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Congenital and perinatal infections: How to prevent sequelaes in neonates and children

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Published in
Frontiers in Pediatrics

ISSN 1664-8714
ISBN 978-2-83251-471-9
DOI 10.3389/978-2-83251-471-9

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Congenital and perinatal infections: How to prevent sequelaes in neonates and children

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Citation

Auriti, C., De Rose, D. U., Paola, R. M., Giuffre, M., Tzialla, C., eds. (2023). *Congenital and perinatal infections: How to prevent sequelaes in neonates and children*. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83251-471-9

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frontiers | Frontiers in Pediatrics

TYPE Editorial
PUBLISHED 13 February 2023
DOI 10.3389/fped.2023.1142636

OPEN ACCESS

EDITED AND REVIEWED BY
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SPECIALTY SECTION
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

RECEIVED 11 January 2023
ACCEPTED 30 January 2023
PUBLISHED 13 February 2023

CITATION
De Rose DU, Ronchetti MP, Tzialla C, Giuffré M and Auriti C (2023) Editorial: Congenital and perinatal infections: How to prevent sequelaes in neonates and children.
Front. Pediatr. 11:1142636.
doi: 10.3389/fped.2023.1142636

Editorial: Congenital and perinatal infections: How to prevent sequelaes in neonates and children

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KEYWORDS

cytomegalovirus, toxoplasma, congenital syphilis, neonatal sepsis, COVID-19

Editorial on the Research Topic
Congenital and perinatal infections: How to prevent sequelaes in neonates and children

The current severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) pandemic has overwhelmingly absorbed attention and health resources for 2 years, allowing us to reflect that infections are a permanent health and social problem, causing morbidity and mortality. They require organization, important prevention measures, and containment. This is particularly true in the neonatal age, where infections remain a complex problem with serious consequences.

The reduction in under-5 mortality by more than 50% observed in the last 25 years shows us that healthcare has achieved unprecedented goals and successes. However, neonates represent a different story: neonatal mortality decreased much more slowly in the same period (1). Therefore, reducing neonatal mortality should be at the heart of international policies to contrast this trend. Considering that infections are still one of the leading causes of neonatal death worldwide after prematurity and perinatal asphyxia, we cannot eliminate neonatal mortality without eliminating avoidable infections. Most of these cases are sepsis, and the "get to zero infections" must not be a goal of high-income countries alone. Beyond mortality, neonatal sepsis is still burdened by a high rate of neurodevelopmental disability (2). Further studies are needed to explore the neurodevelopmental outcomes according to the different sepsis risk assessment and management approaches in term and preterm infants. The changing epidemiology of involved bacteria and the new antibiotic resistance data should also be kept in mind to improve outcomes (3).

Some infections are contracted from the mother during pregnancy and transmitted to the fetus (congenital infections), during labor and childbirth (perinatal infections), and throughout breastfeeding (postnatal infections). The microorganisms most frequently responsible for these categories of infections are not just bacteria: Cytomegalovirus, Toxoplasma gondii, Treponema pallidum, Hepatitis B and C viruses, Human Immunodeficiency Virus, Parvovirus B19, Rubella, and non-polio Enterovirus. Currently, these infections are known under the acronym TORCH (T for Toxoplasmosis, O for other Agents, R for Rubella, C for Cytomegalovirus, and H for Herpes viruses). The SARS-CoV-2 and Zika virus, until now little or known at all, have attracted attention, the first due to the pandemic diffusion and the unknown transmissibility to the fetus when contracted during pregnancy; the second for the teratogenic potential, which appears increasingly clear. These

infections in pregnancy may lead to spontaneous abortion, fetal death, or intrauterine growth retardation or can cause congenital anomalies and sequelae of different severity (4).

Specific risk factors may influence the incidence of the transmission to the fetus and the severity of sequelae: timing of infection in pregnancy, order of the infection (primary or reinfection or chronic one), duration of maternal rupture of membranes, route of delivery, socio-economic conditions, and breastfeeding. Many of the harmful effects of these infections on the newborn could be reduced or sometimes eliminated if screening and prevention activities are practiced in a timely manner and if access to prevention services is simple, even for more disadvantaged women.

Prenatal screening programs for congenital infections can help avoid mother-to-fetus transmission and advise appropriate and prompt treatment and/or counseling to prevent major sequelae in neonates and children. To date, fetal infections are avoidable, totally or in part, by specific drugs, vaccines, or passive immunization administered to pregnant women; many diagnostic tests can help doctors with appropriate prenatal counseling, guiding families with therapies and decisions. For example, a recent meta-analysis confirmed that a negative amniocentesis in pregnant women with Cytomegalovirus (CMV) infection ensures the lack of fetal insult and long-term sequelae to the child, even if the transmission has occurred (5).

Concerning congenital and perinatal infections, neonates with overt symptoms at delivery have a poorer prognosis than asymptomatic ones; however, long-term sequelae (mental and sensorineural sequelae) may occur also in infected children, which are asymptomatic at birth, after the first year of life (6). Most ophthalmological and auditory anomalies might be also progressive. New viruses also, such as Zika and SARS-CoV-2, are confirmed to be responsible for potential disabilities, with motor abnormalities and epilepsy in infants and children with evidence of congenital Zika Virus infection (7), and individual developmental disorders and abnormal ophthalmological findings after exposure to maternal SARS-CoV-2 infection in pregnancy (8, 9). Furthermore, viral infections may also cause autism spectrum disorders (ASDs) through direct teratogenic effects and indirect impacts on the developing brain from inflammation or maternal immune activation. Long-term monitoring is mandatory for children whose mothers report an inflammatory episode of viral infection at any time during pregnancy (10). In the absence of a long-term follow-up, sequelae in neonates and children are frequently unanticipated with a possible poor prognosis.

In the case of mothers with a history of certain or suspected infections in pregnancy, a timely screening of their neonates in the first weeks after birth can be crucial to diagnose congenital infections and to determine the infection course, as in congenital CMV infections (11). Because of the rapid method for detecting the DNA - CMV in saliva and the efficacy of the antiviral therapy in symptomatic infants, CMV screening is cost-effective (12). Interestingly, many cases without prenatal/neonatal signs of congenital CMV infection or maternal history of CMV infection can be identified only by universal screening (13). Without it some infected children who can develop late neurological sequelae may go unnoticed, depriving them of early access to instrumental and therapeutic measures. Therefore, further studies are needed to guide public health institutions to improve the outcomes of tomorrow's adults.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers in Pediatrics

ORIGINAL RESEARCH
published: 23 February 2022
doi: 10.3389/fped.2022.802071

The Clinical Characteristics and Serological Outcomes of Infants With Confirmed or Suspected Congenital Syphilis in Shanghai, China: A Hospital-Based Study

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Background: Congenital syphilis (CS) is the infection of an infant or fetus with *Treponema pallidum.* The aim of this study was to investigate the clinical features and outcomes of serology reversion in infants diagnosed with confirmed or suspected congenital syphilis (CS).

Methods: Infants admitted to the neonatal department of Children's Hospital of Fudan University from 2013 to 2016 who met the case definition of CS or suspected CS were included in this study. Follow-up was performed in an outpatient clinic until reversion to non-reactivity of both toluidine red unheated serum test (TRUST) and Treponemal pallidum particle agglutination (TPPA). Follow-up data were collected until up to the end of 2019, when the last infant with CS reached 3 years of age.

Results: In total, 682 infants were enrolled in this study, including 63 in the CS group and 619 in the suspected CS group. Forty-seven infants (74.6%) in the CS group had symptoms, and 57 (90.5%) had abnormal laboratory and/or long bone X-ray findings. By 6 months of age, TRUST results were negative in 53.3% of the infants with CS and in 100% of the infants with suspected CS. All the infants in the CS group returned to TRUST non-reactivity by 18 months of age. The TPPA results at 18 months of age showed that only 10.0% (3/30) of the patients in the CS group returned to non-reactivity, while a 99.6% (548/550) non-reactivity rate was observed in the suspected CS group. All the infants in the CS group returned to 19S-IgM-TPPA non-reactivity by 6 months of age.

Conclusions: Although CS is an burdensome disease that may cause fetal and neonatal death, CS responds well to treatment when diagnosed and treated promptly, even when symptoms or lab/X-ray findings are present at birth.

Keywords: syphilis, congenital, *Treponema pallidum*, infant, newborn, syphilis serodiagnosis, prognosis

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *26 October 2021*
Accepted: *07 January 2022*
Published: *23 February 2022*

Citation:
Dai Y, Zhai G, Zhang S, Chen C, Li Z and Shi W (2022) The Clinical Characteristics and Serological Outcomes of Infants With Confirmed or Suspected Congenital Syphilis in Shanghai, China: A Hospital-Based Study. Front. Pediatr. 10:802071. doi: 10.3389/fped.2022.802071

INTRODUCTION

Congenital syphilis (CS) is the infection of an infant or fetus with *Treponema pallidum,* and this infection is acquired during pregnancy from a mother with untreated or inadequately treated syphilis (1). CS can cause miscarriage, stillbirth, or infant death (2–4), and can also cause severe birth defects, including bone deformity, severe anemia, and nerve problems, including blindness or deafness (5). CS can be effectively prevented by universal syphilis screening at an early stage of pregnancy and treatment of those infected with penicillin (6–8), but the number of reported CS cases in China has increased nearly 25-fold, from 468 in 2000 to 12,042 in 2011. Large proportions of women with infections are not diagnosed and treated at early stages of pregnancy, and this may contribute to the increasing incidence of CS. Data from China's Information System of Prevention of Mother-to-Child Transmission of Syphilis Management showed that 79.1% of syphilis-infected women in China received antenatal care at or before 37 weeks of gestation in 2013; however, 55.4% of syphilis-infected women received no treatment or initiated treatment after 37 weeks of gestation (9). CS has become a public health problem in China, and its impact on children's health has raised widespread concern. In 2010, China issued the 2010–2020 Plan for Syphilis Control and Prevention to reach the goal of reducing the incidence of the mother-to-child transmission (MTCT) of syphilis to below 15 cases per 100,000 live births by 2020 (10). The release of the National Implementation Guidelines on preventing mother-to-child transmission of HIV, Syphilis, and Hepatitis B Programme in 2011 (11, 12), which was revised in 2015 and 2020 (13, 14), indicated the beginning of the commitment by the national government to eliminate the MTCT of CS. Between 2011 and 2018, the incidence of CS was significantly reduced from 91.6 cases per 100,000 live births to 18.4 cases per 100,000 (15).

The majority of infants with CS may appear normal and have no clinical or laboratory evidence of infection at birth; however, these infants may develop symptoms of disease months to years later if left untreated. The diagnosis of CS is established by the observation of spirochetes in body fluids or tissue and suggested by serologic test results. *T. pallidum* may be identified by dark field microscopy, polymerase chain reaction (PCR) testing, and fluorescent antibody or silver staining of mucocutaneous lesions, nasal discharge, vesicular fluid, amniotic fluid, placenta, umbilical cord, or tissue obtained at autopsy (16). The interpretation of reactive serological tests of the infant may be complicated by the passive transfer of maternal non-treponemal and treponemal IgG antibodies through the placenta to the fetus. Therefore, the diagnosis and management of CS is complex and requires the determination of the maternal stage of infection, adequacy of maternal treatment, and maternal response to treatment. According to Chinese guideline, all infants born to seropositive mothers require complete evaluation. Infants with confirmed CS are referred to specific health institutions for adequate treatment and long-term follow-up. Those who cannot be diagnosed of confirmed CS at birth should receive preventative treatment and are also followed up until 18 months of age when the diagnosis of CS is either confirmed or ruled out (13). **Figure 1** showed the 2015 national guideline of algorithm for evaluation, treatment and follow-up of infants born to mothers with reactive serologic tests for syphilis (13).

Many studies on CS have been performed and have played an essential role in the implementation of strategies for its prevention. More recently, however, few published reports have focused on the clinical aspects of the disease. In this study, infants with confirmed or suspected CS who were admitted to Children's Hospital of Fudan University from January 2013 to December 2016 were enrolled. Serological follow-up was performed from birth as per national guidelines. The clinical characteristics and outcomes of serological reversion were compared between the confirmed CS group and the suspected CS group.

METHODS

Patient Cohort

The study was conducted at Children's Hospital of Fudan University, a comprehensive tertiary pediatric hospital that combines medical care, teaching and research. It is located in Shanghai, one of the largest cities in China, with a total population of more than 30 million residents. Children's Hospital of Fudan University is one of the three designated medical centers for the management of CS in Shanghai and has admitted more than 95% of infants born in Shanghai to mothers with seroreactive syphilis. Infants admitted to the neonatal department of Children's Hospital of Fudan University from January 2013 to December 2016 who met the case definition of CS or suspected CS were included in the study. Data on newborn infants were collected throughout their stay in the neonatal ward. Data on infants' mothers, including antenatal screens and treatment, were collected according to the referral form from the maternal hospital. This study was approved by the Ethics Committee of the Children's Hospital of Fudan University and conducted in agreement with the ethical principles in the Declaration of Helsinki [No. 2019(241)].

Serum samples were collected immediately after admission and examined with the following tests: *Treponemal pallidum* particle agglutination (TPPA; Fujirebio Inc, Tokyo, Japan), 19S-IgM-TPPA (Euroimmun Medizinische Labordiagnostika AG, Lubeck, Germany), toluidine red unheated serum test (TRUST; Shanghai Rongsheng BioTech Co, Ltd, Shanghai, China), and TRUST titer if it was positive. For the purpose of comparison, mothers' serum samples were also collected at infant admission and examined with the TPPA and TRUST tests.

Diagnosis and Treatment of Confirmed CS and Suspected CS

According to the national guidelines (13, 17), any neonate born to mothers who have reactive serologic test for syphilis during pregnancy as having confirmed CS if they met any one of the following laboratory criteria: (1) a positive darkfield test or PCR of lesions or body fluid; or (2) 19S-IgM-TPPA reactivity; or (3) a serum quantitative non-treponemal serologic titer that was 4-fold higher than the mother's titer. As demonstration of *Treponemal pallidum* by dark field microscopy or PCR were not available in

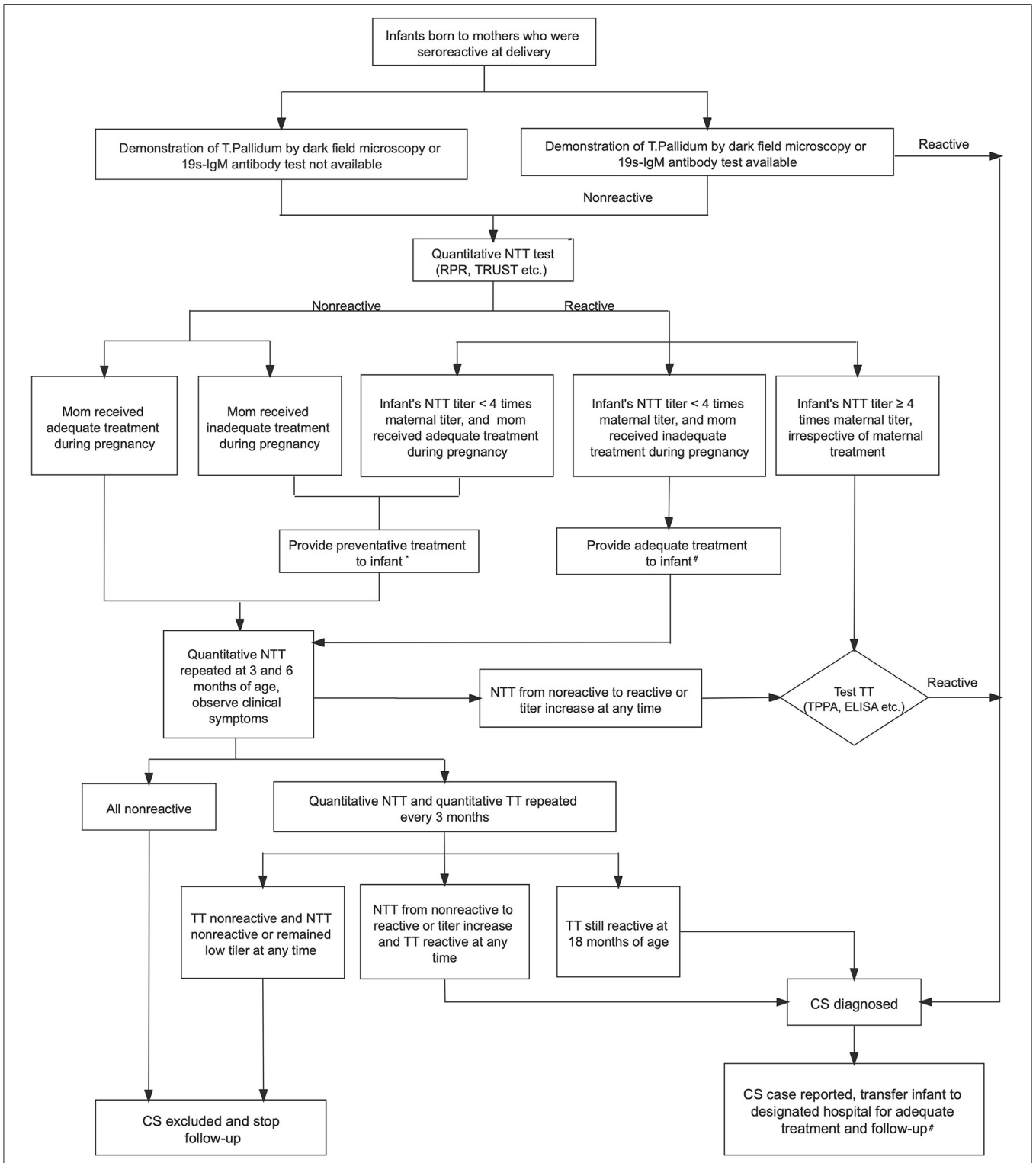

FIGURE 1 | Algorithm for evaluation, treatment and follow-up of infants born to mothers with reactive serologic tests for syphilis in China (13). NTT, non-treponemal test; RPR, rapid plasma regain; TRUST, toluidine red unheated serum test; TT, treponemal test; TPPA, treponemal pallidum particle agglutination; ELISA, enzyme-linked immuno-sorbent assay; CS, congenital syphilis; CSF, cerebrospinal fluid. *Preventative treatment for patients with suspected CS: Benzathine penicillin G 50,000 U/kg IM (single dose). #Adequate treatment for patients with confirmed CS: (1) First-line therapy option: Aqueous penicillin G 50,000 U/kg IV q 12 h (≤1 wk of age), q 8 h (>1 wk), or procaine penicillin G 50,000 U/kg IM daily for 10–14 days. (2) Second-line therapy option (only if CSF is normal): Benzathine penicillin G 50,000 U/kg IM (single dose). (3) Infants should be treated as CSF abnormal if CSF examination unavailable.

our hospital, we defined infants as having confirmed CS if they met above criteria (2) or (3).

Infants born to mothers who had received adequate treatment but were TRUST positive or infants born to mothers who had not received treatment or had received inadequate treatment require preventative treatment with a single dose of intramuscular benzathine penicillin according to the national guidelines. Therefore, we defined these infants who are eligible to receive preventative treatment for syphilis as cases of suspected CS. We defined mothers as having received inadequate treatment if the mother had (1) treatment with a non-penicillin regimen; (2) adequate treatment but non-treponemal antibody titers increased or did not decrease at least 4-fold, or there was insufficient serological follow-up; (3) treatment administered <30 days before delivery; and (4) undocumented treatment.

Infants with non-reactive TRUST results and mothers who received adequate treatment during pregnancy were unlikely to have CS. We administered preventative treatment to these infants and provided follow-up for up to 6 months according to national guidelines, but these infants were not included in this study.

All infants born to syphilis-seropositive pregnant women were evaluated for clinical evidence (e.g., skin rash, hepatosplenomegaly, cholestatic jaundice), laboratory abnormalities [e.g., elevated liver transaminase, elevated conjugated bilirubin, anemia, thrombocytopenia, leukocytosis, elevated C reactive protein (CRP), proteinuria]. Long bone radiographs were reviewed by pediatric radiologists and determined to be consistent with CS if they demonstrated changes of osteochondritis or perichondritis (17). Cerebrospinal fluid (CSF) examination was performed for infants with confirmed CS and was considered abnormal if CSF white blood cell counts was > 25/ml, CSF protein was > 400 mg/dL and/or CSF Venereal Disease Research Laboratory (VDRL) was reactive (17).

According to national guidelines, infants with confirmed CS should receive a single dose of intramuscular benzathine penicillin if the CSF examination results are normal; otherwise, infants should receive intravenous aqueous penicillin G or procaine penicillin G intramuscular injection for 10–14 days. Infants with suspected CS should receive preventative treatment with a single dose of intramuscular benzathine penicillin. Intramuscular benzathine penicillin was not available in our hospital until 2017; therefore, both confirmed and suspected CS patients were treated with aqueous penicillin G during this study period. Infants with confirmed CS were treated for 14 days, while those with suspected CS were treated for 7 days.

Follow-Up at the Outpatient Clinic

All the infants discharged after treatment were referred for follow-up at the neonatal clinic of Children's Hospital of Fudan University at 2, 4, 6, 9, 12, 18, and 24 months of age and then yearly until reversion to non-reactivity of both TRUST and TPPA. Follow-up data were also retrospectively collected until the end of 2019, when the last CS infant reached 3 years of age.

Statistical Analysis

The data are presented as percentages or means (standard deviation, SD) when applicable. Independent-sample *t*-tests were used to compare the continuous parametric variables between the two groups. A continuous-calibration chi-square test was used to compare the rate between the two groups. $P < 0.05$ were considered statistically significant. Statistical analysis was conducted by using SPSS version 20.0 (SPSS Inc.).

RESULTS

Characteristics of the Patients

Between January 2013 and December 2016, 1,190 infants born to mothers who had seropositive syphilis were admitted to the neonatal department of Children's Hospital of Fudan University for further evaluation and treatment. Among these patients, 508 infants born to mothers with adequate treatment and with non-reactive TRUST were excluded from this study. Totally 682 infants were included in this study. Of these, 63 infants met the criteria for case definition of CS (including 56 cases had positive 19S-IgM-TPPA, and seven had both positive 19S-IgM-TPPA and 4-fold higher TRUST titers than that of the mother); this group was termed the CS group. Twenty-three infants in the CS group, whose mothers were not tested for syphilis, were admitted for different reasons, including prematurity, respiratory distress, skin rash and jaundice, at a median day of life 5. The other 619 infants met the criteria for case definition of suspected CS; this group was termed the suspected CS group. The flow chart of the study infants is shown in **Figure 2**.

The proportion of women who received adequate treatment between the two groups was extremely low, with 7.8% infants in the suspected CS group and none in the CS group whose mothers received adequate treatment during pregnancy. 52 out of 63 (82.5%) infants in the CS group were born to mothers who did not receive any treatment. Comparison between the CS group and suspected CS group showed that there were significantly more infants with gestational age <37 weeks, the birth weights were significantly lower, and there were more infants characterized as being small for gestational age (SGA) among the infants in the CS group. There was no sex difference between the two groups. The TRUST titers of the suspected CS group were all moderate and low (≤1:16), whereas 82.5% of the CS group had high titers (≥1:32).

Basic characteristics of confirmed CS and suspected CS cases and their mothers are shown in **Table 1**.

None of the infants in the suspected CS group died. After a 7-day course of aqueous penicillin treatment, 550 of 619 (90.0%) infants completed the follow-up.

In the CS group, one infant died of CS-related multiple organ failure on the first day of life, and two infants died of pneumonia in infancy after discharge. Of the other 60 infants who survived to discharge and received a 14-day course of aqueous penicillin treatment, 30 infants were lost to follow-up, and the other 30 infants were followed up for at least 18 months.

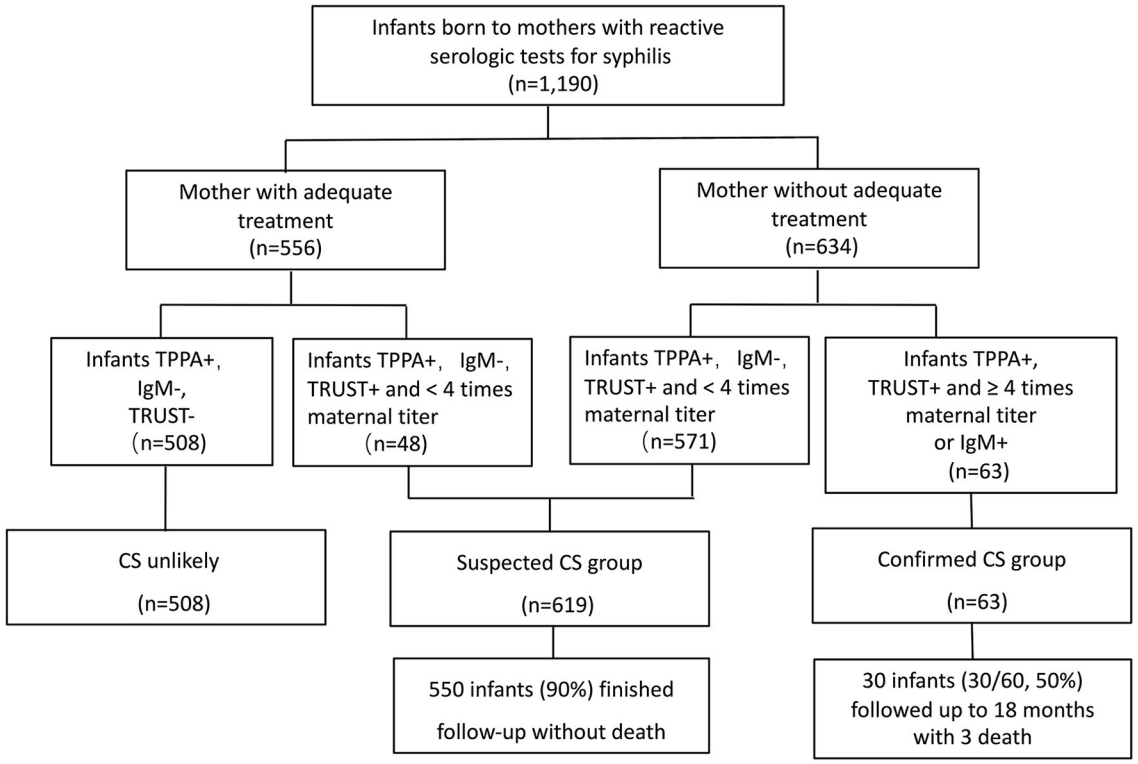

FIGURE 2 | The flow chart for study groups. TPPA, treponemal pallidum particle agglutination; TRUST, toluidine red unheated serum test; CS, congenital syphilis.

Clinical Features in Infants Diagnosed With Confirmed CS in the Neonatal Period

Table 2 showed the clinical picture of 63 infants with confirmed CS. Among the 63 infants with CS, the percentages of prematurity, low birth weight and SGA were 61.9%, 52.4%, and 19.0%, respectively. Forty-seven infants (74.6%) in the CS group had symptoms at birth or within the first 28 days of life, and these symptoms are listed in **Table 3**. The most common symptoms were cholestatic jaundice and hepatomegaly, followed by splenomegaly and cutaneous lesions, all accounting for more than 50% of the total cases. Cutaneous lesions were the earliest clinical symptoms, with 22 (88.0%) of the 25 patients presenting at birth and the other 3 patients presenting at day of life 3, 7, and 25. Cutaneous lesions showed a variety of manifestations, but most patients with these lesions (21 patients, 84%) had characteristic pemphigoid lesions, others had erythema or red maculopapules, and one had eczema-like skin lesions. Symptoms with a 25–50% probability of occurrence were pneumonia, hepatitis, and gastrointestinal symptoms (including abdominal distension in 6 patients, hematochezia in 1 patient, and necrotizing enterocolitis $\geq$ stage 2 in 5 patients). Among the 23 patients diagnosed with pneumonia, 6 required invasive mechanical ventilation, 7 required non-invasive mechanical ventilation, 8 required nasal catheter oxygen inhalation, and 2 did not require any respiratory support. Rare symptoms include fever, petechia, nephrotic syndrome, pseudoparalysis (failure to move an extremity secondary to pain), rhinitis, vitreous opacity, and chorioretinitis, all of which occurred in fewer than 10% of the patients.

Abnormal laboratory and/or X-ray findings were identified in 57 out of 63 (90.5%) patients in the CS group. Long bone abnormalities, including lucent bands under the provisional calcification zone of the long bone metaphysis ($n = 9$), irregular metaphysis with serrated appearance ($n = 37$), and periosteal reaction ($n = 2$), occurred in 48 patients. There was one patient with severe metaphyseal osseous destruction accompanied by pathological fracture of the distal left femur. The infant showed signs of pseudoparalysis. Hematological abnormalities were detected in 44 of the 63 infants (69.8%); among them, 39 infants had leukocytosis with a median white blood cell (WBC) of 26.3×10^9 cells/L (range $12.11 \sim 56.3 \times 10^9$ cells/L), one had leukopenia with WBC count of 3.5×10^9 cells/L, 27 had thrombocytopenia with a median platelet count of 66×10^9 cells/L (range $27 \sim 138 \times 10^9$ cells/L) and 20 had anemia with a median hemoglobin level of 118.5 g/L (range $80 \sim 137$ g/L). CRP was increased in 32 of 63 infants with a median value of 85.5 mg/L (range $15 \sim$ maximum, normal range <8 mg/L, maximum 160 mg/L). CSF abnormalities were observed in 31.6% (18/57) of infants, and proteinuria was detected in 4 infants. Abnormal lab or long bone X-ray present at neonatal period in 63 infants with confirmed CS are also described in **Table 3**.

TABLE 1 | Basic characteristics of confirmed CS and suspected CS cases.

Basic characteristics	Suspected CS group ($n = 619$)	CS group ($n = 63$)	*P*
Maternal syphilis management	48 (7.8)	0 (0)	0.042
Adequate treatment, *n* (%)			
No treatment, *n* (%)	189 (30.5)	52 (82.5)	0.000
Inadequate treatment, *n* (%)	382 (61.7)	11 (17.5)	0.000
Non-penicillin treatment, *n* (%)	129 (20.8)	2 (3.2)	
Non-treponemal test did not decreased Fourfold or increased or lack follow-up, *n* (%)	203 (32.8)	3 (4.8)	
Treatment within 30 days before delivery, *n* (%)	13 (2.1)	3 (4.8)	
Non-documented treatment, *n* (%)	37 (6.0)	3 (4.8)	
Characteristics of infants	335 (54.1)	38 (60.3)	0.346
Male sex, *n* (%)			
GA, mean (SD), wk	39.0 (1.6)	35.6 (3.2)	0.000
GA < 37 wk, *n* (%)	46 (7.4)	39 (61.9)	0.000
GA 34^0-36^6 wk, *n* (%)	38 (6.1)	22 (34.9)	0.000
GA 32^0-33^6 wk, *n* (%)	6 (1.0)	9 (14.3)	0.000
GA <32 wk, *n* (%)	2 (0.3)	8 (12.7)	0.000
BW, mean (SD), g	3,337.2 (517.7)	2,443.9 (620.0)	0.000
BW <2,500 g, *n* (%)	31 (5.0)	33 (52.4)	0.000
BW 1,500–2,499 g, *n* (%)	31 (5.0)	28 (44.4)	0.000
BW <1,500 g, *n* (%)	0 (0)	5 (7.9)	0.000
SGA, *n* (%)	28 (4.5)	12 (19.0)	0.000
TRUST non-reactive, *n* (%)	213 (34.4)	0 (0)	0.000
TRUST ≤ 1:4, *n* (%)	368 (59.5)	4 (6.3)	0.000
TRUST 1:8 or 1:16, *n* (%)	38 (6.1)	7 (11.1)	0.212
TRUST ≥ 1:32, *n* (%)	0 (0)	52 (82.5)	0.000

CS, congenital syphilis; GA, gestational age; BW,birth weight; SGA, small for gestational age; TRUST, toluidine red unheated serum test.

Serology Follow-Up of Two Groups

Serum TRUST result were positive at birth in 100.0% (63/63) of the infants in the CS group and 65.6% (406/619) of the infants in the suspected CS group. There were no cases of serum TRUST results changing from negative to positive or the titer increasing during follow-up. Posttreatment TRUST reversion to non-reactivity was documented in 30 patients in the CS group and 382 patients in the suspected CS group. Posttreatment TRUST reversion tended to occur earlier in infants without CS. By 6 months of age, TRUST results were negative in 53.3% of the infants with CS and in 100% of the infants in the suspected CS group. All the infants in the CS group returned to TRUST non-reactivity by 18 months of age. The cumulative percentage of reversion to TRUST non-reactivity in two groups is shown in **Figure 3**.

In the suspected CS group, 548 out of 550 (99.6%) infants showed TPPA non-reactivity before 18 months of age. These infants were then excluded from the diagnosis of CS and follow-up was discontinued. The other 2 out of 550 (0.4%) infants had positive TPPA results after 18 months of age, and both were reported as confirmed CS cases at this point according to national guidelines.

The TPPA results at 18 months of age showed that only 10.0% (3/30) of the patients in the CS group returned to non-reactivity. We were able to identify the age of reversion to non-reactivity in the other 3 infants in the CS group. TPPA non-reactivity occurred at 3, 5, and 6 years of age in these 3 infants. The cumulative percentage of reversion to TPPA non-reactivity in two groups is shown in **Figure 4**.

Posttreatment 19S-IgM-TPPA reversion to non-reactivity occurred at 2 and 4 months of age in 16 (53.3%) and 12 (40.0%) infants, respectively. All the infants returned to 19S-IgM-TPPA non-reactivity by 6 months of age.

DISCUSSION

Much has been written about CS, but in recent years, only a few studies have addressed its clinical course. This study, in addition to presenting the clinical characteristics, presents a unique comparison of serology reversion between a group of newborn infants meeting the standard case definition of CS and a group of infants with suspected CS.

Definitive diagnosis of CS is made when spirochetes are identified by darkfield microscopy, fluorescent antibody, or other specific stains in specimens from body fluids or tissue, however, most clinical settings lack the capacity to perform direct detection (18). A survey in Latin America and the Caribbean found that

TABLE 2 | The clinical pictures of 63 infants with confirmed CS.

Patient number	Sex	BW, g	GA, wk	Admission age	Clinical pictures	Long bone X-ray*	CSF#
1	M	1,570	31	First day	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions, GI manifestations, hepatitis	Abnormal	Normal
2	M	1,400	32	4 d	Cholestatic jaundice, pneumonitis, GI manifestations	Abnormal	CSF: WBC 47 × 10^9 /L
3	M	3,400	38	First day	Asymptomatic	Normal	Normal
4	M	3,200	38	First day	Cholestatic jaundice, cutaneous lesions, hepatitis	Abnormal	Normal
5	F	2,275	37	First day	Asymptomatic	Abnormal	Normal
6	M	2,500	38	First day	Asymptomatic	Normal	Normal
7	F	2,990	35	12 d	Cholestatic jaundice, hepatosplenomegaly, pneumonitis, hepatitis	Abnormal	Normal
8	M	2,700	38	6 d	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions, GI manifestations, hepatitis	Abnormal	Normal
9	F	1,700	32	First day	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions, pneumonitis, fever	Abnormal	NA
10	M	1,800	36	5 d	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions	Abnormal	Normal
11	M	2,900	38	First day	Asymptomatic	Abnormal	Normal
12	F	3,000	39	9 d	Cutaneous lesions	Abnormal	Normal
13	M	1,780	33	First day	Hepatosplenomegaly, cutaneous lesions, pneumonitis, GI manifestations	NA	NA
14	M	2,000	31	First day	Cutaneous lesions, pneumonitis	NA	NA
15	F	3,080	39	First day	Cutaneous lesions	Abnormal	CSF VDRL+
16	F	1,900	35	9 d	Hepatosplenomegaly	NA	Normal
17	M	2,650	35	First day	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions, pneumonitis	Abnormal	Normal
18	F	2,220	37	First day	Cutaneous lesions	Abnormal	CSF VDRL+
19	M	2,415	33	First day	Cholestatic jaundice, hepatomegaly, pneumonitis, petechiae	Abnormal	Normal
20	F	3,050	38	First day	Asymptomatic	Abnormal	Normal
21	M	2,100	34	4 d	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions, GI manifestations, hepatitis	Abnormal	Normal
22	F	2,100	34	First day	Hepatosplenomegaly	Abnormal	Normal
23	F	1,300	27	First day	Asymptomatic	Abnormal	CSF VDRL+
24	M	3,500	38	28 d	Cholestatic jaundice, hepatitis	Abnormal	Normal
25	F	2,100	32	First day	Cutaneous lesions, GI manifestations	Abnormal	Normal
26	M	3,250	38	28 d	Cutaneous lesions, fever	Abnormal	Normal
27	F	1,545	34	First day	Cutaneous lesions	Abnormal	CSF VDRL+
28	M	2,475	39	First day	Asymptomatic	Normal	Normal
29	M	1,830	35	First day	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions, hepatitis	Abnormal	CSF VDRL+
30	M	2,500	34	First day	Cholestatic jaundice, hepatosplenomegaly, hepatitis	Abnormal	Normal
31	M	1,980	29	First day	Cholestatic jaundice, hepatosplenomegaly, pneumonitis	Abnormal	Normal
32	F	3,420	40	First day	Asymptomatic	Abnormal	Normal
33	M	2,385	36	First day	Asymptomatic	Normal	CSF VDRL+
34	F	3,000	36	First day	Cutaneous lesions, pneumonitis	Abnormal	Normal
35	M	3,250	36	First day	Asymptomatic	Normal	CSF VDRL+
36	M	3,700	40	First day	Asymptomatic	Normal	Normal
37	F	2,500	35	3 d	Hepatomegaly, GI manifestations	Normal	Normal
38	F	2,590	38	First day	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions, pneumonitis, hepatitis	Abnormal	CSF VDRL+
39	M	2,440	34	First day	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions	Abnormal	Normal
40	F	3,300	40	First day	Asymptomatic	Normal	Normal
41	M	1,900	30	10 d	Cholestatic jaundice, rhinitis, vitreous opacity	Abnormal	CSF:WBC132 × 10^9/L, protein 2,919 mg/L

(Continued)

TABLE 2 | Continued

Patient number	Sex	BW, g	GA, wk	Admission age	Clinical pictures	Long bone X-ray*	CSF#
42	F	2,480	37	First day	Hepatosplenomegaly, pneumonitis, GI manifestations	NA	NA
43	M	2,700	39	First day	Asymptomatic	Abnormal	Normal
44	M	2,000	34	First day	Asymptomatic	Abnormal	Normal
45	M	2,050	31	First day	Cholestatic jaundice, hepatitis	Abnormal	CSF VDRL+
46	F	2,200	35	22 d	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions, pneumonitis, GI manifestations, hepatitis	Abnormal	CSF VDRL+
47	F	2,000	33	28 d	Cholestatic jaundice, hepatosplenomegaly, hepatitis	Abnormal	Normal
48	F	1,520	32	First day	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions, pneumonitis, GI manifestations, fever, chorioretinitis	Abnormal	NA
49	M	2,550	36	First day	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions, pneumonitis, petechiae, nephrotic syndrome	Abnormal	CSF VDRL+
50	F	2,820	36	First day	Cholestatic jaundice, hepatosplenomegaly, GI manifestations, hepatitis	Abnormal	CSF VDRL+
51	M	2,650	35	5 d	Cholestatic jaundice, pneumonitis, pseudoparalysis	Abnormal	CSF VDRL+
52	M	3,560	39	First day	Asymptomatic	Abnormal	Normal
53	M	1,440	32	27 d	Cholestatic jaundice, hepatosplenomegaly, hepatitis	Abnormal	Normal
54	F	3,500	41	First day	Asymptomatic	Normal	Normal
55	F	2,200	36	First day	Cholestatic jaundice, hepatosplenomegaly, cutaneous lesions, pneumonitis, hepatitis	Abnormal	Normal
56	M	2,430	34	First day	Cholestatic jaundice, hepatomegaly, cutaneous lesions, pneumonitis, hepatitis	Abnormal	Normal
57	F	2,460	36	First day	Asymptomatic	Abnormal	Normal
58	M	1,520	28	First day	Cholestatic jaundice, hepatosplenomegaly, pneumonitis	Abnormal	CSF VDRL+
59	M	2,800	37	First day	Pneumonitis	NA	Normal
60	M	2,085	32	First day	Hepatomegaly, pneumonitis	NA	NA
61	M	2,500	38	6 d	Cholestatic jaundice, pneumonitis, hepatitis, nephrotic syndrome	Abnormal	CSF VDRL+
62	M	1,420	30	First day	Hepatosplenomegaly, cutaneous lesions, pneumonitis, GI manifestations	Abnormal	CSF:WBC42 × 10^9/L, VDRL+
63	M	2,800	38	First day	Cholestatic jaundice, hepatomegaly, hepatitis	Abnormal	Normal

BW, birth weight; GA, gestational age; CSF, cerebrospinal fluid; GI, gastrointestinal; NA, non-applicable; VDRL, venereal disease research laboratory; WBC, white blood cell; F, female; M, male.

**Among the 63 patients, 57 received long bone X-ray. Patient number 13, 14, 16, 42, 59 refused long bone X-ray. Patient number 60 was too severe to complete the long bone X-ray and died on the first day of life.*

#Among the 63 patients, 57 received CSF examination. Patient number 9, 13, 14, 42, 48 refused CSF examination. Patient number 60 was too severe to complete the CSF test and died on the first day of life.

only two of 69 national reference and large clinical laboratories facilities still performed darkfield or direct fluorescent antibody staining for *T. pallidum* (19). PCR techniques are increasingly used; however, there is as yet no commercially available or internationally approved test for *T. pallidum* (20). Instead, most clinical laboratories utilize serological testing to infer a diagnosis of CS. Because maternal non-treponemal and treponemal IgG antibodies can be transferred from mother to infant, treponemal testing of infant serum is difficult to interpret and is not recommended (21). The finding of an infant's serum quantitative non-treponemal titer that is 4-fold higher than the maternal titer is confirmatory for CS. But the absence of such finding does not exclude the diagnosis as most infants with CS have titers that are equal to or less than the maternal titer. As anti-treponemal IgM does not cross the placenta, it is suggestive of congenital infection if detected in serum of neonatal infants. However, the use of anti-treponemal IgM is still controversial owing to limited availability of tests and inconclusive data thus far on sensitivity; their use in diagnosing CS is recommended in European (20), WHO (22), and Chinese guidelines (13), but not US Centers for Disease Control and Prevention (CDC) (21). Due to unavailability of direct detection techniques in our hospital, the confirmed CS cases were all detected by serology results. Of the 63 confirmed CS cases, 56 had positive anti-treponemal IgM, and the other 7 cases had both positive anti-treponemal IgM and 4-fold higher TRUST titers than that of the mother.

Physicians should be aware of the diverse clinical features of CS and be highly aware of CS so that a correct diagnosis can be made and treatment can be initiated early. In this study, compared to the infants who had suspected CS, infants diagnosed with confirmed CS in the neonatal period exhibited more preterm births, lower birth weights and more SGA infants. In a cross-sectional study conducted in ten maternity hospitals in Brazil, Araújo et al. found the outcome of prematurity in 15.3% of the reported cases of CS, and the non-treatment of the pregnant women or treatment with drugs other than

TABLE 3 | Symptoms and abnormal lab or long bone X-ray presented at neonatal period in 63 infants with confirmed CS.

Symptom	$n = 47$ (%*)	Abnormal lab or long bone X-ray	$n = 63$ (%#)
Cholestatic jaundice	30 (63.8)	Abnormal long bone X-ray§	48 (84.2)
Hepatomegaly	30 (63.8)	Hematologic abnormalities	44 (69.8)
Splenomegaly	25 (53.2)	Thrombocytopenia	27 (42.9)
Cutaneous lesions	25 (53.2)	Anemia	20 (31.7)
Pneumonitis	23 (48.9)	Leukocytosis	39 (61.9)
Hepatitis	18 (38.3)	Leukopenia	1 (1.6)
Gastrointestinal manifestations	12 (25.5)	Elevated CRP	32 (50.8)
Fever	3 (6.4)	Abnormal CSF	18 (31.6)
Petechiae	2 (4.3)	Reactive VDRL	16 (28.1)
Nephrotic syndrome	2 (4.3)	Elevated WBC count or protein	3 (5.3)
Pseudoparalysis	1 (2.1)	Proteinuria	4 (6.3)
Rhinitis	1 (2.1)		
Vitreous opacity	1 (2.1)		
Chorioretinitis	1 (2.1)		

CRP, C reactive protein; CSF, cerebrospinal fluid; VDRL, venereal disease research laboratory; WBC, white blood cell.

**Percentages calculated on the 47 symptomatic patients. Numbers do not reflect the frequency of symptoms among all infants with CS.*

#Percentages calculated on the 63 patients with CS.

§Among the 63 children, 57 received long bone X-ray and CSF examination. Parents of the other 5 patients refused to be examined; One death was too severe to complete the long bone X-ray and CSF test. Percentages calculated on the 57 patients with long bone X-ray and CSF results. Abnormal X-ray was defined as osteochondritis or perichondritis of long bone.

Thrombocytopenia: platelet count $< 150 \times 10^9$ /L; Anemia: hemoglobin < 145 g/L; Leukocytosis: WBC count $>10 \times 10^9$ /L; Leukopenia: WBC count $< 4 \times 10^9$ /L; Elevated CRP: CRP > 8 mg/L; Abnormal CSF: leukocytes > 25/ml or protein > 400 mg/L in cerebrospinal fluid or VDRL reactive. Proteinuria: urine protein > 30 mg/dl for urinalysis.

penicillin during prenatal care (OR 3.52; 95%CI: 1.74–7.13; $p < 0.001$) were associated with higher chances of prematurity (23). In our study, more than half infants with CS were born premature, and none of the mothers received adequate treatment during pregnancy.

The 63 patients with CS had a variety of clinical manifestations, ranging from asymptomatic and without laboratory abnormalities to varying degrees of symptoms. In a serious case, the patient died of CS-related multiple organ failure in the early postnatal period, and the opportunity for treatment was lost. Similar to previous reports, the most common symptoms were cholestatic jaundice, hepatosplenomegaly, and skin lesions, all present in more than 50% of patients. Typical skin lesions, often present at birth, are frequently the indication that leads to clinical suspicion of CS. CS could also present as an atypical rash. For example, in this study, a case of an eczema-like rash commonly seen in infancy was misdiagnosed as eczema, and the diagnosis of CS was delayed until 2 weeks of life. Leung et al. reported a 2-week-old male infant with CS whose cutaneous manifestations included diffuse, erythematous keratoderma with desquamation and fissures on his hands and feet, multiple linear scaly fissures at the corners of his mouth, and onychauxis of his fingernails and toe nails, which have not been previously reported in CS (24). This suggests that for children born with rash, especially in premature babies with low birth weight or SGA, regardless of the mother's history or treatment history, the possibility of CS should be carefully considered.

Similar to previous reports, long bone abnormalities were the most common symptom and were detected in 48 out of 57 (84.2%) CS infants who had conclusive X-ray evaluation; additionally, these long bone abnormalities were most common in infants with symptomatic CS. In 6 out of 16 infants with asymptomatic CS, long bone abnormalities were the only manifestations. Although common, severe bone destructions are rare reported, and often misdiagnosed as non-accidental trauma (25–28). Only one patient in our study showed severe metaphyseal osseous destruction accompanied by fracture; this infant showed signs of pseudoparalysis. Most long bone lesions are asymptomatic and require X-ray detection. Since long bone damage is relatively specific to CS and is often used to distinguish postnatal acquired syphilis, long bone X-rays are helpful in the diagnosis of CS in patients suspected of having CS.

Hematological abnormalities, including anemia, thrombocytopenia, leukopenia, or leukocytosis, are non-specific and need to be differentiated from other causes. Tiffany Lee reported a 5-week-old male CS infant who presented with fever and a complete blood count (CBC) image suggesting malignant disease (e.g., severe leucoerythroblastic anemia with hemoglobin 1.9 g/dL, leukocytosis with WBC 53.7×10^9 cells/L) (29). Thus, it is not surprising that CS is often misdiagnosed as congenital leukemia at first. Our study also found that a significant proportion of infants with CS had elevated CRP levels, with a median value of 85.5 mg/L, which was rarely reported in other studies. This suggests that in cases of unexplained CRP elevation, where there is no evidence of bacterial infection, the possibility of CS should be carefully considered.

Congenital neurosyphilis often results in significant neurologic morbidity in infants and children. Early identification and implementation of treatment are important in improving developmental outcomes and quality of life (30). In our study, CSF abnormalities, including reactive VDRL or elevated WBC count or protein levels, were detected in 31.6% (18/57) of infants with CS, while none of these infants had neurological symptoms. The diagnosis of congenital neurosyphilis is difficult to establish since the majority of infants with CS do not manifest any abnormalities on neurologic examination (31). Currently, central nervous system invasion by *T. pallidum* is usually inferred from CSF abnormalities, such as reactive VDRL, pleocytosis, and elevated protein levels (32). Although a positive VDRL in CSF is considered specific for neurosyphilis, it has limited sensitivity (33–35). Moreover, a reactive CSF VDRL in neonates may be caused by passive transfer of non-treponemal IgG antibodies from serum into the CSF. By using rabbit infectivity testing of the CSF to detect *T. pallidum* infection of the central nervous system in infants born to mothers with syphilis, Michelow et al. found that invasion of the central nervous system by *T.*

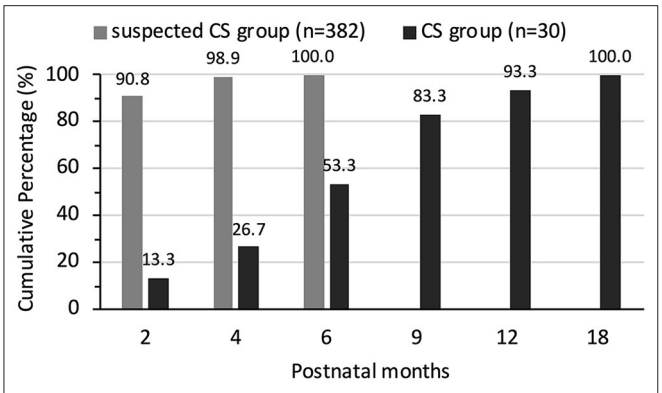

FIGURE 3 | The cumulative percentage of reversion to TRUST non-reactivity in suspected CS group and CS group.

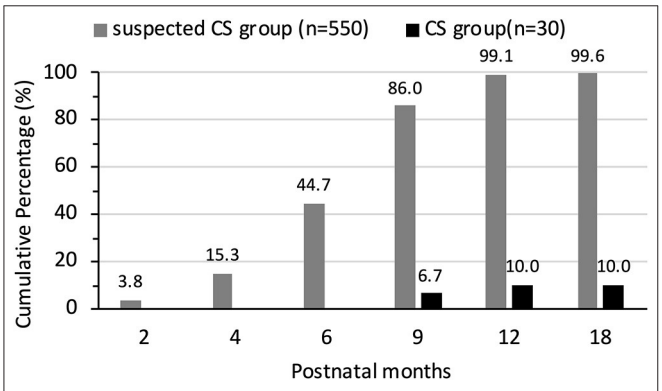

FIGURE 4 | The cumulative percentage of reversion to TPPA non-reactivity in suspected CS group and CS group.

pallidum occurs in 41% of infants who have clinical, laboratory, or radiographic abnormalities consistent with CS and in 60% of those who have an abnormal physical examination consistent with the diagnosis of CS; these findings indicate that central nervous system involvement is common among infants infected with syphilis (36). Therefore, once clinical, laboratory, or radiographic evaluation supports a diagnosis of congenital syphilis, therapy that is effective against central nervous system disease is warranted regardless of the results of CSF analyses (16).

In our study, the patients were divided into confirmed CS group and suspected CS group in accordance with national guideline, which were similar to the US CDC case definition of Scenario 1 (Proven or highly probable CS) and Scenario 2 (Possible CS), respectively (21). However, in terms of treatment, there are some differences between the national guideline and US CDC guideline. According to national guideline, infants with confirmed CS could receive a single dose of intramuscular benzathine penicillin if the CSF examination results are normal. While the US CDC recommended more enhanced treatment with either aqueous penicillin G or procaine penicillin G for 10 days, for infants with proven or highly probable CS, regardless of the results of CSF. In terms of infants with suspected CS, the national guidelines recommended preventative treatment with a single dose of intramuscular benzathine penicillin; while the US CDC recommended that infants with possible CS should also receive a 10 day course of aqueous penicillin G or procaine penicillin G. A single intramuscular dose of benzathine penicillin is an alternative treatment choice for infants with possible CS only if complete evaluation (i.e. CSF examination, long-bone radiographs, and CBC with platelets) was done and all normal, and follow-up is assured according to US CDC. Because successful neurosyphilis treatment requires the presence of adequate, prolonged CSF concentrations of a treponemicidal antimicrobial, benzathine penicillin should not be used to infants with neurosyphilis as it does not reliably achieve sufficient concentrations in CSF (37). Intravenous administration of aqueous penicillin G achieves adequate CSF levels and is the treatment choice for neurosyphilis. Due to the difficultly in diagnosing congenital neurosyphilis as described above, infants with confirmed CS in our study all received 14 days of aqueous penicillin G regardless of the results of CSF, which was similar to the recommendation of US CDC. Procaine penicillin G injections also achieve treponemicidal levels in CSF, but the regimen is difficult to complete because of the need for multiple intramuscular injections that can be painful to neonates. Infants in suspected CS group in this study were also treated with aqueous penicillin G, instead of one dose intramuscular benzathine penicillin as recommended by national guideline, due to the shortage of this medicine in this study period. Although benzathine penicillin is recommended as the first choice of preventing maternal-to-child transmission of syphilis and CS, the shortage of this medicine worldwide presents a major challenge in the treatment of syphilis. In a multi-country survey evaluating the shortages of benzathine penicillin for prevention of mother-to-child transmission of syphilis, 39 (41%) countries and territories reported a shortage of the medicine (38). China is not among the 39 countries with a shortage of benzathine penicillin. However, benzathine penicillin was still unavailable in many hospitals in China. A survey conducted among 948 hospitals in Shandong Province, China, reported the benzathine penicillin availability for syphilis treatment was only 45.0% in 2012, and slightly increased to 56.4% in 2018 (39). In our hospital, benzathine penicillin is unavailable until 2017, therefore, both confirmed or suspected CS cases were treated with aqueous penicillin G during the study period.

Serologic follow-up is recommended for monitoring response posttreatment as well as in suspected cases with a normal clinical examination and investigation. After adequate treatment, there was no patient with increased TRUST titers. Posttreatment TRUST reversion to non-reactivity was documented in 100% (30/30) of the patients in the CS group, although it tended to occur later than in the infants without CS. By 6 months of age, TRUST results were negative in 53.3% of the infants with CS and in 100% of infants with suspected CS. TPPA is a serum test for *Treponema pallidum*, which can passively cross the placenta. Testing for TPPA after 12 to 18 months of age has been proposed by the US CDC and national guidelines for epidemiologic surveillance purposes, as a reactive treponemal test after the disappearance of passively acquired maternal

antibodies is evidence that the child had actually been infected with *T. pallidum*. In this study, after prophylactic treatment, 548 out of 550 (99.6%) infants showed TPPA non-reactivity before 18 months of age, and were then excluded from the diagnosis of CS. Only 2 (0.4%) out of 550 patients had positive TPPA results after 18 months of age, and both infants were asymptomatic during follow-up. According to national guidelines, these 2 infants were reported as confirmed CS cases at this point. In contrast, only 10% of infants with CS showed seroreversion in their treponemal tests by 18 months. In children with CS, similar to adults with syphilis, TPPA reactivity often occurs for life even after effective treatment, so it is not used as an indicator of efficacy. However, a Canadian case series of infants with CS reported that 69% of infants showed seroreversion in their treponemal test by 18 months and that infants who did not show seroreversion were more likely to have had delayed treatment (40). Early treatment is likely to alter the antibody response, making TPPA reactivity disappear over time (41).

The vagaries of maternal histories and symptoms, the lack of symptoms in newborns, and the potential consequences of delayed or missed diagnosis of CS demand a "safety first" approach to both diagnosis and treatment. On the other hand, we found that most infants with CS, when diagnosed and treated promptly, even those with symptoms or lab/X-ray findings at birth, responded well to treatment and had good outcomes. Therefore, the prognosis of children who are adequately treated for CS can be considered favorable in the absence of a very severe disease at birth and of other risk factors, especially preterm birth.

There are some limitations of our study. The main limitation was that we do not have information on infant in whom *T. pallidum* is identified by PCR, dark field microscopy, fluorescent antibody, or other specific stains in specimens from lesions to confirm CS cases, due to unavailability of these techniques in our hospital. All of our CS cases are defined by serology results, which may inevitably cause the misclassification. Secondly, infants in suspected CS group in this study were also treated with aqueous penicillin G, instead of one dose intramuscular benzathine penicillin as recommended by national guideline. Although both medicines are equally effective in the treatment of CS, aqueous penicillin G has a shorter half-life and requires a 7-day course of administration compared to a single dose of intramuscular benzathine penicillin, increasing the length of hospital stay in children with suspected CS. The third limitation was the high rate of loss to long-term follow-up in the CS group. Long-term monitoring of children with CS is difficult to implement because of low compliance by their mothers, who often lack motivation to seek health care. In our study, a high percentage of infants with CS were born to mothers who did not receive antenatal care. Lastly, the retrospective nature of this study inevitably generates inconsistencies in data collection.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Children's Hospital of Fudan University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YD, GZ, ZL, and WS: study design. YD, GZ, SZ, and CC: collection and analysis and interpretation of data. YD, GZ, SZ, CC, ZL, and WS: manuscript preparation and final approval. All authors contributed to the article and approved the submitted version.

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frontiers | Frontiers in Pediatrics

CASE REPORT
published: 06 April 2022
doi: 10.3389/fped.2022.866762

Case Report: Fatal Outcome for a Preterm Newborn With Meningitis Caused by Extended-Spectrum β-Lactamase-Producing *Escherichia coli* Sequence Type 1193

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OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *31 January 2022*
Accepted: *11 March 2022*
Published: *06 April 2022*

Citation:
Oldendorff F, Linnér A, Finder M, Eisenlauer P, Kjellberg M, Giske CG and Nordberg V (2022) Case Report: Fatal Outcome for a Preterm Newborn With Meningitis Caused by Extended-Spectrum β-Lactamase-Producing Escherichia coli Sequence Type 1193. Front. Pediatr. 10:866762. doi: 10.3389/fped.2022.866762

Introduction: In this case report, we describe an extended-spectrum beta-lactamase (ESBL) – *Escherichia coli (E. coli)* strain of sequence type (ST) 1193, a novel, virulent, multidrug-resistant (MDR) clone with a rapid global spread. ST 1193 has been more commonly associated with invasive disease than other ESBL-*E. coli* STs. To our knowledge, this is the first known case in Sweden where a newborn died of an ESBL-*E. coli* ST 1193 meningitis. We emphasize that the clinical knowledge about the properties of certain MDR-clones should be increased.

Case Report: A moderately preterm boy was born after preterm prolonged rupture of membranes. The mother had an ESBL-*E. coli* urinary tract infection during pregnancy. At 36 h of age he developed signs of infection and was given first-line therapy for early onset sepsis. Thereafter he developed seizures. The treatment was changed to cover suspected meningitis. Culture showed growth of the same ESBL- *E. coli* ST 1193 strain in the child's blood and cerebrospinal fluid, as well as in the mother's urine. Antibiotics were adapted. His condition deteriorated and he developed fulminant septic shock with treatment-resistant seizures. The boy passed away at 3 days of age.

Conclusion: This case highlights the risk of delay in diagnosis when a marking for carriage of MDR-bacteria is falsely removed from a medical record of a pregnant women. Further, it demonstrates that ESBL-*E. coli* ST 1193 infection in neonates can be fatal. Thus, studies regarding virulence factors of ESBL-*E. coli* infections in pregnant women and their children are needed to understand the association between this infection and severe invasive disease in newborn children.

Keywords: neonatology, meningitis, extended-spectrum β-lactamase, sepsis, case report

INTRODUCTION

Neonatal meningitis caused by Gram-negative bacteria (GNB) is a feared condition with a high mortality and morbidity rate, with *Escherichia coli (E. coli)* being one of the most common causative bacteria (1, 2). *E. coli* strains are divided into four phylogroups according to their virulence determinants, where phylogroup B2 is associated with invasive disease such as pediatric sepsis and meningitis (3, 4). Further, there is evidence of a higher mortality in newborns with sepsis caused extended-spectrum beta-lactamase (ESBL)-producing GNB, compared to non-resistant strains (5). In our case of neonatal meningitis, the *E. coli* strain was seen to belong to the phylogroup B2 and sequence type (ST) 1193. *E. coli* ST 1193, first described in 2012, is a virulent, multi drug resistant (MDR) clone with characteristics that have contributed to its rapid global spread and a high capacity for invasiveness, which belongs to a previously unknown clonal complex (cc) (6–8). To our knowledge, this is the first fatal case of ESBL-*E. coli* ST 1193 neonatal meningitis in Sweden.

Individuals carrying MDR may be affected by the carriage to a greater or lesser extent depending on the virulence factors carried by the strain. Virulence factors contribute to the strain's greater propensity to cause local or invasive infection and to colonize the gut for longer or shorter time periods (9). It has been shown that gut colonization of community-acquired strains does not cause infection as frequently as virulent strains from hospital settings (10). Moreover, a high degree of maternal-neonatal transmission of MDR has been seen, as well as an increasing MDR prevalence in the community (11–13). In Sweden no molecular characterization is presently carried out of ESBL-*E. coli* isolated from the urine of pregnant women.

CASE REPORT

This case describes a male patient born vaginally at 36 weeks + 2 days after preterm prolonged rupture of membranes (32 h) with a birthweight of 2.8 kg and Apgar score of 9-10-10. Umbilical cord blood-gas analysis, performed after birth, showed normal potential of hydrogen (pH), base excess (BE), and lactate, see Timeline (**Figure 1**). The pregnancy had been normal, apart from several urinary tract infections (UTIs) during the second trimester. The UTIs had been treated by the primary health-care center, where growth of ESBL-*E. coli* in the urine had been detected and the mothers medical record had been marked to signal colonization with MDR bacteria. Further molecular characterization was not performed, as it is not routine on ESBL-*E. coli* isolates from urine. After treatment with pivmecillinam twice and nitrofurantoin twice, a control culture of the mother's urine was negative, which led to the removal of the warning in the medical record by the treating physician. At time of delivery, the mother was given benzylpenicillin intrapartum, as per routine in case of prolonged rupture of membrane.

The newborn patient presented with respiratory distress at 1 h of age and fluctuating tachypnoea during the first 20 h of age. The patient was grunting, hard to comfort and had frequent stools. Nonetheless, the patient had a normal respiratory rate (RR) and oxygen saturation (SaO2), normal blood-sugar levels (lowest value 2.8 mmol/L) and a normal body temperature. Thus, the pediatrician on call assessed that the patient was not tachypneic and clinically stable. No blood test was taken, and no further examinations were made at that point. The patient's condition was unchanged during the first day. However, at 36 h of age, he was found pale and distressed, whereupon he was admitted to the neonatal unit with a suspected infection.

Upon admittance the patient had a temperature of 36.8°C, Median Arterial Pressure (MAP) 43 mmHg, RR 65/minute, SaO2 100% and capillary refill of 3–5 s (chest-foot). Blood tests showed an elevated C-reactive protein (CRP) 85 mg/L (n.v 0–3), a low white blood count (WBC) of 0.8×10^9/L (n.v 5–25) and normal levels of blood platelets (TPK) 144×10^9 (n.v 85–475). Initial venous blood-gas analysis showed pH 7.25 (7.32–7.43); pCO2 5.8 kPa (5.3–6.6); glucose 2.9 mmol/L (n.v 4.0–6.0); lactate 7.5 mmol/L (n.v 0.5–2.3) and BE −8 mmol/L (n.v −3–3). Benzylpenicillin (100 mg/kg/day) and amikacin (18 mg/kg/day), was given as recommended first-line therapy for early neonatal sepsis. Sepsis work-up was done and the mother's chart was checked for risk factors for infection. A maternal urinary culture taken, on the day of delivery, showed the growth of *E. coli* with ongoing susceptibility testing. There were no signs of earlier ESBL- *E. coli* colonization or infection in the maternal medical record, since the MDR-marking previously had been removed. Seven hours after admission, the patient developed seizures with electrographic correlates and phenobarbital and later midazolam was given. A lumbar puncture was performed, and cerebrospinal fluid (CSF) analysis showed pleocytosis with WBC of 1639×10^6/L (n.v 0–5), RBC of 125×10^6/L (n.v 0–1), polymorphonuclear leukocytes 781×10^6/L (n.v 0–1), monomorphonuclear leukocytes 858×10^6/L (n.v 0–5), lactate 8.2 mmol/L (n.v 1.1–2.4) and glucose of <0.7 mmol/L. The patient deteriorated into severe metabolic acidosis with pH 7.06 (n.v 7.32–7.43); lactate 15 mmol/L (n.v. 0.5–2.3) and BE −16 mmol/L (n.v −3–3). The patient received mechanical ventilation and antimicrobial therapy was changed to cefotaxime (150 mg/kg/day), ampicillin (300 mg/kg/day) and acyclovir (60 mg/kg/day), according to guidelines with suspicion of meningitis.

A Gram-negative rod grew rapidly in blood (3.7 h) and a few hours later it was found to be an *E. coli*. At that time, cefotaxime was changed to meropenem (40 mg/kg/day). An hour later, it was reported to be an ESBL- *E. coli* strain, susceptible to carbapenems (i.e., imipenem, meropenem and ertapenem, but resistant to cefotaxime, ceftazidime, gentamicin, and ciprofloxacin) according to European Committee on Antimicrobial Susceptibility Testing (EUCAST)'s breakpoints. Thus, the strain was resistant to earlier applied treatment in this case. The next day, the same bacterial strain grew in the child's cerebrospinal fluid and in the mother's urine. New blood tests showed an increasing CRP 119 mg/L (n.v 0–3), continuously low WBC 1.6×10^9/L (n.v 5–25) and decreasing TPK 22×10^9 (n.v 85–475). The patient proceeded into to severe respiratory failure despite increased supportive mechanical ventilation. Hypotension was treated with fresh frozen plasma and inotropes, with no effect on urine production.

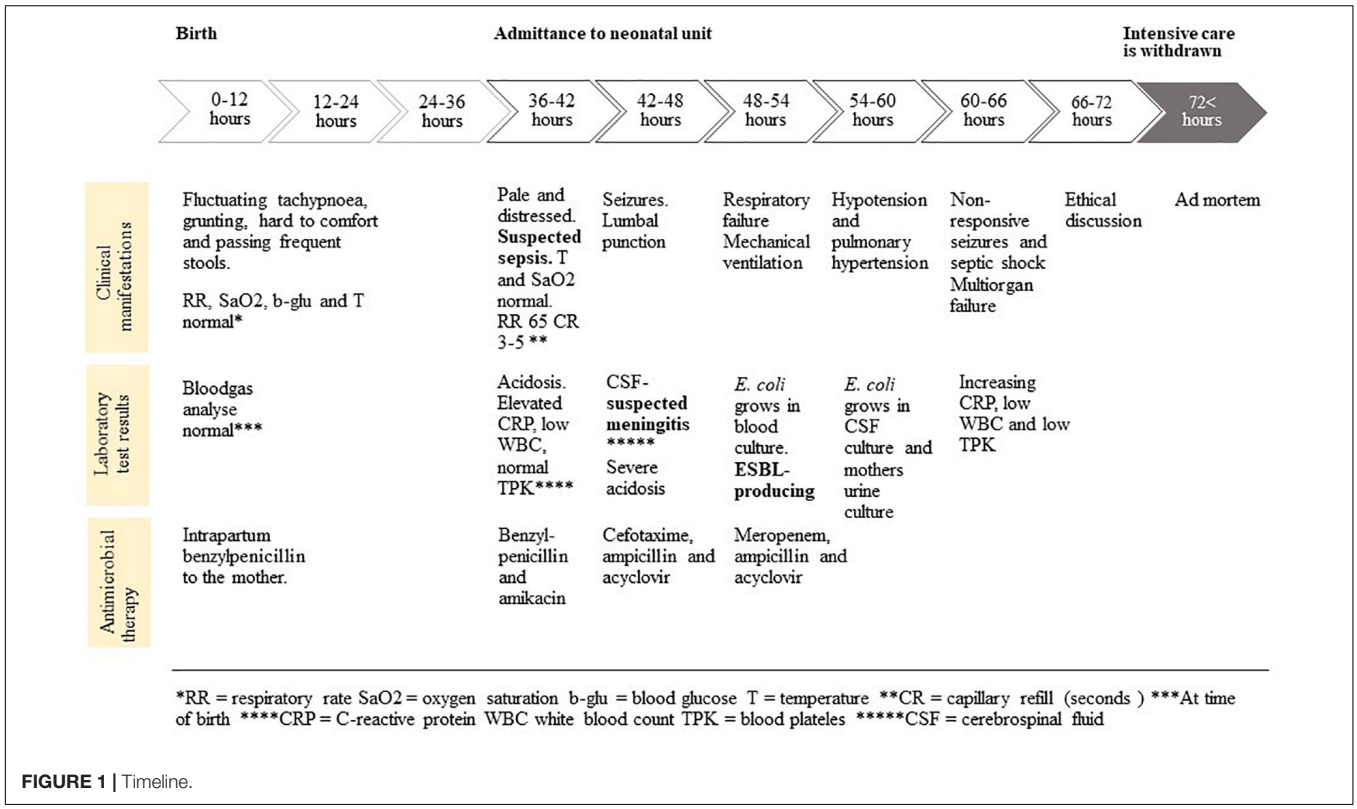

FIGURE 1 | Timeline.

Echocardiography confirmed pulmonary hypertension. Seizures, non-responsive to treatment, increased as the clinical picture of fulminant septic shock developed. Due to poor prognosis, the intensive care was withdrawn from the patient at 3 days of age. This followed an ethical discussion and was in consent with the parents.

DIAGNOSTIC ASSESSMENT

Samples of the blood- and cerebrospinal fluid from the child as well as a urine sample from the mother were analyzed. The bacteria grew on blood agar plates at 37°C after 3.7 h and were thereafter typed as *E. coli* with Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS). Susceptibility testing of the *E. coli* was performed with disk diffusion, which 2.5 days after the samples had been taken, showed that the strain was ESBL-producing with resistance to cefotaxime, ceftazidime, gentamicin and ciprofloxacin. The bacteria showed susceptibility to carbapenems and amikacin. Epidemiological typing was thereafter performed with Multi-Locus Sequence Typing (MLST), showing that the *E. coli* in all three samples belonged to ST 1193.

DISCUSSION

An ESBL-*E. coli* that has successfully caused a maternal UTI could be considered a potentially virulent clone and likely to colonize the maternal gut for a considerable amount of time. Maternal intestinal MDR-colonization is not treated with antibiotic prophylaxis. MDR-colonization could be considered a risk factor for early onset MDR-sepsis in and therefore be considered a risk factor for early onset MDR-sepsis in newborn children, particularly when having caused an infection during pregnancy. Awareness of this could lead to a quicker recognition of, and testing for, an infection in these children and increase the chance of correct treatment early on. Early blood-culturing could be considered in these cases. Moreover, it should be highlighted that some ST's of ESBL- *E. coli* are more virulent than others and therefore at higher risk of causing invasive disease in neonates (3, 4). This case underlines the importance of not falsely removing markings for MDR-colonization from medical records, since it risks causing delay in treatment. In this case, if knowing that the mother previously had been infected with an ESBL-*E. coli*, the antibiotic treatment could have been broadened earlier. However, it might not have changed the fatal outcome for this patient, due to the rapid progress into septic shock.

E. coli ST 1193 is a highly virulent strain with increased global spreading, which has until now, been seldom described in neonates. In a recent study of neonatal invasive *E. coli* and their molecular characteristics in China, ST 1193 was the most frequently detected clone and 33% of the *E. coli* ST 1193 was ESBL-producing (14). Further, there are data on a rapid increase in *E. coli* ST 1193 in isolates from young adults in the United States and the first-time prevalence of *E. coli* ST 1193 in neonates in the country was described in 2019 (15). We have, in this case report, described the first known case in Sweden, where a newborn died because of an *E. coli* ST 1193 meningitis. To our

knowledge, only one fatal case of neonatal meningitis caused by *E. coli* ST 1193 has globally been described earlier, with a similar fulminant course as in our case (16).

From this case we conclude that studies regarding virulence factors of ESBL-*E. coli* infections in pregnant women and their children are needed to find which newborn babies that could be at risk for severe invasive disease. Such studies might lead to new recommendations in the management of the neonates born to ESBL- *E. coli* colonized mothers, i.e., interventions including intensifying the observations of vital parameters of the newborn in the postnatal ward and a new increased awareness of potential severe invasive neonatal infection. Further, it highlights the risk of delay in diagnosis when a marking for a MDR bacteria is removed from a medical record.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

FO, AL, and VN wrote the first draft of the manuscript. FO and VN coordinated the work around the manuscript. AL and FO extracted the original information from the patients medical records. MF, MK, PE, and CG wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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frontiers | Frontiers in Pediatrics

MINI REVIEW
published: 29 April 2022
doi: 10.3389/fped.2022.883953

Coronavirus Disease 2019 Vaccination During Pregnancy and Breastfeeding: A Review of Evidence and Current Recommendations in Europe, North America, and Australasia

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In the late 2020s, less than 1 year into the coronavirus disease 2019 (COVID-19) pandemic, several anti-severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines were introduced on a worldwide scale, with a significant positive impact on the consequences of the disease for several high-risk population groups. In the case of most bacterial or viral respiratory infections, pregnant women are at increased risk of complications, however, neither pregnant nor breastfeeding women were included in the first round of randomized clinical trials evaluating the safety and effectiveness of COVID-19 vaccines, because of safety and ethical concerns. Nevertheless, most anti-SARS-CoV-2 vaccines have not been expressly contraindicated during pregnancy or breastfeeding, and observational data on immune response, adverse effects, and clinical efficacy in pregnant and breastfeeding women have been progressively gathered during 2021. The vast majority of these data is reassuring for what concerns side effects for women and infants and points out the efficacy of vaccines in protecting women against COVID-19-related complications. Despite this, the hesitancy of pregnant and breastfeeding women at being vaccinated is still real. In this mini-review, we resume the available data on the clinical consequences of COVID-19 in pregnant women, as well as adverse effects, systemic and mucosal immune response, and clinical effectiveness of COVID-19 vaccines in pregnant and breastfeeding women. Moreover, we offer an updated overview of European, North American, and Australasian recommendations concerning COVID-19 vaccination in pregnant and breastfeeding women, in order to safely ensure the highest protection of women and their infants.

Keywords: COVID-19, vaccine, pregnancy, breastfeeding, newborn, infant, immune response, safety

OPEN ACCESS

Edited by:
Cinzia Auriti, Bambino Gesù Children's Hospital (IRCCS), Italy

Reviewed by:
Youjia Zhong, National University Hospital, Singapore

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Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *25 February 2022*
Accepted: *05 April 2022*
Published: *29 April 2022*

Citation:
Pietrasanta C, Ronchi A, Crippa BL, Artieri G, Ballerini C, Crimi R, Mosca F and Pugni L (2022) Coronavirus Disease 2019 Vaccination During Pregnancy and Breastfeeding: A Review of Evidence and Current Recommendations in Europe, North America, and Australasia. Front. Pediatr. 10:883953. doi: 10.3389/fped.2022.883953

INTRODUCTION

Since the end of 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has been representing the greatest challenge for healthcare systems worldwide (1). Coronavirus disease 2019 (COVID-19) pandemic impacted vigorously also perinatal medicine: here, pregnant infected women present a higher risk of admission to an intensive care unit (ICU), invasive ventilation, and need for extra corporeal membrane oxygenation compared

to non-pregnant reproductive-aged women, while neonates born to infected mothers, or infected themselves, can also suffer from adverse outcomes such as prematurity or respiratory distress syndrome (2, 3). The rapid introduction of anti-SARS-CoV-2 vaccines in the late 2020s has dramatically changed the trajectories of virus impact on several categories of patients, particularly the most vulnerable ones (4, 5). Pregnant women were initially excluded from clinical trials on COVID-19 vaccines, for theoretical safety and ethical concerns (6, 7). However, the progressive gathering of robust observational data from cohorts of women vaccinated during pregnancy allowed the scientific community to rapidly clarify several unresolved issues. Nevertheless, more than 1 year after the introduction of vaccines worldwide, safety concerns of pregnant or breastfeeding women are still reported as the main reason to refuse COVID-19 vaccination (8, 9), and their vaccination rates are consistently lower than those of the general population of an equivalent age (10). This attitude has been favored, to some extent, by the fact that recommendations by different national and international regulatory authorities regarding the use of anti-SARS-CoV-2 vaccines in pregnant and breastfeeding women have been repeatedly modified and amended (11). The initial (and justifiable) prudence quickly gave way to first, a more permissive and then encouraging recommendations to vaccinate both pregnant and lactating women. The vaccination offer, with country-by-country variations, was initially limited to at-risk categories such as obese, diabetic, and healthcare workers, but was rapidly extended to all pregnant and breastfeeding women.

In this "Questions and Answers" mini-review, we will explore the most updated evidence supporting the practice of COVID-19 vaccination in pregnant and breastfeeding women, and clarify major concerns that still now undermine the achievement of high vaccination rates, and summarize the current recommendations in Europe, North America, and Australasia.

At present (February 2022), 10 COVID-19 vaccines have been granted Emergency Use Listing by the WHO (complete and updated list available at https://extranet.who.int/pqweb/vaccines/vaccinescovid-19-vaccine-eul-issued), but may be approved or not for clinical use in different countries. For reasons of convenience, this review will be focused on data and recommendations existing for two mRNA vaccines (Comirnaty by Pfizer BioNTech Manufacturing GmbH, and Spikevax by Moderna Biotech), two non-replicating viral vector vaccines (Vaxzevria by Astrazeneca AB, and Ad26.COV2-S [recombinant] by J and J/Janssen), and one recombinant protein subunit (Spike) vaccine (Nuvaxovid by Novavax).

IS ANTI-SEVERE ACUTE RESPIRATORY SYNDROME CORONAVIRUS 2 (SARS-COV-2) VACCINATION USEFUL DURING PREGNANCY?

The answer to this question can now be grounded in robust scientific data: a large meta-analysis (12) by a British research team collected available data on over 64,000 pregnant women infected with SARS-CoV-2, showing that pregnant women with infection have a significantly higher risk (unadjusted for confounders) of hospitalization in the ICU (OR: 2.13, 95% CI: 1.53–2.95), of mechanical ventilation (OR: 2.59, 95% CI: 2.28–2.94), and of extracorporeal circulation (OR: 2.02, 95% CI: 1.22–3.34) compared to infected, non-pregnant women. In addition, infection during pregnancy increases the risk of maternal death, preeclampsia, premature birth, and intrauterine death, with odds ratios ramping up as the severity of maternal disease increases (13). Compared to the amount of data available for pregnant women, those regarding SARS-CoV-2 infection in neonates are significantly more limited and less robust. Vertical transmission rates of SARS-CoV-2 from an infected mother to her fetus during pregnancy are currently estimated at around 2–3%, based on a neonatal screening strategy consisting solely of rt-PCR for SARS-CoV-2 RNA on nasopharyngeal swabs (14, 15). After the WHO enacted more accurate definitions for confirmed, probable, or unlikely vertical transmission (16), including longitudinal analysis of multiple sterile and non-sterile body sites, the precise vertical transmission rate of COVID-19 remains to be established in large cohorts. Nevertheless, the impact of SARS-CoV-2 on neonates is not limited to the vertical transmission from an infected mother: indeed, data from the Swedish neonatal registry clearly showed that maternal infection during pregnancy can worsen neonatal outcomes independently of the vertical transmission of the virus. Neonates born to mothers with perinatal COVID-19 present an increased risk of resuscitation in the delivery room, mechanical ventilation, persistent pulmonary hypertension, and of jaundice requiring treatment compared to neonates born to unaffected women (3).

Moreover, neonates can acquire SARS-CoV-2 infection from their mothers even after birth, through a horizontal airborne transmission that seems favored by severe maternal COVID-19, possibly sustained by a high viral load (17). Most of these data were collected in 2020 or early 2021, during the first two waves of the pandemic, when the original strain of the virus or variants whose severity has been later de-escalated, such as the Alpha (B.1.1.7), were prevalent in Europe, North America, and Australasia. In late 2021, limited but worrying data regarding the impact of the Delta (B.1.617.2) variant on pregnant women raised significant concern among healthcare providers, as it was clearly shown that Delta infection during pregnancy significantly increased the proportion of severe or critical disease (36 vs. 13%, aRR: 2.76, 95% CI: 1.73–4.40) and ICU admissions (29 vs. 8%, aRR: 3.42, 95% CI: 1.91–6.11) compared to the pre-Delta period (18). In slightly more than 2 months, the Omicron variant (B.1.1.529) then blew away the Delta all over the world throughout the winter of 2021–2022. The impact of Omicron on perinatal medicine is yet to be established, and the interpretation of data after 2 years of pandemic and the introduction of COVID-19 vaccination may be confused by pre-existing natural or vaccine-induced immunity (19). However, it is nowadays clear that safe and effective anti-SARS-CoV-2 vaccines administered to pregnant women might greatly reduce the negative impact of COVID-19 on both pregnant women and newborns (20), as will be discussed more in detail in the next sections.

TABLE 1 | National and supranational selected recommendations regarding coronavirus disease 2019 (COVID-19) vaccination during pregnancy and breastfeeding.

Location	Vaccines approved (EUA*) for pregnant women	Vaccines preferred for pregnant women	Timing of vaccination	Booster dose
EU[**a]	- Pfizer-BioNTech: BNT162b2 - Moderna: mRNA-1273 - Janssen: Ad26.COV2.S - AstraZeneca: ChAdOx1-S - Novavax: NVX-CoV2373	- Pfizer-BioNTech: BNT162b2 - Moderna: mRNA-1273	- Before pregnancy (any time) - During pregnancy (any trimester**) - After pregnancy (any time)	No specific recommendation
UK[b]	- Pfizer-BioNTech: BNT162b2 - Moderna: mRNA-1273 - Janssen: Ad26.COV2.S - AstraZeneca: ChAdOx1-S	- Pfizer-BioNTech: BNT162b2 - Moderna: mRNA-1273	- Before pregnancy (any time) - During pregnancy (any trimester) - After pregnancy (any time)	Yes, at any time. mRNA vaccines preferred (even after non-replicating viral vector vaccine cycle)
US[c]	- Pfizer-BioNTech: BNT162b2 - Moderna: mRNA-1273 - Janssen: Ad26.COV2.S	- Pfizer-BioNTech: BNT162b2 - Moderna: mRNA-1273	- Before pregnancy (any time) - During pregnancy (any trimester) - After pregnancy (any time)	Yes, at any time. mRNA vaccines preferred (even after non-replicating viral vector vaccine cycle)
Canada[d]	- Pfizer-BioNTech: BNT162b2 - Moderna: mRNA-1273 - Janssen: Ad26.COV2.S - AstraZeneca: ChAdOx1-S - Novavax: NVX-CoV2373 - Medicago-GSK: CoVLP	- Pfizer-BioNTech: BNT162b2 - Moderna: mRNA-1273	Before pregnancy (any time) - During pregnancy (any trimester) - After pregnancy (any time)	Yes, at any time.
Australia and NZ[e]	- Pfizer-BioNTech: BNT162b2 - Moderna: mRNA-1273 - AstraZeneca: ChAdOx1-S	- Pfizer-BioNTech: BNT162b2 - Moderna: mRNA-1273	- Before pregnancy (any time) - During pregnancy (any trimester) - After pregnancy (any time)	Yes, at any time. mRNA vaccines preferred (even after non-replicating viral vector vaccine cycle)

Emergency Use Authorization.

**Country-specific differences may exist (e.g., Italy: vaccination during the second and third trimester of pregnancy is recommended. For vaccination during the first trimester a cos-benefit analysis is encouraged).*

Online references, accessed March 15, 2022:

[a] *https://www.ema.europa.eu/en/news/covid-19-latest-safety-data-provide-reassurance-about-use-mrna-vaccines-during-pregnancy.*

[b] *https://www.rcog.org.uk/media/kbknl3z3/2022-03-07-coronavirus-covid-19-infection-in-pregnancy-v15.pdf.*

[c] *https://www.cdc.gov/coronavirus/2019-ncov/need-extra-precautions/pregnant-people.html; https://www.acog.org/-/media/project/acog/acogorg/files/pdfs/clinical-guidance/practice-advisory/covid19vaccine-conversationguide-121520-v2.pdf*

[d] *https://sogc.org/common/Uploaded%20files/Latest%20News/SOGC_Statement_COVID-19_Vaccination_in_Pregnancy.pdf.*

[e] *https://ranzcog.edu.au/statements-guidelines/covid-19-statement/covid-19-vaccination-information.*

IS VACCINATION DURING PREGNANCY SAFE FOR THE MOTHER AND THE INFANT?

Safety concerns are still reported as the main reason to refuse COVID-19 vaccination during pregnancy (21, 22). The hesitancy of pregnant women to receive vaccines that have been developed faster than any other in history, and for which there is no long-term safety data, is more than justifiable. Therefore clear, evidence-based data are necessary to promote the highest possible adherence to current recommendations. Until February 2022, the US-based "V-safe" register collected self-reported data from over 198,000 women who were pregnant at the moment of COVID-19 vaccination (23). Similarly, more than 100,000 women were reported to have received the COVID-19 vaccine in the United Kingdom (24). These data are constantly updated and have not raised concerns regarding possible serious adverse events caused by or strictly related to vaccination. In particular, the administration of mRNA vaccines (Comirnaty by Pfizer—BioNTech and Spikevax by Moderna) is not associated with a higher incidence of prematurity, with the delivery of neonates small for gestational age (SGA), nor with increased proportions of congenital malformations compared to the standard incidence in non-vaccinated women (25). Furthermore, two large US-based observational studies demonstrated that the cumulative risk of spontaneous abortion, from conception to the 19th week of pregnancy, after COVID-19 vaccination during the first trimester was 14.1% (CI: 12.1–16.1%), in line with that of historical cohorts of unvaccinated women (26, 27). The risk remained stable regardless of the week of administration of the first dose. Recently, data from two large population-based observational retrospective cohorts evaluating outcomes in more than 250,000 pregnancies from Canada, Sweden and Norway were extremely reassuring, especially for pregnant women vaccinated with mRNA vaccines in the second or third trimester of pregnancy. Specifically, in the cohort study conducted in Ontario, Fell et al. reported that COVID-19 vaccination during pregnancy was not significantly associated with increased risk of postpartum hemorrhage, chorioamnionitis, cesarean delivery, admission to neonatal intensive care unit, or low Apgar score compared to vaccination after pregnancy and to no vaccination, even when adjusted for confounding factors (28). Similarly, data from Scandinavian registries showed how vaccination during pregnancy was not significantly associated with increased risk of preterm birth, stillbirth, small gestational age, low Apgar score, or neonatal admission to intensive care unit (29).

Like any other individual, pregnant women can suffer from post-injection side effects, which are more common after the second or third vaccine dose compared to the first for mRNA

vaccines, and apparently more frequent after the first dose for adenovirus (Ad) vector-based vaccines (30). However, the reported rates of side effects after receiving mRNA COVID-19 vaccines do not significantly differ from those of non-pregnant women (25). Among post-injection side effects, fever after the second dose is reported by 46% of women after the Moderna vaccine and by 24.8% after the Pfizer vaccine (25). As maternal fever during the first trimester can be associated with an increased relative risk of congenital malformation (e.g., cleft lip and neural tube closure defects) (31), some national regulatory agencies such as the Italian Ministry of Health recommended a case-by-case evaluation before the administration of COVID-19 vaccines during the first trimester of pregnancy and endorsed a full recommendation only for vaccination in the second and third trimester. However, considering the risks associated with COVID-19 during pregnancy, other authorities such as the Royal College of Obstetricians and Gynecologists (United Kingdom), the American College of Obstetricians and Gynecologists (united states), the Society of Obstetrician and Gynecology of Canada, the Royal Australian and New Zealand College of Obstetricians and Gynecologists opted anyway for an "as early as possible" advice, recommending pregnant women to receive vaccination (first, second, or booster dose) at any time during pregnancy.

Current national recommendations from selected regulatory agencies worldwide are summarized in **Table 1**. Safety data concerning the vaccination of pregnant women with the non-replicating viral vector vaccines Janssen (Ad26.COV2-S, J&J/Janssen) and VaxVeria (ChAdOx1-S, AstraZeneca), and with the S-protein recombinant adjuvanted Nuvaxovid (Novavax) are greatly limited compared to those available for mRNA vaccines. In non-pregnant adults, both Ad26.COV2-S and Nuvaxovid are associated with slightly lower rates of post-injection side effects compared to both Moderna and Pfizer mRNA vaccines (32, 33), while animal studies have shown no clear adverse effect of vaccination on pregnancy or neonatal outcomes. However, observational data on pregnant women are limited and do not enable most regulatory agencies to fully recommend their use during pregnancy. Thrombosis with thrombocytopenia syndrome (TTS) is a serious but extremely rare condition occurring in approximately 1–2/100,000 doses of Janssen or VaxVeria vaccine administered to females aged 30–39 years (34). A warning about the possibility of TTS occurrence after administration of ad vector-based COVID-19 vaccines has been included in the Emergency Use Authorization (EUA) of most countries. The use of ad vector-based COVID-19 vaccines (Janssen in the US, Janssen, and VaxVeria in the EU and United Kingdom), as well as of Nuvaxovid in pregnant women is currently allowed worldwide, but all regulatory agencies state that mRNA vaccine should be routinely preferred unless a clear contraindication or a strong preference of the patient exists (see **Table 1**). To date, there is no evidence that pregnant or postpartum women are at higher risk of vaccine-induced TTS than non-pregnant age-matched women (35). However, considering that pregnancy itself increases the risk of thrombosis four to fivefold (36), the risk of TTS in pregnant women who receive Janssen or VaxVeria vaccines deserves further evaluation.

IS VACCINATION DURING PREGNANCY EFFECTIVE FOR THE MOTHER AND THE INFANT?

COVID-19 vaccination of pregnant and breastfeeding women has been shown to induce humoral and cell-mediated responses akin to that induced in young non-pregnant women (37). Vaccination-induced serum antibodies, both anti-Spike (anti-S) and anti-Receptor-Binding Domain (RBD), are mainly of the IgG class and persist for at least 6–9 months after maternal vaccination (38). Pending the results of randomized trials conducted on pregnant women, observational data from two large Israeli cohorts have shown a significant decrease in COVID-19 cases among pregnant women after vaccination, and overall efficacy of around 96% (CI: 89–100%) for confirmed infection, of 97% (CI: 91–100%) for symptomatic confirmed infection and of 89% (CI: 43–100%) for hospitalization due to COVID-19, starting from 7 days after the administration of the second dose (39, 40). These data mainly reflect the effectiveness against B1.1.7 (Alpha) variant and the original SARS-CoV-2 strain, which were dominant in Israel during the study period. During the last trimester of pregnancy, vaccine-induced IgG is actively transferred to the fetus *via* the placenta through Fc receptors. The maternal serum/cord transfer ratio positively correlates with the distance between the end of the maternal vaccination cycle and the time of delivery and can exceed 1 (41, 42). It is now clear that anti-S and anti-RBD IgG induced by maternal vaccination during pregnancy can persist in neonatal serum up to at least 6 months, a time span much longer than that of antibodies induced by maternal natural infection (43), while the effective clinical protection against COVID-19 hospitalization conferred to infants aged < 6 months has been recently estimated for the first time, in a case-control study, and seems equal to 61% (95% CI: 31–78%) (44). COVID-19 vaccination during pregnancy also induces the production of IgG, IgA, and IgM antibodies in breast milk, although in much lower quantities than in serum and probably not persistent for such a long time (45, 46). Indeed, akin to what occurs for several other vaccines administered intramuscularly (47), the activation of mucosal plasma cells of the mammary gland induced by COVID-19 vaccines may be limited, as highlighted by the relative low (compared to IgG) amount of SARS-CoV-2—specific IgA recovered in the breast milk of vaccinated women (46). Whether breast milk vaccine-induced antibodies may confer some degree of protection to breastfed infants is certainly conceivable, but not proven yet.

IS VACCINATION DURING BREASTFEEDING SAFE AND EFFECTIVE FOR THE MOTHER AND THE INFANT?

Available scientific evidence and recommendation by the included regulatory agencies worldwide do not contraindicate the administration of COVID-19 vaccines (either first, second, or booster dose) during breastfeeding (**Table 1**). For breastfeeding mothers, the clinical efficacy of the COVID-19 vaccine is

not expected to be different from other women, although data from observational cohorts or randomized control trials are still pending. Moreover, lactating women are encouraged not to interrupt breastfeeding before or after vaccination. For lactating mothers, indeed, the expected side effects are the same as those found in non-lactating women, and even in this population, they occur with a slightly higher incidence for the Moderna vaccine than for Pfizer (48). During the 48–72 h after vaccination, breastfeeding women may experience a transient reduction in milk production, a side effect that appears to occur more frequently after the second dose of vaccine, and to a greater extent (23.4% of women) for the Moderna vaccine than for Pfizer (8%) (48). No significant side effect (fever, rash, cough, behavioral change, vomiting, or diarrhea) has been recorded in infants breastfed by vaccinated women within 72 h after BNT162b2 vaccination as recently reported in a prospective study (49). Importantly, there is no clear evidence that mRNA-based vaccines do not significantly diffuse to breast milk, where the vaccine mRNA sequence has not been detected (50, 51), or has been detected in minimal amounts, with no presumable biological activity and high susceptibility to rapid enzymatic degradation (49). Maternal vaccination during breastfeeding is effective in inducing SARS-CoV-2 specific serum antibodies of IgG, IgM, and IgA class in the mother, despite delayed kinetics of antibody titers and FcR-binding capacity compared to non-pregnant or non-lactating ones (45, 46). Specific and neutralizing antibodies are also induced in breast milk, albeit in significantly lower amounts as compared to serum concentrations and, specifically for IgA, in lower amounts as compared to natural infection (37). Akin to maternal vaccination during pregnancy, the capacity of breast milk antibodies induced by maternal vaccination during breastfeeding to confer some mucosal protection against SARS-CoV-2 infection to breastfed infants is yet to be established.

IS VACCINATION DURING PREGNANCY AND BREASTFEEDING ALSO EFFECTIVE AGAINST THE DELTA AND OMICRON VARIANTS?

Most data concerning the immunological and clinical efficacy of COVID-19 vaccines in pregnant and lactating women were obtained in the "pre-Delta" period, in epidemiological contexts dominated by viral variants, such as the Alpha (B.1.1.7), the Beta (B.1.351), or the Gamma (P.1) variant in South America, that are not prevailing anymore. Between May and December 2021, the Delta variant (B.1.617.2) became predominant in Europe, North America, and Australasia, while since the end of 2021 the newly emerged Omicron (B.1.1.529) has been responsible for more than 90% of COVID-19 cases in the same areas (52). Both the Delta and the Omicron variants were classified by the WHO as "variants of concern" (VOC), because of their increased contagiousness and/or virulence compared to both the original SARS-CoV-2 strain and most previous variants. SARS-CoV-2 genome is extremely prone to the acquisition of new mutations, which are also favored by the velocity of virus diffusion in the twenty-first century. Consequently, the global epidemiological scenario changes continuously, imposing a non-stop reassessment of previously acquired data. The first available evidence suggested that the Delta variant might worsen the outcomes of the infected pregnant woman compared to other viral variants, increasing the rate of severe infections, the need for hospitalization, and, as reported in a small case series, the probability of placentitis and fetal demise (53, 54). The short period of Omicron prevalence has not allowed yet to collect robust epidemiological data for pregnant and lactating women. For what concerns vaccine efficacy, data on women of childbearing age have recently been published from the United Kingdom, where over 380,000 individuals over the age of 18 have been vaccinated in an epidemiological setting dominated by the Delta variant (55). The efficacy of the Pfizer vaccine was reduced against a symptomatic or high viral load (Ct $<$ 30) Delta variant infections compared to the Alpha strain of SARS-CoV-2, but was still equal to 84% (95% CI: 82–86%). Further analysis also revealed that vaccine effectiveness tended to wane by about 22% every 30 days after the receipt of the second dose. For what concerns the efficacy of vaccines administered during pregnancy against the Omicron variant, the first available data suggest that the neutralizing capability of serum from vaccinated pregnant women might be lower for the Omicron variant as compared to wild type SARS-CoV-2, or Beta and Delta variants (56). However, another recent report confirmed a reduced RBD recognition and Fc-receptor binding against the Omicron variant but highlighted the preservation of Omicron Spike-specific antibodies, that may continue to attenuate disease severity in pregnant women (57). Translating this evidence into the clinical scenario is not easy, and data are still scarce: the first available report was recently published as a Morbidity and Mortality Weekly Report (MMWR) by the Centers for Disease Control and Prevention (CDC): it is a case-control study on infant protection after maternal vaccination, reporting effectiveness of maternal vaccination during pregnancy against COVID-19 Delta and Omicron hospitalization in infants aged $<$ 6 months of 61% (95% CI: 31–78%) (44). Further data on the effectiveness of maternal outcomes are yet to be collected. Finally, there is currently no available evidence regarding the effectiveness of vaccines in breastfeeding women against VOCs; however, for women, this is reasonably expected not to be different from that of non-breastfeeding women in their childbearing age. Conversely, it has been recently shown that the efficacy of breast milk antibodies (mainly IgA) in binding to most VOCs, including the Delta variant, was reduced compared to the original Wuhan-Hu-1 strain, possibly highlighting reduced clinical protection of breastfed neonates after maternal vaccination during breastfeeding (58).

CONCLUSION

The administration of COVID-19 vaccines to pregnant or breastfeeding women is safe in the vast majority of cases and

is proving effective in reducing adverse consequences of SARS-CoV-2 infection for mothers and their infants. Every effort should be made by national and supranational authorities to provide clear and harmonized recommendations to pregnant and breastfeeding women, in order to achieve the highest possible immunization coverage in this high-risk population group.

AUTHOR CONTRIBUTIONS

CP, AR, FM, and LP conceived the article. CP, AR, BC, GA, CB, and RC revised literature and recommendations. CP wrote the first draft. All authors revised the draft, provided insightful contributions, and agreed to be accountable for the content of the work.

ACKNOWLEDGMENTS

We are grateful to ASGENAR (Associazione Genitori Neonati ad Alto Rischio ONLUS) for their precious support to the activity of the Infectious Disease Research Group of the Neonatal Intensive Care Unit, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 20 May 2022
doi: 10.3389/fped.2022.881516

Clinical and Neurodevelopmental Characteristics of *Enterovirus* and *Parechovirus* Meningitis in Neonates

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Background: Non-polio-enteroviruses (EV) and human parechoviruses (HPeV) are small RNA viruses, which in newborns cause infections with a wide range of severity. Today molecular biology tools allow us to diagnose viral meningitis in neonates, sparing patients from useless antibiotics. Data on neurodevelopmental outcome of children who contract enterovirus meningitis in early childhood are still limited in the literature.

Aims: To evaluate the neurodevelopmental outcome of newborns with documented *enterovirus* and *parechovirus* meningitis contracted within the first months of life.

Methods: *Enterovirus* and *parechovirus* were detected on cerebrospinal fluid (CSF) and plasma by RT-PCR. The virological typing was done according to WHO recommendations. During the hospitalization each neonate underwent many diagnostic and instrumental examinations, to evaluate any neurological lesions attributable to the infection. After the discharge children entered in an outpatient interdisciplinary assessment process, comprehensive of the administration of Bayley III scales up to 12 months old.

Results: We observed longitudinally 30 children, born at term (mean GA 39.7 ± 0.8 weeks, mean birthweight was 3,457 ± 405 grams), who contracted *enterovirus* and *parechovirus* meningitis within the first month of life (mean age at diagnosis was 15.8 ± 7.33 days). We were able to perform the genetic typing only on 15/30 (50.0%) cerebrospinal fluid (CSF) samples from 15 neonates. We found MRI anomalies in 9/26 observed neonates (34.6%): one of them presented brainstem abnormality that are specific of enteroviral central nervous system (CNS) involvement. During the follow up children displayed an overall normal neurodevelopment and no deficit in visual and hearing areas. The mean cognitive (105.19 ± 8.71), speech (100.23 ± 8.22) and motor (97.00 ± 8.98) composite scores, assessed by Bayley III, were normal in 29/30 (96.7%). Despite this, children with pathological brain magnetic resonance imaging (MRI) scored significantly lower ($p = 0.01$) than children with normal brain MRI on cognitive subscale at 12 months of life.

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *22 February 2022*
Accepted: *02 May 2022*
Published: *20 May 2022*

Citation:
Bucci S, Coltella L, Martini L, Santisi A, De Rose DU, Piccioni L, Campi F, Ronchetti MP, Longo D, Lucignani G, Dotta A and Auriti C (2022) Clinical and Neurodevelopmental Characteristics of Enterovirus and Parechovirus Meningitis in Neonates. Front. Pediatr. 10:881516. doi: 10.3389/fped.2022.881516

Conclusions: Early enterovirus infections can be associated to brain MRI abnormalities, more frequently the earlier the infection. Although within a normal range, our children with pathological brain MRI scored significantly lower than those with normal brain MRI on cognitive subscale at 12 months of life.

Keywords: neurodevelopment, outcomes, viral meningitis, viruses, newborns, infants

INTRODUCTION

Non-polio *enteroviruses* (EV) and human *parechoviruses* (HPeV) are small RNA viruses, both within the family of *Picornaviridae*, causing frequently infections in the neonate (1).

Enteroviruses' capsid proteins (VP1, VP2, VP3, and VP4) determine the antigenicity and cellular penetration capacity of the virus and identify the serotype. *Enteroviruses* were traditionally divided into subgroups, based on their replication properties in cell cultures or animal models: they included *polioviruses* (PV), *coxsackie A* (CVA) *and B* (CVB), and *echoviruses* (ECHO for Enteric Cytopathogenic Human Orphan). The subsequently identified enteroviruses were defined by numbers (EV 68–71) (2). *Parechoviruses* were firstly labeled as *echoviruses* 22 and 23; now they have been reclassified and currently constitute HPeV genotypes 1 and 2. To date, 16 HPeV types have been identified (2). Enteroviral infections are transmitted by the fecal-oral and respiratory routes and mainly circulate in summer and autumn in temperate climates (3). Perinatal transmission is well-documented and occurs intrapartum (exposure to blood and/or maternal genital secretions) or postpartum (fecal-oral and/or respiratory samples). Intrauterine and ascending transmission are possible, but less common, and transmission of non-polio HEVs through breast milk has been hypothesized (4). The average incubation period is about 3–6 days for non-polio EVs (except for acute hemorrhagic conjunctivitis with 24–72 h), whereas the HPeV incubation period is unknown still now. The elimination of EVs and HPeVs persists for 1–3 weeks from the respiratory tract and for weeks or months in the stool; both can survive on environmental surfaces for several days (1).

Studies on neonates, especially concerning HPeV infections, are usually based on small case-series or case report, reflecting the lack of knowledge on their circulation in the neonatal age (5–10). In the United Kingdom, the combined incidence of EV and HPeV meningitis in neonates is 0.79/ 1,000 live births and 0.04/1000 live births, respectively, as recently estimated in an elegant study by Kadambari et al. (11). It appears more than double than bacterial meningitis and $<$ 1% of infected infants present complications or death attributable to the infection. The infection in neonates may present a wide range of severity: from pauci-symptomatic forms to meningitis with clear cerebrospinal fluid (11, 12), up to severe acute hepatitis with high mortality rates, especially in those born preterm (13).

The main risk factor for neonatal infection reported in the literature is the absence or the low titer of neutralizing antibodies in the mother. Risk factors for serious illness are maternal exposure, preterm birth and the onset of symptoms in early life (14). Severe illness can occur in infants infected with specific types of EVs (including echovirus 11 and *coxsackievirus B5*) and HPeV (such as *Human Parechovirus* 3), because their neurological tropism and virulence (15, 16).

Neonatal case series reported in the literature are scarce and knowledges on the long-term outcomes of enteroviral meningitis contracted during the neonatal age are poor (11, 17, 18).

We evaluated the neurological development of a group of children with documented *Enterovirus* and *Parechovirus* viral meningitis contracted within the first month of life. We aimed to determine any physical growth deficit, sensory defects, developmental, cognitive, prelinguistic and motor deficit during the 12-months follow-up of these babies.

METHODS

Population

During current clinical activity we prospectively collected all clinical and laboratory data relating to newborns hospitalized with defined enterovirus meningeal infection and followed up 12 months at the outpatient service of Bambino Gesù Children's Hospital (Rome, Italy) from 2015 to 2021. Data of patients were obtained from medical records.

According to our neonatal department protocols, all infants admitted to the Neonatal Intensive Care Unit (NICU) with fever undergo contact isolation and laboratory sepsis work-up: complete blood counts, blood chemistry tests, blood culture, urinalysis, urine culture, C-reactive protein (CRP), procalcitonin (PCT), rectal swab for multidrug-resistant bacteria, nasal swab for *Staphylococcus aureus*. The lumbar tap is routinely performed as part of the initial sepsis work-up in all febrile neonates and infants. We examine the cerebrospinal fluid (CSF) samples for routine cell counts and chemistry and culture. We also performed real-time polymerase chain reaction (RT-PCR) on CSF, to detect the genome of viruses that most frequently cause meningoencephalitis in the newborn, such as *herpes virus* 1–2–6, *cytomegalovirus, enterovirus and parechovirus.*

Definition of Viral Meningitis

Viral meningitis was defined on the basis of two or more of the following clinical signs (fever $>$ or $=$ 38°C, irritability, crying exhaustion, skin marbling, presence of skin rash, abdominal distension, diarrhea, poor feeding, bulging of anterior fontanel), associated with RT-PCR positivity of CSF and/or blood for RNA enteroviruses, and a CSF culture negative for bacteria. The clinical presentation of *parechovirus* central nervous system (CNS) infection is similar to that of enteroviral CNS infection, but with RT-PCR positivity of CSF and/or blood for *parechovirus.*

Although CSF pleocytosis is commonly absent in infants under 30 days of age in EV-positive infants (19), its presence may be observed in about half of cases of EV meningitis (20). Therefore, we also collected data on the CSF pleocytosis.

Virological Testing

Nucleic acids were extracted from 400 μl of CSF and plasma samples, using the automatic platform QIAsymphony (Qiagen) and the Virus/Pathogen Midi kit (Qiagen), according to manufacturer's instructions. RT-PCR was performed on 7,900 HT Real Time PCR System (Applied Biosystems) with Enterovirus R-gene Argene (Biomérieux) or Parechovirus R-gene (Biomérieux), targeting the highly conserved sequences of the terminal 5' non-coding region of Enteroviruses or Parechoviruses, according to manufacturer's instructions.

Enterovirus RNA positive samples, after the extraction step, were submitted to sequencing analysis of part of the VP1 genomic region, coding for one of the capsid proteins, according to WHO recommendations (21).

The targeted region of the viral RNA sequence was reverse transcribed to single stranded complementary DNA (cDNA) via primer extension with the Multiscribe Reverse Transcriptase enzyme (Thermofisher Scientific) on Gene Amp PCR System 9,700 (Applied Biosystem), using four different primers.

Amplifications were carried on with AmpliTaq GoldTM DNA Polymerases (Applied Biosystem) on Gene Amp PCR System 9,700, using two sets of primers.

Sequencing reaction was set on with AN89 and AN88 primers and the Big Dye terminator v3.1 ready reaction mix (Life Technologies). Sequencing analysis was carried on ABI PRISM 3130 XL Genetic Analyzer (Applied Biosystem) according to manufacturer's conditions.

The sequences obtained were then compared with the published prototype sequences to identify unknown enteroviruses through comparison of partial VP1 sequence data (22).

Management of Patients With Viral Meningitis During Hospitalization

In our Neonatal Intensive Care Unit all infants with meningitis of any etiology are monitored with amplitude-integrated electroencephalogram (aEEG) in the first 48–72 h of infection. Then the monitoring is suspended if the newborn improves and there are no suspected tracing anomalies. In case of suspected anomalies of the aEEG, or if the aEEG instrument is not available, an electroencephalogram (EEG) is performed, to rule out seizures. Immediately after carrying out the microbiological cultures, we start empiric antibiotics. If the diagnosis is of enteroviral meningitis, we suspend antibiotics and start immunoglobulins intravenously for three days, according to our local protocol. All of the infants described in this report followed this path of diagnosis and therapy.

Neuroimaging

In our Unit all neonates with enteroviral meningitis undergo brain ultrasound (US) during the hospitalization. In newborns enrolled in this study the MRI examination was performed only in 26/30 (86.7%) newborns, as the protocol for the management of viral meningitis has changed over time. In the past MRI on neonates with viral meningitis was performed as a second level exam, if the brain US examination was pathological. The introduction of the mattress technique for brain MRI currently makes it possible to extend the examination to newborns with normal US, to study them better. The mattress technique allows to avoid sevoflurane sedation, that we administer in order to acquire MRI images with a good quality, only in the case of the child's restlessness. To date we did not observe any side effects. For this procedure we always require the informed consent of the patients' parents. MRI exam is generally performed within 7–15 days after the onset of the infection by 3 T scanner (MAGNETOM Skyra, Siemens, Erlangen, Germany).

Follow-Up at 12 Months

After discharge from the NICU, children enter in an outpatient multidisciplinary follow-up, with quarterly clinical and instrumental assessments up to 12 months of age. All infants of this cohort were regularly evaluated to early intercept the presence of a neurological disability: the presence of a cerebral palsy (CP) was defined according to Bax et al. (23). To evaluate neurodevelopmental outcomes, we used the Bayley Development Scale for Toddlers and Infants Third Edition (BSDI-III, 2006) (24). This scale provided scores for three major development domains: cognition, language, and motor. Scores between 85 and 115 indicate normal development, while scores below 85 (-1 SD) indicate a developmental delay in the domain evaluated. The assessments were administered to patients by a developmental psychologist (S.B.), trained in BSID test procedures. The examiner was blinded to the patients' MRI findings and neonatal course. The psychologist assessed neurodevelopmental outcomes until 12 months. Children with scores within the normal range in all three domains were considered normal; children with a score below 85 (-1 SD) in at least one of the three domains were considered affected by a neurodevelopmental delay (25).

Visual function and retinal diseases were assessed by repeated examinations of the fundus and functional tests (electroretinogram–ERG—and visual evoked potentials –VEP—when necessary). Normal vision was defined as the "absence of any detectable pathology of the visual system"; mild abnormal vision as "the presence of a mild impairment which allowed useful vision", and severe visual impairment as "a child functionally blind or perceives light only" (26).

Hearing function was explored by brainstem auditory evoked potentials (BAEPs). Auditory global function was defined as normal in "absence of any detectable pathology", as mild if requiring hearing aids, or as severe if functionally deaf (uncorrected even with aids) (27).

Statistical Analysis

Data are shown as numbers and percentages for categorical variables, whereas continuous variables are expressed as mean ± standard deviation (SD). Pearson correlations were performed to investigate the relationship between the results of the Bayley III composite score (cognitive, language, and motor) and the clinical characteristics of the infants' diagnosis at admission.

TABLE 1 | Clinical characteristics and length of hospital stay of neonates with viral meningitis.

Clinical characteristics of enrolled children	*n* = 30
Gender (males) [*n* (%)]	17 (56.7)
Gestational age (week) (mean ± SD)	39.7 ± 0.8
Birth weight (g) (mean ± SD)	3457 ± 405
Cesarean section [*n* (%)]	10 (33.3)
Apgar 5' (mean ± SD)	10 ± 0.2
Age on NICU admission (days) (mean ± SD)	15.8 ± 7.33
Symptoms [*n* Yes (%)]	
Fever	30 (100)
Irritability	11 (36.7)
Poor feeding	7 (23.3)
Complaining behavior	7 (23.3)
Diarrhea	2 (6.7)
Rhinitis	2 (6.7)
Rush	1 (3.3)
Marbled skin	1 (3.3)
Exhaustible crying	1 (3.3)
Nuchal stiffness	1 (3.3)
Hyporeactivity	1 (3.3)
Length of hospital stay (mean ± SD)	10.41 ± 5.21

TABLE 2 | Characteristics of CSF in neonates with meningitis.

Chemical characteristics	n = 30
CSF appearance [*n* (%)]	
Clear	26 (86.7)
Almost clear	2 (6.7)
Opalescent	2 (6.7)
White blood cell count [n (%)]	
Normal	13 (43.3)
Abnormal with monocyte prevalence	7 (23.3)
Pathological	10 (33.3)
Protein level [*n* (%)]	
Normal	6 (20.0)
Pathological	24 (80.0)
Glucose levels [*n* (%)]	
Normal	16 (53.3)
Pathological	14 (46.7)

Normal values at our laboratory: CSF white blood cell count 0-3 cells/mm3; CSF protein level 58-150 mg/dl; glucose levels 32-82 mg/dl.

Neurodevelopmental assessment in children with normal and pathological MRI were compared using ANOVA. Multiple logistic regression analysis was used to determine the most important predictors of pathological MRI. The odds ratio and 95% confidence interval for each variable were calculated. A $p < 0.05$ was considered statistically significant. Statistical analysis was performed using software SPSS (version 20 for Windows).

RESULTS

Clinical Characteristics and Course

We longitudinally observed 30 children, full-term born, who contracted *enterovirus* and p*arechovirus* meningitis within the first month of life (mean age at diagnosis was 15.80 ± 7.33 days). Mean gestational age was 39.7 ± 0.8 weeks and mean birthweight was 3,457 ± 405 grams. Viral genome typing was performed on 15/30 (50.0%) neonates. *Echoviruses* (7, 9, 11, 18, 20 and 30) and *coxsackie v*iruses (A9 and B5) RNA were detected in 12/15 cerebrospinal fluid samples while *parechovirus* RNA on 3/15 samples. Two of those three babies with *parechovirus* meningitis showed abnormal brain MRI.

The **Table 1** shows main clinical characteristics of our population of neonates and their length of hospital stay.

The most frequent symptom at the onset of the infection (100% of neonates) was high fever, even up to 39.2°C, associated with marked irritability during examination procedures and poor feeding.

All infants underwent aEEG monitoring, without seizure finding. Seizures were also ruled out by EEG in a total of seventeen infants (54.8%) with dubious aEEG trace.

One of those children with a human herpesvirus 6 (HHV6) coinfection, had epileptic anomalies on the electroencephalographic trace, in particular low waves in the parieto-temporal site and a poor background organization. The diagnosis of epilepsy was never made by neurologists, the EEG trace progressively improved, and anticonvulsant therapy was never necessary. Currently this child is fine and presents a normal neurodevelopmental outcome. Due to the presence of HHV6 co-infection we started therapy with intravenous Ganciclovir and then with oral Valganciclovir.

In summary, 29/30 patients (96.7%) had EEG and/or aEEG trace with no signs of seizures.

Characteristics of the Cerebrospinal Fluid

In 55% of our infants we found an alteration of the CSF. Of these, 32% had frankly pathological liquor, with a predominance of mononuclear cells. The three infants with *parechovirus* meningitis had CSF with less than 5 white blood cells and normal levels of protein and glucose. Pleocytosis was not associated with a more serious course of acute disease nor with worse long-term outcomes. **Table 2** shows the CSF characteristics of our patients.

Biological Typing of Viruses

In all 30 enrolled patients we identified viral RNA. We were able to perform the genetic typing only on 15/30 (50.0%) CSF samples. In 9 of them we typed *echoviruses* (7,9,18,11,20,30): e*chovirus* 30 in three and *echovirus* 18 in two samples; the other virus typed were c*oxsackie* B5 in one patient's CSF and *coxsackie* A9 in two patients' CSF. Three infants had *parechovirus* meningitis (**Supplementary Table 1**). One infant with *enterovirus* meningitis had *HHV6* co-infection. The absence of viral DNA determined with the analysis of the child's hair bulb allowed us to exclude the chromosomal integration of the viral genome.

We did not isolate EV71, which seems to be the most neurotropic and the one most frequently involved in the genesis of brainstem encephalitis (28).

TABLE 3 | Logistic regression models of MRI results.

Predictor	eß (Odds ratio)	95% CI	*p*-value
Age at admission to the NICU (days)	0.788	0.634–0.978	0.031
Birth weight	1.003	0.999–1.006	0.106
CSF white blood cell count	1.001	0.995–1.007	0.725
CSF protein level	1.063	0.942–1.199	0.325
CSF glucose levels	1.144	0.945–1.384	0.168

In our patient with brainstem lesions, we isolated *echovirus* 30, but such lesions were considered secondary to perinatal asphyxia, according to the radiological characteristics and the history of the patient.

Neuroimaging

All 30 patients underwent brain ultrasound (US), while only 26 (86.7%) also performed brain MRI. The NICU protocol concerning viral meningitis has been modified over time. At the beginning the brain MRI was performed only in newborns with brain US abnormalities. Subsequently we decided to carry out the examination to all patients, having introduced the heated mattress technique, which avoids the sedation of the baby. We did not detect abnormalities in all newborns by brain ultrasounds. Conversely, we found non-specific brain abnormalities in 9/26 newborns (34.6%) by, which was performed on patients between 12 and 65 days of life (mean 38 ± 26 days). Among 9 neonates with MRI anomalies, dural contrast enhancement was found in 3/9 (33.3%) neonates (one of them with echovirus 20 infection); diffuse white matter alterations, probably due to delay of myelination, in 2/9 (22.2%) neonates (one had an infection by *echovirus* 7); cytotoxic oedema in 1/9 (11.1%) and periventricular microbleeds in 1/9 (11.1%) neonates (infected by *coxsackievirus* B5). Moreover, 1/9 (11.1%) neonate with *echovirus* 11 infection presented brainstem alterations and 1/9 (11.1%) with *echovirus* 30 showed mildly hyper-intense signals on T2-weighted images in the head of right caudate nucleus. Among the 17 patients with normal MRI one presented a subdural effusion, perhaps due to a difficult delivery, but the MRI was considered normal by neuro-radiologists. Analyzing data by logistic regression model (**Table 3**), pathological brain MRI was associated with age at admission to the NICU: the younger the infant, the greater the likelihood of brain MRI abnormalities (OR: 0.788; 95% CI: 0.634 −0.978; $p = 0.031$).

Follow-Up at 12 Months

During the clinical follow-up, children displayed normal weight growth rates. The neurodevelopmental assessment by Bayley-III is presented in **Table 4**. At 12 months of life we found that most babies (96.7%) had no sequelae on cognitive, language, and motor BSDI-III composite score results. None had a cerebral palsy. Only one child, with previous perinatal asphyxia, had a mild delay in fine and gross motor skills and in receptive language. All infants reported to have a normal vision and a normal hearing function.

Correlations between Bayley III composite score (cognitive, language, and motor) and the clinical characteristics of the infants' diagnosis at admission are reported in **Supplementary Table 2**.

TABLE 4 | Neurodevelopmental outcomes of 30 neonates at 12 months of age.

Neurodevelopmental assessment	*n* = 30
Bayley-III scales (mean ± SD)	
Cognitive	105.19 ± 8.71
Language	100.23 ± 8.22
Motor	97.00 ± 8.98
Abnormal cognitive results, *n* (%)	0 (0)
Abnormal language results, *n* (%)	1 (3.3)
Abnormal motor results, *n* (%)	1 (3.3)

TABLE 5 | Neurodevelopmental outcomes of 30 neonates at 12 months of age.

	Normal MRI	Pathological MRI	F	*p*-value
Bayley III cognitive scale	109.55 ± 8.20	100.56 ± 7.68	6.289	0.022
Bayley III language scale	102.18 ± 8.38	99.33 ± 9.69	0.497	0.490
Bayley III motor scale	100.64 ± 8.03	95.25 ± 10.90	1.548	0.230

Children with pathological MRI during hospitalization scored significantly lower than children with normal MRI on cognitive Bayley III subscale at Neurodevelopmental assessment at one year of age, even if all the scores obtained fell within the normal range ($F = 6.289$; $p = 0.022$) (**Table 5**).

DISCUSSION

Viral meningitis is very common within 90 days of life. The frequency of diagnosis has increased many times over the past 10 years, thanks to the spread of molecular biology techniques. This phenomenon has been observed in all European countries (11).

A proper and early detection of etiology of viral meningitis by molecular biology could help to identify biological types associated with more severe conditions and monitor their associated disease burden and allows to spare the improper use of antibiotics (29, 30).

As in neonates at term and in young infants enteroviral meningitis usually has a benign course, it is not thoroughly studied, mainly with regard to long-term outcomes. Data on children affected after the acute phase of the disease and hospital discharge are scarce. The British Pediatric Surveillance Unit reports that only 38% (254/668) of children with EV and 46% (16/35) of children with HPeV meningitis were seen by hospital clinicians within 12 months of discharge from the ward, for an evaluation of the infection outcomes (11).

In this case series of 30 neonates we have found that 9/26 (34.6%) children, who became infected soon after birth presented pathological brain MRI during hospitalization. The difference of 10-12 days in carrying out the MRI does not change the prognostic value of this examination in meningitis as lesions, after the acute phase of the inflammatory injury, do not regress completely and show the same signal characteristics.

These slight anomalies seem to be associated with a cognitive Bayley III score at 12 months, lower than in children with normal brain MRI, despite within the normal range. The linguistic and motor scores seem to be not affected. We found a mild delay in fine and gross motor skills and in receptive language only in one child, who suffered asphyxia at birth. It has been reported that *Enterovirus* encephalomyelitis has characteristic lesion located in the posterior portions of the brain stem, substantia nigra and dentate nucleus (31): in our cohort, only one of our patients presented brainstem alterations and at 2 years of age he still has still difficulties in language skills.

Kurz et al. described that some infants with *human parechovirus* (mainly genotype 3) could have a cerebral hemorrhage, but we did not find any hemorrhages; however, our HPeV infection cases have not been typed and it could be a milder genotype (32).

The available studies on neurological long-term outcomes in neonates with early meningeal enteroviral infection have been carried out on very small numbers of patients. The largest longitudinal follow-up study comes from the United Kingdom, carried out from July 2014 to July 2015 and included 668 cases in infants < 3 months old and followed up till 12 months of life (11). In this case series, no child showed sensorineural deficits, three patients presented seizures, one myocarditis and one hypotonia of the lower limbs, with an estimated risk of long-term sequelae of 0.6% (4/666, 95% CI, 0.2–1.5%).

A systematic review and meta-analysis about *parechovirus* CNS infection presented an increasing proportion of children with neurological sequalae over time (33): conclusions seem in line with those presented in this report.

Furthermore, Van Hinsbergh et al. recently highlighted the importance of follow-up of these infants to detect subtle neurodevelopmental delay and start early interventions (34).

Studies reporting outcomes after viral meningitis were markedly heterogeneous in age of infection, length of follow-up, inclusion of case controls, and outcomes measured, making it difficult to compare data (17).

Limitations of this study include the single-center site and the small sample size. However, we evaluated infants with viral meningitis in the first month using always the same neurodevelopmental test. We confirm that most infants with EV and HPeV meningitis don't go toward marked linguistic and motor deficits but have a lower cognitive score than children with no brain MRI abnormalities, despite within normal ranges.

CONCLUSIONS

Our study evidenced that early *enterovirus and parechovirus* infections can be associated with slight brain MRI abnormalities, more frequently the earlier the infection. Children with abnormal brain MRI during hospitalization may have long term cognitive Bayley III subscale score lower than children with normal brain MRI at the neurodevelopmental assessment. Despite the overall favorable outcome, children with *enterovirus and parechovirus* meningitis in the first month of life should undergo a multidisciplinary follow-up. It is important to carry out the early diagnosis of developmental defects in order to undertake early rehabilitation interventions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Scientific Directorate, Bambino Gesù Children's Hospital, Rome. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

SB, CA, and LM conceptualized and designed the study, designed the data collection instruments, collected data, contributed to the interpretation of the results, reviewed, and revised the manuscript. LC and LP performed virology testing and revised the manuscript. DD performed literature search, designed the data collection instruments, collected data, analyzed data, and drafted the initial manuscript. AS and MR followed up patients and revised the manuscript. FC and AD collected data and revised the manuscript. DL and GL interpreted brain MRI and revised the manuscript. CA conceptualized and designed the study, supervised data collection, contributed to the interpretation of the results, reviewed, and revised the manuscript. All authors approved the final manuscript as submitted and agree to be accountable for all aspects of the work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2022.881516/full#supplementary-material

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The reviewer AB declared past co-authorships with one of the authors AD and the absence of any ongoing collaboration with any of the authors to the handling editor.

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frontiers | Frontiers in Pediatrics

ORIGINAL RESEARCH
published: 06 July 2022
doi: 10.3389/fped.2022.909646

Universal Newborn Screening for Congenital Cytomegalovirus Infection – From Infant to Maternal Infection: A Prospective Multicenter Study

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Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *31 March 2022*
Accepted: *13 June 2022*
Published: *06 July 2022*

Citation:
Chiereghin A, Pavia C, Turello G, Borgatti EC, Baiesi Pillastrini F, Gabrielli L, Gibertoni D, Marsico C, De Paschale M, Manco MT, Ruscitto A, Pogliani L, Bellini M, Porta A, Parola L, Scarasciulli ML, Calvario A, Capozza M, Capretti MG, Laforgia N, Clerici P and Lazzarotto T (2022) Universal Newborn Screening for Congenital Cytomegalovirus Infection – From Infant to Maternal Infection: A Prospective Multicenter Study. Front. Pediatr. 10:909646. doi: 10.3389/fped.2022.909646

Introduction: Most infants at risk for cytomegalovirus (CMV)-associated sensorineural hearing loss (SNHL) are unrecognized because of the absence of a universal neonatal CMV screening. The search of CMV-DNA by molecular methods in salivary swabs was demonstrated to be a reliable approach. This study describes the results obtained by carrying out a universal screening for congenital CMV (cCMV) infection including all live-born newborns in three Italian sites, as well as the therapeutic interventions and clinical outcome of the CMV-infected neonates. Moreover, CMV maternal infection's characteristics were evaluated.

Methods: To confirm or exclude cCMV infection, a CMV-DNA-positive result on a first salivary swab was followed by repeated saliva and urine samples collected within 21 days of age. Breast milk samples were also collected. The search of CMV-DNA was performed with a single automated quantitative commercial real-time PCR assay, regardless of the type of samples used.

Results: A total of 3,151 newborns were enrolled; 21 (0.66%) of them were congenitally infected (median saliva viral load at screening, 6.65 [range, 5.03–7.17] $\log_{10}$ IU/ml). Very low/low viral load in screening saliva samples (median value, 1.87 [range, 1.14–2.59] $\log_{10}$ IU/ml) was associated with false-positive results ($n = 54$; 1.7%). CMV-DNA was detected in almost half of the breast milk samples of mother–infant pairs with a false-positive result, suggesting that contamination from breast milk may not be the only explanation in the study population. cCMV infection confirmation with the search of

CMV-DNA in a urine sample proved to be the gold standard strategy, since false-positive results were observed in 4/54 (7.5%) of the repeated saliva samples. Symptomatic cCMV infection was observed in 3/21 (14.3%) infants; notably, one (4.7%) developed moderate unilateral SNHL at 5 months after birth. Finally, two symptomatic cCMV infections were associated with primary maternal infection acquired in the first trimester of gestation; one newborn with severe cCMV symptoms was born to a mother with no CMV checkups in pregnancy.

Conclusion: Without universal neonatal CMV screening, some infected infants who develop late neurological sequelae may not be recognized and, consequently, they are not able to benefit early from instrumental and therapeutic interventions to limit and/or treat CMV disease.

Keywords: universal newborn screening, congenital CMV infection, CMV maternal infection, salivary swabs, false positive results, levels of CMV-DNA

INTRODUCTION

Congenital cytomegalovirus (cCMV) infection is a huge public health problem causing neurodevelopmental sequelae, including neurological disability and sensorineural hearing loss (SNHL) (1). The burden of CMV mother-to-child transmission is not completely realized since CMV-related clinical symptoms often do not manifest at birth (2). It has indeed been estimated that almost 10% of the asymptomatic congenitally CMV-infected neonates later develop hearing loss (3). Therefore, most infants at risk for CMV-associated SNHL are unrecognized because of the absence of a universal neonatal CMV screening (4) that could allow early detection of congenitally infected infants and consequently prompt interventions, improving the infants' clinical outcomes (5). Newborn screening for cCMV infection appeared to be cost-effective, as reported by Gantt and colleagues: they evaluated large prospective cohorts in the United States and reported that universal neonatal CMV screening generated larger net savings and the greatest opportunity to provide directed care (6). For large-scale universal neonatal cCMV screening, saliva samples obtained by buccal swabs seem to be an appropriate and non-invasive type of specimen to be analyzed by nucleic acid amplification test (NAAT), considering the high titers of CMV shed by congenitally infected newborns in the saliva and the easiness of specimen sampling (3, 7–10). However, a limitation of saliva samples is the possible contamination due to CMV-DNA present in the genital secretions in the birth canal or in milk from the last breastfeeding (11).

This prospective multicenter study aimed to assess the potential benefit of newborn screening for cCMV to early identify infected newborns and their clinical spectrum and analyze the association between neonatal CMV infection/disease and maternal CMV infection in pregnancy. Moreover, this study investigated if a viral DNA cutoff value in CMV-DNA-positive saliva samples collected within 21 days of life can discriminate a congenitally infected newborn from a non-congenitally infected newborn.

Finally, the study evaluated if a repeated saliva sample might replace the gold standard, represented by neonatal urine sample (11), as confirmatory testing for the diagnosis of cCMV infection.

METHODS

Study Design

Neonatal cCMV screening was offered at birth to all the live newborns born in the period between 12 February 2019 and 21 July 2020 in 3 Italian sites (i.e., IRCCS Azienda Ospedaliero-Universitaria di Bologna, ASST Ovest Milanese, Hospitals of Legnano and Magenta [Milan], and Azienda Ospedaliero-Universitaria di Bari). The study population included newborns whose parents/guardians agreed to screen for cCMV infection and gave written consent for the inclusion in the study.

The sample size estimation carried out in the study design phase assessed that 20 positive subjects were needed to achieve 0.80 power for a one-sample proportion test in which $p0 = 0.50$ was the null hypothesis of the no-discrimination curve and $p1 = 0.80$ was the expected area under the curve (AUC) of the molecular assay. After dividing this result by the expected cCMV prevalence in the population (0.64%) and accounting for an expected 1.3% of false-positive results (12–14), it was estimated that at least 3,125 subjects should have been recruited for this study.

The flowchart of the cCMV screening and the algorithm used for the interpretation of the molecular results is summarized in **Figure 1**. Briefly, infants with CMV-DNA-positive screening results on a first salivary swab were recalled within 21 days of age for both a repeated saliva sample and a urine sample to confirm or exclude cCMV infection. Furthermore, in order to identify in the saliva screening samples a potential CMV-DNA contamination derived from breastfeeding, a breast milk sample was collected from mothers of these infants.

Infants diagnosed with cCMV infection underwent clinical, laboratory, and instrumental evaluations during the first month of life to define infection as symptomatic or asymptomatic and were followed up at least for the first 12 months of life (15).

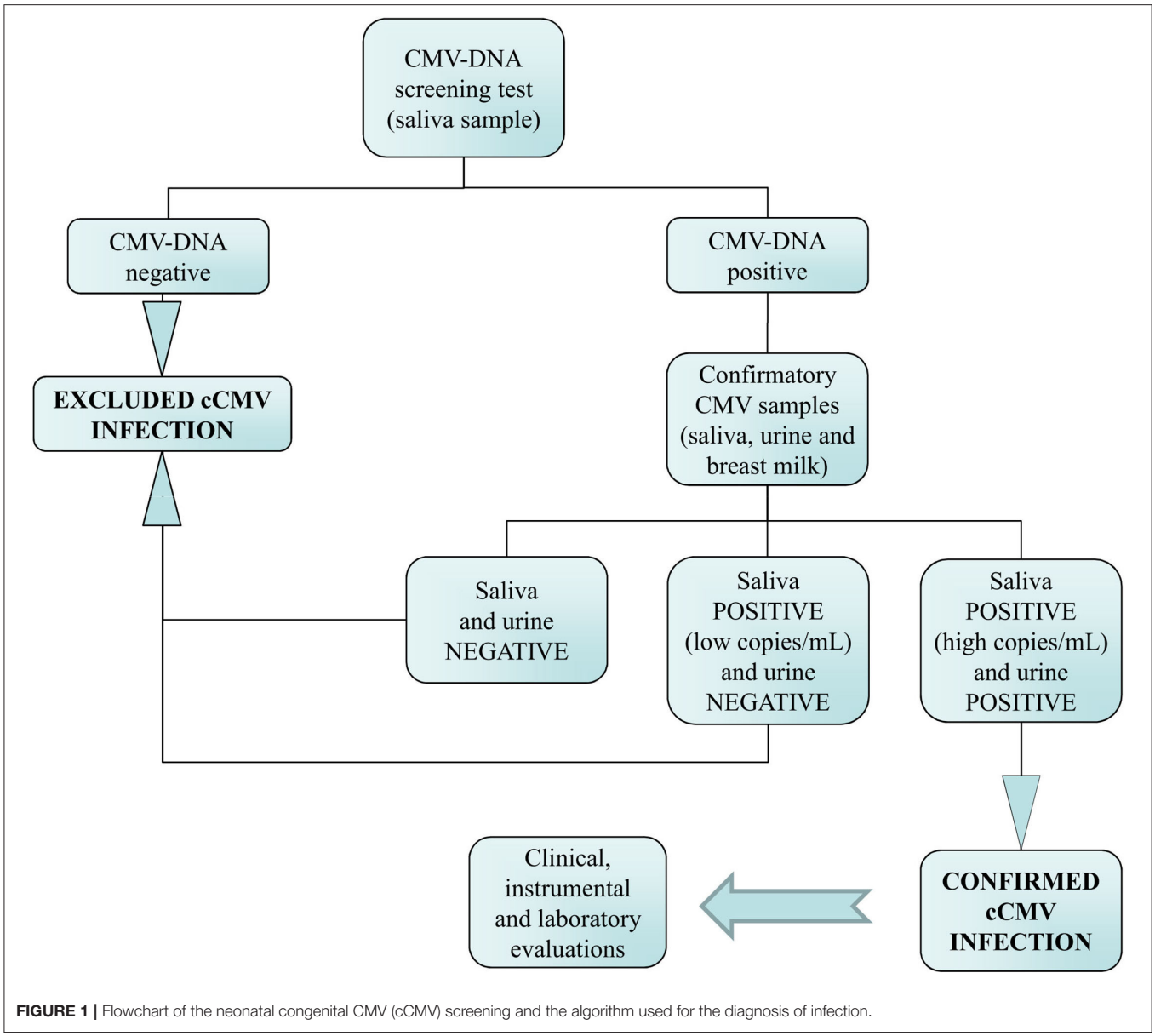

FIGURE 1 | Flowchart of the neonatal congenital CMV (cCMV) screening and the algorithm used for the diagnosis of infection.

Maternal CMV-serostatus before or during pregnancy was evaluated for all the neonates enrolled, i.e., values of anti-CMV immunoglobulin G (IgG) and anti-CMV immunoglobulin M (IgM) (positive/negative/equivocal) as well as anti-CMV IgG avidity indexes (low/moderate/high) were collected consulting patient's medical records. Serological data, routinely obtained at the three sites, i.e., using LIAISON® CMV IgG, IgM, and IgG Avidity II assays (DiaSorin S.p.A., Saluggia, Italy), were interpreted according to the manufacturer's instruction. Maternal primary and non-primary infections were defined, as previously reported (4).

The study was approved by all three centers' Ethics Committee (i.e., Comitato Etico Indipendente di Area Vasta Emilia Centro, Comitato Etico Milano Area 3, Comitato Etico indipendente, and AOU Policlinico di Bari). Parents or guardians provided written consent prior to the inclusion of the infants into the study.

Sample Collection and Storage

Saliva samples from neonates were collected by swabbing inside the mouth using a sterile flocked swab (FLOQSwabs®, Copan, Brescia, Italy); after collection, the swabs were immediately placed in the Universal Transport Medium (UTM®, Copan, Brescia, Italy).

Urine samples and breast milk samples were collected in a sterile container without a medium transport.

In the three centers, samples were investigated by using a single-automated quantitative commercial PCR assay (refer to below) within 48 h after collection; in addition, samples were consistently handled in terms of collection, transport, and storage

conditions in order to minimize the quantification variability due to the pre-analytical phase.

Molecular Assay

Saliva, urine, and breast milk samples were extracted, amplified, and quantified on the ELITeInGenius platform (ELITechGroup Molecular Diagnostics, Turin, Italy), a fully automated sample-to-result PCR system.

DNA was extracted from saliva, urine, and breast milk (200 µl of each body fluid eluted in 100 µl of elution buffer) using the ELITeInGenius total nucleic acid extraction kit (ELITechGroup Molecular Diagnostics, Turin, Italy) specifications with all parameters pre-programmed. CMV-DNA was detected and quantified with the real-time PCR assay CMV ELITe MGB kit (ElitechGroup Molecular Diagnostics, Turin, Italy), according to the manufacturer's package insert. Extraction, amplification, detection, and fully automated PCR analyses were performed on the ELITeIngenius System (ELITechGroup Molecular Diagnostics, Turin, Italy) in accordance with the manufacturer's specifications with onboard automation.

The viral load was reported as number of IU (International Unit)/ml for all body fluids examined. In association with the ELITeIngenius platform, the lower and upper limits of the detection of PCR assay for saliva samples were 44 IU/ml (220 gEq/ml) and 10^6 IU/ml (5×10^7 gEq/ml), respectively, the lower and upper limits for urine sample were 151 IU/ml (216 gEq/ml) and 3.5×10^7 IU/ml (5×10^7 gEq/ml), respectively, and the lower and upper limits for breast milk sample were 250 gEq/ml and 2.5×10^7gEq/ml, respectively.

Statistical Analysis

The study population characteristics were summarized using absolute frequencies and percentages and mean (standard deviation) for categorical and continuous variables, respectively. Variables representing elapsed time were summarized using median and range. Viral loads were transformed from IU/ml into $\log_{10}$ IU/ml to reduce data skewness. Comparisons between independent subgroups of samples, specifically false-positive vs. true-positive samples, were performed using the Mann–Whitney U-test; viral loads detected in the same patients at screening and confirmation tests were compared using the Wilcoxon matched-pairs test.

Statistical significance was set at $\alpha = 0.05$. G*Power version 3.1.9.2 was used for sample size estimation; Stata version 15.1, JASP version 0.16.0.0, and GraphPad Prism version 9 were used for other analyses.

RESULTS

Neonates Enrolled

During the study period, a total of 3,151 newborns were enrolled. All infants were born at a gestational age above 34 weeks, had no significant perinatal complications, and were enrolled mainly in the first 72 h of life. A small proportion of them (2%) required ventilation after birth with a bag-mask or Neopuff. None of the enrolled neonates required further steps of resuscitation, i.e., chest compression, endotracheal intubation, or drugs. Notably, 84 out of the 3,235 (2.6%) parents approached for this study refused the CMV newborn screening.

TABLE 1 | Baseline characteristics of the study population at the time of enrollment.

Sex, number (%)	
Female	1,536 (48.7)
Male	1,615 (51.3)
Age	2 (1.13)
Mean value in days (SD)	
Time elapsed from the saliva sample collection and the last breastfeeding (data were available for 73.2% [$n = 2,307$] of the study population)	
Median value in h (range)	2.0 (0–15.1*)
Number (%) of mothers who received tests for CMV-specific antibodies before or during pregnancy and classified on the base of serological results (data were available for 91.6% [$n = 2,887$] of the study population)	
Anti-CMV IgG positive and IgM negative**	2,014 (69.8)
Anti-CMV IgG negative and IgM negative	821 (28.4)
Anti-CMV IgG positive and IgM positive	52 (1.8)

*Breastfeeding with only breast milk was considered, and artificial feeding was not included. **Only this CMV-serostatus was considered if performed before pregnancy.*

The baseline characteristics of the newborns at the time of enrollment and the maternal serological results available before or during pregnancy are summarized in **Table 1**.

Regarding maternal CMV immunity, a total of 2,014 (69.8%) mothers were CMV IgG positive and CMV IgM negative, 821 (28.4%) mothers were CMV IgG and IgM negative, and 52 (1.8%) mothers were CMV IgG and IgM positive. Maternal serological status was significantly different in the three study centers (χ^2 test, $p < 0.001$), i.e., Bologna showed a higher proportion of women with CMV IgG- and IgM-negative results than Legnano-Magenta and Bari (33.2 vs. 25.7 vs. 22.2%, respectively) as well as a higher proportion of women with CMV IgG- and IgM-positive results than Legnano-Magenta and Bari (3.4 vs. 0.7 vs. 0.3%, respectively).

Congenital CMV Infection Screening Results

The first saliva specimen was collected from the infants at a mean age of 2.25 days (SD, 1.13). Among the 3,151 investigated screening specimens, 3,076 (97.6%) samples were found to be CMV-DNA negative, and the cCMV infection was excluded. The remaining 75 (2.3%) saliva samples resulted positive for the detection of CMV-DNA (median viral load, 1.87 $\log_{10}$ IU/ml; range, 1.87–7.17 $\log_{10}$ IU/ml) and underwent confirmatory testing.

The distribution of the CMV-DNA load in the saliva samples suggested that the 75 infants with positive results could be divided into 3 groups, namely, very low viral load group (i.e., viral load < 1.87 $\log_{10}$ IU/ml) consisting of 53 (70.7%) infants, low viral load group consisting of 1 (1.3%) infant with a CMV-DNA value of 2.59 $\log_{10}$ IU/ml, and high viral load group (i.e., viral load of at least 5.03 $\log_{10}$ IU/ml) consisting of the remaining 21 (28.0%) infants (**Figure 2**).

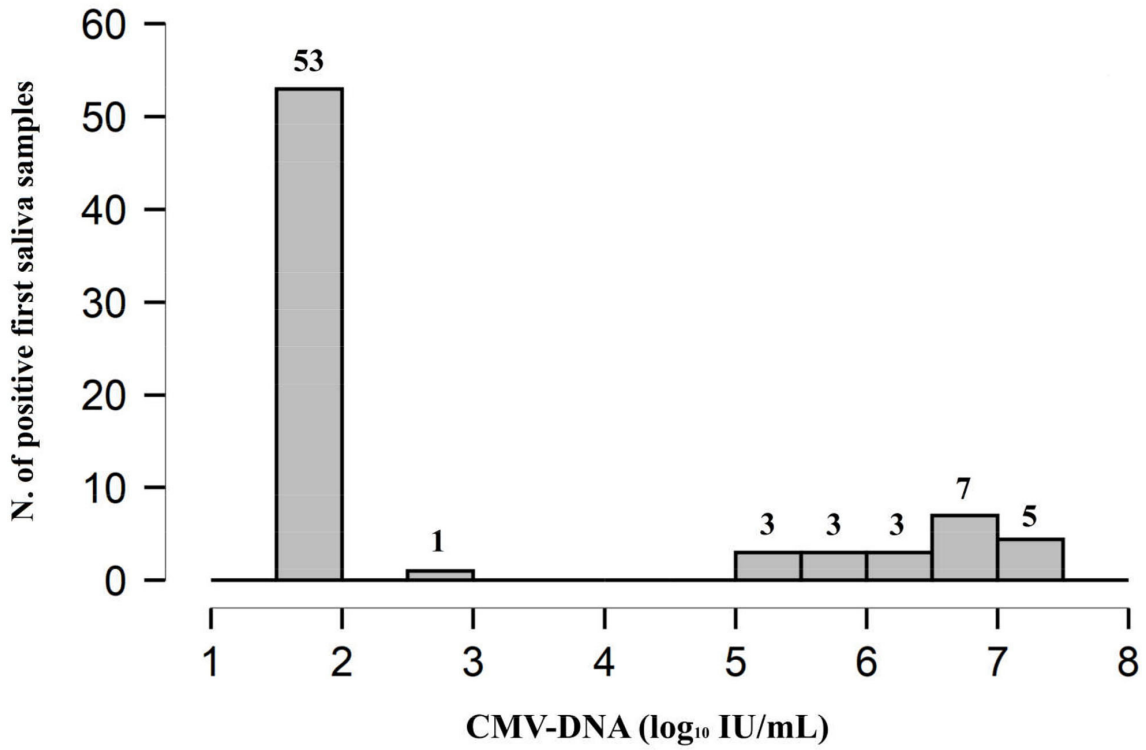

FIGURE 2 | Distribution plot of CMV-DNA load in the 75 (2.3%) positive saliva screening samples. The remaining 3,076 (97.6%) samples were found to be CMV-DNA negative at screening.

Congenital CMV Infection Confirmatory Results

According to protocol, in order to diagnose cCMV infection, a second saliva sample and a urine sample were collected within 21 days of age (median 3, range, 1–18) from all the 75 positive infants; the mean age of the infants at the time of collection was 7.2 days (SD, 4.8). The results obtained by investigating the samples for confirmatory diagnosis are reported in **Figure 3**.

The 53 CMV-positive infants with very low viral load along with the single infant with low viral load at the screening had a CMV-negative urine sample and, therefore, were not confirmed to be congenitally CMV-infected; these were identified as false-positive first saliva samples. The repeated saliva sample was CMV-DNA negative in 50 (92.6%; 50/54) patients, including the unique patient with low viral load in the first saliva sample; the remaining four samples (7.4%; 4/54) resulted low CMV-DNA positive. All the 21 CMV-positive infants with high viral load from screening testing were confirmed to have cCMV infection since high CMV-DNA levels were detected in the urine samples; these first saliva samples were identified as true positive. Of note, among these 21 infants, all the repeated saliva samples resulted CMV-DNA positive with high viral load (**Figure 3**).

The CMV-DNA loads detected in the screening saliva samples of the 21 confirmed congenitally CMV-infected infants (true positive) were higher than those detected in the 54 infants for whom the cCMV infection was excluded by confirmatory testing (false positive). The median CMV-DNA values were, respectively, 6.65 vs. 1.87 $\log_{10}$ IU/ml (Mann–Whitney U-test: $p < 0.001$; **Figure 4**).

Finally, the viral load of the 21 CMV-DNA saliva samples that resulted positive on both screening and confirmation testing was very similar at the screening and confirmation tests (median value, 6.33 vs. 6.29 $\log_{10}$ IU/ml; Wilcoxon matched-pairs test: $p = 0.373$).

Potential CMV-DNA Contamination Results

The time elapsed from the last breastfeeding and the collection of the saliva samples that resulted false positive (with very low/low CMV-DNA levels) at the time of screening was the same as that observed in the saliva samples that resulted CMV-DNA negative at this time point (i.e., median time in h, 2; Mann–Whitney U-test: $p = 0.527$); data were available for 64.8 and 73.5% of cases (35/54 samples and 2,262/3,076 samples, respectively).

By investigating the 39 (72.2%) available breast milk samples collected from the 54 mother–infant pairs with false-positive saliva results at the time of screening, 20 (51.3%) breast milk samples resulted CMV-DNA negative, and 19 (48.7%) samples resulted CMV-DNA positive (median viral load, 3.23 gEq/ml; range, 2.39–5.96 gEq/ml).

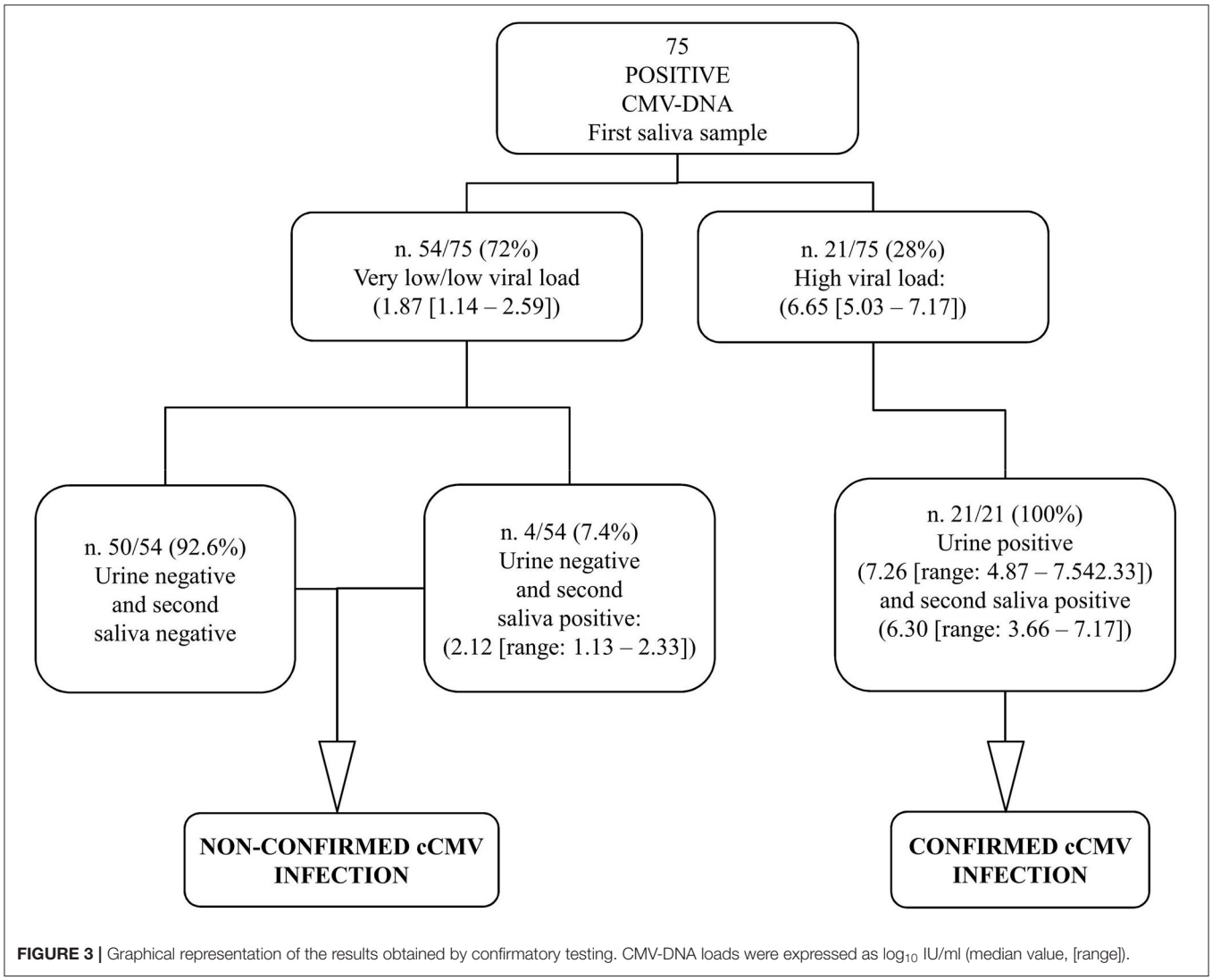

FIGURE 3 | Graphical representation of the results obtained by confirmatory testing. CMV-DNA loads were expressed as $\log_{10}$ IU/ml (median value, [range]).

Characteristics of Congenital and Maternal CMV Infection

The overall incidence of cCMV infection in the study population was 0.66% (21/3,151 infants). In particular, in Bologna, a higher incidence (1.08%) than in Legnano-Magenta (0.36%) and in Bari (0.24%) centers was observed (Fisher's exact test: $p = 0.049$).

Among the 21 congenitally infected infants, clinical and instrumental findings were consistent with cCMV infection for 2 (9.5%) infants who were classified as symptomatic at birth and underwent valganciclovir (VGCV) treatment for 6 months; the remaining 19 (90.5%) infants were asymptomatic. Among the 19 infants, 1 (5.3%) infant developed SNHL at 5 months of age and the remaining 18 (94.7%) infants remained asymptomatic during the follow-up period (median time, 365 [range, 365–429] days). Overall, the incidence of symptomatic cCMV infection was equal to 14.3% (three neonates).

The clinical characteristics of the 21 cCMV infections along with the type and timing of maternal CMV infections are reported in **Table 2**. Among the 21 cCMV-infected newborns' mothers, 9 (42.9%) mothers were CMV IgG and IgM positive, 7 (33.3%) mothers were CMV IgG positive and CMV IgM negative, and 2 (9.5%) mothers were CMV IgG- and IgM-negative (in both cases, a last serological testing was carried out at 27 weeks of gestation). The remaining 3 (14.3%) mothers did not receive any serological test for CMV before or during pregnancy. Available serological data allowed us to define maternal CMV infection as primary in 10 (47.6%) newborns; 50% of these were observed in the third trimester. Seven (33.3%) newborns were born from non-primary maternal infection and in 6 cases (85.7%), it was not possible to define the onset of maternal CMV infection during the gestation. In the remaining cases (4.8%), given that only a result of CMV IgG positive and IgM negative in 22 weeks of gestation was available, it was not possible to define the type of maternal CMV infection.

Finally, the proportion of maternal CMV-specific IgG- and IgM-positive results, evaluated separately, was compared between the neonates with and without cCMV infection; a

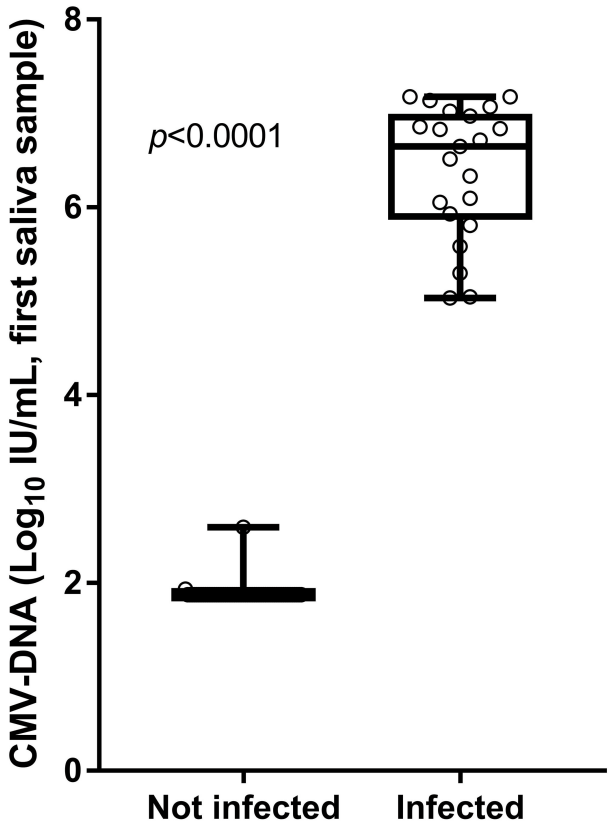

FIGURE 4 | Viral loads detected in screening saliva samples from infants with and without cCMV infection.

statistical significance was observed only for the IgM maternal positivity (**Table 3**).

DISCUSSION

The results obtained by carrying out a universal screening for cCMV infection of all live-born neonates in three different centers in Italy by using the saliva PCR assay, as well as the therapeutic interventions and the clinical outcome of the CMV-infected neonates were described; the characteristics of the maternal CMV infection were also evaluated.

A very high (97.4%) parental acceptability of newborn screening was observed. In the study population, the overall incidence of cCMV infection was equal to 0.66%, in line with literature data (12–14). Of note, a different incidence of cCMV infection among the three study centers was found, with the highest percentage (1.08%) observed in the Bologna one, reflecting the different distribution of maternal CMV immunity among the three centers (16). Particularly, CMV IgM-positive results were associated with a higher proportion of congenitally CMV-infected neonates than uninfected ($P < 0.001$), and the highest percentage of mothers with a positive value of anti-CMV IgM was observed in the Bologna center. This finding is likely because this center is a large national referral center for diagnosis and counseling of CMV infection during pregnancy as well as for the clinical management of congenitally infected newborns. Overall, maternal CMV immunity was known in a very high percentage of cases (91.6%).

At the time of screening, cCMV infection was excluded in almost all cases (97.62%) by means of PCR-negative first saliva sample result. The remaining 2.38% of cases underwent confirmatory investigations. In particular, all (100%) the neonates with a high viral load (at least 5.03 $\log_{10}$ IU/ml) in screening saliva samples were confirmed to be CMV congenitally infected by means of high levels of CMV-DNA detected in urine samples, as well as in the repeated saliva samples. In contrast, in all the neonates with low and very low viral load ($\leq$ 2.59 $\log_{10}$ IU/ml) in screening saliva sample, cCMV infection was excluded by means of PCR-negative urine sample. These findings confirm those suggested by other authors (16, 17) that the evaluation of viral load measured in the first saliva sample could be helpful to discriminate between true-positive and false-positive results. False-positive results were associated with low viral loads, whereas high DNA levels were found only in true-positive samples ($P < 0.001$). Specifically, by comparing the highest value of viral load found at the time of screening in the false-positive saliva samples (2.59 $\log_{10}$ IU/ml) with the lowest one found in the true-positive saliva samples (5.03 $\log_{10}$ IU/ml), a difference in CMV-DNA load equal to 2.44 $\log_{10}$ IU/ml was observed. Considering these results, it is reasonable to suggest that a saliva viral load of $\leq$ 2.59 $\log_{10}$ IU/ml may be indicative of a newborn without cCMV infection and is most likely a result of contamination; however, low viral load in neonatal saliva could potentially be observed in case of intrauterine CMV transmission at the end of the third trimester, as previously reported in neonatal urine by Exler et al. (18). In 7.4% of the infants with a CMV-DNA very low/low positive first saliva sample, the repeated saliva samples also resulted CMV-DNA positive with very low viral load (i.e., the maximum value of CMV-DNA detected was equal to 2.34 $\log_{10}$ IU/ml). These findings in agreement with recent literature (17–20) showed that a definitive diagnosis of cCMV, avoiding unnecessary tests and waste of resources, is to be confirmed by investigating urine sample that remains the gold standard for the diagnosis of cCMV infection. However, the collection of urine samples can be difficult and time-consuming compared to the saliva collection (8, 9). Therefore, saliva sampling in newborn screening programs is easier and more practical. In this study, in line with others (16, 20, 21), a low overall percentage (1.7%) of false-positive saliva screening samples was observed, confirming the suitability of this testing methodology. It is known that a potential CMV-DNA contamination may result from the breastfeeding (9, 22, 23). In this regard, the same median interval from the last breastfeeding and the screening salivary swab collection was observed in the false-positive samples and the true-negative samples, and CMV-DNA was detected in only almost half of the breast milk samples of mother–infant pairs with a false-positive result, suggesting that contamination from breast milk may not be the unique explanation in our study population. Of note, a large number of false-positive results at the time of screening were observed in the early period of the study (data not shown) and this led

TABLE 2 | Characteristics of the 21 neonates with cCMV infection: symptoms, antiviral therapy, and maternal CMV infection.

N. of infants	Symptoms at birth	Symptoms during the follow-up period	Clinical symptoms	Antiviral therapy administration	Maternal CMV serostatus		Weeks of gestation at the moment of execution of the first serological tests	Type of maternal infection	Onset of maternal CMV infection Trimester of pregnancy
					Anti-CMV IgG/ IgM	Anti-CMV IgG avidity index			
1	**YES**	NO (symptoms at birth persist)	Bilateral SNHL profound on the right moderate on the left	YES*	+/+	Low	10 weeks	Primary	I trimester
1	NO	**YES** At 5 months after birth	Moderate unilateral SNHL	NO	+/+	Low	11 weeks	Primary	I trimester
1	NO	NO	NO	NO	+/+	NM	24 weeks	Primary	II trimester
2	NO	NO	NO	NO	+/+	Low/moderate	26 weeks both	Primary	II trimester
1	NO	NO	NO	NO	+/+	NM	31 weeks	Primary	III trimester
2	NO	NO	NO	NO	+/+	Low	35 weeks both	Primary	III trimester
2	NO	NO	NO	NO	–/–	/	27 weeks both	Primary	III trimester
1	NO	NO	NO	NO	+/+	High	10 weeks	Non-primary	I trimester
6	NO	NO	NO	NO	+/–	NA	range, 9 – 25weeks	Non-primary	Undefined
1	NO	NO	NO	NO	+/–	NA	22 weeks	Active^ infection not defined	Undefined
2	NO	NO	NO	NO	NA				
1	**YES**	NO (symptoms at birth persist)	Profound unilateral SNHL CNS involvement	YES*	NA				

*+, positive; –, negative; SNHL, sensorineural hearing loss; CNS, central nervous system; NM, not measurable due to low anti-CMV IgG levels; NA, not available. *Standard dose, oral valganciclovir 16 mg/kg twice daily/6 months; ^Maternal positive CMV-DNAemia.*

TABLE 3 | CMV immunity assessed before or during pregnancy of the study participants' mothers.

Maternal CMV-specific antibodies	*N*. 2,869 NO cCMV infection *n* (%)	*N*. 18 YES cCMV infection *n* (%)	*p*-value*
IgG positive	2,055 (71.5)	16 (88.9)	0.121
IgM positive	43 (1.5)	9 (50.0)	**<0.001**

*Data were available for 85.7% (18/21) and 67% (2,869/3,130) of the infants with and without cCMV infection, respectively. *Fisher's exact test.*

to setting up a strict operational procedure, consistent between all the centers, to avoid any potential contamination during specimen collection.

In line with literature data (15, 24), most infected neonates were asymptomatic at birth and during follow-up. Two (9.5%) infants showed symptoms consistent with cCMV infection at birth and received a 6-month course of antiviral therapy, i.e., one case with severe disease (central nervous system involvement and profound unilateral SNHL) and one case with isolated bilateral SNHL (moderate on one side and profound on the other side). These symptomatic newborns at birth would have also been identified by a CMV-targeted screening, since both of them would have failed the universal newborn hearing screening. One asymptomatic neonate developed moderate unilateral SNHL at 5 months of age. Considering both early- and late-onset symptoms, the percentage of symptomatic CMV infection in the study population was 14.3%.

Of note, 10 out of the 21 (47.6%) congenitally infected infants would not have undergone early virological, instrumental, and clinical evaluation for cCMV infection. Of note, in a phase II international, multi-center, double-blind, placebo-controlled trial of 6 weeks of oral VGCV or 6 weeks of placebo for infants/toddlers with cCMV infection and hearing loss for 1 month through 3 years of age that aimed to assess whether the 6-week course of oral VGCV could stabilize the hearing function (ClinicalTrials.gov Identifier: NCT01649869), no difference in the placebo and treatment groups in terms of the hearing outcome was observed, suggesting that a delayed diagnosis of cCMV after the neonatal period is a missed opportunity for antiviral treatment, which has been demonstrated to confer some benefits in terms of hearing function and neurodevelopmental outcome only if started in the first month of life (25). Due to the increase in the workload of health workers and the expense of molecular testing for CMV of neonatal screening programs, national studies are needed to evaluate the feasibility and cost-effectiveness of an Italian universal newborn CMV screening. By analyzing the outcome of the neonatal infection in relation to the type of CMV maternal infection (the information was available in 80.9% of cases), similar to Faure-Bardon and colleagues (26), it was observed that the two cases of primary maternal infection in the first trimester were both associated with cCMV symptomatic infection; the remaining symptomatic neonate was born to a mother with no CMV-checkups in pregnancy. cCMV infections were a result of non-primary maternal CMV infection in approximately one-quarter of the defined cases and were all asymptomatic. Whether the risk of symptomatic cCMV infection, especially that resulting in hearing loss, differs following primary or non-primary maternal infection has been a matter of debate in the past years. Nevertheless, recent evidence suggests that there is no difference in the risk of hearing loss according to the type of maternal CMV infection (16, 27–29).

The strengths of this study were the large prospective sample size evaluated, the adoption of a single-automated molecular method and a single experimental approach both common to all the study centers, and the timing of the collection of the cCMV infection confirmatory samples that occurred in all cases and for all types of samples inside the timeframe recommended of 21 days of age (11) as well as the maternal CMV immunity that was known in almost all cases of study participants and the availability of all the clinical information about the congenitally infected neonates identified during the CMV screening. The limitations are that the type of maternal infection was unknown in 19.0% of the cCMV infections, including one symptomatic case, and the number of cCMV-infected newborns was too small for evaluating the risk of hearing loss according to the type of maternal CMV infection.

In conclusion, in our setting of neonatal CMV screening, the percentage of false-positive results in saliva samples was low. In particular, low positivity for CMV-DNA in saliva samples was associated with false-positive results. This finding could be communicated to parents by limiting their stress and anxiety while waiting for the confirmatory diagnostics. Screening of cCMV infection by saliva molecular testing and subsequent confirmation with the search of CMV-DNA in a urine sample, rather than in a repeated saliva sample, proved to be the gold standard strategy. Finally, despite universal neonatal screening for CMV is not currently recommended by any public health body (4) and infected neonates are, therefore, identified only because of suspected maternal infection during pregnancy or symptoms and signs associated with cCMV at birth, our findings confirmed literature data reporting that without neonatal screening, some infected infants at risk to develop neurological sequelae may not be recognized earlier.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Indipendente di Area Vasta Emilia Centro, Comitato Etico Milano Area 3, Comitato Etico indipendente, AOU Policlinico di Bari. Written informed

consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ACh collected and analyzed the data and wrote, reviewed, and edited the manuscript. CP, MS, and ACa performed the analyses and collected and analyzed the data. GT participated in analyzing the data and contributed to writing the first draft of some sections of the manuscript. EB, LG, and MD participated in collecting and analyzing the data. DG performed formal analysis. FB and MM performed analyses and participated in collecting results. CM, AR, LPo, MB, AP, LPa, MCapo, MCapr, and NL clinically managed the infants and provided related information. PC supervised the research and contributed to the data analysis. TL conceptualized and supervised the research, analyzed the data, and reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by ELITechGroup Molecular Diagnostics, Turin, Italy.

ACKNOWLEDGMENTS

The authors would like to thank Dr. Cristina Olivo (ELITechGroup Molecular Diagnostics, Turin, Italy) for the support and the coordination of the three study sites and the Linguistic Consultant, Lucy Scioscia, for editing the English language text.

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frontiers | Frontiers in Pediatrics

REVIEW
published: 06 July 2022
doi: 10.3389/fped.2022.894573

Congenital Toxoplasmosis: The State of the Art

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Infection with the protozoan parasite *Toxoplasma gondii* occurs worldwide and usually causes no symptoms. However, a primary infection of pregnant women, may infect the fetus by transplacental transmission. The risk of mother-to-child transmission depends on week of pregnancy at the time of maternal infection: it is low in the first trimester, may reach 90% in the last days of pregnancy. Inversely, however, fetal disease is more severe when infection occurs early in pregnancy than later. Systematic serologic testing in pregnant women who have no antibodies at the beginning of pregnancy, can accurately reveal active maternal infection. Therefore, the risk of fetal infection should be assessed and preventive treatment with spiramycin must be introduced as soon as possible to reduce the risk of mother-to-child transmission, and the severity of fetal infection. When maternal infection is confirmed, prenatal diagnosis with Polymerase Chain Reaction (PCR) on amniotic fluid is recommended. If fetal infection is certain, the maternal treatment is changed to a combination of pyrimethamine-sulfonamide and folinic acid. Congenitally infected newborns are usually asymptomatic at birth, but at risk for tardive sequelae, such as blindness. When congenital infection is evident, disease include retinochoroiditis, cerebral calcifications, hydrocephalus, neurocognitive impairment. The diagnosis of congenital infection must be confirmed at birth and management, specific therapy, and follow-up with multidisciplinary counseling, must be guaranteed.

Keywords: congenital infections, *Toxoplasma gondii*, chorioretinitis, diagnosis, follow-up, pregnancy, neonate

OPEN ACCESS

Edited by:
Offer Erez,
Ha'Emek Medical Center, Israel

Reviewed by:
Drucilla Jane Roberts,
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Specialty section:
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

Received: *11 March 2022*
Accepted: *20 May 2022*
Published: *06 July 2022*

Citation:
Bollani L, Auriti C, Achille C, Garofoli F, De Rose DU, Meroni V, Salvatori G and Tzialla C (2022) Congenital Toxoplasmosis: The State of the Art. Front. Pediatr. 10:894573. doi: 10.3389/fped.2022.894573

INTRODUCTION

Toxoplasmosis is a systemic and cosmopolitan disease affecting about one third of the world population. The causative agent is *Toxoplasma gondii*, an obligate intracellular protozoan parasite, whose replication occurs in the intestine of cats and other felines, the only definitive hosts. Warm-blooded animals and humans are intermediate hosts.

Different strains have been identified, three main designated as type I, II, and III and other atypical, which differ in virulence and epidemiological pattern of occurrence. In Europe, 95% of human infecting *T.gondii* are type II, whereas in North America type II represents 43.9%, type III accounted for 18.2% and atypical strains accounted for the rest (1, 2). A recent study on genetic analyses of atypical strains revealed that a fourth clonal lineage (type 12 lineage) it is the dominant strain in wildlife of North America and accounts for the 46.7% of the isolated strains (3). The parasite genotype may play a role in determining the severity of disease: in South America the strains show greater genetic variability and are usually much more virulent (4, 5).

The infection is acquired mainly through the ingestion of raw or undercooked meat containing still viable cysts, through the ingestion of water, fruit, vegetables, shellfish, or by contact with earth contaminated by oocysts excreted in the feces of infected cats (**Figure 1**). *T. gondii* can also be transmitted *via* blood or leukocytes from immunocompetent and immunocompromised donors. The parasite persists lifelong as cysts in intermediate host (6).

With few exceptions, the acute phase in the immunocompetent adult is usually a subclinical or benign disease. In a minority there may be malaise, low-grade fever, and lymphadenopathy and chorioretinitis.

Primary infection induces the production of specific antibodies and lifelong immunity, but toxoplasmosis can reactivate in immunocompromised individuals (e.g., AIDS or treatment with corticosteroids).

In humans, the seroprevalence of *T. gondii* antibodies increases with age and varies considerably according to geographical location, health education, hygiene, food habits and climatic conditions, it decreased in the last decade due to a greater intake of frozen meat, better hygiene, progressive urbanization (7).

In Europe the IgG seroprevalence ranges from 30 to 50%, and in United States is about 9.1% of women of childbearing age. In South America, the prevalence varies from 30 to 80%, reaching 100% in the most advanced age groups of the poorest populations (8, 9). The overall global prevalence of acute infection in pregnant women is 1.1% but is higher in Eastern-Mediterranean region than in European region (7). The global IgM e IgG seroprevalence in pregnant women is 1.9 and 32.9%, respectively, with statistically significant differences between WHO regions (10).

Congenital toxoplasmosis occurs when maternal infection is acquired for the first time in pregnancy. During the phase of parasitemia, *T. gondii* may cross the placenta and enters the fetal circulation with a risk of fetal infection that increases with gestational age: this results in congenital infection affecting 25–30% of women treated during pregnancy (11). At 6, 18- and 30-weeks' gestation of pregnancy, the risk of fetal infection is 2.2, 23, and 56%, respectively (12).

Incidence of acute infection among *T. gondii* seronegative pregnant women varies by geographic location; it is estimated in Austria, Sweden, France, and United States to be 0.8, 0.5, 2.1, and 0.2 per 1,000, respectively (8, 13, 14). The global incidence rate of congenital infection is estimated to be 1.5 cases/1,000 live births with higher burdens in South America, in some Middle Eastern countries and low-income countries and lower burdens in European countries (15). In particular incidence of congenital toxoplasmosis is 1.0/10,000 in Austria, 0.5/10,000 in United States and 2.9/10,000 live births in France (8, 13, 14).

The likelihood of fetal infection is low in the early pregnancy but increases in later stages. The chance of transmission increases by 12% per week of maternal gestation starting at 13 weeks of gestation (16, 17). The protective role of the placenta is more effective in the first trimester, allowing to the passage of parasites in less than 10% of cases. With the increasing of the vascularity, the placental barrier becomes more and more permeable, leading to parasite transmission in around 30% of cases in the second trimester, in 60–70% of cases in the third trimester, even more in the last weeks of gestation (18).

The mechanisms of *T.gondii* vertical transmission remain unclear, in particular the role of placenta-derived cytokines or chemokines. The syncytiotrophoblast has distinct resistance to T. *gondii* infection, at the level of attachment and post-entry replication, while cytotrophoblasts and extravillous trophoblasts seem not displaying the same kind of resistance, suggesting cell-type specific differences in mechanisms of resistance. In addition, trophoblasts respond to T. *gondii* infection through the specific induction of various cytokines and chemokines, including the robust induction of the regulatory T cell chemokine (19, 20).

The severity of fetal infection decreases with the increase of gestational age. During the first and second trimester, the infection may lead to miscarriage (around 3% of all cases) or still birth. Infected infants frequently show severe symptoms of congenital toxoplasmosis, with neurological disorders and ocular lesions, while in the latest phase of pregnancy the neonatal disease may be less severe or asymptomatic.

The higher risk of early and long term (within 3 years) clinical signs occurs in women who seroconverted between 24 and 30 weeks of gestation (about 10%); for infections occurring in the second and third trimester, the minimum risk is not less than 5% (12, 21). Therefore, dating maternal infection during pregnancy is of great relevance to establish the extent of fetal risks.

In this narrative literature review we provide an updated overview on diagnosis, therapy, and follow-up of toxoplasmosis in pregnancy and neonatal age.

METHODS

We searched PubMed[1] for cohort, cross-sectional and case-control studies, reviews, expert consensus as well as case series or case reports published as articles or letters to the editor describing neonates with congenital toxoplasmosis. An extensive literature search has been performed up to 6 March 2022. The following keywords "Congenital Toxoplasmosis" AND "neonate" OR "infant" were searched as entry terms. We excluded all retrieved articles written in non-English language. Additional studies were identified by authors based on their knowledge on the field, if not already included by literature search.

NEONATAL CLINICAL MANIFESTATIONS

In neonatal age, congenital toxoplasmosis is asymptomatic in 85% of cases. Infected newborn appears normal at the clinical examination, but he is at risk of developing ocular lesions later in life. In such a situation, it is hard to make a diagnosis without information on maternal serologic profile.

The classic triad described by Wolf in 1939 (hydrocephalus, intracranial calcifications, and chorioretinitis) is observed very rarely in present times. Neonatal manifestations, if present, may

[1]https://pubmed.ncbi.nlm.nih.gov/

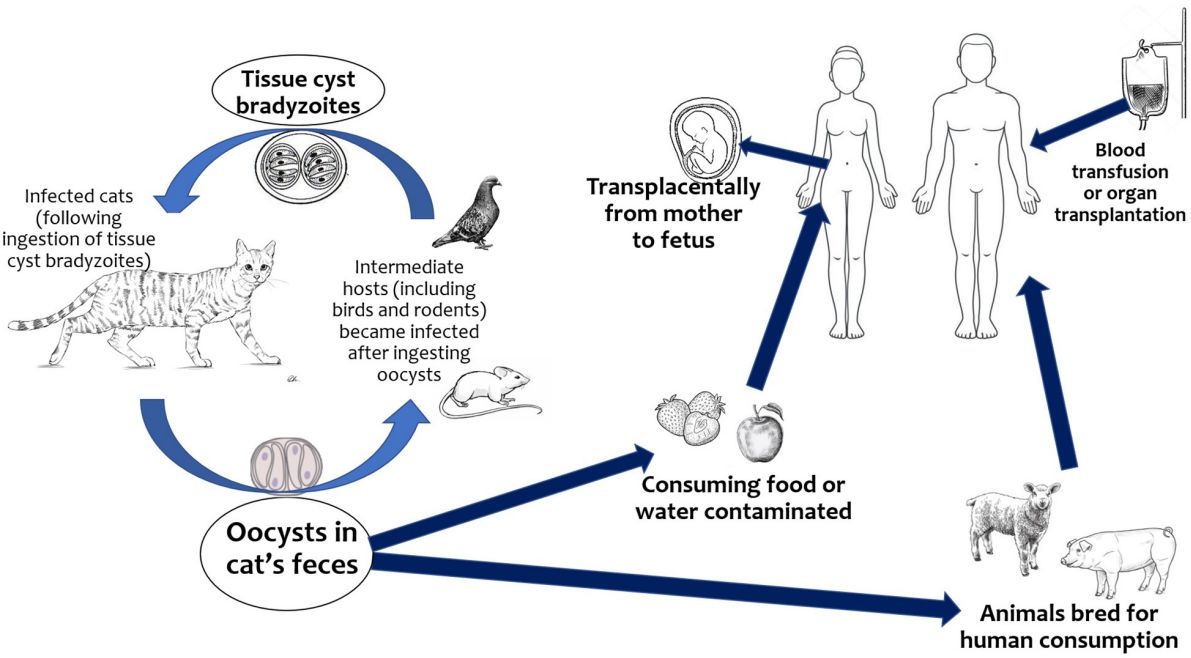

FIGURE 1 | Life cycle of *Toxoplasma gondii*.

include hydrocephalus, microcephaly, intracranial calcifications, chorioretinitis, cataracts, convulsions, nystagmus, jaundice, petechiae, anemia, enlarged liver, and spleen, prematurity and severe intrauterine growth restriction with abnormally low birth weight (14, 21, 22) (**Table 1**). However, none of these symptoms is pathognomonic for toxoplasmosis and may suggest other congenital infections (CMV, Herpes simplex, rubella, syphilis) (6).

The prevalence and severity of principal signs of disease are significantly different in the United States, France, other Western European countries, Israel, and South America (23–30). The reported rate of severe congenital toxoplasmosis is higher in the United States and South America in comparison to European countries due to different and more virulent *T. gondii* strains implicated and in the absence of antepartum treatment in the United States (1, 14, 31–34). In Israel the proportion of severe disease is higher than in Europe probably because of the lack of systematic prenatal screening and treatment but is lower in comparison to United States (30).

A systematic review of cohort studies by SYROCOT (Systematic Review on Congenital Toxoplasmosis study group) shows that, during the first year of life, 19% of infected infants developed at least one of two clinical manifestations: 14% had ocular lesions and 9% had intracranial lesions or both (26).

Among ocular manifestations, the most frequent is chorioretinitis and the retinal lesions are usually in the posterior pole (22, 35). Most frequently occurs after a reactivation of the infection and in cases where the macula is involved there may be a loss of visual function (22, 36, 37). Worsening of central vision, because of macula involvement, may recover after resolution of the inflammation (22). Chorioretinitis is commonly recurrent and relapsing, but these episodes are rarely associated with systemic signs or symptoms (22). For any additional week of gestation, in case of maternal primary infection, the risk of chorioretinitis decreased by 3% but increases by 2.1 times when maternal primary infection occurred before 20 weeks of gestation and by 3.6 times in infants with additional clinical manifestations at birth (38).

Other ocular disorders, reported apart from recurrent focal chorioretinitis, that can contribute to visual impairment are strabismus, microphthalmia, cataract, retinal detachment, optic nerve atrophy, iridocyclitis, nystagmus, and glaucoma (22, 36). Some of these manifestations develop as a consequence of

TABLE 1 | Clinical features reported to be associated with congenital toxoplasmosis (14, 21, 22).

Systemic signs	Preterm birth*, small for gestational age*, rash* (petechial, blueberry muffin), sepsis like illness*, hepato/splenomegaly*, myocarditis*, hepatitis*, hepatic calcifications* jaundice, temperature instability, pneumonitis, lymphadenopathy
Laboratory abnormalities	Anemia*, thrombocytopenia*, CSF abnormalities like pleocytosis, elevated protein, eosinophilia, hypoglycorrhachia* increased level of liver enzymes or bilirubin level
Neurological signs	Macro or microcephaly*, hydrocephalus*, hypotonia*, palsies*, seizures*, psychomotor retardation*, spasticity*, SNHL*, intracranial calcifications*
Ocular signs	Amblyopia*, cataract*, chorioretinitis*, nystagmus*, optic nerve atrophy*, strabismus*, retinal scarring*, visual impairment*, microphthalmia, microcornea

CSF, cerebrospinal fluid; SNHL, sensorineural hearing loss. Symptoms considered to be more common are indicated by an asterisk.

retinal lesions or can be related to neurological involvement like hydrocephalus (36). Indeed, is reported that infants with severe ocular manifestations also present with severe cerebral damage (35).

The ocular manifestations in congenital infected infants in Brazil are more severe than in the United States and Europe (24, 35, 39). Brazilian infants developed chorioretinitis more frequently and the lesions are multiple, larger, and more likely located in the posterior pole than the European infants (24). Several studies suggest that the marked difference in prevalence and severity of ocular involvement in Brazil is due to different prevention protocols and infection with atypical *T. gondii* strains more virulent which are predominate in Brazil but are rarely found in other countries (24, 40–42).

Involvement of the central nervous system is demonstrated by calcifications that follow the phenomena of vasculitis and necrosis and affect mainly the periaqueductal and periventricular regions. Sometimes the hydrocephalus may be the only manifestation of congenital toxoplasmosis; observational data show a frequency of 31% when the mother did not receive any therapy, compared to 0.8% in the newborns of treated women (16). In the SYROCORT study the risk of intracranial manifestations was higher in Brazilian and Colombian infants in comparison to other countries (26).

DIAGNOSIS OF TOXOPLASMOSIS IN PREGNANCY

In pregnant women, when present, symptoms of toxoplasmosis are mild and non-specific (asthenia, low-grade fever, myalgia, and usually laterocervical lymphadenopathy), therefore, the diagnosis relies only on serological tests; in immunocompetent subjects, IgG, IgM, IgA, and IgE antibodies can be detected just after two weeks from the infection.

There is no consensus about serological screening for *T. gondii* IgG and IgM antibodies in pregnant women (5). It is rare in United States (8, 14), not currently recommended in Canada (43) but mandatory in France and Austria at the first trimester prenatal visit (13, 44, 45). The prenatal screening allows to identify anti-*toxoplasma* IgG seronegative women at risk of acquiring the infection in which the primary prevention is mandatory (5, 13, 44, 45).

Serum conversion is the most accurate marker of maternal infection (6). Positive IgG and negative IgM antibodies in the first and second trimester suggest that the infection has been acquired before the current pregnancy. When the first analysis is done during the third trimester, a negative IgM result cannot rule out an infection acquired in early pregnancy, since IgM could have become undetectable in a short time. Negative IgM rules out infection in the first two trimesters, but positive IgM is not always a certain marker of recent infection because IgM can persist beyond to years or can be non-specific IgM (6, 46, 47) (**Table 2**).

Reference laboratories use second level tests: IgG avidity test, and immunoblotting test essential tools to help to date the infection (6, 48, 49). A high IgG avidity index suggests that the infection was contracted at least 16 weeks before; a low or intermediate avidity index is considered markers of recent/acute infection (50, 51). Furthermore, avidity grows more slowly if pregnant woman is subjected to a specific therapy, since treatment could delay IgG appearance and reduce avidity maturation (48).

The immunoblotting test for IgG and IgM could be used to evaluate IgM specificity and allows an earlier identification of specific IgG (6).

The monthly screening during pregnancy, until delivery, allows a timely diagnosis of the maternal infection, so as to start an early specific treatment, in an attempt to prevent transmission, or to reduce the risk of serious injury.

Prenatal Microbiology Diagnosis

PCR techniques for detection of *T. gondii* DNA in amniotic fluid has revolutionized prenatal diagnosis of congenital toxoplasmosis. First, it makes possible an early diagnosis since PCR has a specificity and positive predictive value of 100%, furthermore, it avoids more invasive procedures on the fetus (17, 47).

However, amniocentesis should be performed only after the 18th week of pregnancy, four weeks after the estimated date of infection in a pregnant woman (17). A negative result does not fully exclude the presence of congenital toxoplasmosis, since negative predictive value is 98,1%; the rare false-negative antenatal diagnoses could be due to delayed transplacental transmission of parasites after amniocentesis or to very low parasite densities in amniotic fluid (52, 53). The risk of procedure-related fetal loss (or preterm delivery in more advanced gestation) is estimated to be less than 0.1% (54).

Early diagnosis of maternal infection plays a key role in clinical counseling, in assessing fetal risk and treatment options, and in planning prenatal diagnosis.

Treatment of *Toxoplasma gondii* Infection During Pregnancy

There are many discrepancies in studies published between 1999 and 2006 on the efficacy of prenatal treatment in reducing the incidence and severity of congenital toxoplasmosis (55, 56). Since 2007, observational studies have evidenced a greater efficacy of the therapy when it is undertaken as soon as possible (ideally within 3 weeks from seroconversion) in order to prevent the transmission of the parasite to the fetus and reduce the risk and severity of fetal infection (**Figure 2**) (13, 26, 57–59).

In primary maternal *Toxoplasma* infection, acquired during the first 18 weeks of gestation, it is recommended the treatment with spiramycin, a macrolide that reaches significant placental concentration, and can reduce the frequency of vertical transmission, but is not effective for the treatment of fetal infection (60). A first randomized clinical trial describes a lower placental transmission rate by the association of pyrimethamine and sulfadiazine with folinic acid versus spiramycin, but enrollment was discontinued because of low enrollment and lack of additional funding (61). The combination of spyramicine and trimethoprim-sulfamethoxazole can cross the placenta and kill

TABLE 2 | Results of serological tests during pregnancy and their interpretation.

Scenario	IgG antibodies	IgM antibodies	Interpretation	Comment
1	Negative	Negative	Absence of immunity	Monthly serologic follow-up and 1 month after delivery
2	Positive	Negative	Infection acquired before pregnancy	- Repeat tests after 1 month to confirm previous infection - Stop follow-up if only IgG antibodies are positive
3	Negative	Positive	Initial seroconversion or IgM falsely positive	- Repeat test weekly - Second level tests - Eventual prenatal diagnosis - Neonatal follow-up
4	Positive	Positive	Acute infection or Persistence of IgM	- Date infection - Second level tests - Eventual prenatal diagnosis - Neonatal follow-up

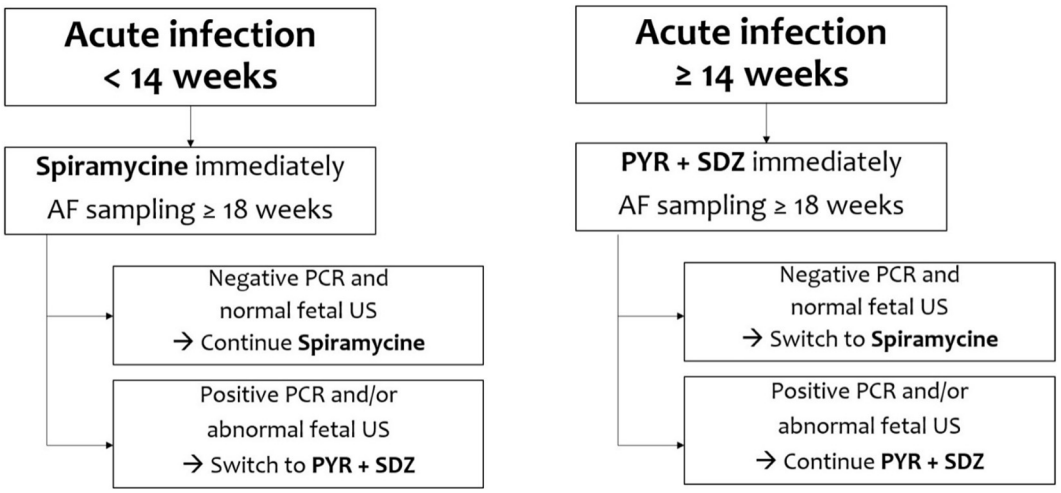

FIGURE 2 | Pharmacological prevention of mother-to-child transmission.

parasites in fetal tissues, therefore it seems to be more effective in reducing the risk of maternal-fetal transmission of *T. gondii* than spyramicine alone (62). When PCR is positive due to an infection acquired after the 18 weeks of gestation, the current gold standard is the association of pyrimethamine, sulfonamides and folinic acid (**Table 3**). This treatment cannot be used before 14 weeks of gestation for the potential risks of teratogenicity (17).

TABLE 3 | Pyrimethamine–sulfonamides combinations for mothers.

Anti-*Toxoplasma* drug	Regimen use
Pyrimethamine	1 Tablet of 50 mg daily
Sulfadiazine	3 Tablets of 500 mg twice daily
Folinic acid	2 Capsules of 25 mg per week
OR	
Sulfadoxine–pyrimethamine combination	Capsules equivalent to Fansidar (500 mg/25 mg) must be prepared: 2 capsules per week
Folinic acid	2 Capsules of 25 mg per week

Adapted from Treatment Recommendations of a French Multidisciplinary Working Group by Peyron et al. (17).

In case of a primary maternal *Toxoplasma* infection, to exclude fetal abnormalities, a monthly ultrasonographic monitoring is recommended until term. When amniocentesis is positive, ultrasounds must be checked every 2 weeks to monitor the brain anatomy of fetus. The main ultrasound findings associated with congenital toxoplasmosis are ventriculomegaly and intracranial calcifications. Prognosis of isolated fetal parenchymal cerebral lesions without ventriculomegaly were not related to neurological damage, instead hydrocephalus is associated with adverse neurological sequelae. The interaction between physicians and families is always important in managing a pregnancy complicated by fetal infection, termination of pregnancy is to be discouraged unless, in the opinion of experts, there is evidence of serious sequelae affecting the fetus (17, 63).

MANAGEMENT OF NEONATES AT BIRTH

All neonates at risk of congenital toxoplasmosis (proven maternal infection, with or without prenatal diagnosis) must perform a complete clinical and neurological check-up at birth, specific seroimmunological tests, direct and indirect

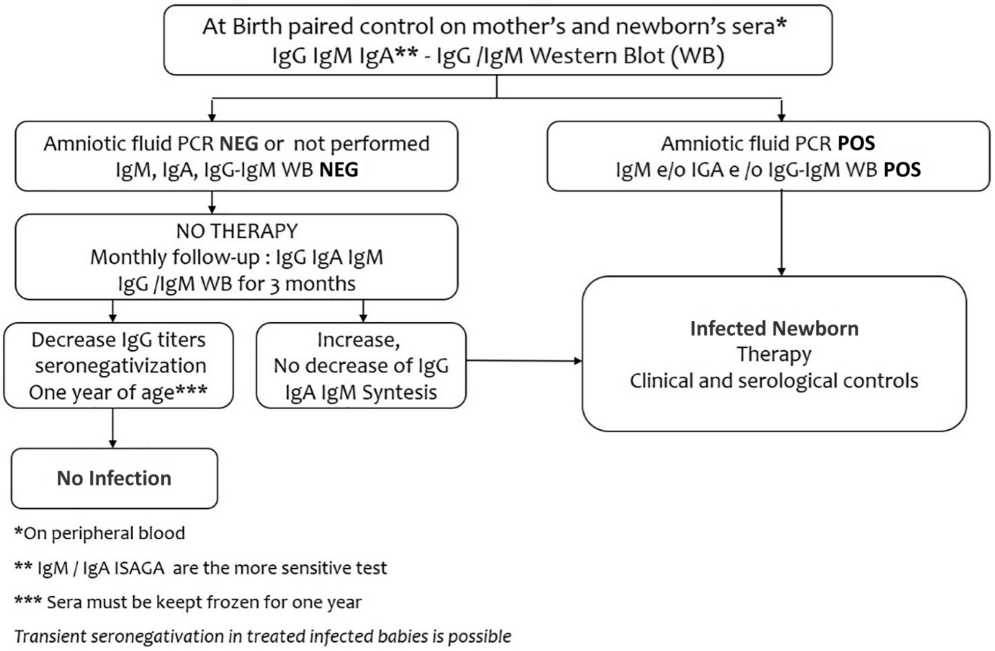

FIGURE 3 | Serological screening of congenital toxoplasmosis.

dilated fundoscopy (to exclude chorioretinitis, or associated ophthalmological pathologies), and transfontanellar ultrasound examination (to rule out any ventricular dilatation, cerebral calcifications, porencephaly) (64).

Hepatic and cardiac ultrasound, brain computed tomography (CT) or magnetic resonance imaging (MRI) and electroencephalographic monitoring (EEG), are helpful when clinical and neurological symptoms are severe.

Postnatal Diagnosis

IgM and IgA anti-Toxoplasma antibodies by Enzyme-Linked Immunosorbent Assay method (ELISA) or Immunosorbent Agglutination Assay (ISAGA) on a peripheral neonatal blood sample, represent the best markers of congenital infection, since these specific antibodies are produced by the neonate and cannot cross the placental barrier (**Figure 3**). With traditional tests for IgM and IgA antibodies, it is possible to diagnose at birth only 75% of infected newborns (6). IgM ISAGA is the more sensitive and specific test for the detection of Toxoplasma IgM (49). The absence of specific IgM and IgA antibodies does not exclude the infection since they may be not produced by congenitally infected infant in the first month of life (47). Furthermore, when maternal infection occurs in the late pregnancy, testing at birth may be falsely negative (17).

The IgG antibodies cross the placenta, so they are not a marker of congenital infection. Maternal anti-Toxoplasma IgG decline to disappear within 6–12 months. The persistence of IgG antibodies up to one year or their increase in the first months, lead to the diagnosis of congenital toxoplasmosis, whereas their negativity at one year, in subjects who did not receive any therapy, excludes the infection (65).

In newborns at risk of congenital toxoplasmosis, with positive IgG and negative IgM and IgA tests, a comparative mother-infant Western blot (WB) allows the early detection of synthesized neonatal antibodies, which have different antigenic specificity from maternal ones (66). Western blot has an excellent specificity (97–100%) and, in addition to traditional tests, will identify up to 96–98% of infected newborns. However, after the third month of life, the test becomes non-specific (67).

In infected infants, Interferon γ release assay (IGRA) may be used to evidence lymphocytes activation and secretion of interferon γ, following *in vitro* stimulation with *T. gondii* antigens (68).

If at birth there are insufficient data for the diagnosis, a definitive response can now be obtained much faster than in the past since confirmatory testing can be validated by reference laboratories within the first 60-90 days of life in almost all cases since Western blotting has been shown to establish diagnosis up to 3 months earlier than conventional serological methods (67, 69).

Treatment of Infected Neonates

The efficacy of therapeutic protocols does not have the support of randomized controlled trials. Observational data describe that congenital toxoplasmosis has a good outcome and results in a normal neurological development when the treatment begins as soon as possible, both in pregnant mother and in newborn. Accordingly, delaying the therapy and/or neglecting subclinical infection increase the risk of serious disabilities (70, 71).

Synergistic effect of the combination therapy with pyrimethamine and sulfonamides against experimental toxoplasmosis, was observed in mice in the early 1950s. Studies performed decades ago provided the basis for the current recommendation for the combination of pyrimethamine with sulfadiazine or sulfadoxine and folinic acid as first-line treatment of toxoplasmosis in humans and, even today, it remains the gold standard.

Pyrimethamine and sulfonamide, because of their action on folate synthesis, act synergistically against *T. gondii*. Both drugs reduce the growth of the rapidly proliferating tachyzoites and prevent their transformation into new cysts, which are unsensitive to this treatment (72). Pyrimethamine is absorbed slowly but completely in the gastrointestinal tract. The serum half-life in the newborn is about 60 hours, and in cerebrospinal fluid reaches a concentration of about 10–20% of serum levels (17). Sulfadiazine seems to be the most active sulfonamide; its plasma half-life of 12–19 h makes it preferable to other sulfonamide and its concentration in the cerebrospinal fluid reaches 50% of the plasma concentration. It is excreted by the kidney, and its poor solubility can give crystalluria, which can be avoided with good patient hydration. The sulfadoxine, half-life of 120–195 h, allows for a simpler administration scheme (17).

There is no clear evidence about the comparative efficacy of the different postnatal treatment protocols, applied by different centers (14, 47, 60, 73). The French ones, brought together in a multidisciplinary team, have provided state-of-the-art care and an algorithm that optimizes outcomes for those suffering from this infection (17). Another protocol, adopted in several European countries, refers to the one published by Rima Mc Leod with a higher or a lower dose of pyrimethamine according to the severity of disease at birth (71).

Treatment should be continued for at least one year since a shorter therapy can lead to severe disabilities (60). Before starting the therapy, a G6PD deficiency must be ruled out, and throughout the whole treatment patients must be monitored clinically and serologically, to check the efficacy of therapy and the possible occurrence of side reactions (72).

Hematological adverse events may affect up to 30% of newborns. Use of antifolates and sulfonamides may result in a gradual bone marrow depression, more frequent in the first two months of life and mainly consistent in a reversible neutropenia. Folinic acid must always be associated for prevention and reduction of the hematological toxicities of the drugs. Sometimes anemia and thrombocytopenia are also present. The blood of patients must be therefore monitored, initially every 15 days, and later once a month. When neutrophils are $<800/mm^3$, the therapy must be temporarily interrupted and resumed with the rising of white blood cells (17). No long-term hematological toxicity or late onset malignancies have been found. Gastrointestinal symptoms such as vomiting, diarrhea and lack of appetite are also occasionally described (74). A small percentage of studies (75) report dermatologic adverse events, including rash. Sulfonamide intolerance causes severe skin manifestations, such as Steven Johnson syndrome, and the treatment must be stopped immediately and permanently (17).

Azithromycin has good tissue and intracellular concentration and demonstrated *in vivo* activity against *T. gondii*. However, we have few data on its use in congenital infection. Again, there are no data in infants for therapy with Macrolides, Clindamycin, Atovaquone and immunotherapy (17, 60, 76).

Finally, we must obviously stress that breastfeeding and vaccination are strongly recommended.

OCULAR TOXOPLASMOSIS

In congenital toxoplasmosis, chorioretinitis may be present even at birth, with chorioretinal scars or focal necrotizing retinitis. It may also appear unpredictably during childhood or adolescence, as a new manifestation or as a reactivation of a previous lesion (37). The symptoms are scotoma, pain, photophobia, blurred vision, and excessive watering of the eye (22).

A delay between maternal seroconversion and the beginning of treatment, and especially the presence of cerebral calcifications, are risk factors of retinochoroiditis during the first 2 years of life (77). In a prospective cohort study on 3 years old children with congenital toxoplasmosis, more than 90% of children with chorioretinitis had normal vision in the best eye, and only 9% had severe bilateral impairment (78).

In the active form, the specific therapy (Pyrimethamine + Sulfadiazine + folinic acid) must be applied for at least 1–2 weeks after resolution of clinical signs, the longest treatment being about 3 months. The efficacy of steroids in ocular toxoplasmosis has not been clearly demonstrated. Currently, they are used in association with the standard therapy only in severe inflammation, or when the lesions are close to the fovea or the optic disk. Corticosteroid therapy, without antiparasitic treatment, may result in large retinal lesions (60, 79).

A recent meta-analysis indicated that the combination of trimethoprim-sulfamethoxazole could be an alternative treatment (80). A long-term, intermittent regimen of this combination can be used in an effort to reduce the recurrence of chorioretinitis (81).

FOLLOW-UP

The follow-up of the newborn with congenital infection is mainly dedicated to the ophthalmological, neurological, auditory and serological aspects.

Ophthalmological Follow-Up

Since the diagnosis of ocular toxoplasmosis remains fundamentally clinical, it is crucial to continue a long-term follow-up in all congenital patients. The risk of ocular affections persists throughout the whole life, even in treated children. In a follow-up with a median of 10.5 years, 29% of infected children born from treated mothers developed at least a new ocular lesion after the first one (82). It is therefore strongly recommended to plan a fundus check, performed by direct ophthalmoscopy, every three months in the first year of life, every six months in the second year and every further year, without age limits.

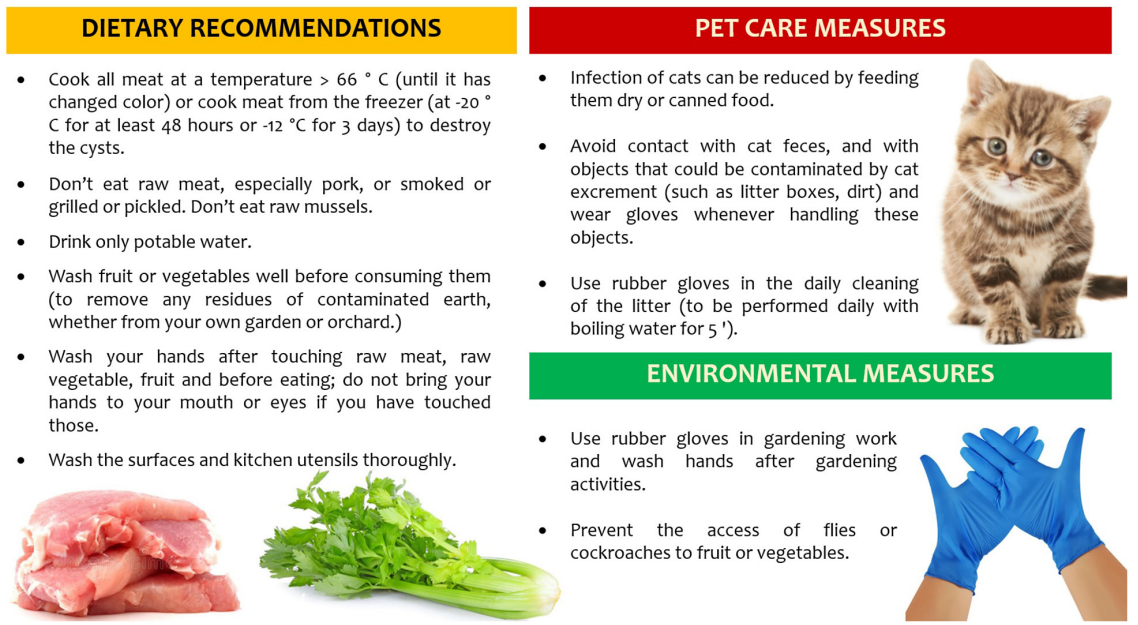

FIGURE 4 | Recommendations to prevent *Toxoplasma* transmission during pregnancy.

When checks are carefully performed and the pathology properly treated, the overall prognosis is satisfactory, and the consequences are rarely severe. The impact of retinochoroiditis and of associated eye pathologies neither reduce the visual performance of affected patients nor compromise the long-term quality of life (83).

Neurological Follow-Up

A full evaluation of the neurobehavioral outcome of the newborn should be done in the first years of life. The predisposing factors for neurological anomalies are a lacked prenatal therapy and the presence at birth of chorioretinitis, possibly already accompanied by severe neurological signs, such as hydrocephalus, convulsions, muscle tone abnormalities. A transfontanellar ultrasound follow-up is recommended, looking for cerebral calcifications and ventricular dilatation.

Without cerebral lesions, there will be no neurological sequelae. In case of isolated calcifications, neurodevelopmental outcome is normal in most children and the calcifications may resolve during therapy (84). Indeed, the National Collaborative Chicago Based Congenital Toxoplasmosis reports that even in severe conditions, extremely rare at birth, a timely prenatal therapy, continued throughout the first year of life, led to a remarkable resolution of the neurological abnormalities (25).

As to the correlation between congenital toxoplasmosis and sensorineural deafness, data are very reassuring, since no infant treated for 12 months with an early specific therapy had sensorineural severe deficits. Accordingly, no association between *T. gondii* infection in pregnancy and hearing loss in offspring is recorded (85, 86). However, correlations between congenital toxoplasmosis and a high prevalence of hearing problems and language delays have been described in Brazil (Congenital Toxoplasmosis Brazilian Group) where the disease is more frequent and severe than in Europe (87).

The long-term impact of congenital toxoplasmosis on life quality and visual performance, was good in most of a cohort of adult individuals, who had been treated in pre-postnatal period. No limitations in cognitive functions were present, and the school level is not affected by this pathology (82).

Serological Follow-Up

When diagnosis is not defined in the prenatal period (if performed) or at birth, and newborn is at risk of congenital infection, a serological follow-up must go on without starting any treatment that may mask the serology.

Serological test must be performed monthly in the first 3 months and after every 2 months, until one year of age. The absence of a congenital infection is defined by the negativization of specific antibodies within the first year of life, in absence of therapy. On the contrary, the increase of IgG or the appearance of IgM and/or IgA antibodies define the congenital infection (18).

When the maternal infection dates in the last weeks of pregnancy, the serology of the newborn at birth can be falsely negative. It is therefore recommended a monthly serological follow up without starting any treatment (18). A transient negativization of the IgG frequently happens during therapy for congenital infection. This however must not lead either to a treatment suspension or to question the diagnosis (17).

After the end of a full year of therapy, the IgG antibody titer may recover in 70–97% of cases. This antibody rebound is considered an antigenic re-expression that occurs when the drug pressure is over; if ophthalmological surveillance is warranted, prosecution of the treatment does not seem necessary (88, 89).

PREVENTION OF PRIMARY *T. GONDII* INFECTION IN PREGNANCY

The knowledge about *T. gondii* life cycle is crucial to reduce exposure to major risk factors, such as raw or undercooked meats (especially lamb and pork), poor hand hygiene and improper cleaning of cooking utensils, consumption of unfiltered water, gardening and other soil contact, exposure to cat litter and travels to high-incidence countries (90). Furthermore, the vaccination of food animals, the decontamination of all meats and products destined for undercooked consumption, and the vaccination of cats are ongoing intervention strategies to reduce *T. gondii* disease burden, across the general population.

However, special considerations must apply to all pregnant women, where health education approaches are mainly aimed at the prevention of congenital toxoplasmosis. Dietary recommendations and behavioral factors, including pet care and environmental measures, can help to reduce the risk of acquiring toxoplasmosis in pregnant women or women of childbearing age (91). Randomized controlled trials have shown little evidence that prenatal education has a positive effect in reducing seroconversion for toxoplasmosis in pregnancy (92). A recent systematic review on hygiene measures as primary prevention of toxoplasmosis during pregnancy suggests the efficacy of health education on *toxoplasma*-related knowledge, behavior, and risk of seroconversion in pregnancy (93).

Health care professionals must be aware of the risk factors and of the recommendations to give to seronegative pregnant women at the beginning of pregnancy (**Figure 4**): the knowledge of these preventive measures will have a positive impact.

CONCLUSION

The correct hygienic-sanitary education of the seronegative pregnant woman (primary prevention), the systematic serological screening in pregnancy (secondary prevention) allowing early diagnosis and therapy of pregnant mother, the treatment of congenital infection and the follow-up of the newborn, have proved to be the cornerstones for addressing a public health problem such as congenital toxoplasmosis.

The development of new techniques for diagnosis of maternal infection, prenatal diagnosis and a therapeutic approach to limit vertical transmission and fetal injury of toxoplasmosis, can all concur for positive outcomes regarding health and quality of life.

Remarkable differences exist in public-health policies; research should be performed to assess the burden of congenital toxoplasmosis and the cost of care of the disease in each country (45). Furthermore, there is a need for implemented information programs and homogeneous guidelines, and more reference centers for the management of maternal or congenital infection, and for the post-natal follow-up of neonates with suspected congenital toxoplasmosis.

Despite great advances in basic clinical and scientific research, many questions remain to be addressed. We stress the necessity of less expensive serological tests, new drugs with less toxicity, more efficacy (allowing for a shorter treatment cycle), and able to eliminate resistant cysts (94, 95). Furthermore, improved pediatric formulations for established drugs would be useful (72).

Toxoplasmosis is still among the main infections that can be transmitted from mother to child and, when untreated, may have serious consequences; therefore, we must alert the health policy makers, not to make congenital toxoplasmosis a neglected disease.

AUTHOR CONTRIBUTIONS

LB and CT performed literature search and wrote the first draft of manuscript. CAu, CAc, FG, DD, and GS critically revised the manuscript. All authors approved the submitted and final versions of the manuscript.

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frontiers | Frontiers in Pediatrics

TYPE Original Research
PUBLISHED 19 July 2022
DOI 10.3389/fped.2022.885926

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

RECEIVED 28 February 2022
ACCEPTED 28 June 2022
PUBLISHED 19 July 2022

CITATION
Scaramuzzino F, Di Pastena M, Chiurchiu S, Romani L, De Luca M, Lucignani G, Amodio D, Seccia A, Marsella P, Grimaldi Capitello T, Longo D, Palma P, Lancella L, Bernardi S, Rossi P and Calo Carducci FI (2022) Secondary cytomegalovirus infections: How much do we still not know? Comparison of children with symptomatic congenital cytomegalovirus born to mothers with primary and secondary infection. *Front. Pediatr.* 10:885926. doi: 10.3389/fped.2022.885926

Secondary cytomegalovirus infections: How much do we still not know? Comparison of children with symptomatic congenital cytomegalovirus born to mothers with primary and secondary infection

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Congenital cytomegalovirus (cCMV) infection can follow primary and secondary maternal infection. Growing evidence indicate that secondary maternal infections contribute to a much greater proportion of symptomatic cCMV than was previously thought. We performed a monocentric retrospective study of babies with cCMV evaluated from August 2004 to February 2021; we compared data of symptomatic children born to mothers with primary or secondary infection, both at birth and during follow up. Among the 145 babies with available data about maternal infection, 53 were classified as having symptomatic cCMV and were included in the study: 40 babies were born to mothers with primary infection and 13 babies were born to mothers with secondary infection. Analyzing data at birth, we found no statistical differences in the rate of clinical findings in the two groups, except for unilateral sensorineural hearing loss (SNHL) which was significantly more frequent in patients born to mother with secondary infection than in those born to mother with primary infection (46.2 vs. 17.5%, $P = 0.037$). During follow up, we found a higher rate of many sequelae (tetraparesis, epilepsy, motor and speech delay, and unilateral SNHL) in the group of children born to mothers with secondary infection, with a statistical difference for tetraparesis and unilateral SNHL. Otherwise, only children born to mothers with

primary infection presented bilateral SNHL both at birth and follow up. Our data suggest that the risk of symptomatic cCMV and long-term sequelae is similar in children born to mother with primary and secondary CMV infection; it is important to pay appropriate attention to seropositive mothers in order to prevent reinfection and to detect and possibly treat infected babies.

KEYWORDS

congenital cytomegalovirus infection, CMV, secondary infection, pregnancy, symptoms, sequelae

Introduction

Congenital cytomegalovirus (cCMV) is the most common congenital infection affecting 0.5–2% of all live births, the leading non-genetic cause of SNHL, and a major cause of neurological disability (1).

Intrauterine transmission of CMV can follow primary or secondary maternal infection; the latter condition can be the result of either reactivation of latent CMV infection or reinfection with a different CMV strain during pregnancy (2).

This feature explains why the rate of cCMV infections increases with the seroprevalence of maternal populations, ranging from 0.3% in populations with 30% seroprevalence to approximately 2% in populations with 98% seroprevalence (3).

Transmission rates after primary maternal infection increase during pregnancy from 20–30% in the first trimester to 70% in the third trimester (4). Transmission rates by trimester due to maternal secondary infection are hard to assess because the diagnosis of this type of infection is difficult and consenquently it is not known how many women have reactivation or reinfection during pregnancy and how many congenital infections result from reactivation or reinfection (5).

In the past, symptomatic cCMV was thought to occur almost exclusively after primary maternal infection (6). Therefore, preventive measures for cCMV infection have been focused mainly on seronegative women. Nowadays, a growing body of evidence suggests that secondary maternal infection contributes to a much greater proportion of symptomatic cCMV than was previously thought (3, 5).

Thus, we performed a retrospective study analyzing data of symptomatic children with cCMV, comparing those born to mothers with primary infection with those born to mothers with secondary infection, both at birth and at follow-up.

Materials and methods

Study population

We performed a monocentric retrospective observational study of babies with cCMV evaluated in a tertiary care Pediatric Academic Hospital, without maternity ward, from August 2004 to February 2021. Children were referred to our center because of the presence of symptoms at birth consistent with cCMV or because of the serological evidence of maternal infection during pregnancy. We excluded from this study children with severe comorbidities and/or other congenital and perinatal infections.

Considering the retrospective nature of the analysis, the current study did not require the approval of the local ethics committee according to current legislation, but a notification was sent.

Data were retrospectively analyzed in line with personal data protection policies.

Maternal infection

We collected data about the type of maternal infection. Primary maternal infection was defined in presence of seroconversion from negative to positive CMV IgG during pregnancy or presumed in presence of CMV IgM and a low CMV IgG-avidity. Secondary maternal infection was defined when previous infection was documented and presumed when at the beginning of the pregnancy women presented with positive IgG and negative IgM.

The time of maternal infection was estabilished according to the time of appearance of antibodies, without considering the lag beetween the infection and the antibody appearance. For secondary infection group the time of maternal infection was not assessed.

Evaluation at birth

Diagnosis of cCMV infection was based either on detection of CMV DNA in urine and/or blood samples collected within the 21st day of life or on viral DNA detection on a Guthrie Card after the 21st day of life (7, 8).

We conducted the following investigations in all children with a confirmed diagnosis of cCMV: complete blood count, liver enzymes, conjugated bilirubin, renal function,

cranial ultrasound scanning (CrUSS), abdominal ultrasound, audiological, ophthalmic and neurological assessment; we performed cerebral Magnetic Resonance Imaging (MRI) in babies with clinically detectable neurologic findings or CrUSS abnormalities until 2019; since January 2019 we started to perform cerebral MRI in all patients with cCMV and to consider isolated MRI abnormalities as sign of CNS involvement.

Babies were categorized at birth as "symptomatic" or "asymptomatic" according to the European Expert Consensus Statement on Diagnosis and Management of Congenital Cytomegalovirus (9).

We considered the nonspecific findings detected on CrUSS and cerebral MRI, such as lenticulostriated vasculopathy (LSV), as signs of CNS disease and consequently defined children as "symptomatic"; however, we did not treat children who presented those findings as the only sign of CMV disease (10).

Treatment

We treated only babies with evidence of CNS disease, life-threatening disease, severe single-organ disease or multiorgan involvement; those patients received either intravenous ganciclovir (12 mg/kg/day divided into two daily doses) or oral valganciclovir (32 mg/kg/day divided into two daily doses) or a combination of both from the diagnosis of symptomatic disease, for a total of 6 weeks until 2015. Patients born after the publication of Kimberlin's study in 2015 received antiviral treatment for 6 months, in accordance with the evidence that emerged from the study (11).

To monitor for signs of toxicity, all treated babies underwent full blood count, liver function tests, urea, creatinine, and electrolytes weekly for the first 4 weeks and then at least monthly until completion of treatment course.

Follow up

After discharge, all babies underwent a 6 years follow-up including pediatric clinical evaluation, audiologic, ophthalmic and neurodevelopmental assessment. Children with severe neurological disability (e.g., tetraparesis) were followed up longer.

All children were routinely screened for SNHL. Audiological evaluation was performed at birth and every 4 months until the age of 12 months, then twice a year during second and third year of life, thereafter by an annual audiometric surveillance. If a sensorineural hearing loss was detected, audiometric tests were recorded more frequently and longer.

Babies younger than 18 months of age were studied with objective tests: these included tympanometry, transient-evoked otoacoustic emissions (TEOAEs) and auditory brainstem response (ABR) assessment; from 2012 automated ABR (AABR) was introduced. Older patients underwent tympanometry, acoustic stapedius reflex threshold measurements, TEOAEs and puretone audiometry. The latter one was conducted with an age-specific test (behavioral observation audiometry in young children or visual reinforcement audiometry in older ones) and transducer (speakers for toddlers and earphones for more collaborating patients). Puretone audiometry was used to collect air and, if needed and possible, bone conduction thresholds. Older children with a suspicion of hearing loss, not compliant with puretone audiometry, underwent ABR testing for threshold under sedation.

Hearing loss was defined as absence of TEOAEs, an air conduction threshold >25 decibels hearing level (dB HL) on ABR, >35 dB HL on AABR, or >20 dB HL on age-specific puretone audiometry. Hearing loss was considered as sensorineural if the air-bone gap was <10 dB.

Neurodevelopmental assessment was performed using Bayley-III Scale to observe verbal, motor and fluid intelligent abilities until 3 years of age; WPPSI-III Scale to ages 4–7 years to parameterized general language, verbal, fluid intelligence and processing speed.

We considered similar constructs: Verbal Scale (Bayley-III)/General Language Scale (WPPSIIII); Cognitive Scale (Bayley-III)/Performance Scale (WPPSIIII); Motor Scale (Bayley-III)/Processing speed Scale (WPPSIIII). We considered a score below 85 IQ in each verbal, motor and cognitive index for each neurodevelopmental scale to be abnormal.

Statistical analysis

We compared data of children born to mothers with primary infection with those born to mothers with secondary infection, both at birth and during follow up. Statistical analysis was performed using the Statistical Package for Social Science (SPSS). The Chi-square and the Fisher exact test were used to assess statistical significance of clinical data and outcome measures. *P*-values <0.05 were considered statistically significant.

Results

We performed a monocentric retrospective observational study of babies with cCMV evaluated in our center from August 2004 to February 2021. In total, we identified 175 babies with cCMV:

- 118 born to mothers with primary infection (67.4 %)
- 27 born to mothers with secondary infection (15.4 %)
- 30, with unavailable data about maternal infection (17.1%)

We included in our study only babies with available data about maternal infection. Thus, 145 babies were considered in our study:

- 118 born to mothers with primary infection (81.4%)
- 27 born to mothers with secondary infection (18.6%)

The mean follow-up for children born to mothers with primary and secondary infection was 37.2 ± 20.5 months and 55 ± 48.7 months, respectively.

At birth 53 infants were classified as having a symptomatic cCMV infection: 40 babies born to mothers with primary infection and 13 babies born to mothers with secondary infection. No significant difference was found in the rate of symptomatic infection at birth between the two groups of children (33.9 vs 48.1%, $P = 0165$).

In the group of symptomatic children born to mothers with primary infection, 22 were tested for CMV infection in the 1st day of life, 4 in the 2nd, 1 in the 5th, 3 in the 8th, 2 in the 10th, 1 in the 11th, 1 in the 12th, 2 in the 13th, 2 in the 15th, 1 in the 17th, 1 in the 20th day of life. CMV-PCR resulted positive on both blood and urine in 12 patients, on blood in 7 patients (not tested on urine), on urine in 21 patients.

In the group of symptomatic children born to mothers with secondary infection 1 was tested for CMV infection in the 1st day of life, 1 in the 2nd, 2 in the 3th, 4 in the 10th, 2 in the 12th, 3 in the 15th day of life. CMV-PCR resulted positive on both blood and urine in 2 patients, on blood in 5 patients (not tested on urine), on urine in 6 patients.

In the group of children born to mothers with primary infection, the time of maternal infection was mainly the first trimester of pregnancy (45% of cases); the percentage of infections acquired in subsequent trimesters was lower and decreasing (32.5% and 20%). Of the 18 women infected during the first trimester, 3 were diagnosed because of seroconversion, 15 because of positivity of IgM and low avidity IgG; of the 13 patients infected during the secondary trimester, 12 were diagnosed because of seroconversion, 1 because of positivity of IgM and low avidity IgG (tested at 22 weeks of gestational age); in all the 8 patients infected during the third trimester seroconversion was documented.

In the group of mothers with secondary infection, 8 were known to be CMV seropositive prior to current pregnancy and 5 presented at the beginning of pregnancy (6–10 weeks of gestational age) with positive IgG and negative IgM.

Table 1 shows the signs and symptoms presented at birth. We found only one symptom with a significant difference between the two groups: the rate of unilateral SNHL, which was most common in children born to mothers with secondary infection (46.2 vs. 17.5%, $P = 0.037$).

Only children born to mothers with primary infection had bilateral SNHL.

TABLE 1 Signs and symptoms presented at birth by 53 symptomatic children divided according to the type of maternal CMV infection (primary or secondary).

	Maternal primary infection ($n = 40$)	Maternal secondary infection ($n = 13$)	*P*
IUGR	2 (5%)	1 (7.7%)	1
Congenital hydrocephalus	0	1 (7.7%)	0.245
SGA*	1 (2.5%)	1 (7.7%)	0.434
Prematurity	6 (15%)	2 (15.4%)	1
Apgar <7 at 1 min	2 (10%)	2 (15.4%)	0.249
Microcephaly**	2 (5%)	2 (15.4%)	0.249
Neurological signs	11 (27.5%)	6 (46.2%)	0.211
Chorioretinitis	0	1 (7.7%)	0.245
Thrombocytopenia	4 (10%)	1 (7.7%)	1
Anemia	1 (2.5%)	0	1
Splenomegaly	3 (7.5%)	0	0.567
Hypertransaminasemia	3 (7.5%)	3 (23%)	0.15
Hyperbilirubinemia	3 (7.5%)	0	0.567
Hepatomegaly	4 (10%)	0	0.561
Unilateral SNHL	(17.5%)	6 (46.2%)	0.037
Bilateral SNHL	4 (10%)	0	0.561
Abnormal CrUSS	20 (50%)	8 (61.5%)	0.469
Abnormal MRI	14/21 (66.7%)	3/6 (50%)	0.638

*SGA (Small for Gestational Age): defined as birthweight below the 10th percentile for gestational age at delivery.
**Microcephaly: defined as age-appropriate head circumference below two SD.

Regarding prenatal signs of fetal disease, 5% of children born to mothers with primary infection and 7.7% of children born to mothers with secondary infection presented intrauterine growth restriction (IUGR); one child born to mother with secondary infection showed a congenital hydrocephalus on fetal ultrasound later confirmed by fetal MRI.

Neurological signs, e.g. hypotone, hypertone and seizures, were presented by 27.5% of children born to mothers with primary infection and by 46.2% of children born to mothers with secondary infection.

In babies born to mothers with primary or secondary infection, abnormal CrUSS was found in 50% and 61.5%, respectively. In particular, we analized the rate of the following findings at CrUSS in both groups without finding any significant difference: calcification (15 vs. 7.7%, $P = 0.666$), cysts/pseudocysts (17.5 vs. 30%, $P = 0.432$), ventriculomegaly (2.5 vs. 15.4%, $P = 0.145$), white matter abnormalities (7.5 vs. 23, $P = 0.15$), LSV (17.5 vs. 30.8%, $P = 0.432$).

Table 2 shows the incidence of each sequela in the two groups of patients. We found a higher rate of many sequelae (tetraparesis, epilepsy, motor delay, speech delay, and unilateral SNHL) in the group of children born to mother with secondary

TABLE 2 Outcome in 53 symptomatic patients divided according to the type of maternal CMV infection.

	Maternal primary infection ($n = 40$)	Maternal secondary infection ($n = 13$)	*P*
Tetraparesis	0	4 (30.8%)	0.002
Epilepsy	1 (2.5%)	1 (7.7%)	0.434
Motor delay	9 (22.5%)	5 (38.5%)	0.257
Speech delay	12 (30%)	7 (53.8%)	0.119
Cognitive delay	0	0	1
Any neurodevelopmental impairment	18 (45%)	7 (53,8%)	0.579
Unilateral SNHL	5 (12.5%)	6 (46.2%)	0.009
Bilateral SNHL	6 (15%)	0	0.317
Any sensorineural hearing impairment	11 (27.5%)	6 (46.2%)	0.306

infection, with a statistical difference for tetraparesis and unilateral SNHL; in particular, tetraparesis was presented by 30.8% of the children born to mothers with secondary infection but was not presented in the other group. Among the 5 children with unilateral SNHL born to mothers with primary infection, deafness was profound in all cases; among the 6 born to mothers with secondary infection, deafness was profound in 2 children and mild in 4 children.

The only sequela presented only by the group of children born to mothers with primary infection was bilateral SNHL: 6 babies presented bilateral and profound SNHL and required cochlear implantation.

Motor delay, speech delay and cognitive delay were observed with Bayley III and WPPSI III neurocognitive scale.

The neurocognitive sequela observed with Bayley III and WPPSI III scales in children born to mothers with primary infection showed: Linguistic-Verbal Scale Mean IQ 95 with a range score between IQ 60 and QI 120, speech delay in 30% of score cases; Mean Motor Scale-Processing speed IQ 96 with a range score between IQ 52 and IQ 118, motor delay in 22.5% of score cases. Mean Cognitive-Performance Scale IQ 105 with a range score between IQ 85 and IQ 115.

The neurocognitive sequela observed with Bayley III and WPPSI III scales in children born to mothers with secondary infection showed: Linguistic-Verbal Scale Mean IQ 95 with a range score between QI 60 and QI 120, speech delay in 53.8% of score cases; Mean Motor-Processing Speed Scale IQ 96 with a range score between 52–118, motor delay in 38.5% of score cases; Mean Cognitive-Performance Scale IQ 100.8 with a range score between IQ 85 and IQ 105.

We treated 57.5% of babies born to mothers with primary infection and 84.6% of babies born to mothers with secondary infection; no statistical difference in the rate of treated babies was found between the two group *(P = 0.102)*.

Table 3 shows the outcome of symptomatic children born to mothers with primary infection divided by trimester of maternal infection. The majority of neurodevelopmental and auditory sequelae were found in babies born to mothers infected in the first trimester of pregnancy, who developed sequelae in 66.7% of cases. Nevertheless, three babies born to mothers infected at second trimester (23%) and one infected at third trimester (12.5%) reported severe sequelae.

Regarding the incidence of sequelae in babies born to mothers with secondary infection, we have scanty data about the trimester of reactivation/reinfection thus it is not possible to draw any conclusion.

Discussion

To date, we are not aware of the real incidence of secondary CMV infection during pregnancy. Thus, we do not know how many congenital infections result from this condition and what is the exact frequency and full spectrum of signs, symptoms and sequelae presented by children born to mothers with non-primary infection (12).

A population-based prediction model found that secondary infections are responsible for the majority of cCMV infections as well as CMV-related hearing loss (3).

A recent meta-analysis, including 879 children, indicated that symptomatic cCMV infection at birth is not associated with type of maternal infection and that the risk of long-term sequelae is similar in children born to mother with primary and non-primary CMV infection (5).

In this study, we compared data from symptomatic children born to mothers with primary infection with those born to mothers with secondary infection, both at birth and during long-term follow-up (37.2 and 55 months, respectively), assessing whether there were differences in the rate of signs and symptoms at birth, abnormal findings on neuroimaging, or sequelae at follow-up.

We found no significant differences in the rate of symptomatic children at birth between the two groups (33.9 vs. 48.1%, $P = 0.165$). Giannattasio et al. described similar results: they found 46.2% symptomatic newborns to mother with primary infection and 60% symptomatic newborns to mother with secondary infection (13). They reported a significantly higher rate of neurological signs, chorioretinitis and thrombocytopenia in children born to mothers with secondary infection, whereas we found no statistical differences in the rate of clinical findings at birth in the two groups, with the only exception of SNHL which was significantly more common in children born to mothers with secondary infection. In our cohort, neurological signs were the most frequent of all signs/symptoms presented at birth in both groups, with a higher

TABLE 3 Outcome of symptomatic children born to mothers with primary infection divided according to trimester of infection.

Trimester of maternal infection	Symptomatic babies for each trimester (total $n = 40$)	Any sequela	Neurodevelopment impairment	Unilateral SNHL	Bilateral SNHL
I	18 (45%)	12 (66.7%)	8 (44%)	2 (11%)	4 (22%)
II	13 (32.5%)	3 (23%)	1 (7.7%)	1 (7.7%)	2 (15%)
III	8 (20%)	1 (12.5%)	1 (12.5%)	1 (12.5%)	0
Unknown	1 (2.5%)	0	0	0	0

rate in the group of children born to mothers with secondary infection without reaching statistical significance.

Considering unilateral and bilateral SNHL together, the incidence in the two groups was similar; 27.5% children born to mothers with primary infection and 46.2% children born to mothers with secondary infection presented SNHL at birth. Consistently, Ross et al. reported in a cohort of 300 children, 124 born to mothers with either presumed or confirmed secondary infection and 176 born to mothers with either presumed or confirmed primary CMV infection, a similar rate for hearing loss in the two groups; they considered both symptomatic and asymptomatic children in their study (14).

Regarding neuroimaging, 50% of babies born to mothers with primary infection and 61.5% of those born to mothers with secondary infection had an abnormal finding at CrUSS. We found a higher incidence of cysts/pseudocysts, ventriculomegaly, white matter abnormalities and LSV in children born to mother with secondary infection; instead, calcifications were prevalent in the group of children born to mothers with primary infection. None of the findings had a significantly different rate in the two groups. Hadar et al. reported a significantly higher rate of abnormal finding at CrUSS in babies born to mothers with primary infection; however, in their cohort there was a much higher number of symptomatic children born to mothers with primary infection than children born to mothers with secondary infection (95 vs. 12) (15). In addition, the type of pathologic findings at CrUSS was not specified. In the cohort of patients described by Giannattasio et al. there was a similar rate of brain abnormalities in the group of children born to mothers with primary infection and those born to mothers with secondary infection (13).

MRI was performed in 20 babies born to mothers with primary infection and 6 babies born to mothers with secondary infection; the exam reported abnormal findings in 66.7% and 50% of those babies, respectively, without statistically significant differences.

During follow up, we found a similar rate of neurodevelopmental sequelae in the two groups, as previously reported by Puhakka et al. (13), Giannattasio et al. (16), and Coscia et al. (17). Nethertheless, analyzing each sequela separately (tetraparesis, epilepsy, motor and speech delay) we found a statistical difference for tetraparesis that was more frequent in children born to mothers with secondary infection (30 vs. 0%).

Considering audiological impairment, we found that the higher rate of unilateral SNHL in children born to mothers with secondary infection was maintained at follow-up; moreover, none of these children developed bilateral SNHL. In our cohort only children born to mothers with primary infection presented bilateral SNHL: 4 children at birth and 6 during follow-up. Consistently, Ross et al. reported in their cohort that fewer children in the secondary infection group had bilateral hearing loss compared with the primary infection group, and significantly fewer children born to mothers with preexisting seroimmunity had progression of their hearing loss compared with those born to mothers without prior immunity (14).

Considering that the likelihood of fetal harm is greater when CMV infection occurs early in pregnancy, we analyzed data from symptomatic newborns divided by trimester of maternal infection (18). We assessed the long-term outcome of symptomatic children born to mothers with primary infection, divided by trimester of maternal infection. As expected, we found that symptomatic children born to mothers who acquired the infection in the first trimester developed sequelae in 66.7% of cases. Surprisingly, we also found that 23% and 12.5% of the symptomatic children born to mothers infected during the second and the third trimester, respectively, reported severe sequelae at follow up. Our results differ from those reported by the meta-analysis of Chatzakis et al. They found that severe sequelae are common only when maternal infection occurs in the periconceptional period and in the first trimester (29% and 19%, respectively), decreasing to ∼1% after this point (18).

The important difference in the rate of sequelae could be explained by the fact that our cohort is composed only by symptomatic infants, who more often have an unfavorable outcome. Moreover, the number of patients is too small to draw numerical conclusions. Nonetheless, we want to highlight that primary maternal infections acquired in the second and third trimesters of pregnancy can lead to long-term sequelae in

children, although to a lesser extent than those acquired in the first one.

This retrospective study presents several limitations. First of all, the numbers on which comparison are performed are small and thus not conclusive. The proportion of symptomatic patients in the cohort is very high and the frequency and severity of sequelae in the symptomatic group is also higher than expected from the literature. This could be explained by the fact that many of those children were referred to a tertiary center because of the severity of symptoms. This selection bias particularly affects the group of children born to mothers with secondary infection; many of these children were tested at birth for CMV because of suggestive symptoms of infection, even without documented reinfection/reactivation of the virus during pregnancy.

Furthermore, the population included is extremely heterogeneous both because of the different criteria adopted over the years to define a "symptomatic" baby and the different treatment strategies applied over the years, which have a different impact on the long-term outcomes.

Thus, it is not possible to draw firm conclusions from this retrospective study regarding the overall, population-based frequency of disability or impairments due congenital CMV infection from primary or secondary maternal infection. Making those estimates and comparison requires prospective identification of maternal infections during pregnancy and screening newborns for congenital CMV infection.

Another limitation of this retrospective study is that follow up data where not complete for all patients; morover, serological maternal data were available from medical files and sera could not be retrived to perform new standardized test for this study.

In conclusion, our study aims to emphasize that cCMV infections due to secondary maternal infections can be as serious as those due to primary infections, both at birth and during follow up; the less attention often given to seropositive mothers can lead to missed diagnosis of congenital infections in children who may develop serious sequelae. Boppana et al. demonstrated that two-thirds of CMV infection in previously seropositive women were due to exogenous reinfection (19). Thus, increasing hygienic measures in those women may reduce the number of reinfection and, consequently, the number of cCMV. Moreover, paying more attention to seropositive women could allow to detect and possibly treat otherwise unrecognized infected babies.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not provided by the participants' legal guardians/next of kin because of the retrospective nature of the analysis, but a notification to the Ethics Committee was sent. Data were retrospectively analyzed in line with personal data protection policies.

Author contributions

FS participated to the design of the study, analyzed data, interpreted results, and drafted the article. SB, LL, PP, and PR contributed to the revision of draft and interpretation of data. LR, SC, MDL, DA, DL, GL, AS, and PM were involved in the acquisition and analysis of data. MDP and TG performed and revised neurodevelopmental outcomes. FC conceived the study and revised the article critically for important intellectual content.

Acknowledgments

The authors thank the patients and their families for participating in the study and the nurses who gave them loving care and collected the samples: Nadia Iavarone, Tamara Di Marco and Rita Perinelli.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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frontiers | Frontiers in Pediatrics

TYPE Original Research
PUBLISHED 22 July 2022
DOI 10.3389/fped.2022.882416

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

RECEIVED 23 February 2022
ACCEPTED 30 June 2022
PUBLISHED 22 July 2022

CITATION
Berardi A, Zinani I, Bedetti L, Vaccina E, Toschi A, Toni G, Lecis M, Leone F, Monari F, Cozzolino M, Zini T, Boncompagni A, Iughetti L, Miselli F and Lugli L (2022) Should we give antibiotics to neonates with mild non-progressive symptoms? A comparison of serial clinical observation and the neonatal sepsis risk calculator. *Front. Pediatr.* 10:882416. doi: 10.3389/fped.2022.882416

Should we give antibiotics to neonates with mild non-progressive symptoms? A comparison of serial clinical observation and the neonatal sepsis risk calculator

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Objective: To compare two strategies [the neonatal sepsis risk calculator (NSC) and the updated serial clinical observation approach (SCO)] for the management of asymptomatic neonates at risk of early-onset sepsis (EOS) and neonates with mild non-progressive symptoms in the first hours of life.

Methods: This was a single-center, retrospective cohort study conducted over 15 months (01/01/2019–31/03/2020). All live births at ≥34 weeks of gestation were included. Infants were managed using SCO and decisions were compared with those retrospectively projected by the NSC. The proportion of infants recommended for antibiotics or laboratory testing was compared in both strategies. McNemar's non-parametric test was used to assess significant differences in matched proportions.

Results: Among the 3,445 neonates (late-preterm, $n = 178$; full-term, $n = 3,267$) 262 (7.6%) presented with symptoms of suspected EOS. There were no cases of culture-proven EOS. Only 1.9% of the neonates were treated with antibiotics (median antibiotic treatment, 2 days) and 4.0% were evaluated. According to NSC, antibiotics would have been administered in 5.4% of infants (absolute difference between SCO and NSC, 3.51%; 95% CI, 3.14–3.71%; $p < 0.0001$) and 5.6% of infants would have undergone "rule out sepsis" (absolute difference between SCO and NSC, 1.63%, 95% CI 1.10–2.05; $p < 0.0001$).

Conclusion: SCO minimizes laboratory testing and unnecessary antibiotics in infants at risk of EOS or with mild non-progressive symptoms, without the risk of a worse neonatal outcome. The NSC recommends almost three times more antibiotics than the SCO without improving neonatal outcomes.

KEYWORDS

early-onset sepsis, neonatal early-onset sepsis calculator, newborn, serial clinical observation, neonates, perinatal distress

Introduction

The current management of neonates at risk of early-onset sepsis (EOS) remains controversial, and the suggested approaches are heterogeneous (1). EOS rates have declined substantially due to the widespread use of intrapartum antibiotic prophylaxis (IAP). However, the diagnosis of EOS remains challenging because its initial clinical signs may be ambiguous, diagnostic tests are poorly predictive, and delayed antibiotic treatment can have devastating consequences. Therefore, 30–40 uninfected neonates are exposed to unnecessary antibiotics for each infant subsequently confirmed to have EOS (2). However, perinatal antibiotic exposure is a major concern, given the potential long-term effects of changes in the intestinal flora of uninfected neonates (1, 3) and the consensus on the optimal management of neonates considered at risk of EOS is shifting (4, 5).

The American Academy of Pediatrics suggests three alternative approaches for the use of risk factors (RFs) to identify infants at increased risk of EOS (1). First, the categorical RFs assessment (6); such an approach has some limitations and is associated with higher rates of antibiotic treatment (7). Second, the neonatal sepsis risk calculator (NSC). The NSC has a Bayesian approach to create a multivariate model to predict infant-specific EOS risk, derived and validated from a case-control study of blood culture-proven EOS. NSC permit improved delineation of low-risk babies that can be safely managed with observation. Following the adoption of the most recent NSC laboratory workups, the use of empirical antibiotic administration in the first 24 h of life has decreased significantly (from 5.0 to 2.6%) in a recent US multicenter study (8). However, these low rates of antibiotic treatment have rarely been confirmed outside the USA (the country where NSC was created), and antibiotic treatment may reach 8% of neonates elsewhere (9). Finally, increasing evidence has shown that asymptomatic neonates at risk can be safely managed with serial clinical observation (SCO) without antibiotic treatment (10–12). However, for "less symptomatic" infants, it is difficult to allow adequate time to undergo a physiological transition before deciding whether clinical signs are transient or permanent (4).

This study aimed to assess the impact of an updated SCO approach planned to reduce unnecessary antibiotic therapy in "less symptomatic" full-term and late-preterm neonates. The impact on neonatal outcomes, laboratory testing rates, and neonatal intensive care unit (NICU) admissions were also evaluated. Furthermore, we compared management decisions using the updated SCO with those projected through the virtual application of the NSC.

Methods

Study design

This was a retrospective study carried out over 15 months (from January 1st, 2019 to March 31th, 2020), in a single, high-volume tertiary care center (Modena University Hospital, Italy), with ∼3,000 live births/year. This center advocates a recto-vaginal culture screening strategy at 35–37 weeks gestation (6). The project was approved by the local ethics committee (no. 169/2019/OSS*/AOUMO). All infants born at ≥34 weeks gestation were included in the study. To obtain complete information on the rates of antibiotic treatment in the entire population, we included neonates with malformations, metabolic diseases, or surgical complications. Full maternal data (gestational age, mode of delivery, group B Streptococcus status, RFs for EOS, and duration of IAP) were routinely recorded in neonatal charts. The records were collected anonymously in an Excel format with controlled access, assigning each newborn a progressive numerical code.

SCO approach

This approach is directed at asymptomatic infants with RFs (10). A standardized form, signed by each examiner, were used to detail general wellbeing, skin color, and respiratory signs at standard intervals (at ages 1, 3, 6, 12, 18, 24, 36, and 48 h) (13). All asymptomatic neonates with RFs for EOS are usually managed by midwives, nurses or pediatricians in the mother baby unit, where neonates ≥ 35 weeks' gestation "room in"

with their own mother. Asymptomatic neonates with 34 weeks' gestation are usually admitted to intermediate care unit.

Each newborn with symptoms of suspected sepsis is immediately referred to a neonatal care specialist. However, those with mild to moderate disease that requires oxygen support or a high-flow nasal cannula are separated from their mothers and admitted to an intermediate care unit. Severely ill neonates and those undergoing nCPAP or mechanical ventilation are admitted to the NICU. Ampicillin plus an aminoglycoside is administered as empiric therapy for suspected EOS in symptomatic neonates. Before March 2018, white blood cell count (WBC), C-reactive protein (CRP) levels, and blood culture were obtained to rule out sepsis in asymptomatic, chorioamnionitis-exposed neonates or those who developed symptoms of variable severity.

Updated SCO approach

In March 2018, the main neonatal symptoms triggering laboratory evaluation were defined (Table 1A) (14). Neonates with mild, non-progressive symptoms (that can be due to non-infectious diseases, i.e., transient tachypnea of the newborn) who present in the first few hours of life can be reevaluated at 2-h intervals. No laboratory testing is performed, and no antibiotic therapy is given if symptoms remain mild, even after multiple re-evaluations in case of minor criteria. In contrast, the presence of major symptoms (as defined in Table 1A) or worsening of mild symptoms suggest the need for laboratory evaluation. Among patients who undergo a sepsis workup, treatment decisions are left to the discretion of the physician. This updated approach aims to minimize laboratory testing (which, in our experience, strongly influences clinicians' decisions) (10), unnecessary antibiotics, and mother-baby separation.

NSC approach

The NSC is an online tool that quantifies the risk of EOS in infants with a gestational age ≥34 weeks using a pretest probability. Recommendations for antibiotic treatment or neonatal management are derived from an algorithmic framework based on the local incidence of EOS, maternal RFs (gestational age, highest intrapartum temperature, duration of membrane rupture, GBS status, and IAP), and clinical presentation of the infants during the first few hours of life. For each infant, the previous risk of EOS was calculated based on the local incidence of EOS and maternal RFs alone. The prior probability is converted into a posterior probability of EOS in the different categories of infants' clinical presentation (likelihood ratio of 0.41, 5.0, and 21.2 for well-appearing, equivocal, and clinical illness, respectively; Table 1B) (15). The resulting post-test probability of EOS is classified into three risk layers (<0.65, 0.65–1.54, and >1.54 cases/1,000 live births). The management recommendations suggested by the NSC are as follows: (1) no culture, no antibiotics, routine vitals (posterior risk <1/1,000 live births); (2) no culture, no antibiotics, vitals every 4 h for 24 h (posterior risk <1/1,000 live births, but prior risk >1/1,000 live births); (3) blood culture, vitals every 4 h for 24 h (posterior risk 1–3/1,000 live births); (4) strongly consider starting empiric antibiotics, vitals per NICU (posterior risk <3/1,000 live births); and (5) empiric antibiotics, vitals per NICU (posterior risk >3/1,000 live births or clinical signs of illness). In the current study, each infant was retrospectively scored as well as appeared equivocal or clinically ill within 4 h after birth. Recommendations for the management of neonates according to NSC were calculated by assuming an incidence rate of EOS of 0.6/1,000 live births (16).

Statistical analyses

We used MedCalc version 9.3 (MedCalc Software, https://www.medcalc.org). Continuous variables are expressed as mean ± SD or median and interquartile range (IQR), and categorical data are expressed as numbers (percentages). Categorical and continuous variables were compared between patient groups using the χ^2 test, Fisher's exact test, Student's *t*-test, or Mann–Whitney test, as appropriate. All *p*-values refer to two-tailed tests of significance; $p < 0.05$ was considered significant. McNemar's non-parametric test was used to assess significant differences in the matched proportions.

Results

All neonates

During the study period, 3,456 neonates were ≥34 weeks of gestation. Records were available for 3,445 (99.7%) infants, of which 178 were born late preterm and 3,267 were born full-term. The median gestational age was 39.6 weeks and the median birth weight was 3,310 g.

Table 2 shows demographics according to full-term or late-preterm delivery. Vaginal delivery and prenatal vagino-rectal screening were more likely in full-term neonates, while prolonged membrane rupture and IAP were more likely among late preterm neonates. Among the 3,445 infants included in the study, 264 (7.6%) had symptoms of suspected EOS (most were respiratory and already at birth). Table 3 shows the age of presentation of symptoms, NSC scores, and antibiotics administered by comparing full-term and late-preterm neonates. Only 1.9% of the entire cohort was treated with antibiotics (median 2 days), and 4% underwent "rule out sepsis"; 3.1 and 2.3% were admitted to the NICU or intermediate care unit, respectively (neonates who were admitted and were given

TABLE 1 Symptoms and classification of infant's clinical presentation according to serial clinical observation and neonatal sepsis calculator.

(A) Minor and major clinical symptoms and criteria suggesting observation or laboratory evaluation and antibiotic treatment [modified from Berardi et al. (14)].

Minor*	Major
Mild respiratory distress (> 60/m) without the need of respiratory support	Moderate to severe respiratory distress (requiring respiratory support)§ → tachypnoea plus increased respiratory effort
Tachycardia > 160 bpm	Hypoxia, reduced SpO2 saturation
Metabolic acidosis (base excess ≤ −10 mmol/l)	Reduced skin perfusion, Refill time ≥ 3 seconds, Signs of shock
Temperature < 36° or > 37.5 < 38 °C	Temperature ≥ 38 °C
	Grayish, pallor or marbling of the skin color
	Worsening of general wellbeing, apnoea, lethargy, irritability, convulsions

(B) Classification of infant's clinical presentation according to NCI (available at https://neonatalsepsiscalculator.kaiserpermanente.org).

Clinical exam	Description
Clinical illness	1. Persistent need for NCPAP / HFNC / mechanical ventilation (outside of the delivery room)
	2. Hemodynamic instability requiring vasoactive drugs
	3. Neonatal encephalopathy /Perinatal depression:
	- Seizure
	- Apgar Score at 5 min < 5
	4. Need for supplemental O2 > 2 h to maintain oxygen saturations > 90% (outside of the delivery room)
Equivocal	1. Persistent physiologic abnormality > 4 h
	- Tachycardia (HR > 160)
	- Tachypnea (RR > 60)
	- Temperature instability (> 100.4°F or < 97.5 °F)
	- Respiratory distress (grunting, flaring, or retracting) not requiring supplemental O2
	2. Two or more physiologic abnormalities lasting for > 2 h Tachycardia (HR > 160)
	- Tachypnea (RR > 60)
	- Temperature instability (> 100.4°F or < 97.5 °F)
	- Respiratory distress (grunting, flaring, or retracting) not requiring supplemental O2
	Note: abnormality can be intermittent
Well appearing	No persistent physiologic abnormalities

SpO_2, Saturation of peripheral oxygen.
*On the basis of the clinician's judgment, laboratory evaluation can be delayed in the presence of minor, initial, unspecific and non-progressive symptoms during the first 12–24 h of life. Neonates with mild symptoms are re-evaluated at 2-h intervals. The presence of major symptoms and the worsening or persistence (for 12¬24 h) of minor symptoms warrant laboratory evaluation and (eventually) empirical antibiotics, but the decision is left to the clinician's discretion.
§Respiratory support includes mechanical ventilation. However, it does not necessarily include high flow nasal cannula or nasal continuous positive airway pressure.
HFNC, High Flow Nasal Cannula; HR, Heart Rate; NCPAP, Nasal Continuous Positive Airway Pressure; RR, Respiratory Rate.

antibiotics or underwent "rule out sepsis" are reported in the footnote of Table 3).

Among the 200 symptomatic neonates unexposed to antibiotics, 77 (38%) were allowed to "room in" with their mothers within a few hours after birth. The remaining 123 (62%) were admitted to NICU (of which 80 underwent nCPAP) and/or intermediate care unit (of which 67 underwent high-flow nasal cannulation). Symptoms improved substantially within the first 24–48 h of life (median duration 72 h, IQR 24–120). Late-preterm neonates were more likely to have symptoms, undergo SCO, be evaluated, have a higher NSC score, be admitted to the NICU, and be treated with antibiotics. However, the median number of days on antibiotics did not differ between late preterm and full-term neonates.

Figure 1 details neonatal symptoms, Apgar scores and the need for respiratory support among symptomatic neonates. Respiratory symptoms (tachypnea, respiratory distress syndrome, desaturation) were the most common.

Comparison between SCO and NSC

Among 3,445 infants, the following indications were suggested by NSC: no culture, no antibiotics, routine vitals

TABLE 2 Demographics, risk factors for EOS and intrapartum antibiotic prophylaxis.

	Late preterm neonates ($n = 178$)	Full term neonates ($n = 3,267$)	p	All ($n = 3,445$)
Median gestational age, wks	36.14 (35.29–36.57)	39.71 (39.0–40.29)	NA	39.57 (38.86–40.29)
Median birth weight, g	2,582.5 (2,295–2,860)	3,340 (3,060–3,620)	NA	3,310 (3,010–3,605)
Vaginal delivery	96 (53.93)	2,565 (78.49)	<0.0001	2,661 (77.24)
Prenatal vagino-rectal screening	66 (37.07)	3213 (98.35)	<0.0001	3,279 (95.17)
GBS positive screening	13 (19.69)	703 (21.88)	0.7838	716 (21.84)
GBS bacteriuria	0 (0)	46 (1.41)	0.2082	46 (1.34)
Prolonged membrane rupture	41 (23.03)	473 (15.31)	0.0044	514 (15.73)
Maternal temperature ≥ 38° C	2 (0.01)	34 (1.05)	0.9186	36 (1.05)
Previous infant with GBS disease	0 (0)	2 (0.06)	0.2050	2 (0.05)
At least 1 risk factor	178 (100)	503 (15.39)	NA	681 (19.77)
IAP	98 (55.05)	923 (28.25)	<0.0001	1,021 (29.63)
Adequate IAP	59 (60.20)	516 (55.90)	0.4785	575 (56.32)

GBS, group B streptococcus; IAP, Intrapartum Antibiotic Prophylaxis; NA, not assessable; wks, weeks.
Highest maternal temperature and duration of membrane rupture were missing for 28 (0.8%) (1 preterm, 27 full term) and 178 (5.2%, all full term) cases, respectively. Percentages are calculated without missing cases.
Data are presented as median (IQR) and n (%).

($n = 3,238$), strong consideration of starting empiric antibiotics, vitals per NICU ($n = 131$); empiric antibiotics, vitals per NICU ($n = 55$); no culture, no antibiotics, vitals every 4 h for 24 h ($n = 13$); and blood culture, vitals every 4 h for 24 h ($n = 8$).

Table 4 compares the number of "rule out sepsis" evaluations (a) and antibiotic treatments (b) in all neonates. Of the 3,380 neonates who did not start on antibiotics according to the SCO, 3,254 would also have avoided antibiotics according to the NSC. The remaining 126 neonates would have been recommended antibiotics by the NSC, but remained well without treatment. In contrast, five neonates would have avoided antibiotics according to NSC (absolute difference 3.5%; 95% CI, 3.1–3.7%, $p < 0.0001$). According to NSC, antibiotics would have been administered to 5.4% of infants. Of the 138 neonates who were evaluated to rule out sepsis according to the SCO, 118 were also evaluated according to the NSC. Seventy-six neonates would have undergone "rule out sepsis" according to the NSC but were not evaluated. In contrast, 20 neonates would not have been evaluated according to as per NSC but were evaluated according to as per SCO (absolute difference, 1.63%; 95% CI, 1.10–2.05; $p < 0.0001$). According to NSC, 5.6% of infants would have undergone "rule out sepsis."

Table 5 compares the number of "rule out sepsis" evaluations (a) and antibiotic treatments (b) among late-preterm infants. According to NSC, antibiotics would have been administered in 27.0% of late-preterm infants compared to 8.4% according to SCO (absolute difference, 18.6%; 95% CI, 12.3–24.8%; $p < 0.0001$). Similarly, according to NSC, 28.1% of late-preterm infants would have undergone "rule out sepsis," compared to 18.5% according to SCO (absolute difference, 9.6%; 95% CI, 4.4–14.7%; $p < 0.0001$).

Maternal temperature and neonatal symptoms

Among the 110 neonates born after an increased maternal intrapartum temperature (≥37.5°C), 20 developed symptoms. Eight of these 20 patients were treated with antibiotics. For the 12 untreated neonates, the NSC would have suggested the following: empiric antibiotics, vitals per NICU ($n = 7$); blood culture, vitals every 4 h for 24 h ($n = 2$); no culture, no antibiotics, routine vitals ($n = 2$); and strongly consider starting empiric antibiotics and vitals per NICU ($n = 1$). Therefore, 8 of the 12 neonates would have received antibiotics per NSC but remained untreated.

Figure 2 is a box plot showing the distribution of the NSC scores in the three cohorts: (i) all infants in the study population, (ii) infants initiated on antibiotics according to SCO, and (iii) those recommended antibiotics by NSC. The sepsis risk score in the study population was low; infants treated with antibiotics according to SCO had higher sepsis risk scores than those recommended antibiotics by the NSC.

Neonatal outcome

No cases of culture-proven sepsis were found. Six infants were administered antibiotics (≥5 days), and all but one were born to GBS-negative mothers. Three of the six infants had an abnormal blood count, three had elevated CRP, and four underwent lumbar puncture (the CSF was sterile in all cases). Their blood cultures were sterile except for one neonate (born

TABLE 3 Age at presentation of symptoms, NSC score and antibiotics.

	Late preterm neonates ($n = 178$)	Full term neonates ($n = 3,267$)	p	All ($n = 3,445$)
Neonates with symptoms	53 (29.78)	211 (6.46)	<0.0001	264 (7.66)
Neonates with symptoms already at birth	46 (86.79)	134 (63.51)		180 (68.18)
Neonates with symptoms from 1 to 6 h of life	5 (9.43)	44 (20.85)	0.0044	49 (18.56)
Neonates with symptoms after 6 h of life	2 (3.77)	33 (15.64)		35 (13.26)
Serial clinical observation	56 (31.46)	301 (9.21)	<0.0001	357 (10.4)
Evaluation to rule out sepsis †	33 (18.54)	104 (3.18)	<0.0001	137 (3.98)
NSC score < 0.5	117 (70.06)	3,146 (96.30)	<0.0001	3,263 (94.72)
NSC score > 0.51 < 1	11 (6.59)	46 (1.41)	<0.0001	57 (1.65)
NSC score > 1.01 < 3	16 (9.58)	46 (1.41)	<0.0001	62 (1.80)
NSC score > 3.01	23 (13.77)	29 (0.89)	<0.0001	52 (1.51)
Neonates given antibiotics ¶	15 (8.43)	49 (1.49)	< 0.0001	64 (1.86)
Median days on antibiotics	2 (2–2)	3 (2–3)	0.0637	2 (2–3)
Symptomatic neonates admitted to NICU §	35 (19.66)	73 (2.23)	<0.0001	108 (3.13)
Symptomatic neonates admitted to intermediate care unit §	17 (9.55%)	62 (1.90%)	<0.0001	79 (2.30%)
Symptomatic neonates unadmitted to NICU or intermediate care unit ¥	1 (0.06 %)	76 (2.33%)	0.1209	77 (2.24%)

NICU, neonatal intensive care unit; NSC, neonatal sepsis calculator.
† Including white blood cell count, blood culture, and C-reactive protein.
¶ Among 64 neonates receiving antibiotics, 14 underwent therapeutic hypothermia and 6 had surgical prophylaxis.
§ Antibiotics were given to 55 out of 108 symptomatic neonates admitted to NICU and 9 out of 79 admitted to intermediate care unit; "rule out sepsis" was performed in all symptomatic neonates admitted to NICU.
¥ "Rooming in" was allowed within a few hours of birth to all 77 neonates and they were discharged home with a "healthy newborn" code.
Data are presented as median (IQR) or n (%).

to a GBS-positive mother and exposed to inadequate IAP) who developed mild tachypnea (7 h of life) and received ampicillin plus gentamicin. The blood culture yielded *Staphylococcus hominis*, which was considered a contaminant because the subsequent blood culture was sterile.

None of the 3,445 included infants was readmitted within 30 days of birth with a positive blood culture result. Among neonates who developed symptoms after birth, none worsened or had brain lesions due to delayed antibiotic treatment. Two neonates died: the first was affected by an inherited metabolic disease and the second was affected by a congenital diaphragmatic hernia.

Discussion

There is a consensus to administer empirical antibiotics to neonates with suspected EOS symptoms, with or without RFs (17). This strategy is based more on historical customs and practices than on evidence. In most babies, non-specific symptoms during the first hours of life are not due to infection. Unnecessary antibiotics may disrupt the neonatal gut microbiome with long-term consequences and increase the resistance to pathogens. Furthermore, intravenous infusion may complicate extravasation, whereas mother-infant separation for EOS evaluation can delay breastfeeding initiation and increase formula supplementation (7, 18). Therefore, strategies to reduce unnecessary antibiotic use are of interest. This is the first comparison between the NSC and the updated SCO approach (13). Indeed, we defined "minor" and "major" clinical symptoms to guide clinicians in the evaluation and treatment of infants with antibiotics.

Approximately ¾ of the symptomatic neonates were not exposed to antibiotics; 38% were allowed to "room in" with their mother within a few hours after birth. Among the remaining 62% who were admitted to the NICU or to the intermediate care unit, some underwent nCPAP or HFNC several hours after birth. Therefore, the persistence of symptoms beyond 2 h after birth may not be a good criterion for the administration of antibiotics, especially if the symptoms do not worsen or the risk of EOS is very low (i.e., infants born out of labor and with intact membranes). "Equivocal symptoms" or "clinical illness" are common in the first hours of life due to the transition to extrauterine life. The developers of NSC suggested that antibiotics "strongly consider" as a safeguard not to discontinue therapy in clinically symptomatic infants, even if the posterior probability is below the threshold for treatment (< 3 cases/1,000 live births). However, a comparison with our

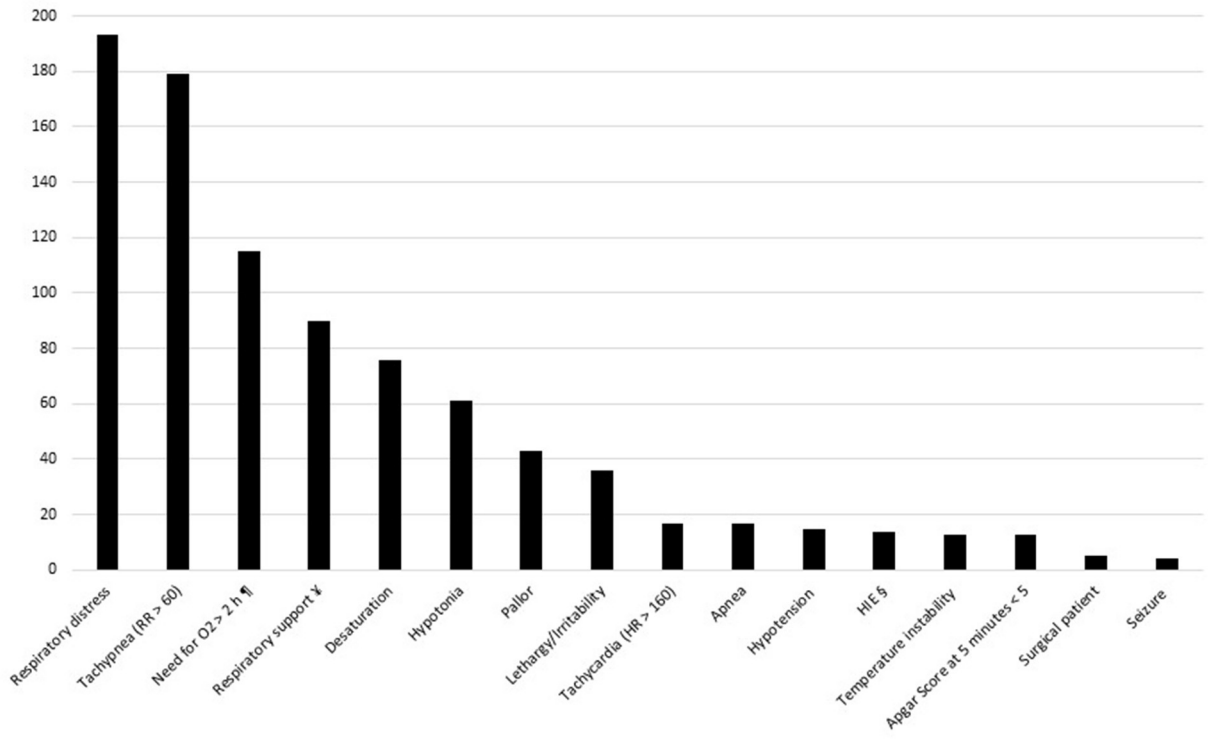

FIGURE 1
Symptoms and respiratory supports among symptomatic neonates. HIE, hypoxic ischemic encephalopathy. ¶ to maintain SpO2 > 90%. ¥ persistent need for NCPAP / HFNC / mechanical ventilation. § hypoxic ischemic encephalopathy requiring hypothermia.

updated SCO approach shows that this recommendation of NSC would substantially increase antibiotic exposure in uninfected infants without improving neonatal outcomes. In particular, our updated SCO approach reduced antibiotic exposure among late preterm infants by two-thirds compared to NSC. The NSC model is associated with increased postnatal antibiotic exposure, especially among infants with RFs for EOS or those with "transitional" symptoms in the first hours of life. Indeed, in some cases, the NSC model can overestimate the absolute risk of EOS; for example, the NSC assumes the same risk of EOS for neonates unexposed or exposed to inadequate IAP (duration <2 h) (19); however, it is known that the risk of developing EOS in asymptomatic IAP-exposed neonates is very low, regardless of the duration of IAP (20).

The low rate of "rule out sepsis" evaluations we performed in infants with mild symptoms probably contributed to the reduction of unnecessary antibiotics. This finding is consistent with recent studies demonstrating the low predictive value of ancillary tests (21) and the reduced (~30%) antibiotic exposure when CRP is excluded from the diagnostic panel of EOS (22). Furthermore, the importance of a positive blood culture obtained from an asymptomatic newborn infant is unclear (23). In our experience, repeated evaluations of asymptomatic infants may even increase the yield of pathogens from blood cultures (often difficult to interpret), thus giving antibiotics even to infants whose symptoms of EOS would likely never appear.

Only 1.9% of our infants were exposed to antibiotics and most received very short courses. Our approach was of utmost benefit in preterm infants, who often have "transient" symptoms in the first few hours of life, compared with full-term infants. Until recently, up to 35% of late-preterm infants received antibiotics (24), thus separating neonates from their mothers. In contrast, only 8% of our preterm neonates received antibiotics, while 70% were allowed to room in with their mothers. However, the overall neonatal antibiotic exposure rates in preterm and full-term neonates could be 30 times higher than necessary, as the incidence of EOS in our NICU is 0.6/1,000 live births (16). More efforts should be made to better identify the infants to be treated. None of the neonates had worse outcomes due to delayed treatment of an infection. Perhaps the SCO strategy is safer in our center, as adherence to recommendations for GBS prevention is very high (25), whereas SCO would be less effective in centers with low adherence to guidelines.

This study had several important limitations. First, the SCO strategy may be safer in our center, where adherence to recommendations for the prevention of GBS is very high, while it could be less effective where adherence to guidelines is low, or the incidence of EOS is higher. Second, the sample size of the

TABLE 4 Comparison of antibiotic use (A) and "rule out sepsis" evaluations (B) as per SCO vs. recommendations of the NSC in the study population. Differences between proportions were analyzed using McNemar's test.

		NSC		
		No antibiotics	**Antibiotics**	**Total (% of study cohort)**
(A) Comparison of antibiotic use				
SCO	No antibiotics	3,254	126	3,380 (98.1)
	Antibiotics	5	60	65 (1.9)
	Total (% of study cohort)	3,259 (94.6)	186 (5.4)	3,445 (100.0)
		NSC		
		No test	**Rule out sepsis**	**Total (% of study cohort)**
(B) Comparison of "rule out sepsis" evaluations				
SCO	Not evaluated	3,231	76	3,307 (96.0)
	Rule out sepsis	20	118	138 (4.0)
	Total (% of study cohort)	3,251 (94.4)	194 (5.6)	3,445 (100.0)

NSC, neonatal sepsis calculator; SCO, serial clinical observation.

TABLE 5 Comparison of antibiotic use (A) and "rule out sepsis" evaluations (B) as per SCO vs. recommendations of the NSC among late-preterm infants.

		NSC		
		No antibiotics	**Antibiotics**	**Total (% of preterm infants)**
(A) Comparison of antibiotic use				
SCO	No antibiotics	130	33	163 (91.6%)
	Antibiotics	0	15	15 (8.4%)
	Total (% of preterm infants)	130 (73.0%)	48 (27.0%)	178 (100%)
		NSC		
		No test	**Rule out sepsis**	**Total (% of preterm infants)**
(B) Comparison of "rule out sepsis" evaluations				
SCO	Not evaluated	127	18	145 (81.5%)
	Rule out sepsis	1	32	33 (18.5%)
	Total (% of preterm infants)	128 (71.9%)	50 (28.1%)	178 (100%)

NSC, neonatal sepsis calculator; SCO, serial clinical observation.
Differences between proportions were analyzed using McNemar's test.

infants in the study was small and we had no cases of culture-proven EOS to define a hypothetical overtreatment index or to confirm the safe management of infected neonates, although this information has already been provided in our previous study (10). In addition, management was based on SCO, whereas the data elements for the NSC were collected retrospectively. This would result in a less accurate identification of the symptoms by which the NSC score was calculated, although newborn charts accurately describe their clinical condition. Finally, the definition of "minor" and "major" symptoms was defined a priori, based on expert opinions in our network. However, a large prospective study including EOS cases and controls may more accurately define which symptoms are most predictive of EOS.

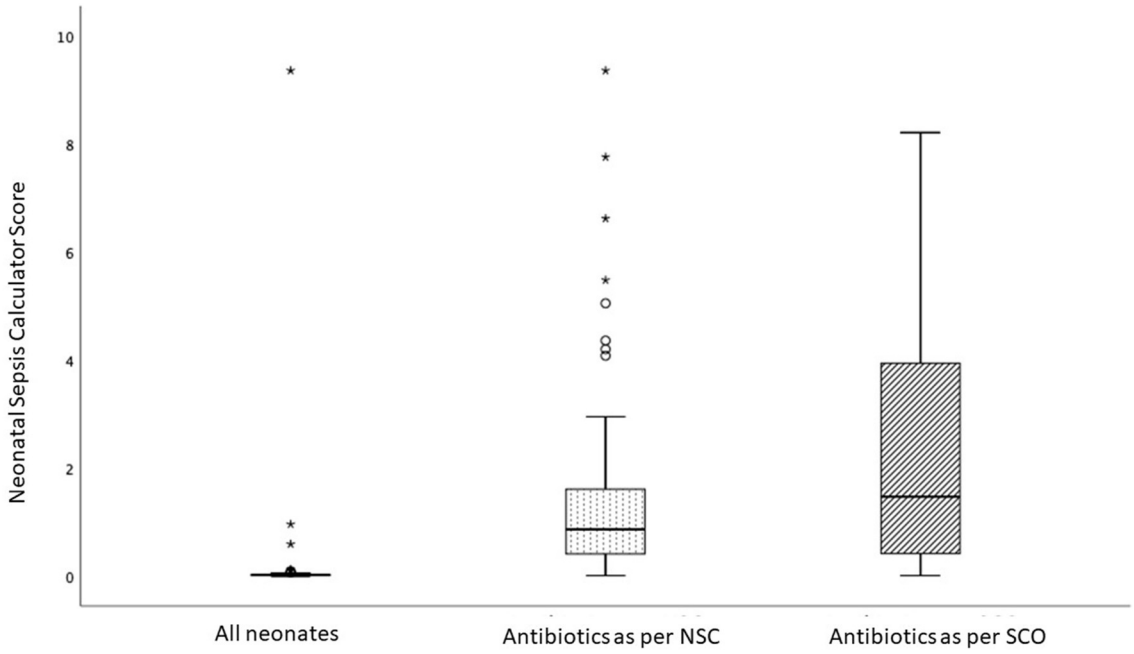

FIGURE 2
Box-and-Whisker plot comparing the score of the Neonatal Sepsis Calculator (NSC) in three groups: all infants in the study (median score = 0.02; IQR 0.02), infants receiving antibiotics as per NSC (median = 0.86; IQR = 1.22) and infants recommended antibiotics by Serial Clinical Observation (SCO) (median = 0.86; IQR = 1.22). The score in the study population was low; infants recommended antibiotics as per SCO had higher scores compared with infants recommended antibiotics as per NSC. Each box bounds the IQR range divided by the median (solid horizontal line); the lower and upper margins of the box represent the 25th and the 75th centile, respectively. The whiskers extend 1.5 times the IQR from the median. A circle (o) is used to mark outliers with values between 1.5 and 3 box lengths from the upper edge of the box; the asterisk (*) is used for extreme outliers (a value more than 3 times the interquartile range).

In conclusion, SCO of asymptomatic infants with RFs for EOS or with "mild, non-progressive symptoms" during the first days of life reduces laboratory evaluation, minimizes unnecessary antibiotics, and avoids separation of the mother from her infant without delaying antibiotic treatment of infected infants. Antibiotic overuse is a planetary emergency and we hope that our experience can help reduce neonatal antibiotic exposure.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Comitato Etico Area Vasta Emilia Nord. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

Author contributions

AB conceptualized the study, drafted the initial manuscript, reviewed and edited it, and supervised the study. IZ and LL contributed to conceptualization, drafted the initial reviewed, and edited the manuscript. LB, EV, AT, GT, ML, FL, FMo, MC, TZ, AB, LI, and FMi contributed to acquisition, analysis and interpretation of data for the work, and reviewed and edited the manuscript. All authors substantially contributed to the work, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Funding

This study was partially supported by the Agenzia Sanitaria Regionale (Emilia-Romagna) Piano Regionale della Prevenzione (2015-2018) C.U.P. n. E43G17000680002.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor CA declared a past co-authorship with one of the authors AB.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2022.882416/full#supplementary-material

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frontiers | Frontiers in Pediatrics

TYPE Brief Research Report
PUBLISHED 15 August 2022
DOI 10.3389/fped.2022.893812

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

RECEIVED 10 March 2022
ACCEPTED 19 July 2022
PUBLISHED 15 August 2022

CITATION
Gallini F, De Rose DU, Coppola M, Pelosi MS, Cota F, Bottoni A, Ricci D, Romeo DM, Spanu T, Maggio L, Mercuri E and Vento G (2022) Maternal *Ureaplasma/Mycoplasma* colonization during pregnancy and neurodevelopmental outcomes for preterm infants. *Front. Pediatr.* 10:893812. doi: 10.3389/fped.2022.893812

Maternal *Ureaplasma/Mycoplasma* colonization during pregnancy and neurodevelopmental outcomes for preterm infants

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Introduction: *Ureaplasma* (*U.*) and *Mycoplasma* (*M.*) species have been related to pregnancy complications (including preterm birth) and worse neonatal outcomes. The aim of our work is to evaluate neurodevelopmental outcomes in preterm infants born to mothers with *Ureaplasma*/*Mycoplasma* colonization during pregnancy.

Methods: Preterm infants with gestational age (GA) of ≤ 30 weeks were included in a retrospective follow-up study. To evaluate the effects of maternal vaginal colonization, we divided preterm infants into two groups: exposed and unexposed infants. All infants were assessed at 24 ± 3 months of age using Griffith's Mental Developmental Scales (GMDS).

Results: Among 254 preterm infants, only 32 infants (12.6%) were exposed to U. /M. colonization during pregnancy. Exposed infants and unexposed ones had a similar Griffith's Developmental Quotient (106 ± 27.2 vs. 108.9 ± 19.5, respectively), without significant differences (p = 0.46). However, exposed infants had a significantly poorer outcome than their unexposed peers in terms of locomotor abilities (100.7 ± 28.3 exposed vs. 111.5 ± 26.1 unexposed, p = 0.03).

Conclusion: For visual and hearing impairment, exposed and unexposed infants had similar incidences of cognitive and motor impairment.

However, exposed infants had significantly lower locomotor scores than unexposed peers.

KEYWORDS

neonate, newborn, neurodevelopment, cognitive, pregnant, prematurity—risk assessment and prevention, motor performance, motor outcomes

Introduction

Ureaplasma (U.) and *Mycoplasma (M.)* species have been related to pregnancy complications (including preterm birth) and worse neonatal outcomes (1, 2). The data from clinical and animal studies, as well as *in vitro* findings, suggest neuroinflammatory patterns in exposed mother/infant pairs (3).

Vaginal swabs can be used to quickly rule out bacterial colonization in mothers during pregnancy. However, given the high frequency of vaginal *Ureaplasma/Mycoplasma* spp. colonization in asymptomatic sexually active women, we still do not know the actual clinical impact of positive maternal swabs regarding neonatal outcomes (4–6).

Different authors investigated their concerns on *Ureaplasma*-driven neuroinflammation in neonates (3). Viscardi et al. described how the presence of *Ureaplasma* was significantly associated with elevated interleukin-1-beta in cord blood and *Ureaplasma* serum-positive infants had a 2.3-fold increased risk of severe intraventricular hemorrhage (IVH) (7). This has been confirmed by Kasper et al., not only for severe IVH, but also when including all the IVH grades (8). Isolation of *Ureaplasma* species from the amniotic cavity cultures at birth also resulted to be significantly associated with an abnormal psychomotor development index, an abnormal neurologic outcome, and a higher probability for diagnosis of cerebral palsy at 2 years of age when compared to patients with negative culture results, according to Berger's findings (9).

The aim of our work was to evaluate neurodevelopmental outcomes in preterm infants born to mothers with *Ureaplasma/Mycoplasma* vaginal colonization during pregnancy.

Materials and methods

Design of the study

A total of 254 preterm infants with gestational age (GA) of $\leq$ 30 weeks (born in our hospital between June 2012 and June 2017) were enrolled in a previous retrospective study about the extra-uterine growth restriction and related neurodevelopmental outcomes, as previously published (10). All included infants regularly attended our follow-up program at least up to 24 months of corrected age (CA), as per our unit protocol (11), up to November 2019. Exclusion criteria were death after discharge, incomplete medical records, congenital malformations, genetic syndromes, large for gestational age (LGA) at birth, non-Italian speaker, lost to follow-up (not present at 2 sequential follow-up visits), and inclusion in any trial that could interfere with growth and outcomes (10). Data of patients were obtained from medical records.

In order to evaluate the effects of maternal intrauterine inflammation, we divided preterm infants into two groups: infants born to mothers with *Ureaplasma/Mycoplasma* colonization during pregnancy (cases) and unexposed infants (controls). We also considered unexposed infants born to mothers who were not screened, and pregnancy was uneventful until delivery (**Figure 1**).

All mothers with the threat of miscarriage or preterm labor were screened for *Ureaplasma/Mycoplasma* colonization; samples from the lower genital tract were obtained using vaginal swabs during physical examinations. Colonization was diagnosed *via* microbial culture or DNA extraction and PCR analysis according to diagnostic conditions employed at the microbiology unit of each laboratory where the mothers went.

All exposed preterm infants received clarithromycin in the first 3 days of life, after baseline respiratory specimens (nasal swabs if in spontaneous breathing or in non-invasive ventilation; bronchoalveolar lavage fluid if intubated) were collected. In our hospital, culture and identification were performed until 2015 using the *Mycoplasma* IST 2 kit (BioMérieux, Craponne, France), and we were able to identify *U. urealyticum* and *M. hominis* (12). Afterward, the method used has been changed into the new molecular Anyplex^{TM} STI-7 Detection kit (Seegene Inc., Seoul, Korea), based on a multiplex real-time PCR method that can identify *M. genitalium*, *M. hominis*, *U. parvum,* and *U. urealyticum* (13).

Antibiotic treatment was stopped when samples resulted negative.

Neonatal outcomes

Data were collected in our follow-up facility care unit, including pregnancy and newborn characteristics.

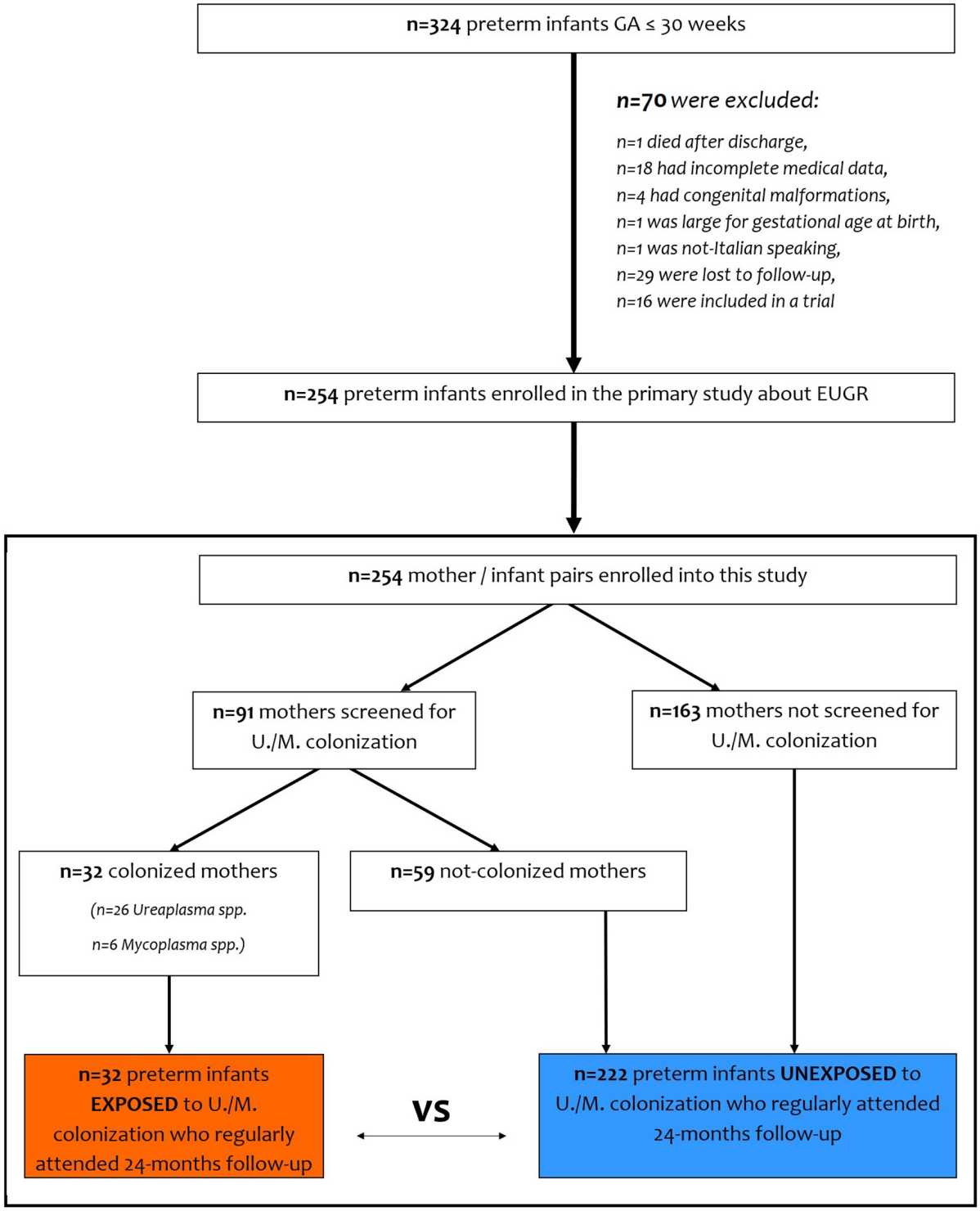

FIGURE 1
Flow-chart of the study design.

The following maternal and neonatal characteristics were collected during the neonatal period: maternal age, antenatal corticosteroids, complete course of antenatal corticosteroids, multiple pregnancy, pathological umbilical artery Doppler parameters, cesarean section, preterm premature rupture of membranes, sex of the neonate, GA,

birthweight, birth head circumference, Apgar at 5 min, number of days on invasive mechanical ventilation, incidence of bronchopulmonary dysplasia (BPD, defined by the need for supplemental oxygen at 36 w of PMA), postnatal steroids, presence of major brain lesions [defined as a grade ≥ III Intraventricular Hemorrhage (IVH) according to Papile or a Cystic Periventricular Leukomalacia (cPVL) according to De Vries (14, 15)], incidence of necrotizing enterocolitis (NEC, defined as a stage ≥ IIA according to Bell's criteria), incidence of early-onset sepsis (EOS, defined as the presence of systemic signs suggestive of infection and positive blood and/or cerebrospinal fluid (CSF) culture before 72 h from birth), incidence of late-onset sepsis (LOS, defined as the presence of systemic signs suggestive of infection and positive blood and/or CSF culture after 72 h from birth), incidence of hemodynamically significant patent ductus arteriosus (PDA, i.e., pharmacologically or surgically treated), and days of parenteral nutrition.

Long-term outcomes

All infants were assessed at 24 ± 3 months of age by an expert pediatric neurologist using Italian-validated translation of Griffith's Mental Developmental Scales (GMDS) (0–2 years) (16).

GMDS yields five subscales: Locomotor, Personal-Social, Hearing and Speech, Eye and Hand Coordination, and Performance. The subscales yield standardized scores for each domain and a composite developmental quotient. The cognitive developmental outcome was classified normal when Griffiths' developmental quotient (GDQ) was > 85; borderline, when GDQ was from 70 to 85; and delayed when GDQ was less than 70 (17).

Conversely, the motor outcome was classified as normal development, "minor neurological dysfunction" (MND, according to Touwen) (18), and cerebral palsy (CP, according to Bax) (19).

The onset of epilepsy was also recorded.

The severity of Retinopathy of prematurity (ROP) was defined as stage of ≥ 3, according to ICROP criteria (20), whereas all infants underwent an Auditory Brain Response test at 3 months CA to identify hearing impairment. Mild hearing loss was defined as the auditory threshold of 15–40 dB, moderate hearing loss if 40–70 dB, severe if 70–90 dB, and deafness if > 90 dB (21).

Disability was classified according to a previous scheme utilized in the EPICure studies (22), classifying outcomes as severe, moderate, and mild or no impairment using defined categories in motor, developmental, sensory, and communication domains.

Statistical analysis and ethical approval

Data are presented as numbers and percentages for categorical variables. Continuous variables are expressed as mean ± standard deviation (SD) if they were normally distributed or as median and interquartile range if normality could not be accepted. Categorical variables were compared using the Fisher's exact test or Chi-square with Yates correction. *T*-test was used to compare cases and controls in terms of Griffiths' development quotient (GDQ) and neurodevelopment impairment (NDI). A p-value < 0.05 was considered significant.

Multivariate analysis (by means of logistic regression in case of binary outcome or linear regression in case of continuous variable outcome) was considered when appropriate, after the correction by GA and the presence of major brain lesions (IVH ≥ III grade or cPVL).

Statistical analysis was performed using software programs Microsoft Excel (2016 for Windows) and STATA/IC (version 15.1 for Windows).

The study was carried out in compliance with the Declaration of Helsinki and its later amendments, approved by the Ethics Committee of Fondazione Policlinico Universitario "Agostino Gemelli," Rome, Italy (in the context of the protocol number 0036181/20 - ID 3244), and written informed consent was obtained from parents for any clinical research purpose about clinical data.

Results

Maternal characteristics and neonatal characteristics of the study patients are reported in **Table 1**. Among 254 preterm infants, only thirty-two infants (12.6%) were reported to be exposed to *U./M.* colonization during pregnancy: among these, 26 were exposed to *Ureaplasma* (10 *U. urealyticum*, 4 *U. parvum*, 12 *U.* spp. Not specified) and 6 were exposed to *Mycoplasma* spp. *Mycoplasma* cases were not typed.

However, only 91 mothers were screened for *U./M.* colonization. If we only consider them, the colonization rate increases up to 32/91 cases (64.8%).

Mean GA at the time of *U./M.* detection was 19.3 ± 7.3 weeks GA (range: 7–29). The two groups of exposed and unexposed infants were similar in all maternal characteristics, except for the administration of antenatal corticosteroids (significantly higher in the exposed group) and twin pregnancies (higher in the unexposed group).

None of the preterm infants had a respiratory culture obtained after birth that resulted positive for *U./M.* spp. Exposed infants had a higher incidence of EOS, but not statistically significant. Furthermore, we identified no cases of early-onset and late-onset sepsis due to *U./M.* spp.

TABLE 1 Maternal characteristics and neonatal characteristics at birth and during NICU stay.

	Whole cohort (n = 254)	*Ureaplasma/Mycoplasma* exposed infants (n = 32)	*Ureaplasma/Mycoplasma* unexposed infants (n = 222)	*P*-value
Maternal characteristics				
Maternal age (years), mean ± *SD* (range)	34.2 ± 5.9	36.2 ± 5.6	34.2 ± 5.9	0.07
Antenatal corticosteroids, *n* (%)	221 (87.0)	27 (84.4)	194 (87.4)	0.58
Complete course of antenatal corticosteroids, *n* (%)	113 (44.5)	22 (68.8)	91 (41.0)	**<0.01**
Multiple birth, *n* (%)	82 (32.3)	2 (6.3)	80 (36.0)	**<0.01**
Pathological umbilical artery Doppler parameters, *n* (%)	55 (21.7)	5 (15.6)	50 (22.5)	1.00
Cesarean section, *n* (%)	214 (84.3)	23 (71.9)	191 (86.0)	0.06
Preterm premature rupture of membranes, *n* (%)	104 (40.9)	18 (56.3)	86 (38.7)	0.08
Neonatal characteristics				
Males, *n* (%)	126 (49.6)	19 (59.4)	107 (48.2)	1.00
Gestational age (weeks), mean ± *SD* (range)	28.6 ± 1.5 (23.5–30.6)	28.1 ± 1.7 (25.1–30.4)	28.4 ± 1.5 (23.5–30.6)	0.30
Birthweight (grams), mean ± *SD* (range)	1,090 ± 283 (500–1,820)	1,124 ± 272 (610–1,680)	1,095 ± 288 (500–1820)	0.59
Birth head circumference (centimeters), mean ± *SD*	25.9 + 2.1	25.7 ± 2.3	25.4 ± 2.0	0.44
Apgar at 5 min, mean ± *SD*	8.4 ± 0.9	8.4 ± 1.1	8.4 ± 0.8	1.00
Number of days on invasive mechanical ventilation, mean ± *SD*	6.7 ± 36.4	5.3 ± 11.6	4.3 ± 10.8	0.63
BPD at 36 weeks, *n* (%)	21 (8.3)	3 (9.4)	18 (8.1)	0.74
Postnatal steroids, *n* (%)	21 (8.3)	4 (12.5)	17 (7.7)	0.32
Patients with major brain lesions, *n* (%)	20 (7.9)	4 (12.5)	16 (7.2)	0.29
ROP ≥ 3, *n* (%)	6 (2.4)	0	6 (2.7)	0.75
NEC ≥ 2, *n* (%)	11 (4.3)	1 (3.1)	10 (4.5)	1.00
Early-onset Sepsis, *n* (%)	15 (5.9)	3 (9.4)	12 (5.4)	0.41
Late-onset Sepsis, *n* (%)	92 (36.2)	8 (25)	84 (37.8)	0.17
Hemodynamically Significant PDA, *n* (%)	65 (25.6)	5 (15.6)	60 (27.0)	0.27
Number of days of parenteral nutrition, mean ± *SD*	22.5 ± 17.6	18.4 ± 14.7	22.8 ± 16.7	0.16

Bold values are those that resulted to be statistically significant.

In Table 2, we show how neurodevelopmental outcomes at 24 months CA were similar in exposed and unexposed infants to *U./M.* colonization during pregnancy.

Indeed, exposed infants and unexposed ones had a similar GDQ (106 ± 27.2 vs. 108.9 ± 19.5, respectively), without significant differences (p = 0.46). Analyzing Griffith's subscales, exposed infants had a significantly poorer outcome than their unexposed peers in terms of locomotor abilities (subscale A, 100.7 ± 28.3 exposed vs. 111.5 ± 26.1 unexposed, p = 0.03). Conversely, unexposed infants achieved similar results in other subscales: Personal-Social interactions (subscale B), Hearing and Language assessment (subscale C), Eye and Hand Coordination (subscale D), and Performance scale (subscale E) (Table 3). We tested the result of locomotor abilities with linear regression to verify if the effect of maternal colonization was independent of confounding factors. Maternal colonization was still significantly associated with the locomotor score after correcting with GA, IVH ≥ III grade, and cPLV (OR −10.3; CI −20.5/−0.04; p = 0.04).

Discussion

The detection of *Mycoplasma* and *Ureaplasma* spp. in vaginal cultures has been associated with spontaneous miscarriage, preterm birth, and chorioamnionitis (23). After

TABLE 2 Neurodevelopmental outcomes at 24 months CA in exposed and unexposed infants to *U./M.* colonization during pregnancy.

	Whole cohort (*n* = 254)	*Ureaplasma/Mycoplasma* exposed infants (*n* = 32)	*Ureaplasma/Mycoplasma* unexposed infants (*n* = 222)	*P*-value
Cognitive impairment at 24 months CA				
No cognitive impairment, *n* (%)	231 (90.9)	27 (84.4)	204 (91.9)	0.18
Mild, *n* (%)	14 (5.5)	3 (9.4)	11 (5.0)	0.40
Moderate, *n* (%)	4 (1.6)	2 (6.3)	2 (0.9)	0.08
Severe, *n* (%)	5 (2.0)	0	5 (2.3)	0.86
Motor impairment at 24 months CA				
No motor impairment, *n* (%)	207 (81.5)	28 (87.5)	179 (80.6)	0.47
MND, *n* (%)	26 (10.2)	1 (3.1)	25 (11.3)	0.22
Ambulatory cerebral palsy, *n* (%)	13 (5.1)	1 (3.1)	12 (5.4)	0.91
Not ambulatory cerebral palsy, *n* (%)	8 (3.1)	2 (6.3)	6 (2.7)	0.27
Visual impairment at 24 months CA				
No visual impairment, *n* (%)	225 (88.6)	31 (96.9)	194 (87.4)	0.14
Strabismus/refraction defects, *n* (%)	19 (7.5)	1 (3.1)	18 (8.1)	0.48
Hypovision, *n* (%)	10 (3.9)	0	10 (4.5)	0.62
Blindness, *n* (%)	0	0	0	1.00
Hearing impairment at 24 months CA				
No hearing impairment, *n* (%)	229 (90.2)	29 (90.6)	200 (90.1)	0.92
Mild (uncorrected), *n* (%)	12 (4.7)	2 (6.3)	10 (4.5)	0.65
Moderate (corrected with hearing aids), *n* (%)	11 (4.3)	1 (3.1)	10 (4.5)	0.74
Deep, *n* (%)	2 (0.8)	0	2 (0.9)	0.59
Disability at 24 months CA				
No, *n* (%)	207 (81.5%)	25 (78.1)	182 (82.0)	0.63
Mild, *n* (%)	23 (9.1)	4 (12.5)	19 (8.6)	0.51
Moderate, *n* (%)	11 (4.3)	1 (3.1)	10 (4.5)	0.72
Severe, *n* (%)	13 (5.1)	2 (6.3)	11 (5.0)	0.67

TABLE 3 Griffiths' at 24 months CA in exposed and unexposed infants to *U./M.* colonization during pregnancy.

	Ureaplasma/Mycoplasma exposed infants (*n* = 32)	*Ureaplasma/Mycoplasma* unexposed infants (*n* = 222)	*P*-value
Griffiths' developmental quotient (GDQ)	106.0 ± 27.2	108.9 ± 19.5	0.46
Griffiths' subscales			
A: Locomotor abilities	100.7 ± 28.3	111.5 ± 26.1	**0.03**
B: Personal-Social interactions	115.2 ± 25.3	116.1 ± 35.8	0.89
C: Hearing and Language assessment	98.5 ± 28.0	94.9 ± 33.2	0.56
D: Eye and Hand Coordination	101.9 ± 17.0	101.9 ± 25.1	1.00
E: Performance scale	117.2 ± 26.9	119.7 ± 35.2	0.70

Bold values are those that resulted to be statistically significant.

preterm birth, perinatal *Ureaplasma* exposure might have a role in the development of neonatal inflammation, infection, and lung damage (24).

In this study we compared neonatal outcomes following maternal colonization during pregnancy due to *Mycoplasma* and *Ureaplasma*, comparing two groups with similar characteristics, in terms of GA and birthweight. We found no significant differences in the incidence of BPD at 36 w and other comorbidities, according to maternal colonization. However, the

higher percentage of a complete course of antenatal steroids in the exposed group could have contributed to this finding.

The association of BPD with *Ureaplasma* spp., as reported by other studies, probably depends on a perinatal intrauterine infection (25) or a high-degree maternal colonization ($\geq 10^4$ colony-changing units/ml) (26), rather than a single finding during pregnancy.

Ureaplasma spp. stimulate the release of tumor necrosis factor-alpha (TNFα), interleukin-1beta (IL-1β), interleukin-8 (IL-8), monocyte chemoattractant-1 (MCP-1), transforming growth factor-beta 1 (TGFβ1), and other mediators by various cell types *in vitro,* and *Ureaplasma* spp. colonization is associated with increased concentrations of these cytokines in tracheal aspirates during the first week of life in infants who develop BPD (27). Interleukin-6 (IL-6) stimulates the local antigen-specific immune response and especially exerts important anti-inflammatory effects in the lungs. By partially blocking the IL-6 response to lipopolysaccharide, *Ureaplasma urealyticum* might neutralize the downregulation of proinflammatory cytokines (28). The multiple banded antigen (MBA) is a surface lipoprotein that is the predominant pathogen-associated molecular pattern (PAMP) detected by the host immune system and has been proposed as the major *Ureaplasma* virulence factor (29).

Ureaplasma exposure can be the first "hit," downregulating the host response, while microbial dysbiosis changes in the relative abundance of *Proteobacteria* and *Firmicutes*, and reduced *Lactobacilli* may be linked to the progression and severity of BPD (28). More research on microbiome optimization in preterm infants at risk for BPD is needed (30).

Exposed infants had a higher global incidence of early-onset sepsis from other microorganisms than *U./M.*, although not significantly. This is in line with findings by Kasper et al., who found a relationship between the bacterial load of *Ureaplasma* in amniotic fluid and an increased intrauterine inflammatory response (31).

Concerning neurodevelopmental outcomes at 2 years, *Ureaplasma*-mediated brain injury is probably due to cytokine activation of the central nervous system immune response, with a fivefold increased risk for severe IVH in case of *Ureaplasma*-positive sera and increased serum IL-1β (32).

In our study, exposed and unexposed infants to maternal *Ureaplasma* during pregnancy had similar incidences of cognitive and motor impairment, as for visual and hearing impairment. We observed no differences in terms of disability. These findings are similar to those reported by Viscardi et al. who recently published the 2-year outcomes of a double-blind, placebo-controlled randomized trial of azithromycin to eradicate *Ureaplasma* respiratory colonization in preterm infants. They did not observe strong evidence of a difference in long-term neurodevelopment outcomes (assessed using the Bayley Scales of Infant and Toddler Development, third edition) in preterm infants treated with azithromycin in the first week of life compared to placebo (33).

However, we observed significantly lower scores in Griffith's subscale concerning locomotor abilities in exposed infants. The biological plausibility of this result is based on *Ureaplasma*-driven systemic inflammation, well-confirmed *in vitro* and in animal data with retarded myelination, impaired brain growth, microglia activation, decreased astrocyte numbers, and increased oligodendrocytes (3). Therefore, exposed infants should be carefully evaluated already during the NICU stay, ruling out the presence of major brain lesions and monitoring the growth of the main cerebral structures (34, 35).

The main limitations of our study are the retrospective single-center design with a small sample of exposed infants, the lack of data about maternal antibiotic treatment administered during pregnancy, the lack of complete data about chorioamnionitis and placental pathology with evidence of inflammation in colonized mother/infant pairs, and the microbial culture initially used to identify these microorganisms in neonates (rather than the current molecular method). Furthermore, infants whose mothers have not been screened were also included in the unexposed group, and some colonized mothers may have been missed due to a single sampling during pregnancy and their infants could have been misclassified.

Finally, we found no significant differences according to the week of GA at diagnosis of *U./M.* spp., probably due to the small sample size of exposed infants. We speculated that, probably, later colonization could have a greater weight in influencing outcomes of preterm infants. To the best of our knowledge, there are no available studies that have yet investigated this aspect.

In conclusion, we analyzed a homogeneous cohort of preterm infants, without significant differences in maternal and neonatal characteristics between the exposed and unexposed groups, in the context of a correct follow-up study. We found that exposed infants to maternal *U./M.* colonization had significantly lower locomotor scores than unexposed peers.

Further prospective multicenter studies in a larger population of preterm infants are needed to better understand if maternal colonization with *Ureaplasma* and *Mycoplasma* could affect neonatal outcomes or not, sparing improper use of antibiotics in mothers and infants.

Data availability statement

The original contributions presented in this study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics Committee of IRCCS Fondazione

Policlinico Universitario "Agostino Gemelli" (Rome, Italy)—in the context of the protocol number 0036181/20 - ID 3244. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

FG and DD conceptualized and designed the study, designed the data collection instruments, enrolled subjects, collected the data, analyzed the data, and drafted the initial manuscript. MC, MP, FC, and AB collected the data, analyzed the data, and revised the manuscript. DR, DMR, TS, LM, and EM critically reviewed the manuscript. GV coordinated and supervised the work and critically reviewed the manuscript for important intellectual content. All authors approved the final manuscript as submitted and agreed to be accountable for all the aspects of the work.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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frontiers | Frontiers in Pediatrics

TYPE Brief Research Report
PUBLISHED 23 August 2022
DOI 10.3389/fped.2022.900249

OPEN ACCESS

EDITED BY
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SPECIALTY SECTION
This article was submitted to Neonatology, a section of the journal Frontiers in Pediatrics

RECEIVED 20 March 2022
ACCEPTED 27 July 2022
PUBLISHED 23 August 2022

CITATION
Huang T, Cai W, Ni C, Lai S, Lin S and Wang Q (2022) Changes in cytomegalovirus load in the breast milk of very/extremely premature infants and the effect of pasteurization and freeze–thawing on reducing viral load.
Front. Pediatr. 10:900249.
doi: 10.3389/fped.2022.900249

Changes in cytomegalovirus load in the breast milk of very/extremely premature infants and the effect of pasteurization and freeze–thawing on reducing viral load

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Objective: This study aimed to clarify the change in Cytomegalovirus (CMV) loads in breast milk (BM) of very/extremely premature infants (VPI/EPI) with birth weight < 1,500 g after birth, and to compare the effectiveness of pasteurization and freeze–thawing methods in reducing the CMV load of BM.

Methods: Breast milk samples were collected and tested every 2 weeks by fluorescence quantitative polymerase chain reaction (FQ-PCR). We determined CMV load in BM before and after pasteurizing, and freeze-thawing.

Results: Cytomegalovirus DNA can already be detected in colostrum. The viral load gradually increased in the first 4 weeks, peaked in the 4th to 6th weeks, and gradually decreased thereafter. The viral load gradually returned to the initial level approximately 10–12 weeks postpartum. During the peak period of the CMV load in BM, the viral load was higher in the EPI than the VPI ($P < 0.05$). The average CMV load (logarithmic [LG]) in the pasteurization group was significantly lower than that in the raw BM group. The average CMV load in the freeze-thawed BM group was significantly lower than that in the raw BM group. The mean CMV load in the pasteurized BM group was lower than that in the freeze–thawed BM group, but the difference was not statistically significant. The CMV-DNA clearance rate in pasteurized was higher than in freeze–thawed ($P < 0.05$).

Conclusion: The CMV detoxification rate in BM is high and the peak load period is mainly between 4 and 6 weeks. The CMV load values detected are higher than the threshold values (7×10^3 copy number/mL) of CMV infection that are reported in the literature as a concern. Both the freeze-thaw and pasteurization techniques can effectively reduce the CMV load.

KEYWORDS

CMV DNA, breast milk, very/extremely premature infants, pasteurization, freeze-thawing, CMV infection

Introduction

The Cytomegalovirus-immunoglobulin G (CMV-IgG) positivity rate in the serum of women of childbearing age in China is as high as 90% (1, 2). After delivery, CMV can be reactivated in the mammary gland. Preterm infants, especially very/extremely premature infants (VPI/EPI), with birth weight < 1,500 g, lack maternal antibodies obtained through the placenta, and their immune function are immature. Breastfeeding has become the main cause of acquired CMV infection, which may cause multiple organ damage, and even death in severe cases (3).

Breast milk (BM) provides the optimal diet for newborns, particularly those born prematurely. However, how to breastfeed VPI/EPI with CMV DNA positive BM remains a point of contention. There have been only limited investigations on the rules of CMV detoxification in VPI/EPI, and there is still contention regarding the most effective therapies for CMV DNA-positive BM. The CMV load level in the breast milk of VPI/EPI was evaluated at different times after birth in this study to better understand the CMV infection features and apply that knowledge to the CMV detoxifying practices for BM. A strategy to reduce CMV load in breast milk was discovered by comparing the reduction of CMV load in breast milk following pasteurization and freeze-thaw treatment.

Methods

Research object and grouping

The inclusion criteria of the study were as follows: newborns hospitalized in the neonatal department of our hospital from January 2020 to December 2020 who met the following three requirements: (1) hospitalized within 24 h after birth; (2) VPI/EPI with birth weight < 1,500 g (gestational age at birth 28–32 weeks/gestational age at birth < 28 weeks); and (3) breastfeeding during hospitalization. The exclusion criteria were as follows: (1) congenital abnormalities or malformations; (2) death occurring before the study was completed; and (3) failure to submit BM for examination according to study design specifications.

The Experimental Group comprised nursing mothers of VPI/EPI included in the study. We collected BM every 2 weeks until 12 weeks postpartum or discharge. Overall, 100 CMV DNA-positive milk samples were collected at 2, 4, 6, 8, and 10 weeks postpartum, with 20 samples in each period. The collected milk was equally divided into three groups: the fresh BM group, the pasteurization group, and the freeze-thaw treatment group.

This study was approved by the Ethics Committee of the Fujian Provincial Maternity and Child Hospital. Informed consent was obtained from the parents of the children.

Collection and treatment of specimens

Breast milk was collected from very/extremely preterm mothers enrolled in the study at 0–5 days, 2, 4, 6, 8, 10, and 12 weeks after birth, or until discharge. Each time, 3–15 mL of BM was collected and evenly divided into three disposable sterile test tubes.

In the Raw BM group, milk samples were stored at 4°C. In the pasteurized BM groups, the samples were heated in a 62.5°C water bath for 30 s. In the freeze–thawed BM groups, the samples were frozen at −20°C for 72 h, melted naturally at 4°C, and then rewarmed in a warm water bath at 40°C for 30 min.

Outcome criteria

After the fluorescence quantitative polymerase chain reaction (FQ-PCR) instrument generated the curve, the specific quantitative value of CMV load was extracted from BM samples using the automated extraction system. The result was judged as negative when the quantitative value was <500 copies/mL, and positive when the quantitative value suggested by the BM sample was >500 copies/mL. Subsequently, the load of CMV DNA test values was obtained from the standard curve suggested by the system.

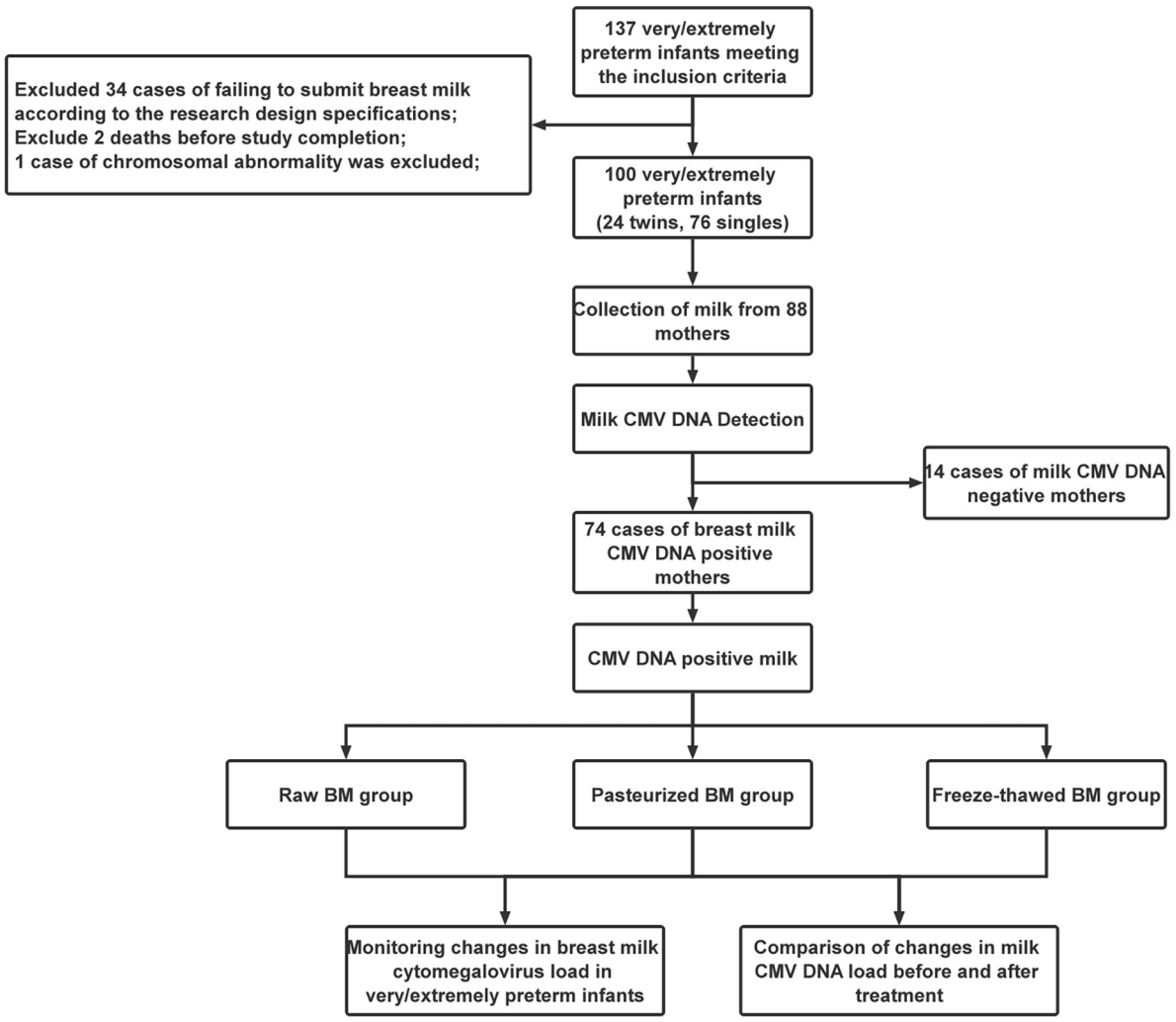

FIGURE 1
Flow Chart of the study.

TABLE 1 Comparison of positive rates of cytomegalovirus (CMV) DNA in very/extremely premature infants (VPI/EPI) breast milk (BM).

	<28 week $N = 13$	28–32 week $N = 75$	χ^2	P
Total positive rate (N, %)	10 (76.9)	64 (85.3)	–[b]	0.427
Colostrum positive rate (N, %)	2 (15.4)	16 (21.3)	–[b]	1.000

[b]Applies Fisher's exact probability method.

Definition

The CMV clearance rate was defined as the number of BM samples that initially gave a positive CMV test result and then a negative result, divided by the total number of CMV-positive breast milk samples. For this calculation, a conversion was defined when the CMV load dropped below 500 copies/mL.

The CMV detoxification rate was defined as the number of BM samples with a positive CMV DNA test divided by the total number of BM samples.

Statistical analyses

Statistical analyses were performed using SPSS 26 software. The CMV DNA load value was logarithmic (LG). The number of cases and the percentage of the two groups were expressed (N, %). Comparisons between groups were performed using the chi-square test or Fisher's exact probability test. If the measurement data of the two groups met the normality and homogeneity of variance, the data were expressed as the mean ± standard deviation, and the T-test was adopted. If the measurement data of the two groups did not conform to normal distribution or uneven variance, the data were described by the median

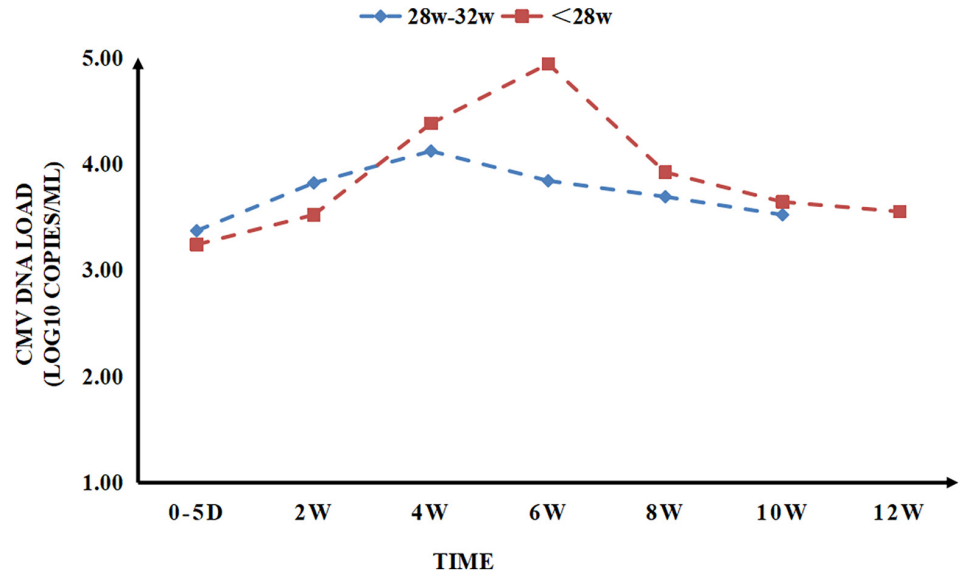

FIGURE 2
Cytomegalovirus (CMV) detoxification rule of very/extremely premature infants (VPI/EPI).

TABLE 2 Comparison of cytomegalovirus (CMV) load in breast milk during peak detoxification periods.

	<28 week $N = 13$	28–32 week $N = 75$	t	P
Breast milk CMV DNA load (Log_{10} copies/mL)	4.94 ± 0.52	4.12 ± 0.68	2.842	0.006

and quartile spacing [M(Q)], and the comparison between groups was performed using the Mann–Whitney U test. If the measurement data of the three groups met the standard of normality and homogeneity of variance, they were expressed as the mean ± standard deviation. One-way ANOVA was used, and SNK (Student–Newman–Keuls) was used for pial comparison between groups. If the measurement data of the three groups did not conform to normal distribution or uneven variance, they were expressed as median and quartile spacing [M(Q)], and the Kruskal–Wallis H test was used. The P-value represents the difference between the data sets, and $P < 0.05$ was considered statistically significant.

Results

Characteristics of the study population

Initially, 137 VPI/EPI who met the inclusion criteria were enrolled for this study, but 37 were subsequently excluded, including 34 cases of failure to submit BM according to study design specifications, two deaths before study completion, and one chromosomal abnormality. Finally, 100 premature infants from 88 different mothers were enrolled in the study. Of these, there were 24 cases of twin premature infants and 76 cases of single premature infants. Figure 1 shows the flow of the study.

Cytomegalovirus transmission from breast milk

Cytomegalovirus DNA positivity rate in breast milk

In this study, we collected BM samples from 88 mothers. CMV DNA of BM was tested every 2 weeks until 12 weeks after delivery, or until discharge. The positivity rate of CMV DNA in BM was 84.1% (74/88), including a rate of 76.9% (10/13) in the BM of EPI and 85.3% (64/75) for VPI. There was no significant difference between the groups ($P > 0.05$, Table 1).

Variation in cytomegalovirus load in breast milk

In our study, CMV DNA could already be detected in colostrum (0–5 days), and the earliest detection time was 3 days after delivery. CMV load showed a gradually increasing trend in the first 4 weeks and peaked at about 4–6 weeks, subsequently beginning to decline gradually and dropping to colostrum level about 10–12 weeks after delivery (Figure 2). With the data collected once every 2 weeks, a peak of CMV load emerged in the preterm infants: the EPI peaked at week 6, and the VPI peaked at week 4. At the peaked CMV load in preterm infants, the CMV load in EPI was higher than that in VPI, and the difference was statistically significant ($P < 0.05$, Table 2).

Changes in cytomegalovirus load in breast milk after different treatments

One hundred BM samples were collected from 88 very/extremely preterm mothers. We collected 20 samples in each postpartum period (2, 4, 6, 8, and 10 weeks). The average load value of CMV in the Raw BM group was 1.1×10^4 copies/mL, that in the pasteurized BM group was 3.5×10^3 copies/mL, and that in the freeze–thawed BM group was 4.9×10^3 copies/mL. We used the logarithm (LG) for the numerical CMV viral load. The average CMV load (LG) in the pasteurization group was significantly lower than that in the raw BM group ($P < 0.05$). The average CMV load (LG) in the freeze–thawed BM group was significantly lower than that in the raw BM group ($P < 0.05$). The mean CMV load (LG) in the pasteurized BM group was lower than that in the freeze–thawed BM group, but the difference was not statistically significant (**Table 3**).

The CMV DNA negative conversion rate in the pasteurized BM group was 16% (16/100), and the CMV DNA negative conversion rate in the freeze–thawed BM group was 7% (7/100). The difference between the two groups was statistically significant ($P < 0.05$; **Table 4**).

TABLE 3 Comparison of cytomegalovirus (CMV) DNA load before and after breast milk treatment.

Group	*N*	CMV DNA load (Log_{10} copies/mL)	*F*	*P*
Raw BM	100	$4.06 \pm 0.67^{*ab}$	13.447	0.000003
Pasteurized BM	100	3.55 ± 0.78		
Freeze-thawed BM	100	3.69 ± 0.71		

P-value: one-way ANOVA; SNK-q test was used for pairwise comparison.
^{*a}The raw BM group compared with the pasteurized BM group, $P < 0.05$.
^{*b}The raw BM group compared with the freeze–thawed BM group, $P < 0.05$.

TABLE 4 Cytomegalovirus (CMV) DNA clearance rate after pasteurization and freeze–thawing of breast milk (BM).

	Pasteurization $N = 100$	Freezing-thawing $N = 100$	χ^2	*P*
CMV DNA clearance rate (*N*, %)	16 (16.0)	7 (7.0)	3.979	0.046

Discussion

Cytomegalovirus transmission in breast milk

The positivity rate of CMV-IgG in Chinese women has been found to be over 90% (1, 2). CMV can be locally reactivated and shed by the infected maternal mammary gland (4, 5). Studies have found that the positivity rate of CMV in BM fluctuates between 56.1 and 96% (6–9). In this study, the positivity rate of CMV DNA in the BM of VPI/EPI was 84.1%, which is quite high. There was no difference in the positivity rate of CMV DNA in BM between VPI and EPI.

Detoxification of CMV in BM generally begins mainly at 10 days postpartum. The viral load increased gradually with age, reaching a peak at approximately 4–8 weeks, and decreasing significantly at 9–12 weeks (5, 10–12). In this study, the positive rate of CMV DNA detected in the colostrum of VPI/EPI was 20.1%, which first appeared on the 3rd day after delivery. The CMV load gradually increased in the 4th week, peaked at approximately 4–6 weeks, and then gradually decreased to the colostrum level at approximately 10–12 weeks postpartum. This suggests that the CMV detoxification rules of BM in VPI/EPI can guide the choice of the timing of BM treatment.

Jim et al. (9), Wang et al. (13) showed that breastfeeding with high CMV load and prolonged BM CMV detoxification increased the incidence of CMV infection acquired from BM in premature infants. Domestic studies have found that CMV infection caused by BM transmission is more likely to occur when the BM CMV load is greater than 2.6×10^3 copy number/mL (13). Some scholars also believe that the risk of CMV infection is higher for BM CMV load $> 7 \times 10^3$ copy number/mL, and the peak period for BM detoxification, that is, when the risk of CMV infection may be increasing, is at 4–8 weeks postpartum (1). In this study, the peak CMV load in BM appeared at week 6 in VPI and at week 4 in EPI. At the peak CMV load in BM, the viral load level of EPI was significantly higher than that of VPIs and higher than the threshold value of CMV load proposed in domestic studies (1, 13). This suggests that VPI/EPI are at risk of acquiring CMV infection through breastfeeding, and EPI are at higher risk than VPI.

Effect of pasteurization and freeze-thawing of breast milk on cytomegalovirus load

Currently, the commonly used methods for inactivating BM CMV mainly include freeze–thawing and pasteurization (3, 4, 14). Chiavarini et al. (8) and Hosseini et al. (15) showed that both pasteurization and freeze–thawing effectively reduced the CMV load levels in BM. Pasteurization includes long pasteurization (62.5°C for 30 min) and high-temperature pasteurization (72°C for 5–10 s). Long pasteurization, while effective at reducing the risk of CMV infection, results in the loss of some nutritional and immune components in BM. High-temperature pasteurization has little effect on the active ingredients in BM, but it is not easy to control clinically. Therefore, long pasteurization is still the most commonly used method of BM disinfection recommended by international BM banks (16). Freeze-thawing of BM is also considered to be effective at reducing CMV load levels while avoiding the impact on BM composition. However,

other studies have suggested that freeze–thawing cannot reduce CMV load (17).

In a study, CMV infectivity was quantified using sensitive detection techniques like CMV late mRNA and high-speed centrifugal microculture assays. The results confirmed that late mRNA and viral infectivity were eliminated after pasteurization but not after freeze-thawing (18). Furthermore, the results showed that both techniques can effectively reduce the CMV load level in BM. The CMV load reduction and clearance rates of pasteurized BM are higher than those of freeze–thawed BM. There was still CMV DNA in pasteurized and freeze–thawed BM, and the clearance rate of CMV DNA was not high. Therefore, the evaluation of the viral infectivity of CMV requires further laboratory examination and the support of clinical trial data.

Conclusion

The BM CMV detoxification rate is high in VPI/EPI. During the peak of the BM CMV load, the viral load level was significantly higher for EPI than that of the VPI, and both were significantly higher than the threshold value of viral load leading to CMV infection. Therefore, VPI/EPI are both at risk of acquiring CMV infection through breastfeeding. Both pasteurization and freeze–thawing can effectively reduce the CMV load of BM. The CMV clearance rate of pasteurized BM is higher. VPI and EPI have different BM CMV load peak periods. It is recommended to adopt an individualized breastfeeding strategy in preterm infants according to the change in BM CMV load during the first months after birth.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Ethics Committee of the Fujian Provincial Maternity and Child Hospital. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

WC and TH designed the study and submitted the manuscript. TH and CN collected the data. SLa and QW analyzed the data together. TH and SLi drafted the manuscript. WC supervised this study. All authors read the final version of this article and approved the submitted version.

Funding

This work was supported by the Project of Scientific Research Fund of Fujian Maternal and Child Health Hospital, China (no. YCXM19-20). This funding source played a key supportive role in the study.

Acknowledgments

We express our profound thanks to WC, CN, SLa, SLi, and QW for their generous contributions to the manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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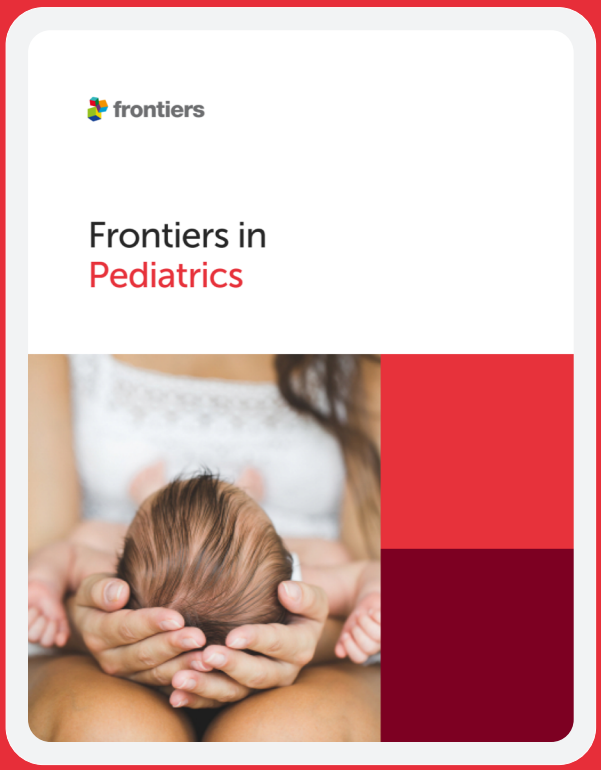

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