Ebola virus disease in pregnancy: clinical, histopathologic and immunohistochemical findings

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ABSTRACT

Here we describe clinicopathologic features of EVD in pregnancy. One woman was infected with Sudan virus had stillbirth and survived in Gulu, Uganda in 2000, and a second woman with Bundibugyo virus had livebirth with maternal and infant death in Isiro, the Democratic Republic of Congo in 2012. Ebolavirus antigen was seen in the syncytiotrophoblast and placental maternal mononuclear cells by immunohistochemistry, and no antigen was seen in fetal placental stromal cells, or in fetal organs. In the Gulu case, ebolavirus antigen localized to malaria pigment-laden macrophages. These data suggest trophoblast infection may be a mechanism of transplacental ebolavirus transmission.

INTRODUCTION

Ebola virus disease (EVD) and Marburg virus disease (MVD) are caused by viruses of the *Ebolavirus* and *Marburgvirus* genera (family *Filoviridae*). Here, we collectively refer to Ebola (EBOV), Sudan (SUDV) and Bundibugyo (BDBV) virus species (all within the *Ebolavirus* genus) as ebolaviruses. Filovirus infection during pregnancy is associated with maternal hemorrhage, pre-term labor, miscarriage and maternal and neonatal death. **Supplementary Table 1** presents a summary of literature to date on filovirus infection in pregnancy, which was also recently reviewed [1, 2]; of 119 cases reported in the scientific literature, maternal death was 85% and there was uniform loss of offspring, whether by miscarriage, stillbirth or neonatal death, including only 18 live births with the longest survival only 19 days of life [3].

With the exception of liver biopsies on patients with MVD in Marburg, Germany, in 1967 [4] and a biopsy to evaluate a periorbital mucormycete fungus coinfection in a patient who survived EVD in the Democratic Republic of the Congo (DRC) in 1995 [5], human pathological studies on patients with filovirus infection have been almost entirely limited to post-mortem samples at the end stage of disease [6], largely confined to skin punch biopsies and core needle biopsies of the liver and spleen.

Despite the severity of filovirus infection in pregnancy for both mother and child, very little is known regarding pathogenesis. Fetal-placental viral tropism has been hypothesized due to recent observations during the 2013-2016 West Africa EBOV outbreak: pregnant women were noted to survive EVD and clear virus from the blood without fetal loss during acute infection, and deliver stillbirths in the subsequent weeks and months with relatively high EBOV RNA levels in placental and fetal tissue swabs [7-9] [10]. We report clinical, histopathologic and immunohistochemical findings of SUDV and BDBV virus infections in two pregnant women and their offspring that help shed light on the pathogenesis of fetal infection and loss in EVD.

METHODS

Patients

Two pregnant women with EVD were cared for in Ebola Treatment Centers (ETC) during ebolavirus outbreaks in Gulu, Uganda, in 2000 [11, 12] and Isiro, DRC in 2012 [13, 14]. Specimens were collected and evaluated during the course of outbreak responses.

Ebolavirus diagnostic testing

Enzyme-linked immunosorbent assays (ELISA) and RT-PCR assays for SUDV in Gulu and RT-PCR assays for BDBV in Isiro were performed as previously described [15, 16] in field laboratories run by the Viral Special Pathogens Branch (VSPB), U.S. Centers for Disease Control and Prevention (CDC), Atlanta, GA. BDBV IgM and IgG enzyme-linked immunosorbent assays (ELISA) were performed by VSPB, CDC, Atlanta, GA.

Histopathology, immunohistochemistry and transmission electron microscopy

Placenta (Gulu and Isiro), fetal tissues (Gulu) and a post-mortem skin biopsy (Isiro) were collected and placed in 10% neutral buffered formalin and transported to the CDC where the samples were processed using standard histological methods. The identification and scoring of malaria pigment was performed as previously described [17]. Immunohistochemistry (IHC) for ebolavirus antigens was performed using a polymer-based indirect immunoalkaline phosphatase detection system for colorimetric detection (Biocare Medical, Concord, CA). Rabbit polyclonal antisera against EBOV, SUDV and Reston virus, and EBOV hyperimmune mouse ascitic fluid (courtesy Thomas Ksiazek, VSPB, CDC), previously shown to detect SUDV and BDBV antigens, were each used at a 1:1000 dilution with appropriate positive and negative controls [18]. On slide embedding and transmission electron microscopy was performed as previously described [19].

RESULTS

Patient #1: Case Presentation, Gulu, Uganda

A 30 year-old housewife in Gulu District of northern Uganda who presented with asthenia, anorexia, abdominal pain, nausea and vomiting, non-bloody diarrhea, and dry cough for 1 day. She reported previous contact with other persons with EVD in her village. Based on the dates provided to her from previous antenatal clinic visits, she was 28 weeks pregnant but reported feeling no fetal movements in the past few days. Vital signs were not taken on admission due to minimal staffing but the next day her axillary temperature was 36.7°C, pulse 120 bpm, and respiratory rate 24 breaths per minute, with oxygen saturation on pulse oximetry of 92 percent. Physical exam revealed conjunctival injection, diffuse abdominal tenderness, and slight pulmonary rales. The patient was clearly pregnant, but no formal obstetric exam was performed. She was placed on intravenous fluids and oral amoxicillin. Her blood tested positive for SUDV by both ELISA antigen assay and nested RT-PCR.

On day four of illness the patient spontaneously delivered a dead but apparently morphologically normal fetus and placenta. The degree of vaginal bleeding did not seem out of the ordinary for a stillbirth. Over the next three days, the patient complained of pain and swelling of the joints, especially the wrists and knees, throat and chest pain, persistent dry cough with dyspnea and, briefly, hiccups. Her wrists and knees were visibly swollen and tender to the touch and rales continued to be noted. She was consistently febrile during this time, with disease severity peaking at day 7 of illness, when her vital signs showed an axillary temperature of 37.8°C, pulse 128 bpm, respiratory rate 30 breaths per minute, and oxygen saturation of 90

percent. She gradually improved and she was discharged on day 13 with normal vital signs and all symptoms resolved.

After the patient's stillbirth, the medical staff explained to her in her native language that there was much to be learned about EVD in pregnant women, and that important knowledge could be gained by performing pathologic examination of the fetus and placenta. The patient agreed to submit those tissues for testing and written informed consent was obtained.

Patient #1: Pathologic Findings, Gulu, Uganda

The placenta had mild subchorionitis and a moderate amount of malaria pigment (hemozoin) in fibrin and within macrophages embedded in fibrin **(Figure 1A)**. No parasitized erythrocytes or malarial intervillous inflammatory infiltrates were present. By electron microscopy, hemozoin crystallites were identified **(Figure 1B)**, but no ebolavirus virions were seen. The umbilical cord was normal.

Immunohistochemistry revealed Ebolavirus antigen in the placenta, primarily within areas of fibrin deposition, localized to embedded maternal mononuclear cells including malaria pigment-laden macrophages **(Figure 1C)**. Focal immunostaining was seen within the syncytiotrophoblast **(Figure 1D)**. The decidua, fetal placental villous stroma, amnion and umbilical cord were negative by IHC and no tissue necrosis or viral inclusions were noted.

Fetal tissues (lung, heart, liver, spleen, kidney, skin, and bone marrow) were well-preserved with minimal autolysis, normal for gestational age and had no necrosis or viral inclusions. All fetal tissues were negative by IHC.

Patient #2: Case Presentation, Isiro, DRC

Several clinical details have been previously published from this patient [13]. She was A 29 year-old housewife, gravida-8 para-7, who was transferred from a health center because of suspicion of EVD by a local clinician who knew that her relative died recently. She was admitted to the ETC on day 4 of illness with fever, fatigue, headache, abdominal pain (with uterine contractions), anorexia, dysphagia, vomiting, diarrhea and muscle and joint pain. The date of her last menstrual period was unknown but she was initially estimated to be 7 months pregnant. Conjunctival injection was noted. Her heart rate was 80 bpm and respiratory rate 20/min. Her cervix was 50% effaced with 4 cm dilation and fetal movement was normal.

Before admission, she was treated with oral artemether-lumefantrine (AL), intravenous quinine, ampicillin, diazepam, cimetidine, and scopolamine. At the ETC, she was treated with oral rehydration and antibiotics (cefixime, presumably) and AL was continued. On the day of admission, she tested positive for BDBV by RT-PCR and ELISA IgM. On day 5 of illness her cervix was at 100% effacement and 8 cm dilation and she was treated with oxytocin. A malaria rapid diagnostic test was positive and AL was continued. That night (day 6 of illness), spontaneous vaginal delivery of a live-born male infant occurred without assistance. The degree of vaginal bleeding did not seem out of the ordinary for a normal delivery, although she had an episode of

black stool some hours later. She was treated with oxytocin, ergometrine, IV fluids, and cefixime, and Plumpy'nut was provided. On day 7 the mother rapidly deteriorated, with wheezing, drowsiness, weakness and a temperature of 38.5°C. Antibiotics were switched to ceftriaxone. The next day she became comatose and died. A *post mortem* skin sample was taken from the mother as part of the routine outbreak response protocol [18].

The infant appeared healthy at birth, with Apgar scores of 8/10/10, and was clinically assessed to be at term based on examination of the nails and soles of the feet. Infant formula was provided, although the baby may have briefly breastfed immediately after delivery. A placental sample was collected to evaluate for BDBV. Blood collected at 1 day of age (the second day of life) was positive for BDBV by RT-PCR with a cycle threshold of 29.2. Over the next few days the baby was noted to be quiet and inactive. He became febrile (38.5°C) on day 4 of age and repeat testing of the blood revealed a cycle threshold on RT-PCR of 17.9 with negative ELISA for IgM and IgG. Over the next few days, the baby had hematemesis and bloody stools. He developed respiratory distress and coma and died on the seventh day of age (8^{th} day of life). No *post mortem* specimens were collected from the infant.

Patient #2: Pathologic findings, Isiro, DRC

In the placenta, scattered atypical maternal macrophages were seen within the intervillous space. These cells had degenerate appearing nuclei, cytoplasmic blebs and small eosinophilic cytoplasmic granules, suggestive of viral inclusions **(Figure 2A)**. The placenta was otherwise normal, and the placental membranes and umbilical cord were not sampled. No malaria

pigment or parasitized erythrocytes were seen. No virions were seen by transmission electron microscopy.

Ebolavirus antigen was seen by IHC within the circulating large atypical maternal mononuclear cells **(Figure 2B)**. Antigen was also present in multiple foci within the villous syncytiotrophoblast **(Figure 2C)**, frequently most intense at the basal aspect. Fetal stromal and endothelial cells were negative by IHC. In the basal plate, immunostaining was prominent within the extravillous trophoblast **(Figure 2D)** with scattered additional cell types likely representing decidual and maternal mononuclear cells. Focally, the lining cells of the maternal vessels of the basal plate (likely endovascular trophoblasts) were positive. Within the placenta, fetal stromal tissue, including villous blood vessels, was negative by IHC.

The post-mortem maternal skin specimen was morphologically normal and IHC negative.

DISCUSSION

Vertical transmission of pathogens can be by transplacental, transvaginal or by breastfeeding routes. Placenta sampling provides the opportunity to study disease processes in living patients and gain insights regarding the mode and mechanism of vertical transmission. In this study, SUDV or BDBV antigen was noted in fetal trophoblast cells, suggesting that these viruses can infect, and potentially cross, the placental epithelial barrier, resulting in transplacental infection of the fetus. Transplacental infection of the fetus by EBOV has been previously documented in stillbirths by virus PCR analysis of amniotic fluid, fetal blood and fetal swab specimens [7, 8].

The immuno-protective role of the placenta may promote the persistence of virus observed in these cases even after virus has been cleared from maternal blood [8, 9].

Several human pathogens can efficiently penetrate the placental barrier and infect the fetus, including some herpesviruses, HIV, Zika virus, *Treponema* and *Toxoplasma*. The trophoblast is the major cellular barrier to fetal infection, and is comprised of two major types: the villous trophoblast, which is directly exposed to maternal blood, and the extravillous trophoblast which invades the maternal decidua and directly contacts maternal cells, including lymphocytes and decidual stromal cells. In this study, both the syncytiotrophoblast (both patients) and the extravillous trophoblast (Isiro) demonstrated ebolavirus antigen by IHC **(Supplementary figure)**.

Findings from the two cases reported here together with the recent reports of EBOV RNA RT-PCR-positive stillbirths in women who have recovered from EVD [7, 8] suggest ebolaviruses have a degree of placental tropism. Ebolavirus entry into cells involves endocytosis and macropinocytosis [20]—both mechanisms that are important for the placental acquisition of maternal nutrients for fetal growth [21]. The NPC1 gene, which is required for ebolavirus cellular infection [22], is expressed in the placental syncytiotrophoblast [23].

Unexpectedly, fetal tissue from the Gulu patient showed no features of ebolavirus infection, suggesting that fetal demise was attributable to processes that occurred early in the course of maternal infection (e.g a systemic inflammatory response), which is consistent with the patient's noting of lack of fetal movement in the days prior to presentation and delivery. Post-

mortem tissue was not available from the baby of the Isiro patient, but he was BDBV RT-PCR positive by day 1 with a qualitative increase by day 4 of age, suggesting that the infant died of EVD. Although the finding of the IHC-positive trophoblast suggests potential transplacental BDBV transmission in the Isiro case, the baby appeared healthy at birth and fetal stromal tissue within the placenta was IHC-negative, so transvaginal infection cannot be excluded. Transplacental and transvaginal ebolavirus infection would not be mutually exclusive, such as in vertical transmission of HIV, in which one third is thought to be intrauterine-transplacental and the remainder transvaginal in the absence of preventative efforts [24].

Identifying infections that occur near or at the time of delivery is particularly important because these may be suitable targets for prevention through procedural or chemotherapeutic interventions. The World Health Organization currently recommends that asymptomatic infants born to mothers with EVD be separated and formula-fed. However, if the infant is confirmed or suspected to be infected, the benefits of breast feeding are thought to outweigh the risks, and breast feeding is thus recommended if the mother is able [25].

The co-localization of malaria pigment and ebolavirus antigen in the placenta of the Gulu patient is a novel finding and suggests that these two pathogens may interact at a cellular level. Filoviruses target monocyte-macrophages, and monocyte-macrophage infiltrates are a hallmark of active placental *Plasmodium falciparum* infection. Similar infiltrates are seen in other organs (particularly the liver and spleen) in non-pregnant individuals with severe malaria, further raising consideration of the potential for pathogen interaction. Of note, the Isiro patient had a

positive malaria rapid diagnostic test on her peripheral blood but no evidence of malaria-related placental pathology, perhaps due to receiving antimalarial treatment with AL; up to one third of documented cases of antenatal malaria do not show evidence of malaria in the placenta [26].

In contrast to the human infections described here, vertical transmission does not appear to occur in Egyptian fruit bats (*Rousettus aegyptiacus*), which are thought to be the natural reservoir of Marburg virus [27]; placentas of four naturally captured Marburg virus RNA-positive Egyptian fruit bats were all PCR negative. This can perhaps be explained by recognition that zoonotic pathogens often have unique maintenance mechanisms in distinct hosts. The cellular structure of placentas is markedly diverse across mammalian species, including whether fetal trophoblasts are directly exposed to maternal blood. Such structural differences may influence the likelihood of pathogen vertical transmission and/or placental tropism in natural versus incidental hosts.

Future sampling of placental tissue is necessary to fully understand the pathogenesis of EVD in pregnant women and their offspring and to ultimately develop ways to prevent or treat infection. In addition, given the very high rates of malaria in many areas where filovirus outbreaks occur and frequent EBOV-malaria co-infection during the 2013-2016 West African outbreak [28], future investigation of the interaction and clinical outcomes associated with these two pathogens should be a priority.

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DISCLAIMER

The findings and conclusions herein are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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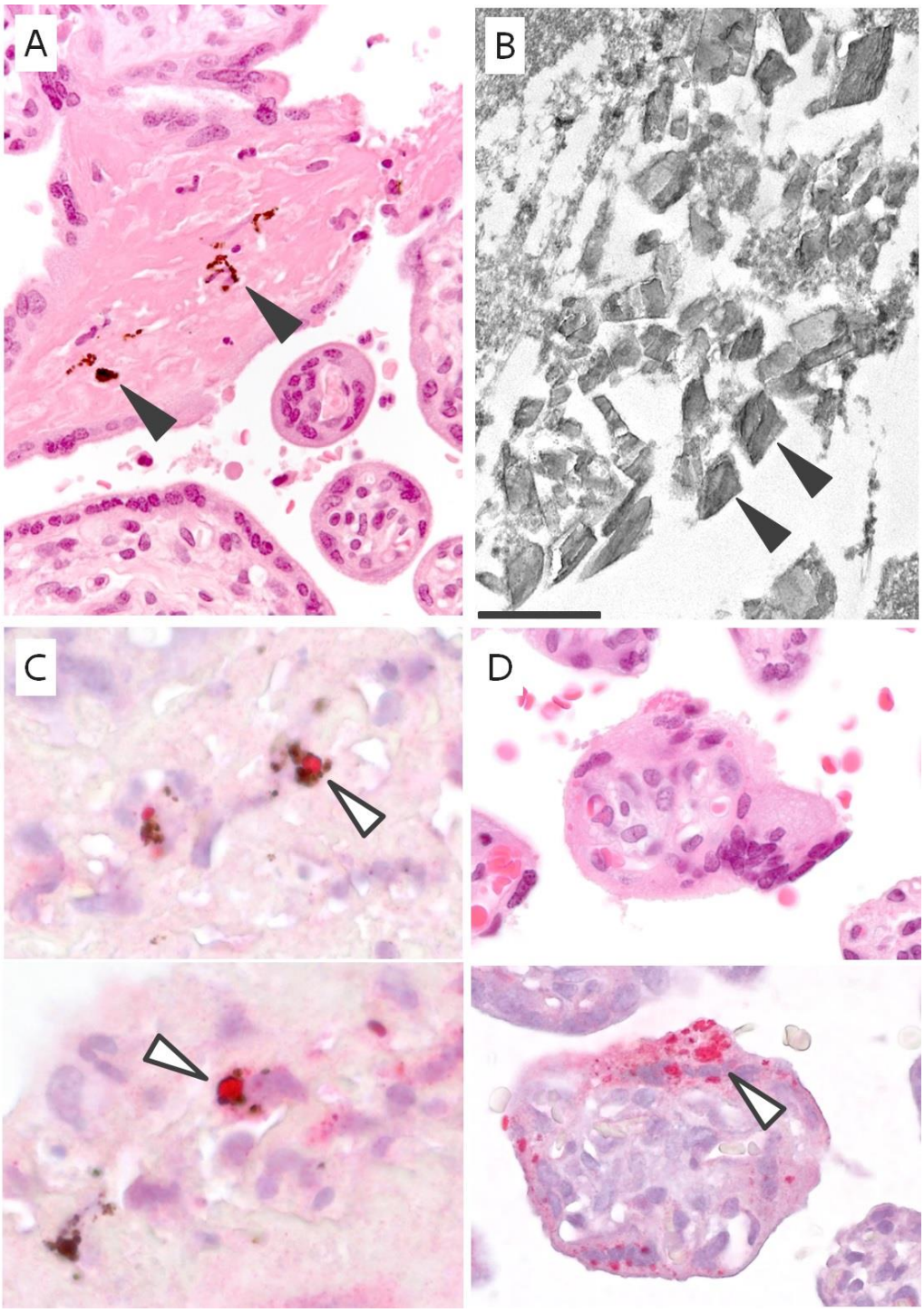

Figure 1: Placental findings from Patient #1. A) Hemozoin (malaria pigment) in fibrin (arrowheads), B) Transmission electron microscopy showing malaria hemozoin crystallites (arrowheads); no ebolavirus virions were identified. Scale bar=500 nm. C) Colocalization of ebolavirus antigen (arrowheads) with malaria pigment. D) Serial sections by H&E (upper) and IHC (lower) showing ebolavirus antigen (arrowhead) localized to the syncytiotrophoblast.

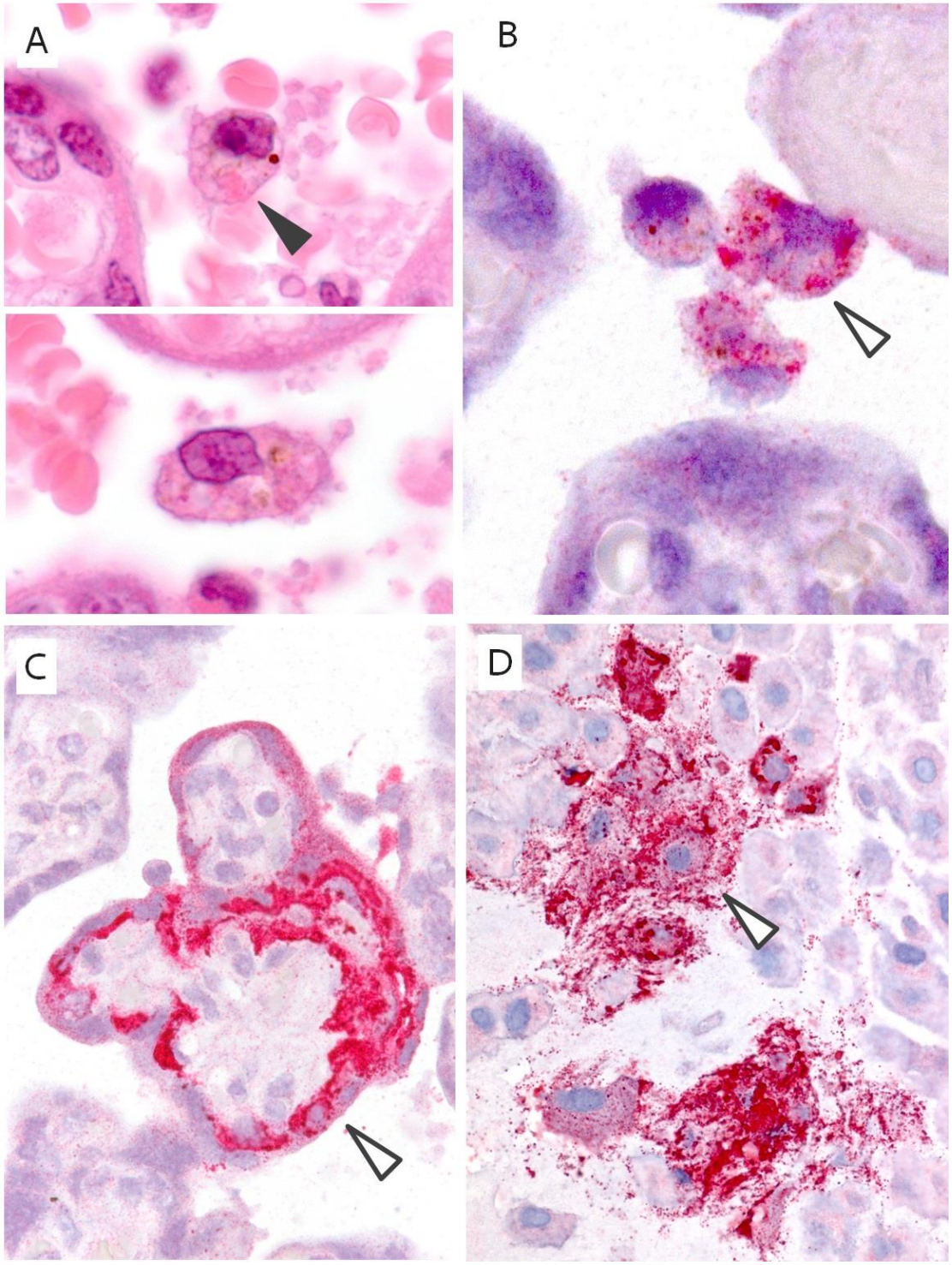

Figure 2: Placental findings from Patient #2. A) Circulating atypical maternal macrophages with vacuolated cytoplasm and eosinophilic cytoplasmic granules suggestive of viral inclusions (arrowhead). By IHC, ebolavirus antigen localization to B) Circulating maternal macrophages; C) syncytiotrophoblast, D) intermediate trophoblast (arrowhead) within the basal plate.