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Prognostic markers of symptomatic congenital human cytomegalovirus infection in fetal blood

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Objective To identify fetal cord blood prognostic markers of symptomatic congenital human cytomegalovirus infection (HCMV).

Design Retrospective observational study.

Setting Fetal medicine unit in Milan and Medical virology unit in Pavia, Italy.

Population HCMV-infected and -uninfected fetuses of mothers with primary HCMV infection during the period 1995–2009.

Methods Overall, 94 blood samples from as many fetuses of 93 pregnant women experiencing primary HCMV infection were examined for multiple immunological, haematological and biochemical markers as well as virological markers. Congenital HCMV infection was diagnosed by detection of virus in amniotic fluid, and symptomatic/asymptomatic infections were determined by ultrasound scans, nuclear magnetic resonance imaging, histopathology or clinical examination at birth. Blood sample markers were retrospectively compared in symptomatic and asymptomatic fetuses with congenital infection.

Main outcome measures A statistical analysis was performed to determine the value of each parameter in predicting outcome.

Results Univariate analysis showed that most nonviral and viral markers were significantly different in symptomatic (n = 16) compared with asymptomatic (n = 31) fetuses. Receiver operator characteristics analysis indicated that, with reference to an established cutoff for each marker, the best nonviral factors for differentiation of symptomatic from asymptomatic congenital infection were β_2-microglobulin and platelet count, and the best virological markers were immunoglobulin M antibody and DNAaemia. β_2-Microglobulin alone or the combination of these four markers reached the optimal diagnostic efficacy.

Conclusions The determination of multiple markers in fetal blood, following virus detection in amniotic fluid samples, is predictive of perinatal outcome in fetuses with HCMV infection.

Keywords β_2-microglobulin, congenital infection, cordocentesis, fetal blood, human cytomegalovirus, prognostic markers.

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Introduction

Human cytomegalovirus (HCMV) is a major cause of congenital infection as well as sensorineural hearing loss and neurodevelopmental delay.[1,2] The risk of mother-to-child HCMV transmission from mothers with primary infection is 32%, whereas this risk drops to 1.4% in case of recurrent infection during pregnancy.[1] The overall prevalence of congenital HCMV infection is 0.64–0.70%. Among congenital infections, the percentage of symptomatic infections at birth ranges from 11.0 to 12.7%. The percentage of congenitally infected symptomatic children with permanent sequelae is estimated to be 40–58%, whereas the percentage of children without symptoms at birth who develop later permanent sequelae is 13.5%.[2]

Clinical management of HCMV infection during pregnancy is based mainly on the detection of HCMV infection in the amniotic fluid and on the generally shared opinion that fetal HCMV infection acquired late in pregnancy does not result in symptomatic newborns,[3] whereas infection

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acquired in the first half of pregnancy carries a higher risk of a symptomatic fetus/newborn.[4] However, the main issue both for mothers-to-be and doctors is whether it is possible to predict which infected fetuses will be symptomatic at birth (or later in life).[5]

The role of ultrasound examination in predicting the presence of symptoms at birth has been emphasised by some authors.[6] However, this has been questioned by other groups who found an ultrasound sensitivity of only 21% in predicting symptomatic newborns.[7] As a result of these discrepancies, new approaches have been proposed based on HCMV DNA quantification in amniotic fluid[8,9] or combined assessment of ultrasound findings and low platelet count in fetal blood samples.[6] However, it seems unlikely that a single test or examination can predict the collective events that might lead to permanent damage.

In the present study, we analysed a retrospective cohort of pregnant women who suffered from primary HCMV infection at different ages of gestation and who underwent amniocentesis and cordocentesis. A panel of nonviral and viral assays was performed according to clinical protocols on fetal blood samples, to integrate multiple markers of fetal organ damage caused by HCMV infection.

Methods

This retrospective study was conducted on a subgroup of 735 pregnant women who were referred to our two centres (Virology Unit, Fondazione IRCCS Policlinico San Matteo University Hospital, Pavia, and Buzzi Childrens' Hospital, Milan, Italy) when primary maternal HCMV infection was suspected.

After collection of an initial accurate medical history, maternal seroconversion was assessed by examination of blood samples drawn to perform serological and virological assays. These assays were performed at the Virology Unit, Fondazione IRCCS Policlinico San Matteo Hospital. The study covered the period between January 1995 and July 2009. From 1995 to 2004, women choosing prenatal diagnosis underwent amniocentesis and cordocentesis on the same occasion. Subsequently, only pregnant women with an amniotic fluid sample positive for HCMV were offered cordocentesis. The study protocol was approved by the Institutional Review Board. Written informed consent was obtained from each woman before invasive procedures (amniocentesis or cordocentesis). Primary HCMV infection and its timing were diagnosed according to decreasing levels of HCMV-specific immunoglobulin M (IgM) antibody, increasing levels of IgG antibody avidity, detection of HCMV and HCMV products in blood, and presence of clinical symptoms and/or abnormal laboratory findings.[10–15] Detection of HCMV fetal infection was performed on amniotic fluid samples by viral culture (determination and quantification of HCMV infectivity) and viral DNA quantification by polymerase chain reaction in multiple amniotic fluid subsamples, as reported previously.[16] Results of viral tests were confirmed on terminated fetuses, or using urine of neonates at birth.

For clinical purposes, pregnant women were counselled on the fetal risks associated with primary maternal infection as well as the possible diagnostic options: prenatal diagnosis of an intrauterine infection by amniocentesis and fetal monitoring by serial ultrasound or magnetic resonance imaging examinations. A symptomatic infection was defined as the presence of an abnormal ultrasound and/or magnetic resonance imaging findings and/or histopathological findings of disseminated infection for fetuses terminated or dead *in utero* or as the presence of clinical symptoms up to 6 months of age for delivered newborns (Table 1).

For the purposes of this study, the following examinations were performed prospectively in fetal blood samples: immunological (relative percentage of CD3+, CD4+, CD8+, CD4+ CD8+ ratio and CD3+ HLA-DR+), haematological (platelet and white blood cell counts, percentage of lymphocytes), biochemical (aspartate and alanine aminotransferases, γ-glutamyl-transpeptidase, β_2-microglobulin). In addition, the following virological parameters were prospectively determined on fetal blood samples, i.e. antigenaemia,[14] viraemia,[15] DNAaemia[12,13] and HCMV-specific IgM antibody.

Results of nonviral and viral assays were compared between infected and noninfected, and between symptomatic and asymptomatic fetuses by the Mann–Whitney *U*-test. For variables that were significantly different between symptomatic and asymptomatic fetuses, a receiver operator characteristics (ROC) analysis was performed to identify an optimal cutoff value to discriminate between symptomatic and asymptomatic fetal infections. The optimal cutoff for each marker was identified as the number expressing the highest value of sensitivity multiplied by specificity. Finally, combinations of optimal cutoffs for different markers were examined to maximise the predictive discriminatory value between symptomatic and asymptomatic infections.

Results

As reported in Figure 1, 93 pregnant women, one of them with a twin pregnancy, were accepted to undergo cordocentesis. A total of 94 fetal blood samples were obtained. Sixty-four fetuses were sampled on the same occasion as amniocentesis and 30 were sampled after detection of HCMV in amniotic fluid by combined tests. In the first group, 27 samples were obtained from fetuses in whom a positive amniotic fluid was assessed, and 37 from fetuses with negative amniotic fluid tests. Cordocentesis was

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Table 1. Characteristics of the 16 symptomatic fetuses/newborns

Fetus/ newborn#	Outcome	Cerebral ultrasound/ MRI alterations	Noncerebral ultrasound alterations	Post-mortem examination	Symptoms at birth
1	TOP	Ventriculomegaly	Hyperechogenic bowel	ND	NA
15	TOP	No	Ascites	ND	NA
18	TOP	Ventriculomegaly	No	ND	NA
54	TOP	Microcephaly	Hyperechogenic bowel	ND	NA
64	Intrauterine death	No	Ascites, hyperechogenic liver, enlarged placenta	ND	NA
65	Intrauterine death	Ventriculomegaly	Hyperechogenic bowel	ND	NA
69	Intra-uterine death	Ventriculomegaly	Hyperechogenic bowel	Kidney and lung HCMV infection	NA
70	TOP	Ventriculomegaly	Ascites	Multiorgan HCMV inclusions, ventriculomegaly, club feet	NA
72	TOP	No	No	Multiorgan HCMV inclusions	NA
78	TOP	MRI alterations	Pelvic effusion	ND	NA
79	Born	MRI alterations	No	NA	Hearing impairment
83	Born	No	Hyperechogenic liver, developmental retardation	NA	Low birthweight, deafness, jaundice, brain damage (MRI)
86	Intra-uterine death	Ventriculomegaly	Developmental retardation, increased nuchal translucency	ND	NA
87	Born	No	Hyperechogenic bowel	NA	Deafness
89	Born	No	No	NA	Low birthweight, deafness, jaundice, brain damage (MRI)
94	TOP	MRI alterations	No	ND	NA

MRI, magnetic resonance imaging; NA, not applicable; ND, not done; TOP, termination of pregnancy.

performed at a median of 22 weeks (interquartile range 21–22) of gestation.

Fifty-seven fetuses were infected, and 37 were noninfected at the combined amniotic fluid examination. Eighteen of these fetuses, 10 with and 8 without evidence of intrauterine infection, were lost to follow up. The final analysis was performed on blood samples of 29 noninfected fetuses, which represent a natural control group, and 47 infected fetuses. Among the 47 infected fetuses analysed, 16 had a symptomatic HCMV infection (Table 1). Of these, four died *in utero*, eight were terminated for signs of HCMV inclusion disease, and four were delivered with hearing impairment ($n = 1$), deafness ($n = 1$) and deafness, jaundice and brain damage ($n = 2$).

Table 2 reports on immunological and biochemical markers that were significantly different between noninfected and infected (both asymptomatic and symptomatic) fetuses. Among the markers determined, there was no significant difference between noninfected and infected fetuses for white blood cell count per microlitre blood and aspartate and alanine aminotransferases. In addition, the comparisons between noninfected, infected asymptomatic and infected symptomatic fetuses for immunological, haematological and biochemical characteristics are shown in Figure 2. White blood cell counts, percentage of lymphocytes and levels of aspartate aminotransferase and β_2-microglobulin were significantly higher ($P < 0.01$) in the symptomatic group, whereas platelet counts were significantly ($P < 0.001$) higher in the asymptomatic group. No significant difference ($P > 0.05$) for CD4+/CD8+ ratio (Figure 2A), % CD3+ HLA-DR+ (Figure 2B), γ-glutamyl-transpeptidase (Figure 2F) and alanine aminotransferase (data not shown) was found between asymptomatic and symptomatic fetuses.

Figure 3 shows the same comparison for virological data. All indices of viral infection evaluated in fetal blood samples were found to be significantly lower in the asymptomatic group ($n = 31$) versus the group of symptomatic ($n = 16$) fetuses, as follows: IgM ratio values, 0 (0–8.6) versus 7.1 (1.2–14.3); antigenaemia values, 1 pp65-positive (0–24) versus 73 pp65-positive (2–370)/2 $\times 10^5$ leucocytes examined; viraemia values, 0 (0–4) versus 2 (0–10) p72-positive inoculated fibroblasts; DNAaemia values, 1.0×10^4

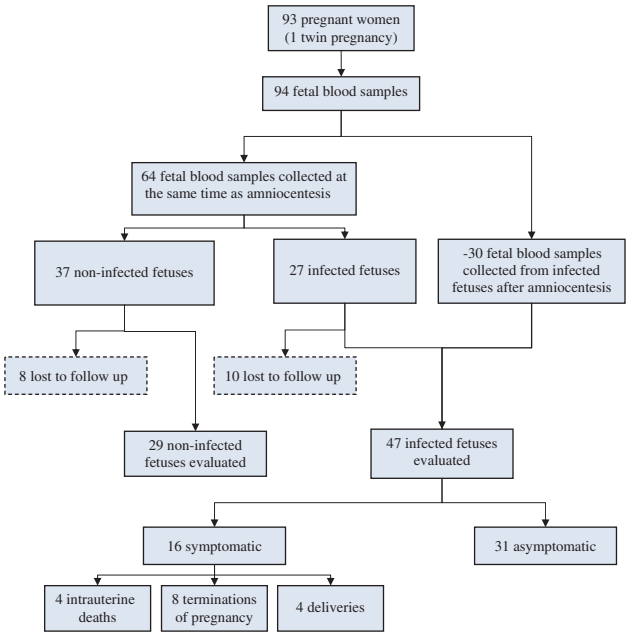

Figure 1. Flow-chart of the 93 pregnant women and their fetuses undergoing cordocentesis in this study.

Table 2. Immunological and biochemical values detected in fetal cord blood samples from 29 HCMV noninfected and 47 HCMV-infected fetuses

Fetal blood parameters	Median values (range)		*P* value
	Noninfected fetuses	Infected fetuses	
CD4+/CD8	2.9 (0.8–6.2)	1.6 (0.7–5.6)	0.001
CD3+ HLA-DR+	1 (1–5)	5 (1–13)	0.001
% Lymphocytes	82 (68–93)	86 (75–94)	0.007
Platelet counts	191 × 10^3/μl (111–267)	124 × 10^3/μl (6–241)	0.001
γ-Glutamyl transpeptidase	40 IU/l (11–144)	93 IU/l (13–587)	0.001
β_2-Microglobulin	3.7 mg/l (3.1–4.8)	10.4 mg/l (1.8–22.4)	0.001

(0–1.0 × 10^5) versus 1.0 × 10^5 (1.0 × 10^4 to 7.6 × 10^6) DNA copies/ml blood or 10^7 peripheral blood leucocytes. In addition, also the DNA copy numbers detected in amniotic fluid samples of asymptomatic fetuses, 9.8 × 10^5 copies/ml (0–1.3 × 10^8) versus symptomatic fetuses, 3.7 × 10^6/ml (1.3 × 10^5 to 1.3 × 10^9) were significantly different ($P = 0.030$). The level of HCMV infectivity in the amniotic fluid of symptomatic fetuses was not significantly different from that of asymptomatic fetuses. However, the number of amniotic fluid samples examined for infectivity was smaller than that examined for viral DNA (Figure 3F). The correlation between viral DNA and infectivity was significant ($P < 0.01$, $r = 0.58$).

For each fetal blood marker with significantly different results between asymptomatic and symptomatic fetuses, a ROC analysis was performed by calculating both the sensitivity and specificity of different cutoffs, and the relevant areas under the curve. The area under the curve (ranging from 0.5, representing no capacity, to 1.0, representing maximal capacity) indicates the capacity of each marker to differentiate between symptomatic and asymptomatic fetuses. Hence, for each marker, the cutoff with the highest sensitivity × specificity product was selected (Table 3). The best nonviral factor for differentiating symptomatic from asymptomatic congenital infections was β_2-microglobulin followed by platelet count, whereas the best virological markers were IgM antibody and DNAaemia. The ROC analysis for these markers is shown in Figure S1. The combination of β_2-microglobulin, IgM antibody and DNAaemia, as well as the combination of β_2-microglobulin, platelet count, IgM antibody and DNAaemia, showed a diagnostic accuracy comparable with that of β_2-microglobulin (Table 3 and Figure S1).

Discussion

Several reports have shown higher median values of viral DNA in amniotic fluid in symptomatic compared with asymptomatic fetuses. However, a high degree of overlap in the results of the two groups was consistently observed.[9,17–21] In particular, whereas a low viral load in amniotic fluid was consistently found to be associated with asymptomatic congenital infection, it was observed that high viral load in amniotic fluid was associated with either symptomatic or asymptomatic congenital infection. Only Guerra *et al.*[22] reported that a DNA level >10^5 copies/ml amniotic fluid was a possible predictor of symptomatic congenital infection. The lack of a close association between HCMV DNA quantification in amniotic fluid and fetal prognosis may be the result of different variables such as gestational age at maternal infection, timing of intrauterine transmission of infection, timing of amniocentesis, and particularly the unfeasibility of follow-up of infection during fetal life.[23]

The present study indicates that many haematological, biochemical and virological markers measured in fetal blood are significantly related not only to infection but also to organ damage caused by HCMV infection and symptomatic postpartum sequelae. It was shown that all assays used to determine viral load, i.e. viraemia, antigenaemia and DNAaemia, were higher in fetuses with abnormal biochemical/haematological and ultrasound findings compared with fetuses with normal findings.[23] In addition, high levels of HCMV-specific IgM antibody were found to correlate with adverse fetal outcome. These findings have been

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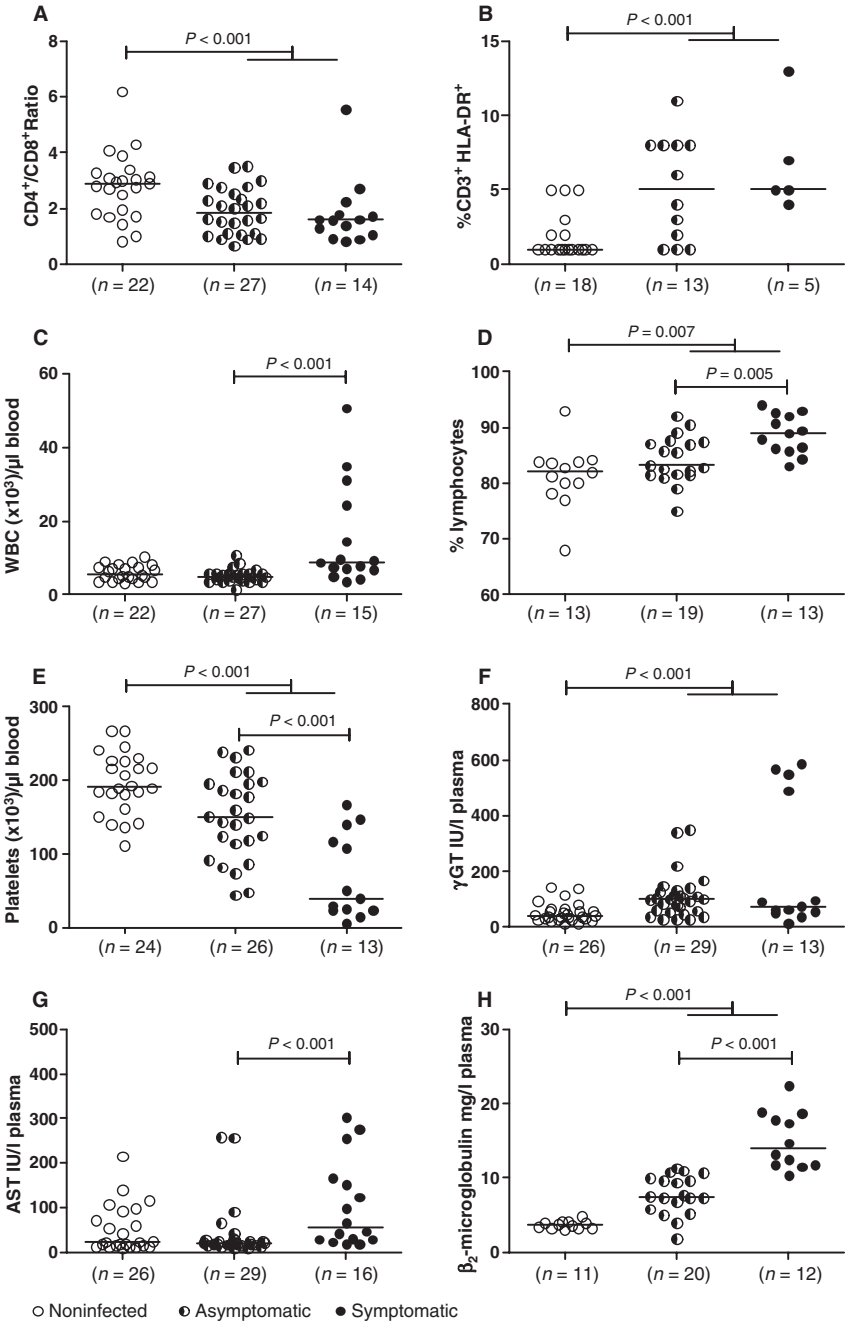

Figure 2. Determination of immunological, haematological and biochemical values in fetal blood of noninfected, asymptomatic and symptomatic fetuses. Horizontal bars at the top of each panel indicate significant differences between data pairs. (A) CD4+/CD8+ ratio; (B) %CD3+ HLA-DR+; (C) WBC, white blood cells; (D) % lymphocytes; (E) platelet count; (F) γGT, γ-glutamyltranspeptidase; (G) AST, aspartate aminotransferase; (H) B^2-microglobulin.

confirmed in the present study. However, all viral markers, as well as haematological and biochemical markers (except for β_2-microglobulin), were of poor clinical value in counselling individual patients.

When ROC analysis was applied to single markers with significant differences between symptomatic and asymptomatic fetuses, only β_2-microglobulin gave a satisfactory predictive indication in all but one fetus (Table 3). No other single viral or nonviral marker was sufficient to reliably counsel pregnant women on the chance of damage to the infected fetus. In particular, as observed for DNA in amniotic fluid, while negative or low levels of IgM and DNAaemia are consistently associated with favourable outcome (and their negative predictive values are >95%),

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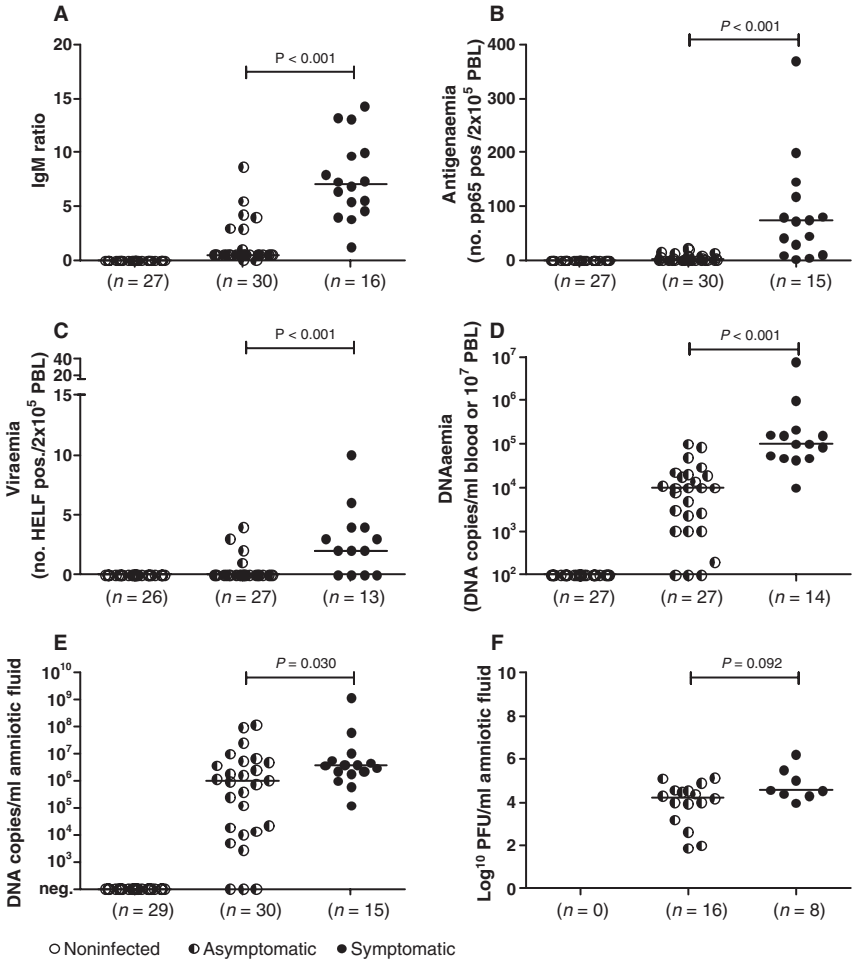

Figure 3. Quantification of virological markers determined in noninfected, asymptomatic and symptomatic fetuses. (A–D) Markers determined in fetal blood; (E) DNA quantification in amniotic fluid; (F) HCMV infectivity in amniotic fluid. Horizontal bars at the top of each panel indicate statistically significant differences between the data pairs examined.

altered levels of the different parameters can be found in both asymptomatic and symptomatic fetuses. However, we tried to verify whether a combination of different parameters could provide a better prediction of symptomatic fetuses than β_2-microglobulin alone. When two virological markers at established cutoffs for IgM ratio of 3.0 and for DNAaemia level of 30 000 DNA copies/ml blood or 10^7 peripheral blood leucocytes, along with one biochemical marker (established cutoff for β_2-microglobulin of 11.5 mg/l), and one haematological marker (established cutoff for platelet count of 50 000/μl blood) were analysed in combination (when at least three out of four were abnormal), the diagnostic efficacy was highly satisfactory with 100% specificity, 100% positive predictive value, 94% negative predictive value and 86% sensitivity. This result was comparable to that provided by β_2-microglobulin alone. However, as irrevocable decisions are to be taken on the basis of prenatal diagnosis results, it is highly desirable not to base these decisions on a single marker. The results of this study indicate that the combination of nonspecific and specific markers may provide the best key to the reliable identification of fetuses at risk of congenital disease as well as fetuses with a favourable outcome. Future studies in a larger number of fetuses should be performed to verify whether the combination of these markers may have a better diagnostic accuracy than β_2-microglobulin alone for prognostic purposes.

Thrombocytopenia had already been reported as an independent factor responsible for unfavourable fetal outcome.[6] Similarly, β_2-microglobulin had already been identified as a marker of congenital HCMV infection in symptomatic fetuses.[24] In addition, the level of IgM antibody to the 70-kDa heat-shock protein in fetal serum was reported to correlate positively with fetal disease.[25]

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Table 3. Prognostic markers of symptomatic congenital HCMV infection (receiver operator characteristics analysis)

Parameter	AUC	Cutoff	Fetuses			Diagnostic accuracy (%)			
			Cutoff	Sympt.	Asympt.	Sens.	Spec.	PPV	NPV
A. Haematological and biochemical markers									
β_2-Microglobulin	0.98	≥11.5 mg/l	Above	11	0	91.7	100	100	95.0
			Below	1	19				
Platelets	0.84	≤50 000/μl	Above	8	2	61.5	92.3	80.0	82.8
			Below	5	24				
White blood cells	0.83	7000/μl	Above	12	3	80.0	88.5	80	88.5
			Below	3	23				
Lymphocytes	0.80	84.5%	Above	12	9	92.3	52.6	57.1	90.9
			Below	1	10				
AST	0.81	31 IU/l	Above	11	5	68.8	82.1	68.8	82.1
			Below	5	23				
B. Viral markers									
IgM ratio	0.95	3.0	Above	15	4	93.7	86.2	78.9	96.2
			Below	1	25				
DNAaemia	0.93	30 000 copies/ml blood or 10^7 PBL	Above	13	3	92.9	88.5	81.3	95.8
			Below	1	23				
Antigenaemia (pp65 pos. PBL)	0.92	$9/2 \times 10^5$ PBL	Above	13	5	86.7	82.8	72.2	92.3
			Below	2	24				
Viraemia (p72 pos. HELF)	0.78	$1/2 \times 10^5$ PBL	Above	9	3	69.2	88.5	75.0	85.2
			Below	4	23				
Combined A and B values									
β_2-Microglobulin + IgM ratio + DNAaemia	0.98	2 out of 3 altered markers	Above	15	2	100.0	92.7	88.2	100.0
			Below	0	26				
β_2-Microglobulin + IgM ratio + DNAemia + platelets	0.99	3 out of 4 altered markers	Above	12	0	85.7	100.0	100.0	93.8
			Below	2	30				

AST, aspartate aminotransferase; Asympt., asymptomatic; AUC, area under curve; HELF, human embryonic lung fibroblasts; NPV, negative predictive value; PBL, peripheral blood leucocytes; PPV, positive predictive value; Sens., sensitivity; Spec., specificity; Sympt., symptomatic.

The previous finding by Romanelli *et al.*[26] that there is no significant difference for some of these parameters between symptomatic and asymptomatic congenital infections may be the result of the small number of fetuses analysed. As reported in this study, both β_2-microglobulin and platelet count have become part of an algorithm (including viral markers of fetal HCMV infection such as IgM ratio and viral load) for prediction of an unfavourable fetal outcome. The prospective use of defined cutoffs might help to integrate a formal algorithm for biological markers with ultrasound findings which have been claimed to predict neonatal outcome[6] with the obvious limitation that anatomical damage might be visible days and weeks after functional irreversible damage has already occurred.[7,26–30]

A limitation in the interpretation of our findings might be based on the small number of symptomatic cases. However, to the best of our knowledge, this is the largest cohort of cordocentesis with a control group of non-infected fetuses diagnosed by amniocentesis and confirmed in neonatal urine samples. The cutoffs we present here and their integrated algorithm might well be used in clinical practice with prudent integration with all other markers available.

Conclusion

In conclusion, the determination of specific virological, haematological and biochemical markers in fetal blood sampled by cordocentesis, once a positive viral detection has been established in amniotic fluid samples, greatly helps to predict fetal neonatal outcome and provides valuable information for pregnant women and their partners in their decision-making regarding continuation or termination of pregnancy. Once primary HCMV infection is diagnosed in a pregnant woman, amniocentesis should be offered to diagnose intrauterine HCMV transmission and, in the case of a positive result, cordocentesis should be discussed for prognostic purposes.

Disclosure of interests

There was no conflict of interest.

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Contribution to authorship

EF contributed to counselling, data collection and analysis; GG and MGR contributed to the conception and design of the study; MF and MZ contributed to virological data analysis and counselling; DL contributed to the statistical analysis; BT and AQ contributed to manage the primary HCMV infection in pregnant women; MR performed the ultrasound examinations; UN was the former leader of the clinical study in Milan; EF is the current leader of the clinical study in Milan.

Details of ethics approval

IRB approval was obtained preliminarily. Informed consent was obtained from each woman before amniocentesis or cordocentesis.

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Supporting information

The following supplementary materials are available for this article:

Figure S1. Receiver–operator characteristics curves of (A) β_2-microglobulin, (B) platelets, (C) IgM antibody, (D) DNAemia, and (E) the combination of three and (F) four parameters.

Additional Supporting Information may be found in the online version of this article.

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