# Analysis of blood from Zika virus-infected fetuses: a prospective case series

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## Summary

**Background** **Zika virus has spread through the Americas and the Caribbean since early 2015 and was rapidly declared a Public Health Emergency of International Concern by WHO because of the potential association with fetal anomalies. We analysed fetal and maternal fluids and tissues in fetuses with confirmed Zika virus infection prospectively monitored in Martinique, a French Caribbean island.**

**Methods** **Since the beginning of the Zika virus outbreak in Martinique, all pregnant women undergo monthly fetal ultrasound examination surveillance. In this study, we prospectively studied all patients with fetal anomalies and a positive amniotic fluid for Zika virus by RT-PCR. Maternal and fetal blood, urine, amniotic fluid, placenta, and fetal tissues were tested for Zika virus by RT-PCR. Fetal blood was analysed to identify haematological and biological anomalies.**

**Findings** **Between Jan 1, 2016, and Nov 10, 2016, we recruited eight cases of Zika virus infection. All but two cases were symptomatic during the first trimester. Fetal anomalies were only detected after 20 weeks' gestation. After an initial positive result, amniocentesis became negative in two cases and fetal blood was transiently Zika virus-positive in six cases. Fetal blood analyses showed a cholestatic pattern, anaemia, and infectious response.**

**Interpretation** **Normalisation of amniotic fluid and fetal blood for Zika virus, as well as maternal blood and urine, shows the limitations of the performance of these investigations, due to the possibility of false negative results. Abnormal fetal blood needs to be investigated further to establish prognostic factors of severe Zika virus infections.**

**Funding** **None.**

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## Introduction

Zika virus infection has been spreading throughout the Americas and the Caribbean since May, 2015. This epidemic has been marked by the discovery of substantial complications of Zika virus infection when contracted in pregnancy. Both experimental and epidemiological data now strongly link Zika virus infection to abnormal fetal development and, in particular, to severe neurological anomalies, similar to the TORCH pathogens: toxoplasmosis, others (syphilis, parvovirus B19, varicella zoster), rubella, cytomegalovirus, and herpes simplex virus.[1,2] Nevertheless, prospective standardised studies are scarce and urgently needed to fully understand the spectrum of anomalies associated with congenital Zika virus infection. As with the TORCH pathogens, the severity of fetal infection with Zika virus probably ranges from severe disease, as the syndrome has been described so far,[3] to moderate and asymptomatic forms. Several biological fetal parameters, such as low platelet count (<100), have been described as prognostic factors of adverse fetal outcomes in congenital cytomegalovirus infections,[4] and it is crucial to determine whether similar parameters exist for congenital Zika virus infections. Furthermore, there is little information about adequate monitoring of affected pregnancies. In particular, the benefit of amniocentesis, which is considered the gold standard for diagnosis of other teratogenic fetal infections,[4] is unclear;[5] data on the predictive values of such an invasive procedure are required.

On Dec 21, 2015, Martinique was the first Caribbean island where an autochthonous Zika virus infection was confirmed by RT-PCR.[6] Since then, the French authorities have implemented active surveillance of all pregnancies with monthly ultrasound. We prospectively monitored eight cases of fetal cerebral anomalies with a confirmed Zika virus infection and assessed biological parameters of infected fetuses.

## Methods

### Study design and patients

This study was a prospective case series assessing all fetuses with anomalies on ultrasound and a positive amniocentesis for Zika virus in Martinique. Martinique is a French Caribbean island with a total population of 385 551, as estimated in 2013.[7] In 2016, 3777 livebirths were registered. The Zika virus epidemic in Martinique lasted from January to November, 2016, with 35 190 potential cases of Zika virus reported.[8] In January 2016, French authorities implemented active surveillance of pregnancies in the French Caribbean territories. At present, all pregnant women in Martinique undergo monthly ultrasound examinations at 12, 18, 22–24, 26–28, 32, and 36 weeks' gestation as opposed to three ultrasounds in routine prenatal care.

## Research in context

### Evidence before this study

Since early 2015, Zika virus has been spreading through the Americas and the Caribbean, leading to the most massive Zika virus epidemic reported so far. An association between infection and the development of fetal anomalies was suspected because of the increased incidence of microcephaly in affected countries. Since then, both in vitro and epidemiological studies have confirmed this association and Zika virus is now considered a teratogenic infectious pathogen. Similarly to other teratogenic pathogens, fetal infections will probably occur in only a fraction of fetuses, among which some will develop severe symptoms, while others will remain asymptomatic. Amniocentesis, ultrasound monitoring, and fetal blood samplings are used to discriminate between these two groups and help parents to decide whether to continue or terminate the pregnancy. By analogy, similar analysis might be useful in the case of maternal Zika virus infection.

We searched PubMed up to Jan 20, 2017, with the terms "Zika" or "Zika virus" or "ZIKV" and "pregnancy" or "pregnant women", as well as "Zika virus" or "Zika" and "amniocentesis" or "amniotic fluid" or "cordocentesis" or "fetal blood sampling", and found 11 studies reporting analysis of amniotic fluid in case of Zika virus infection. No studies reporting "fetal blood sampling" were recovered.

In this study, we report a case series of eight pregnant patients with a confirmed fetal Zika virus infection monitored prospectively and the associated result of ultrasound monitoring, amniocentesis, and fetal blood sampling.

### Added value of this study

To our knowledge, this is the first study to describe biological parameters of fetuses with Zika virus infection. Furthermore, we provide for the first time the evolution of Zika virus RNA detection in the amniotic fluid over time.

### Implications of all the available evidence

The results of amniocentesis changed over time, which might suggest a limited negative predictive value, or else clearance of the virus. This initial description of biological fetal anomalies helps define Zika virus congenital syndrome. Further studies are needed to better define the use of amniocentesis and fetal blood sampling in possible fetal Zika virus infection.

All pregnant women with clinically suspected Zika virus infection (ie, fever or rash) or any fetal abnormalities at ultrasound examination are referred to the Fort-de-France University Hospital Pluridisciplinary Centers for Prenatal Diagnosis, Maternal and Child Care (tertiary reference centre for all Martinique Island), for further management. Maternal or fetal Zika virus infection was confirmed when either a blood or urine sample (from the mother) or amniocentesis was positive for Zika virus RNA, detected by a specific RT-PCR. Gestational age was estimated antenatally from the date of the last menstrual period reported by the mother, and confirmed when available by fetal ultrasonography. The Pluridisciplinary Centers for Prenatal Diagnosis of Martinique approved the management of all patients included in this study, and gave ethics approval. Patients provided written and informed consent.

### Procedures

In our reference Center for Prenatal Diagnosis, all scans were done according to the International Society of Ultrasound in Obstetrics and Gynecology interim guideline[9] with GE Healthcare VE10/E8 machines (Cincinnati, OH, USA) with an abdominal probe (RA4B or RM6C) and a vaginal probe (RIC5-9-D).

All fetal sampling procedures were guided by ultrasound. Diagnostic amniocentesis was done after the identification of fetal anomalies and at the time of delivery (for one patient) or at termination of pregnancy. Termination of pregnancy was permitted at any gestational age at the parent's request, provided that a severe fetal anomaly without any available curative treatment at the time of diagnosis was confirmed by a pluridisciplinary fetal medicine unit. When possible, we sampled fetal blood after or during amniocentesis (as is done for other congenital infections) through percutaneous umbilical blood sampling. Except for one patient, both procedures were done at the time of pregnancy termination (n=7). A placental biopsy was done at birth in all cases.

### Laboratory analysis

Specific laboratory investigations included assessment of maternal and fetal blood, urine, amniotic fluid, and placenta sampling for Zika virus RNA by RT-PCR (RealStar Zika virus RT-PCR Kit 1.0, Altona Diagnostics, Hamburg, Germany). Brain and liver or cerebrospinal fluid were tested for Zika virus RNA by RT-PCR after termination of pregnancy or birth, respectively. In addition, maternal blood was screened for dengue virus and chikungunya virus with Simplexa Dengue RT-PCR assay (Focus Diagnostics, Cypress, CA, USA) and the RealStar Chikungunya RT-PCR kit 1.0 (Altona Diagnostics, Hamburg, Germany). Maternal and fetal serum samples were analysed for Zika virus, chikungunya virus, and dengue virus IgM and IgG with virus-specific IgM antibody capture (MAC) ELISA and IgG antibody capture ELISA (EUROIMMUN, Lubeck, Germany; PANBIO, Alere, Waltham, MA, USA). Positive Zika virus serologies were confirmed by Zika virus neutralisation.

Fetal blood was also analysed for six biochemical and two haematological markers: $\beta_2$microglobulin (immunoturbidimetry), total proteins, albumin, total

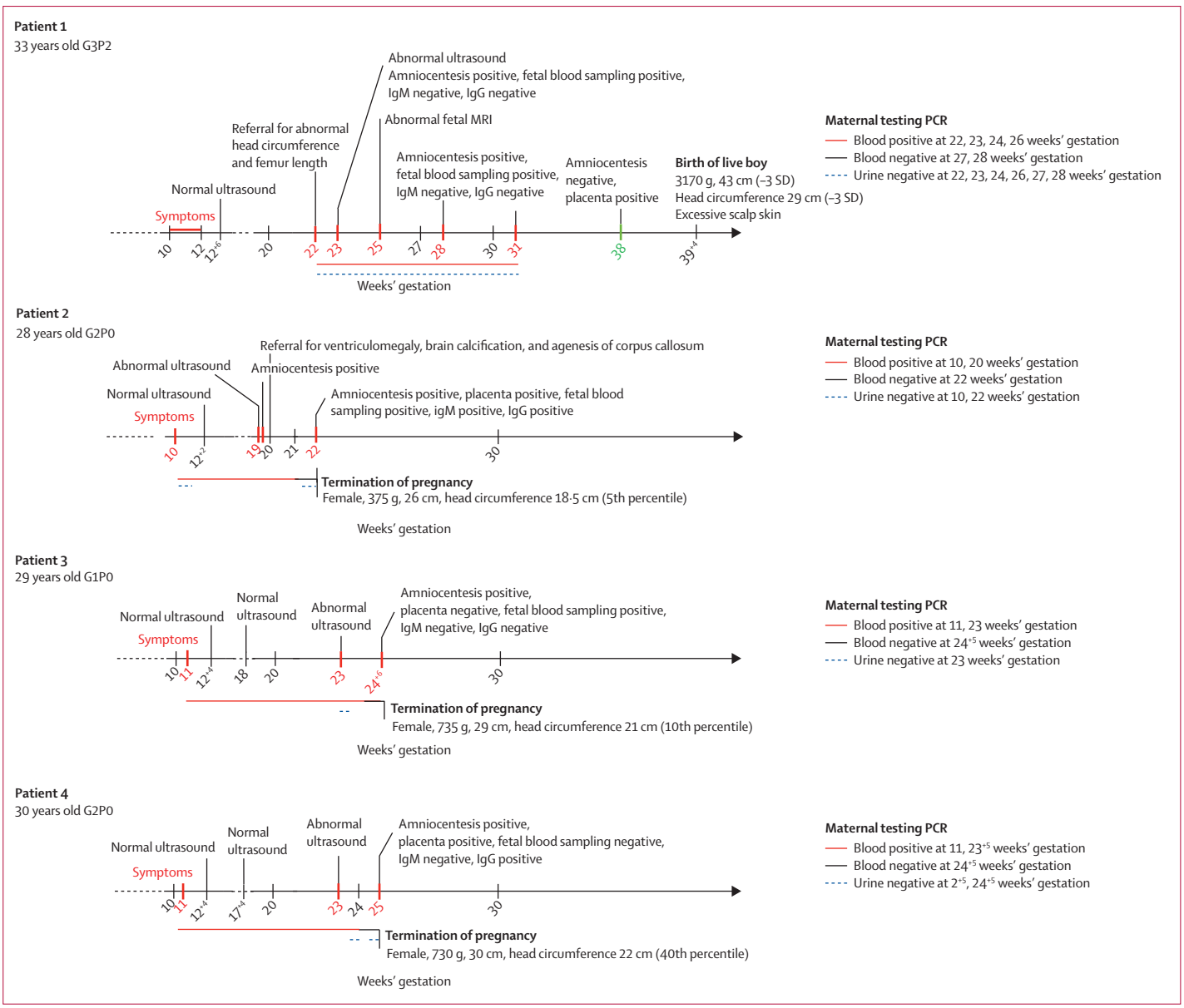


***Figure 1:*** **Cases presentation (1–4)**
This timeline represents the monitoring, laboratory results, and outcomes of the first four cases included in this series. GnPn=Gravida n Para n.

IgM, γ-glutamyl transpeptidase, and aspartate aminotransferase (reagents and platform Olympus AU 400 [Beckman Coulter, Fullerton, CA, USA]), as well as haemoglobin and platelet count. These haematological and biochemical fetal parameters are part of the routine fetal blood analyses in cases of congenital infections, specifically cytomegalovirus.[4,10–12] Normal values of these markers in fetal blood have been defined previously.[13,14]

## Role of the funding source

No funding was obtained for this study. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

## Results

From Jan 1, 2016, to Nov 10, 2016, 551 pregnant women presenting with compatible symptoms were diagnosed with a confirmed Zika virus infection. 14 women presented with fetal anomalies of which nine cases were confirmed to be related to Zika virus. Eight cases were managed in our centre and included in the study; the remaining case was subsequently managed elsewhere.[8] The monitoring timeline, results of maternal and fetal

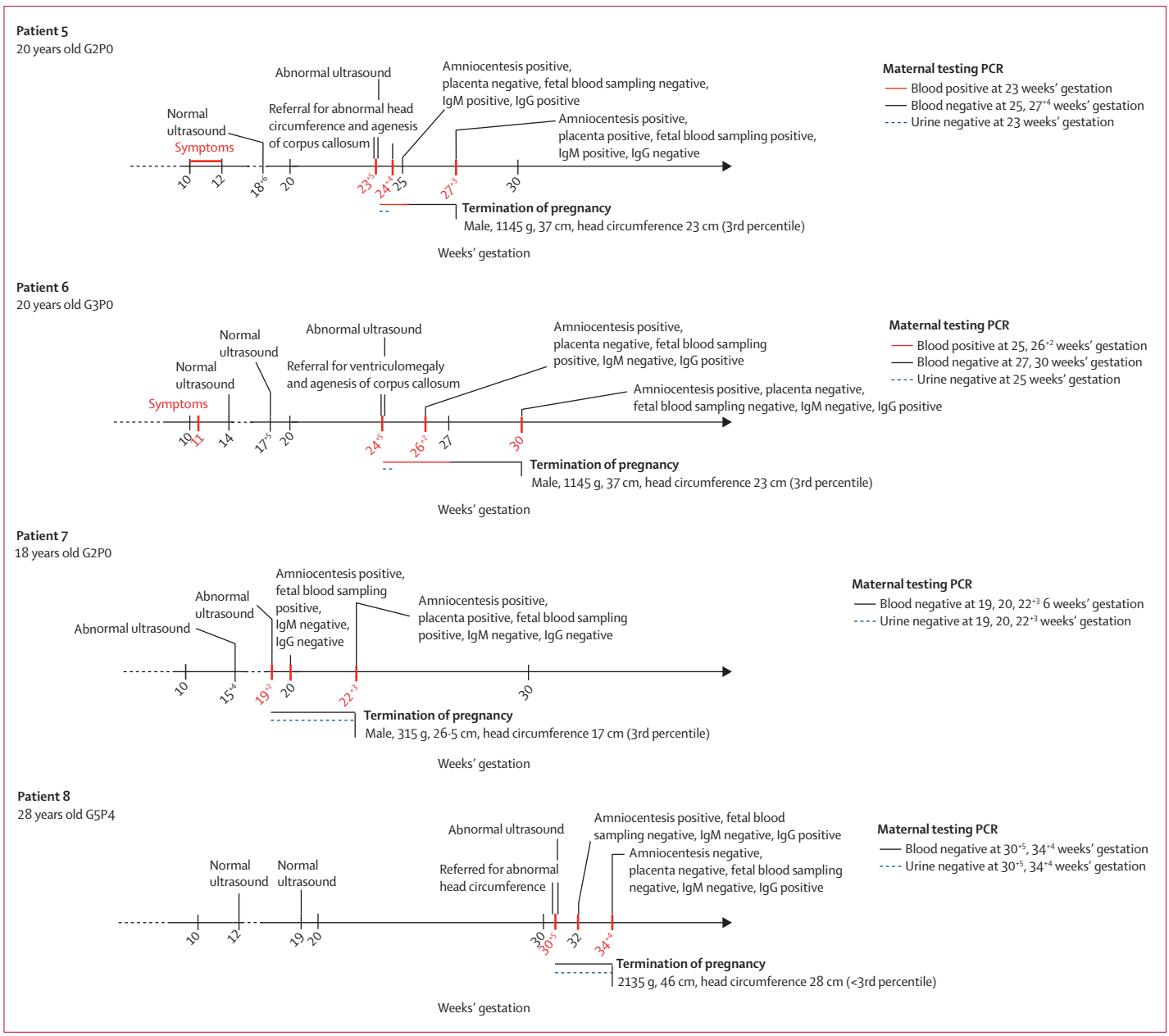


***Figure 2:*** **Cases presentation (5–8)**
This timeline represents the monitoring, laboratory results, and outcomes of the last four cases included in this series. GnPn=Gravida n Para n. IUGR=Intrauterine Growth Restriction.

See Online for appendix

testing, and pregnancy outcomes for each pregnancy are presented in figures 1 and 2. All eight mothers were healthy, not on any drug treatment, with the exception of routine iron supplementation, vitamins, and occasional paracetamol as needed. Six (75%) of eight women (patients 1–6) presented with symptoms consistent with Zika virus infection between 10–12 weeks' gestation. The symptomatic mothers had persistent viraemia that lasted up to 14 weeks, with subsequent abatement at 22–27 weeks' gestation. No viral particles were detected in urine samples. The two remaining patients (patients 7 and 8) were asymptomatic and infection was suspected to have occurred between 7+4 and 9+4 weeks' gestation in patient 7, and between 9+5 and 19 weeks' gestation in patient 8 based on retrospective serological analysis (appendix). Both mothers remained negative for Zika virus RNA in their blood and urine, despite proven fetal infection. Serological screening for other congenital infections was all negative. Active dengue virus and chikungunya virus infections were excluded by the absence of specific IgM. After the diagnosis of Zika virus-associated fetal anomalies, one patient opted to continue

the pregnancy and delivered at 39 weeks' gestation, whereas the remaining women opted for a termination of pregnancy between 22–34 weeks' gestation because of poor prognosis.

All first trimester and early second trimester (15+4–19 weeks' gestation) ultrasounds were normal. Fetal anomalies were first detected between 19 and 30 weeks' gestation (mean 23 weeks' gestation), 9–13 weeks (mean 11·8 weeks) after Zika virus symptoms when recognised (figure 3, appendix).[15–17] Head circumference was in the bottom third percentile in four of eight cases, in the fifth percentile in one case, and normal in the other three cases.[18] In all cases, we noted moderate ventriculomegaly with an atrium of 10–13 mm, cortical atrophy with enlarged pericerebral spaces, absent or short and thin corpus callosum, and multiple brain calcifications, mainly at the junction between cortical and subcortical white matter, thalamic, and basal ganglia areas. The posterior cranial fossa, cerebellum, brainstem, and pons were normal with the exception of two cases with a thin pons and one case with a thin pons and a Blake's pouch cyst. Amniotic fluid volume and placental architecture were normal. No additional anomalies were found except for a shortened femur in case 1 and hepatic calcifications in case 7. Only one fetus had intrauterine growth restriction that was below the fifth percentile. The other fetuses were eutrophic at the time of delivery or termination of pregnancy.

Results of amniocenteses, placental biopsies, and fetal serologies are presented in figures 1 and 2.

In all eight cases, the fetal karyotype was normal and amniotic fluid was negative for cytomegalovirus. Toxoplasmosis, rubella, chikungunya virus, and dengue virus infections were previously excluded by maternal serology. At the time of diagnosis, Zika virus RNA was detected in all amniotic fluid samples. The amniotic fluid was retested in six cases (patients 1, 2, 5, 6, 7, and 8) at the time of delivery or termination of pregnancy: three cases (patients 2, 6, and 7) remained positive for Zika virus infection whereas in three cases (patients 1, 5, and 8) the second test was negative for Zika virus infection (2–10 weeks after the positive sample).

In three cases, placenta biopsies were obtained by chorionic villus sampling during pregnancy and tested negative (patients 1, 5, and 6). In all cases, placental biopsies were obtained at birth or after termination of pregnancy and tested positive for Zika virus RNA in three cases (patients 2, 4, and 7). In the remaining five cases (patients 1, 3, 5, 6, and 8), the placenta tested negative despite detection of Zika virus in the amniotic fluid.

Fetal blood was positive for Zika virus RNA in four cases (patients 1, 2, 6, and 7). When retested at the time of delivery (patient 1) or termination of pregnancy (patients 6 and 7), blood from two fetuses that were previously positive became negative for Zika virus, showing a transient fetal viraemia. In six of the eight cases, results were congruent with maternal testing and all fetal samples taken after the resolution of maternal viraemia were negative, except in one case (patient 7). Flavivirus IgG was detected in seven of eight fetal serum samples and Zika virus-specific IgM was positive in four cases.

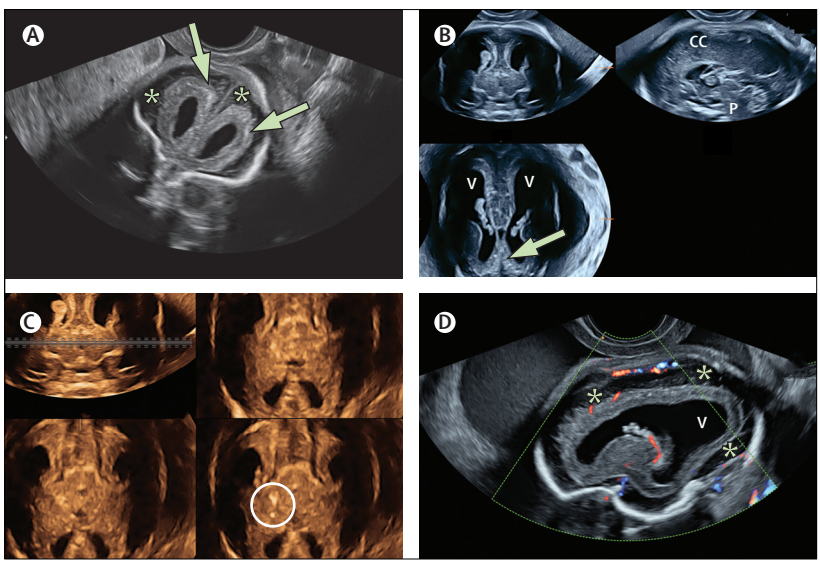


***Figure 3*: Fetal anomalies observed by ultrasound**
Representative vaginal ultrasound images of the fetal brain (case 4): frontal coronal (A), multiplanar frontal (B), transversal (C), and parasagittal (D) at 23 weeks' gestation. Note cortical atrophy with enlarged pericerebral spaces (X), bilateral ventriculomegaly (V), multiple subcortical calcifications (arrow), calcifications in the thalamus (circle), and short and thin corpus callosum (CC, 15 mm anterior–posterior length and 1·2 mm thick). The vermis cerebellum and the pons were normal.

We did post-mortem analysis of fetal tissues in five cases; Zika virus RNA was detected in all brain samples, but not in the liver or lungs. In the only case resulting in a live delivery (patient 1), analysis of urine, blood, and cerebrospinal fluid did not identify any Zika virus RNA, which is in agreement with the final fetal sampling in which both amniocentesis and placenta were negative. At 5 months of age, the child from patient 1 presented with severe microcephaly (–3 SD) whereas height and weight were in the normal range, axial tonus hypotonia, spastic quadriparesy, severely abnormal vision, and otoacoustic emissions.

13 fetal blood samples were analysed because five fetuses were sampled a second time before birth or termination of pregnancy (table). In all cases, at least one of the two biochemical markers for infectious diseases was positive, with an elevated total IgM in all cases and an abnormally high $\beta_2$-microglobulin in seven samples in six patients (patients 1, 2, 4, 5, 6, and 7). Among those who were retested before birth or termination of pregnancy, a rapid decrease in $\beta_2$-microglobulin occurred in three cases (patients 1, 5, and 6), whereas a slight increase occurred in one case (patient 7).

All fetuses had biochemical signs of liver cholestasis (elevated γ-glutamyl transpeptidase) with associated moderate cytolysis (abnormal aspartate aminotransferase)

| | Normal ranges | Case 1 | | Case 2 | Case 3 | Case 4 | Case 5 | | Case 6 | | Case 7 | | Case 8 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 WG | 28 WG | 22 WG | 24+5 WG | 24+5 WG | 24+4 WG | 27+4 WG | 26 WG | 30 WG | 20 WG | 22+3 WG | 32 WG | 34 WG |
| **General parameters** | | | | | | | | | | | | | | |
| Total IgM (mg/L) | <20 | 40 | 250 | 79 | 82 | 265 | 223 | 108 | 76 | 91 | 70 | 60 | 64 | 47 |
| $\beta_2$-microglobulin (mg/mL) | <5 | 8·9 | 4·4 | 8·4 | 4·2 | 7·1 | 5·7 | 3·7 | 5·4 | 4·3 | 9 | 9·5 | 4·2 | 4 |
| **Liver function** | | | | | | | | | | | | | | |
| GGT (IU/L) | <100 | 237 | 222 | 155 | 142 | 135 | 250 | 327 | 342 | 367 | 1766 | 1522 | 348 | 309 |
| AST (IU/L) | <20 | 15 | 91 | 102 | 44 | 18 | 20 | 24 | 31 | 24 | 42 | 28 | 25 | 25 |
| Albumin (g/L) | 20–35 | 23·3 | 25·5 | 23·8 | 27·1 | 26·6 | 28·7 | 29·9 | 27·1 | 30·8 | 24·8 | 25·4 | 30 | 31·6 |
| Total proteins (g/Ll) | 20–30 | 25 | 39 | 32 | 34 | 34 | 34 | 39 | 34 | 39 | 35 | 37 | 43 | 46 |
| **Haematological parameters** | | | | | | | | | | | | | | |
| Haemoglobin (g/100 mL) | >15 | 10·4 | 12·2 | 12·2 | 10·6 | 11·3 | 7·6 | 10·1 | 12 | 12·9 | 13 | 11·3 | 13·4 | 13·2 |
| Platelets (g/L) | >150 | 181 | 190 | 167 | 301 | 189 | 129 | 220 | 159 | 184 | 198 | 197 | 272 | 391 |

WG=weeks' gestation. AST=aspartate aminotransferase. GGT=γ-glutamyl transpeptidase.

***Table:*** **Fetal biological parameters**

in seven cases (table).[13] One patient (patient 5) had severe anaemia (haemoglobin <10 g/100 mL) and the remaining seven patients had moderate anaemia.[14] Except for cases 7 and 8, haemoglobin concentrations increased at follow-up.

## Discussion

In this study, we described a prospective case-series of eight pregnancies with confirmed Zika virus infection and severe fetal anomalies. We observed the diffuse cerebral lesions described in previous reports[22] and describe, for the first time, abnormal fetal biological parameters highly suggestive of hepatic dysfunction and potentially anaemia.

All patients were probably infected with Zika virus in early pregnancy, between 9 and 12 weeks' gestation, though the exact timing remains unclear in two cases because of the absence of suggestive symptoms. The severity of the anomalies in this series accord with previous reports, which suggest that the risk to be highest when infection occurs during the first trimester.[19–21] We noted fetal anomalies soon after supposed Zika virus infection (19–30 weeks' gestation). We observed a range of neurological anomalies, which correlates with other findings.[22] All fetuses except one were eutrophic, similar to the 14% incidence of intrauterine growth restriction reported previously.[22–24]

Six of eight mothers had blood positive for Zika virus with RT-PCR at the time of referral, which persisted up to 14 weeks after suspected initial infection, whereas urine was negative. Zika virus viraemia usually lasts up to 7 days after infection.[25] Prolonged viraemia concomitant with fetal infection has been described previously,[23,26] and might result from viral replication in the fetoplacental compartment and further release into maternal circulation. There is growing evidence that Zika virus crosses the placental barrier and replicates in different cell types of the placenta.[27]

With the exception of one case (patient 7) we observed a correspondence between maternal and fetal viraemia and a delayed viral elimination in amniotic fluid (patients 1, 3, 4, 5, 6, and 8). This chronology supports the hypothesis of fetoplacental driven maintenance of viraemia and the potential of long-lasting maternal viraemia as a marker of ongoing fetal infection.[28]

Amniocentesis was done in all cases, according to present empirical recommendations, after 21 weeks' gestation and at least 6 weeks after maternal exposure.[29] In all cases, Zika virus RNA was detected in amniotic fluid more than 10 weeks after suspected maternal infection, supporting an excellent sensitivity. In three cases, however, repeat sampling at 27+4, 34+4, and 38 weeks' gestation were negative. This finding could be due to false-negative results. Alternatively, it could be related to the development of a fetal immune response eliminating viral particles in the maternofetal circulation, leading to a normalisation of amniocentesis and maternal and fetal viraemia, despite the virus's persistence in the fetus' CNS. The positive and negative predictive values of amniocentesis still need to be defined because no fetuses without anomalies were assessed in this study. Nevertheless, amniocentesis remains the gold standard for diagnosis of fetal infections, such as cytomegalovirus. When done adequately, amniocentesis with subsequent specific cytomegalovirus PCR is associated with a positive predictive values of 100% and negative predictive value of 90% of fetal infection.[30] Larger cohort studies are needed to further describe the performance of amniocentesis in cases of suspected Zika virus infection, although it might be similar to what has been described for cytomegalovirus. Placental sampling detected Zika virus RNA in only three cases, suggesting a limited use for such analysis.

Zika virus RNA was detectable in post-mortem brain tissue, even after becoming undetectable in maternofetal

circulation, placental biopsy, and amniotic fluid. These data confirm the transient nature of viraemia and placental replication,[31] and the preferential establishment of Zika virus within the CNS. Zika virus RNA was not detected in the placenta, blood, and urine samples of the only live birth of this series, which excludes any of these markers as useful negative predictors of Zika virus infection in at-risk populations. This absence is a major difference compared with the long-lasting persistence of cytomegalovirus particles in tissues and secretions of infected newborns.[32]

To our knowledge, this is the first description of fetal blood analyses in cases of Zika virus infection, which suggests potential factors to detect infection. All cases showed an increase of non-specific markers of infectious diseases (ie, total IgM and $\beta_2$-microglubulin). High $\beta_2$-microglubulin followed by a rapid decrease in follow-up sampling, indicates antigen turnover and signal lymphoid cell stimulation, followed by IgM production.[13] Similar changes occur in cytomegalovirus and toxoplasmosis infections and increase in $\beta_2$-microglobulin has a 90% sensibility to confirm fetal toxoplasmosis infection and a 93% sensibility to confirm cytomegalovirus infection.[13,33] Such markers, though non-specific for Zika virus infection, could help differentiate severely and non-severely affected fetuses (eg, in cases of minor sonographic signs, or before microcephaly appears) in ambiguous situations, in particular in cases of late diagnosis, where amniocentesis might be negative.

All fetuses had a persistent cholestasis profile occasionally associated with rapidly resolving hepatocellular dysfunction. Similar dysfunction is also observed in other congenital infections (cytomegalovirus and toxoplasmosis)[34] as well as in other congenital disorders.[35] All fetuses had anaemia (severe in one case). In congenital fetal infections, only parvovirus B19 is known to have an affinity for erythropoid precursor cells leading to hydrops fetalis and intrauterine growth restriction. One case of Zika-virus-associated hydrops fetalis has been described previously.[36] Anaemia in our study seemed to be self-limiting, suggesting it might be a marker of acute fetal infection. We did not record abnormal platelet concentrations, which is common in other congenital infections.

One major limitation of our study is that only symptomatic fetuses were included. Further studies are needed to better define outcomes, biological parameters, and the performance of diagnostic tests in exposed fetuses, especially those without ultrasound anomalies, as well as in cases of late maternal infection. Most exposed fetuses will probably not become infected, and few will develop the severe symptoms that have been described so far.[5,28] Clinicians should be aware that although amniocentesis and subsequent isolation of Zika virus in the amniotic fluid might confirm diagnosis, its absence does not rule out fetal Zika virus infection. Additional parameters are needed to aid diagnosis and should include results of maternal testing, ultrasound findings, and fetal biological parameters. Each procedure should be carefully discussed with the parents-to-be and the medical team.

#### Contributors

BS, MG, AM, CH, EJ, CA, and JLV conceived and designed the study. BS, MG. AM, CH, EJ, CA, and JLV provided care to mothers and collected the clinical data and samples (amniocentesis and fetal blood sampling). FN, RC, and IL-G did the viral investigations and analysis. FM and SD did the fetal biology investigations and analysis. DB, MV, and BS interpreted the results, did the literature review, and provided critical inputs to the manuscript. BS, MV, and DB wrote the first version of the report and all authors critically reviewed and approved the final version.

#### Declaration of interests

We declare no competing interests.

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