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

Rovito, R.

CHAPTER 3

NEONATAL SCREENING PARAMETERS IN INFANTS WITH CONGENITAL CYTOMEGALOVIRUS INFECTION

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ABSTRACT

Congenital Cytomegalovirus infection (cCMV) is the most common cause of congenital infections worldwide that can cause long-term impairment (LTI). The metabolic alterations due to cCMV are largely unknown. This study aims to assess the metabolites included in the neonatal screening in relation to cCMV and cCMV outcome, allowing the identification of prognostic markers for clinical outcome. Essential amino acids, hormones, carnitines and enzymes from dried blood spots (DBS) were analyzed of 102 children with cCMV and 179 children without cCMV, and they were related to symptoms at birth and LTI at 6 years of age. In this cohort, the neonatal screening parameters did not change in relation to cCMV, nor to symptoms at birth or LTI. However, metabolic changes were observed in children born preterm, with lower concentrations of essential amino acids in premature infants with cCMV compared to premature controls. Finally, a higher concentration of palmytoilcarnitine (C16) in the group with higher viral load was observed. Though these data demonstrate limitations in the use of neonatal screening data as predictors for long-term cCMV outcome, the metabolism of preterm neonates with cCMV merits further evaluation.

3.1. INTRODUCTION

Cytomegalovirus (CMV) infection is a common infection with a seroprevalence of almost 50% in the general Dutch population (1). CMV is the most common cause of congenital infections worldwide with an overall birth prevalence of 0.6-0.7% in industrialized countries (2, 3). A significant part of children with congenital CMV infection (cCMV) will have long-term permanent neurological sequelae. Among congenitally CMV-infected children, 12.7% is estimated to be symptomatic at birth with the most common symptoms being petechiae, jaundice, hepatosplenomegaly, thrombocytopenia, chorioretinitis, and microcephaly (2, 3). An estimated 40-58% of these symptomatic children will develop permanent sequelae, such as hearing loss, mental retardation, and developmental delay (3). Approximately 13.5% of the asymptomatic children will develop permanent sequelae as well (3).

cCMV outcome is the result of a complex interplay between viral, maternal, fetal, and child factors. *In vitro*, CMV has been shown to influence different cellular metabolic pathways. The fatty acid biosynthetic pathway of infected cells is highly upregulated in order to sustain the viral envelope production (4-6). In infected cells, the increased glucose uptake and glycolytic activity provide the necessary carbon atoms used for fatty acid biosynthesis (4, 6, 7). The cellular energy requirement is then insured by the increase of glutaminolysis in order to allow the tricarboxylic acid cycle (TCA) to function (8). *In vivo*, few studies have evaluated the metabolic changes occurring in children with cCMV. A recent metabolomics analysis on amniotic fluid (AF) showed that primary CMV infection during pregnancy, irrespective of fetal infection, resulted in the activation of glutamine-glutamate and pyrimidine metabolic pathways and, when comparing asymptomatic CMV-infected newborns to symptomatic CMV-infected newborns, a possible shift in fatty acid biosynthesis was observed (9). Moreover, in a group of congenitally infected children a metabolic fingerprint was identified in urine samples compared to uninfected controls. An increase of ketone bodies (3-hydroxybutyrate and 3-aminoisobutyrate) was observed in the CMV-infected group in an attempt to compensate a general reduced level of ATP (10).

The aim of this study was to assess the metabolites included in the neonatal screening, which is performed in Dried Blood Spots (DBS), in relation to cCMV and cCMV outcome. Importantly, several considerations should be taken into account with respect to the neonatal screening, specifically designed to diagnose rare genetic metabolic disorders. First of all, excluding the metabolic disorders, changes in these metabolites have only been reported in critically ill children. A decrease in thyroid hormones in septic neonates with poor outcome was observed and premature critically ill neonates showed different amino acids profiles, usually with higher concentrations, compared to the healthy controls (11, 12). The majority of newborns with cCMV do not have symptoms at birth or only have mild disease, and the clinical signs of symptoms and LTI included in this cohort are diverse. Therefore, if any changes in metabolites are found, these will most likely be subtle. Second, several factors have been described influencing the analytes measured on DBS, such as fetal blood volume, hematocrit, gestational age, birth weight, maternal factors and storage conditions (13-19). However, despite these potential limitations, this exploratory study was undertaken to study biomarkers in neonatal DBS of cCMV-infected children. This could allow the identification of prognostic markers

for long term outcome of cCMV with a profound impact on parental counselling, postnatal interventions and the potential introduction of neonatal screening for cCMV. For this purpose, the neonatal screening data of a large nation-wide cohort of children with and without cCMV was evaluated in relation to long-term impairment (LTI) at the age of six years.

3.2. MATERIALS AND METHODS

3.2.1. Study population and clinical data

A previously described, nationwide, retrospective cohort was used for this study. A group of 31,484 children born in 2008 in the Netherlands was retrospectively tested for cCMV by PCR for CMV DNA in neonatal DBS at 5 years of age (20). cCMV was diagnosed in 156 children and informed consent for retrieval of medical data was given by parents of 133 children with cCMV and 274 matched controls. After approval by the Medical Ethics Committee of the Leiden University Medical Center, the parents of 102 congenitally CMV-infected children and 197 children without cCMV gave informed consent to retrieve the neonatal screening data of their child. The controls without cCMV are from a gender-, month-of-birth and region-matched control group. Children were defined as symptomatic at birth if they had one or more of the following signs or symptoms in the neonatal period: prematurity, being small for gestational age, microcephaly, hepato- or splenomegaly, generalized petechiae or pupura, hypotonia, abnormal laboratory findings (elevated liver transaminases, hyperbilirubinemia, neutropenia or thrombocytopenia), cerebral ultrasound abnormalities, ophthalmologic abnormalities or neonatal hearing impairment. LTI was defined as the presence of impairment in one or more domain (hearing, visual, neurological, motor, cognitive and speech-language). Because in this cohort maternal seroimmunity to CMV before birth was unknown, it was assumed that cCMV infection could have resulted from either maternal primary or recurrent infection.

3.2.2. DNA extraction from DBS and qPCR of CMV

After a first initial CMV PCR screening performed at the National Institute for Public Health and the Environment (RIVM), a second confirmatory PCR was performed at the Leiden University Medical Center (LUMC) (20). For this purpose, DNA was extracted from DBS by using the QIAamp DNA minikit according to the previously described protocol (21). For each test one full DBS was punched by using an automated DBS puncher (1296-071, Perkin Elmer-Wallac, Zaventem, Belgium). CMV DNA amplification of a 126-bp fragment from the immediate-early antigen region was performed using an internally controlled quantitative real-time PCR as described previously (22, 23) on a CFX96 Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands). The PCR was performed in triplicate, and the CMV viral load expressed in IU/ml.

3.2.3. Neonatal screening data

The genetic metabolic disorders included in the Dutch neonatal screening program in 2008 are listed in Table 1. The screening is carried out on DBS and involves five regional accredited screening laboratories, among which the National Institute for Public Health and the Environment (RIVM). The DBS are collected between 72 and 168 hours after birth and the markers are quantified mainly

Table 1 Genetic disorders included in the Dutch neonatal screening program in 2008.

Disorder	Marker	Quantification method	Incidence[1]
Amino acid disorders			
Glutaricaciduria type 1 (GA I)	C5DC	MS/MS	1: 335455
Isovaleric academia (IVA)	C2, C5	MS/MS	1: 351429
Maple syrup urine disease (MSUD)	Leucine, Valine	MS/MS	1:567692
Homocystinuria (HCU)	Met	MS/MS	1:167727
3-methylcrotonyl-CoA- carboxylase Deficiency (3-MCC)	C5OH	MS/MS	1: 194211
HMG-CoA lyase deficiency	C5OH	MS/MS	1:100000 [34][2]
multiple CoA carboxylase deficiency (MCD)	C5OH	MS/MS	1:200000 [35]
Phenylketonuria (PKU)	Phenylalanine, Tyrosine	MS/MS	1: 11865
Fatty acid oxidation disorders			
Medium chain acylCoA dehydrogenase Deficiency (MCAD)	C8, C10	MS/MS	1: 23730
Long-chain hydroxyacyl-CoA dehydrogenase Deficiency (LCHAD)	C16OH	MS/MS	1:410000
Very long chain acylCoA dehydrogenase Deficiency (VLCAD)	C14:1, C16	MS/MS	1: 144706
Carnitine transporter deficiency (CTD)	C0	MS/MS	1:40000 [36]
Endocrine disorders			
Congenital hypothyroidism (CH)	T4, TSH, TBG	Immunochemistry	1:3000 – 1:4000 [37]
Congenital adrenal hyperplasia (CAH)	17-OHP	AutoDELFIA	1:10000 – 1:20000 [38]
Other			
Galactosemia (GAL)	GALT, TGAL	Enzymatic method	1: 49530
Biotinidase deficiency (BTD)	BIOT	Enzymatic method	1: 49865

[1] Unless otherwise specified the incidence is retrieved from the Dutch Diagnosis Registration Metabolic Diseases (DDRMD) database [39]; [2] for HMG-CoA lyase deficiency the prevalence is reported.

using Tandem Mass Spectrometry (MS/MS) or Immunochemistry as reported in Table 1. The data were provided by the National Institute of Public Health and the Environment (RIVM), Department of Prevention Programs. The cut off values of the parameters used for referral of the child are described in the national guideline for neonatal screening (24). An analyte below or above the cut-off levels would strongly indicate the presence of a rare genetic metabolic disorder, though a confirmatory test would be needed on a second DBS. Thus, the whole cohort of children included in the study was screened for analytes above or below the cut-off levels. Furthermore, among the factors described to influence the metabolites measured on DBS, gestational age and birthweight are the most important and well-characterized (13). These factors were taken into account in the analysis, and the markers were first assessed in premature versus term infants, and then in dysmature versus non-dysmature infants, within the whole cohort. Prematurity was defined as birth before 37 weeks of gestation while dysmaturity as weight at birth less than -2 SD for gestational age. Then, the analysis

of the metabolites in relation to cCMV and cCMV outcome was subsequently stratified for these factors in order to evaluate if they were of significant influence.

3.2.4. Statistics

The differences in the levels of the metabolic markers between the different categories - cCMV status, CMV viral load, symptoms at birth, and LTI - were assessed by using an independent-samples t-test. A Chi-square test was used to assess the differences in the proportion of prematures and dysmatures in relation to cCMV and LTI. Several analytes including C5OH, C5DC, C5 and C16OH showed results below the limit of reliable quantification and were therefore excluded from the analysis. The carnitines included in the present study (C0, C2, C8, C10, C14:1 and C16) identify fatty acid disorders associated to different pathways which are short, medium, long and very long chain fatty acids metabolism. Therefore, in order to gain more insights into these pathways total carnitine (TC), acylcarnitines molar ratio (AFR), short chain index (SCI), medium chain index (MCI) and long chain index (LCI) were additionally calculated as previously described (25), with slight modifications according to the carnitine available in this study. TC was calculated by addition of all available acylcarnitines (AC) and free carnitine (FC); AFR was calculated as the ratio between AC/FC; SCI as C2 divided by TC level; MCI as the sum of C8 and C10 divided by TC level; LCI as the C14 and C16 divided by TC level. A p value <0.05 was considered statistically significant. Due to the exploratory nature of this study, no multiple comparison correction was applied. Data were analyzed by using the Statistical Package for Social Sciences (SPSS, version 23, Chicago, IL, USA).

3.3. RESULTS

3.3.1. Study population and clinical data

The clinical data of the study population are shown in Table 2. Neonatal screening data were retrieved from 102 children with cCMV and 179 non-infected controls. In the control group, 12.4% of children (n = 22) had symptoms at birth and 8.4% (n = 15) showed any LTI. In the children with cCMV, 17.6% (n = 18) had symptoms at birth and 24.5% (n = 25) showed LTI. In this cohort, 10.8% of children with cCMV (n = 11) and 5.1% of children without cCMV (n = 9) were born preterm, while 2% of children with cCMV (n = 2) and 5.6% of children without cCMV (n = 10) were born dysmature, these differences were not significant. However, a significantly higher percentage of prematures was observed in the cCMV-infected group that developed LTI compared to cCMV-infected group that did not (24% and 6.5% respectively, p = 0.024), whereas this was not observed in the control group (data not shown). Additionally, within the cCMV-infected group, all children born premature with birthweight <2500 g developed LTI, and only one premature child with birthweight ≥ 2500 g did so, whereas none of the non-infected premature neonates developed LTI.

3.3.2. Neonatal screening data in relation to congenital CMV infection

First, the metabolic markers were assessed in relation to cCMV. For this purpose, CMV-positive children (n = 102) were compared with CMV-negative children (n = 179), and no significant differences were found in any of the markers included in this study (data not shown).

Table 2. Clinical data in the group with cCMV and controls.

	Congenital CMV infection			No congenital CMV infection		
	Overall n (%) N = 102	Asympt.[1] n (%) N = 84	Sympt.[2] n (%) N = 18	Overall n (%) N = 179[3]	Asympt. n (%) N = 156	Sympt. n (%) N = 22
Gender						
Male	60 (58.8)	48(57.1)	12 (66.7)	96 (53.6)	83 (53.2)	12 (54.5)
Female	42 (41.2)	36 (42.9)	6 (33.3)	83 (46.4)	73 (46.8)	10 (45.5)
Gestational age (weeks)[4]						
<32	1 (1.0)	0 (0.0)	1 (5.6)	2 (1.1)	0 (0.0)	2 (9.1)
32 - < 37	10 (9.8)	0 (0.0)	10 (55.6)	7 (3.9)	0 (0.0)	7 (31.8)
≥ 37	91 (89.2)	84 (100)	7 (38.9)	169 (94.4)	156 (100)	13 (59.1)
Birthweight (g)[5]						
<1500	1 (1.0)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)
1500-2499	5 (5.0)	0 (0.0)	5 (27.8)	10 (5.6)	0 (0.0)	10 (45.4)
≥2500	95 (94.1)	83 (100)	12 (66.7)	168 (94.4)	156 (100)	12 (54.5)
Premature	11 (10.8)	-	11 (61.1)	9 (5.1)	-	9 (40.9)
Dysmature	2 (2.0)	-	2 (11.1)	10 (5.6)	-	10 (45.5)
Long term impairment						
Hearing impairment[6]	3 (2.9)	2 (2.4)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)
Visual impairment[7]	3 (2.9)	3 (3.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Neurological impairment[8]	5 (4.9)	3 (3.6)	2 (11.1)	8 (4.5)	8 (5.1)	0 (0.0)
Motor impairment[9]	11 (10.8)	8 (9.5)	3 (16.7)	2 (1.1)	2 (1.3)	0 (0.0)
Cognitive impairment[10]	4 (3.9)	2 (2.4)	2 (11.1)	2 (1.1)	2 (1.3)	0 (0.0)
Speech/language impairment[11]	17 (16.7)	10 (11.9)	7 (38.9)	9 (5.0)	8 (5.1)	1 (4.5)
One or more impairment[12]	25 (24.5)	15 (17.9)	10 (55.6)	15 (8.4)	14 (9.0)	1 (4.5)

[1] Asymptomatic at birth; [2] Symptomatic at birth; [3] For one CMV-negative child data on symptoms at birth were not available; [4] For one CMV-negative child gestational age was not available; [5] For one CMV-positive and one CMV-negative child birthweight was not available; [6] sensorineural hearing loss; [7] optic nerve atrophy, cortical visual impairment, congenital cataract; [8] cerebral palsy, epilepsy, microcephaly, ADHD, autism; [9] motor impairment (fine, gross or balance) based on test or diagnosis or sensory processing disorder or developmental coordination disorder; [10] cognitive impairment based on test or diagnosis; [11] language impairment based on test or diagnosis, speech-impairment, oral motor skill difficulties or auditory processing disorder; [12] Any long-term impairment, in one or more domains.

Furthermore, as previously mentioned, gestational age and birthweight are important and well known factors influencing the metabolites measured on DBS. Because of the significant percentage of premature and dysmature infants in the group with symptoms at birth, we aimed to evaluate if these variables affected the metabolic markers in this cohort. For this purpose, we compared neonates born premature (n = 20) with non-premature (n = 260), and dysmature neonates (n = 12) with non-dysmature neonates (n = 267) using the total cohort, both children with and without cCMV. A significantly decreased concentration of T4 and biotinidase activity (BIOT) was observed in the premature group compared to non-premature, as well as a significantly increased 17OHP concentration (Table 3) ($p < 0.001$). No differences in concentration were found between dysmature neonates and non-dysmature neonates (data not shown). Given the diversity of clinical signs in the symptomatic children, we next wondered whether prematurity was the main factor driving

Table 3. Metabolic markers in association with prematurity.

	All children[1]			Preterm children[2]			Preterm children with cCMV[3]		
Mean (SE)	Term (n = 260)	Preterm (n = 20)	p-value	cCMV- (n = 9)	cCMV+ (n = 11)	p-value	No LTI (n = 5)	LTI (n = 6)	p-value
Essential amino acids[4]									
Methionine	17.8 (0.3)	19.5 (1.3)	0.150	21.1 (2.2)	18.2 (1.5)	0.269	19.2 (1.2)	17.3 (2.6)	0.564
Leucine	155.4 (2.6)	140.7 (10.0)	0.132	167.7 (15.0)	118.6 (9.5)	0.010	134.6 (16.3)	105.3 (8.8)	0.132
Valine	127.2 (2.2)	114.7 (8.7)	0.128	129.0 (14.7)	102.9 (9.3)	0.137	118.8 (7.4)	89.7 (14.2)	0.121
Phenylalanine	64.7 (1.1)	73.0 (4.0)	0.038	81.7 (6.7)	65.9 (3.7)	0.044	65.8 (2.0)	66.0 (6.9)	0.980
Hormones[5]									
T4[6]	83.7 (1.1)	68.5 (5.0)	<0.001	62.2 (6.7)	73.6 (7.0)	0.267	81.6 (5.4)	66.8 (11.9)	0.321
17OHP[7]	0.81 (0.02)	1.2 (0.1)	<0.001	1.3 (0.2)	1.2 (0.1)	0.399	1.13 (0.06)	1.18 (0.14)	0.769
Carnitines[8]									
C0[9]	17.5 (0.5)	19.3 (1.6)	0.276	21.3 (3.1)	17.7 (1.4)	0.273	16.7 (2.4)	18.6 (1.7)	0.538
C2[10]	19.9 (0.5)	23.3 (2.1)	0.094	25.1 (3.6)	21.7 (2.4)	0.428	19.4 (2.2)	23.7 (4.0)	0.400
C8[11]	0.034 (0.001)	0.036 (0.004)	0.494	0.034 (0.005)	0.037 (0.006)	0.726	0.030 (0.004)	0.043 (0.010)	0.295
C10[12]	0.050 (0.001)	0.056 (0.006)	0.367	0.05 (0.01)	0.06 (0.01)	0.190	0.05 (0.01)	0.07 (0.01)	0.332
C14:1[13]	0.061 (0.003)	0.07 (0.01)	0.362	0.07 (0.02)	0.07 (0.01)	0.823	0.08 (0.02)	0.06 (0.01)	0.344
C16[14]	2.72 (0.06)	2.29 (0.22)	0.053	2.3 (0.4)	2.3 (0.3)	0.880	2.5 (0.4)	2.1 (0.4)	0.461
Enzymes[15]									
BIOT[16]	99.3 (1.4)	85.2 (3.2)	<0.001	78.7 (3.9)	90.5 (4.4)	0.067	101.8 (5.3)	81.0 (3.7)	0.009
GALT[17]	100.5 (1.6)	94.1 (4.8)	0.277	86.7 (8.1)	100.1 (5.4)	0.170	92.4 (9.6)	106.5 (5.1)	0.207

[1] Metabolic markers in relation to prematurity; [2] Metabolic markers in relation to cCMV in premature neonates; [3] Metabolic markers in relation to LTI in cCMV-infected premature neonates; [4] The concentration of essential amino acids is given in µmol/l of blood; [5] The concentration of hormones is given in nmol/l of blood; [6] T4 = Thyroxine; [7] 17OHP = 17-Hydroxyprogesterone, values in log scale; [8] The concentration of carnitine is given in µmol/l of blood; [9] C0 = Free carnitine; [10] C2 = Acetylcarnitine; [11] C8 = Octanoylcarnitine; [12] C10 = Decanoylcarnitine; [13] C14:1 = Tetradecenoylcarnitine; [14] C16 = Palmitoylcarnitine; [15] The enzyme activity is given in % compared to the average of the daily run; [16] BIOT = biotinidase activity; [17] GALT = galactose-1-phosphate uridylyltransferase.

the aforementioned metabolic changes, or rather a combinations of clinical signs. Hence, an additional sensitivity analysis was performed by first comparing the group of symptomatic neonates (n = 40) with the asymptomatic neonates (n = 240) without excluding premature neonates. Then the group of premature was excluded, and symptomatic neonates (n = 20) were compared to asymptomatic neonates (n = 240). The results and significance changed suggesting that the observed differences were mainly driven by prematurity (data not shown).

Therefore, we next assessed the markers in relation to cCMV by stratifying for prematurity. Premature cCMV-negative children (n = 9) were compared to premature cCMV-positive children (n = 11), and term cCMV-negative (n = 169) children were compared to the term cCMV-positive (n = 91). The premature cCMV-positive showed lower levels of essential amino acids compared to premature controls, statistically significant for leucine and phenylalanine (p = 0.01 and p = 0.04 respectively) (Table 3 and Fig. 1). No differences were found when comparing term cCMV-positive with term cCMV-negative (Fig. 1).

Next, in order to assess the influence of CMV viral load on the metabolic markers, the cCMV-positive group was divided into two groups according to the median viral load in DBS, which was 3.1 log (IU/ml), namely low (n = 50) and high viral load (n = 50) group. The high viral load group had significantly higher concentration of palmitoylcarnitine (C16) (p = 0.002) (Table 4 and Fig. 2). This was not influenced by the presence of premature neonates (data not shown). As a

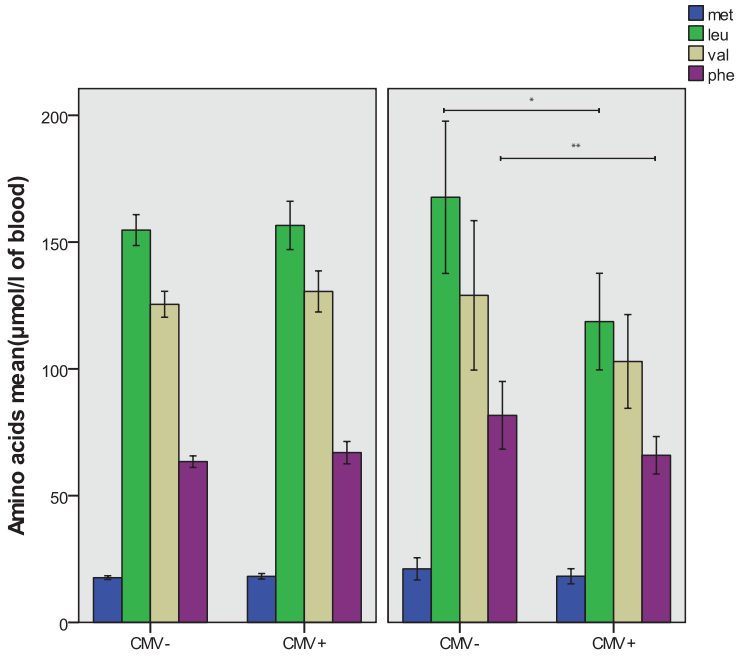

Figure 1. Essential amino acids in relation to cCMV in term and preterm infants. *Left panel:* essential amino acids in CMV-negative and CMV-positive term infants (n=169 and n=91, respectively). *Right panel:* essential amino acids in CMV-negative and CMV-positive pre-term infants (n=9 and n=11, respectively). The mean from each group of infants ± 2SE are shown. *p = 0.01 and **p = 0.04.

Table 4. Metabolic markers in relation to CMV viral load.

	Viral Load cCMV infection[1]		
Mean (SE)	**low load[2] (n=50)**	**high load[3] (n=50)**	**p-value**
Essential amino acids			
Methionine	18.4 (0.8)	18.2 (0.7)	0.846
Leucine	157.7 (6.9)	147.2 (6.1)	0.255
Valine	128.1 (5.2)	127.2 (5.9)	0.911
Phenylalanine	65.5 (2.5)	68.7 (3.2)	0.431
Hormone			
T4	79.9 (2.9)	87.5 (3.1)	0.075
17OHP	0.8 (0.1)	0.91 (0.04)	0.082
Carnitines			
C0	18.1 (1.3)	17.6 (1.0)	0.764
C2	19.0 (1.4)	22.5 (1.6)	0.113
C8	0.033 (0.002)	0.033 (0.003)	0.951
C10	0.049 (0.002)	0.054 (0.003)	0.208
C14:1	0.06 (0.01)	0.06 (0.01)	0.961
C16	2.4 (0.2)	3.1 (0.1)	0.002
Enzymes			
BIOT	99.3 (3.2)	99.3 (2.8)	0.993
GALT	99.0 (3.4)	102.8 (4.1)	0.471
Pathways			
TC[4]	39.81 (2.61)	43.42 (2.47)	0.318
AFR[5]	1.28 (0.06)	1.51 (0.06)	0.008
SCI[6]	0.48 (0.01)	0.51 (0.01)	0.029
MCI[7]	0.0023 (0.0001)	0.0021 (0.0001)	0.164
LCI[8]	0.066 (0.003)	0.076 (0.003)	0.008

[1] CMV viral load measured in DBS. For two infected neonates the DBS was not available, therefore CMV viral load could not be assessed. [2] CMV viral load below the median (3.1 log (IU/ml)); [3] CMV viral load above the median (3.1 log (IU/ml)); [4] Total carnitine (TC); [5] Acylcarnitines molar ratio (AFR); [6] Short chain index (SCI); [7] Medium chain index (MCI); [8] Long chain index (LCI).

result, the acylcarnitine molar ratio (AFR) and the long-chain index (LCI) were increased (both p = 0.008) (Table 4).

Finally, the genetic metabolic disorders, included in the neonatal screening, were assessed in relation to cCMV. Six children without cCMV showed an increased 17-Yhydroxyprogesteron (17OHP) concentration. However, when taking into account the gestational age, only two infants had dubious 17OHP results. Furthermore, one child with cCMV showed a low concentration of free carnitine (C0). The analysis of clinical data of these infants did not show any underlying disorders related to congenital adrenal hyperplasia nor to carnitine transporter deficiency. Therefore, none of the metabolites' concentrations are affected by any of the genetic conditions included in this neonatal screening.

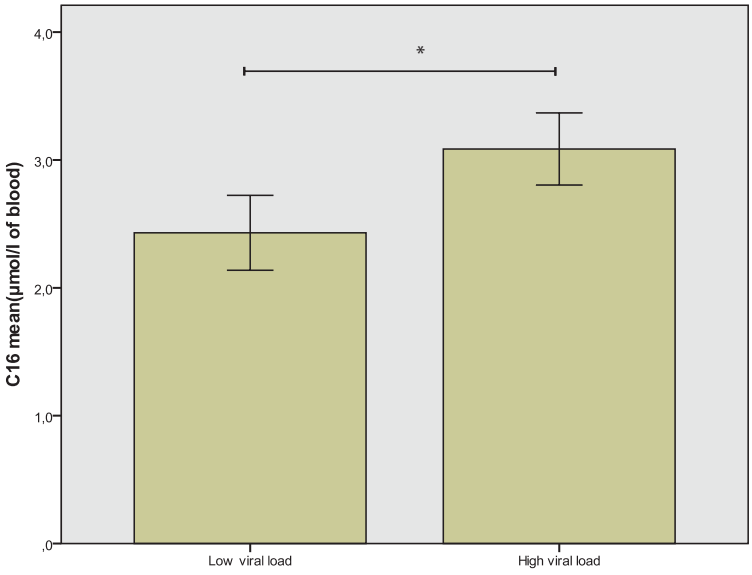

Figure 2. Palmytoilcarnitine (C16) in relation to CMV viral load. *Low viral load:* CMV-positive infants with viral load below the median measured in DBS (n=50). *High viral load:* CMV-positive infants with viral load above the median measured in DBS (n=50). The mean from each group of infants ± 2SE are shown. *p = 0.002.

3.3.3. Neonatal screening data in relation to long-term impairment

Next, neonatal screening data were analysed in the group of children with cCMV in relation to LTI development in one or more of the following domains of impairments: hearing, visual, neurologic, motor, cognitive, and speech-language. Given the previously shown effect of prematurity on different analytes, the analysis was stratified for this factor, and infected premature neonates who developed LTI (n = 6) were compared to infected premature neonates without LTI (n = 5). A lower BIOT was observed in the neonates who developed LTI compared to those who did not (p = 0.009) (Table 3), while this was not observed when comparing infected term neonates who developed LTI (n = 19) with infected term neonates without LTI (n = 72) (data not shown). Finally, in order to establish whether a relationship between the metabolites and LTI development may occur independently of cCMV, the same analysis was performed in the control group. The difference between non-infected premature neonates that developed LTI and those who did not, could not be assessed because none of the nine prematures developed LTI. Whereas no differences were observed when comparing non-infected term neonates who developed LTI (n = 15) with non-infected term neonates who did not (n = 154).

3.4. DISCUSSION

Few studies have evaluated the metabolic effect of cCMV, and this is the first exploratory study on biomarkers in neonatal DBS of cCMV infected children in relation to long-term outcome (9, 10). A reliable biomarker for cCMV long-term outcome that does not require additional neonatal tests

could provide the means to introduce the long debated newborn screening program for cCMV in DBS (26). Indeed, this would define subgroups of children that would benefit from clinical, audiological follow-up and possibly antiviral treatment.

cCMV outcome is the result of a multifactorial process that accounts for viral, maternal, fetal, and child factors. The host metabolism has already been shown to be altered following CMV infection, both *in vivo* and *in vitro* (4, 6-10), therefore it may play a role in cCMV outcome. This exploratory study shows that, overall, there were no differences in concentrations of metabolic and endocrine parameters included in the neonatal screening between cCMV-positive children and controls. Likewise, between cCMV-infected children who developed LTI and cCMV-infected children who did not, suggesting that these neonatal screening data cannot be used as predictors for long-term outcome in the general population. The effects we demonstrated were detected when prematurity was taken into account and, though the numbers of individuals may be a limiting factor, if confirmed in other cohorts, these findings give useful insights into metabolic changes in relation to cCMV in infected preterm neonates. The main metabolic changes concerned lower concentration of essential amino acids in premature cCMV-infected newborns (Fig. 1).

Importantly, gestational age and birthweight are the best characterized variables responsible for the variation of metabolites, mainly attributed to fetal stress and immature functions of liver and kidney. A lower concentration of T4, BIOT and an increased concentration of 17OHP has already been demonstrated in preterm infants (27-31). In this cohort, similar variations were found, indicating that these changes are attributable to prematurity rather than the infection.

Moreover, we aimed to evaluate whether higher CMV viral loads may alter the host metabolism more efficiently than lower viral loads, and found a significantly higher concentration of C16 in the former (Fig. 2). Though this may be a coincidental finding, the relationship between CMV infection and palmitic acids was described before. In a study of pregnant women with primary CMV infection, the palmitic acids was found decreased in AF of transmitters mothers as well as increased in AF of symptomatic neonates (9). Palmitate (16:0), which is the end product of fatty acid synthase pathway, is the precursor of longer chain fatty acids. The CMV envelope is enriched for longer chain fatty acids and its infectivity is reduced by the inhibition of fatty acid elongases (ELOVLs), an enzyme involved in their biosynthesis (32). Therefore, the increase of C16 in the high viral load group may simply reflect the increased viral burden.

Finally, because in this cohort maternal seroimmunity to CMV before birth and trimester of vertical transmission were unknown, the influence of these conditions could not be assessed. Therefore, significant metabolites differences, in relation to cCMV and cCMV outcome, cannot be entirely excluded if such conditions were taken into account. Nevertheless, this cohort study, retrieved from a large population screening, does reflect a real population of newborns with cCMV in all its diversity, ranging from no symptoms at birth and no LTI to symptoms at birth with severe LTI. Furthermore, in view of the described differences in sensitivity of the CMV PCR on DBS (33), it is important note that with the high sensitivity of our PCR (estimated > 85%), high specificity (> 99.9%) and the cCMV birth prevalence of 0.5%, the chance of a CMV false-negative result is 1/1000 (20). Therefore, the influence of the sensitivity of the CMV PCR on DBS on our conclusions can be considered negligible.

In conclusion, although these findings demonstrate limitations in the use of routine neonatal screening data as predictors for long-term cCMV outcome, a possible influence of cCMV in the amino acids metabolism of preterm neonates, and of higher viral loads in the metabolism of longer chain fatty acids were shown. Finally, by considering the use of routine neonatal screening data, this study represents a first step in identifying prognostic markers for cCMV outcome.

3.5. CONFLICTS OF INTEREST

The authors declare no conflict of interest.

3.6. ACKNOWLEDGEMENTS

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