalities almost certainly corresponded to infections in the first trimester, primary or non-primary. The latter cannot be diagnosed by maternal serology although the impact on the fetal brain is similar.

Fifth, asymptomatic CMV infection was not ruled out among all the controls, but we believe this probability is very low considering that congenital infection occurs in around 0.7% of all fetuses/newborns.

Sixth, the halo sign is a subtle sonographic marker that may be interobserver dependent. Although in our study, all the images were evaluated by the same observer, standardized quantitative data (Digital Imaging and Communications in Medicine) would have helped to correlate the intensity of the halo sign and the degree of inflammation. Further studies are warranted to establish different halo types and their relation to prognosis. Moreover, as mentioned previously, we did not obtain MRI studies in all the isolated-halo cases. Finally, under the hypothesis of the halo as a transient sonographic sign, and since prenatal CMV screening is not

recommended, we do not know how many infected fetuses may have been born with a transient halo during the study period.

Finally, regarding treatment, our **study 6** did not compare results between treated and untreated fetuses. Given the difficulties in matching similar cases, we did not compare newborn VL and outcome with a historical cohort of untreated fetuses. The main limitation of **study 7** is that CMV screening was not systematic and the initiation of VCV therapy was discussed on a case-by-case basis. However, the use of VCV would have been instituted in the highest risk situations, which would have tended to limit the effectiveness of VCV. Furthermore, practices have evolved within the same center and between centers over time.

7. CONCLUSIONS

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1. In CMV infected fetuses, thrombocytopenia, and high levels of GGT were associated with severe US/MRI brain abnormalities. Nevertheless, among severely affected fetuses, blood parameters, with exception of GGT, change according to gestational age. Fetal blood markers could be less predictive of brain damage in the third trimester.

2. Increased GGT levels were associated with severe brain damage in CMV-infected and uninfected fetuses. Nevertheless, infection may play a role since the increase was significantly higher among the infected ones. Due to the lower platelet count in CMV-infected fetuses, GPR may improve the detection rate of SBD in this group.

3. In CMV-infected fetuses, FLV corrected for AC and FBV was higher compared to healthy-controls, indicating relative hepatomegaly. Although increased FLV was not correlated with the severity of brain damage, these parameters could potentially be used as a surrogate marker of liver enlargement.

4. Unaffected and mildly affected CMV-infected fetuses with mild involvement showed an underdeveloped cortical maturation compared with healthy controls. These results suggest that congenital CMV infection, even in non-severe affected fetuses, which are typically considered of good prognosis, could be related to altered brain cortical structure.

5. In CMV-infected fetuses, isolated-halo sign was observed exclusively in the second trimester and associated with histological findings that suggested immune-mediated damage, but not severe lesions. Detailed sonographic follow-up and MRI in the early third trimester are required to counsel parents.

6. Fetal CMV lesions remained stable with high-dose maternal VCV. Newborn viral load was unchanged despite treatment duration and fetal/neonatal abnormalities.

7. VCV treatment of pregnant women with primary CMV infection in the first and second trimester reduces the risk of maternal-fetal transmission.

8. REFERENCES

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