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

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## 2 ABSTRACT

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

11

## 12 INTRODUCTION

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

22

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

29  
30 Despite the severity of filovirus infection in pregnancy for both mother and child, very little is  
31 known regarding pathogenesis. Fetal-placental viral tropism has been hypothesized due to  
32 recent observations during the 2013-2016 West Africa EBOV outbreak: pregnant women were  
33 noted to survive EVD and clear virus from the blood without fetal loss during acute infection,  
34 and deliver stillbirths in the subsequent weeks and months with relatively high EBOV RNA levels  
35 in placental and fetal tissue swabs [7-9] [10]. We report clinical, histopathologic and  
36 immunohistochemical findings of SUDV and BDBV virus infections in two pregnant women and  
37 their offspring that help shed light on the pathogenesis of fetal infection and loss in EVD.

38

## 39 **METHODS**

### 40 **Patients**

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

44

45 **Ebolavirus diagnostic testing**

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

51

52 **Histopathology, immunohistochemistry and transmission electron microscopy**

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

64

65

66

67 **RESULTS**

68 **Patient #1: Case Presentation, Gulu, Uganda**

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

80  
81 On day four of illness the patient spontaneously delivered a dead but apparently  
82 morphologically normal fetus and placenta. The degree of vaginal bleeding did not seem out of  
83 the ordinary for a stillbirth. Over the next three days, the patient complained of pain and  
84 swelling of the joints, especially the wrists and knees, throat and chest pain, persistent dry  
85 cough with dyspnea and, briefly, hiccups. Her wrists and knees were visibly swollen and tender  
86 to the touch and rales continued to be noted. She was consistently febrile during this time, with  
87 disease severity peaking at day 7 of illness, when her vital signs showed an axillary temperature  
88 of 37.8°C, pulse 128 bpm, respiratory rate 30 breaths per minute, and oxygen saturation of 90

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

91

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

96

#### 97 **Patient #1: Pathologic Findings, Gulu, Uganda**

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

103

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

109

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

113

#### 114 **Patient #2: Case Presentation, Isiro, DRC**

115 Several clinical details have been previously published from this patient [13]. She was A 29 year-  
116 old housewife, gravida-8 para-7, who was transferred from a health center because of suspicion  
117 of EVD by a local clinician who knew that her relative died recently. She was admitted to the  
118 ETC on day 4 of illness with fever, fatigue, headache, abdominal pain (with uterine  
119 contractions), anorexia, dysphagia, vomiting, diarrhea and muscle and joint pain. The date of  
120 her last menstrual period was unknown but she was initially estimated to be 7 months  
121 pregnant. Conjunctival injection was noted. Her heart rate was 80 bpm and respiratory rate  
122 20/min. Her cervix was 50% effaced with 4 cm dilation and fetal movement was normal.

123

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



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

137

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

148

#### 149 **Patient #2: Pathologic findings, Isiro, DRC**

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

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

156

157 Ebolavirus antigen was seen by IHC within the circulating large atypical maternal mononuclear  
158 cells (**Figure 2B**). Antigen was also present in multiple foci within the villous

159 syncytiotrophoblast (**Figure 2C**), frequently most intense at the basal aspect. Fetal stromal and  
160 endothelial cells were negative by IHC. In the basal plate, immunostaining was prominent

161 within the extravillous trophoblast (**Figure 2D**) with scattered additional cell types likely

162 representing decidual and maternal mononuclear cells. Focally, the lining cells of the maternal

163 vessels of the basal plate (likely endovascular trophoblasts) were positive. Within the placenta,

164 fetal stromal tissue, including villous blood vessels, was negative by IHC.

165

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

167

## 168 **DISCUSSION**

169 Vertical transmission of pathogens can be by transplacental, transvaginal or by breastfeeding

170 routes. Placenta sampling provides the opportunity to study disease processes in living patients

171 and gain insights regarding the mode and mechanism of vertical transmission. In this study,

172 SUDV or BDBV antigen was noted in fetal trophoblast cells, suggesting that these viruses can

173 infect, and potentially cross, the placental epithelial barrier, resulting in transplacental infection

174 of the fetus. Transplacental infection of the fetus by EBOV has been previously documented in

175 stillbirths by virus PCR analysis of amniotic fluid, fetal blood and fetal swab specimens [7, 8].

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

178

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

186

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

193

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

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

206  
207 Identifying infections that occur near or at the time of delivery is particularly important because  
208 these may be suitable targets for prevention through procedural or chemotherapeutic  
209 interventions. The World Health Organization currently recommends that asymptomatic infants  
210 born to mothers with EVD be separated and formula-fed. However, if the infant is confirmed or  
211 suspected to be infected, the benefits of breast feeding are thought to outweigh the risks, and  
212 breast feeding is thus recommended if the mother is able [25].

213  
214 The co-localization of malaria pigment and ebolavirus antigen in the placenta of the Gulu  
215 patient is a novel finding and suggests that these two pathogens may interact at a cellular level.  
216 Filoviruses target monocyte-macrophages, and monocyte-macrophage infiltrates are a hallmark  
217 of active placental *Plasmodium falciparum* infection. Similar infiltrates are seen in other organs  
218 (particularly the liver and spleen) in non-pregnant individuals with severe malaria, further  
219 raising consideration of the potential for pathogen interaction. Of note, the Isiro patient had a

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

224  
225 In contrast to the human infections described here, vertical transmission does not appear to  
226 occur in Egyptian fruit bats (*Rousettus aegyptiacus*), which are thought to be the natural  
227 reservoir of Marburg virus [27]; placentas of four naturally captured Marburg virus RNA-  
228 positive Egyptian fruit bats were all PCR negative. This can perhaps be explained by recognition  
229 that zoonotic pathogens often have unique maintenance mechanisms in distinct hosts. The  
230 cellular structure of placentas is markedly diverse across mammalian species, including whether  
231 fetal trophoblasts are directly exposed to maternal blood. Such structural differences may  
232 influence the likelihood of pathogen vertical transmission and/or placental tropism in natural  
233 versus incidental hosts.

234  
235 Future sampling of placental tissue is necessary to fully understand the pathogenesis of EVD in  
236 pregnant women and their offspring and to ultimately develop ways to prevent or treat  
237 infection. In addition, given the very high rates of malaria in many areas where filovirus  
238 outbreaks occur and frequent EBOV-malaria co-infection during the 2013-2016 West African  
239 outbreak [28], future investigation of the interaction and clinical outcomes associated with  
240 these two pathogens should be a priority.

241

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244

245 **DISCLAIMER**

246 The findings and conclusions herein are those of the authors and do not necessarily represent

247 the official position of the Centers for Disease Control and Prevention.

248

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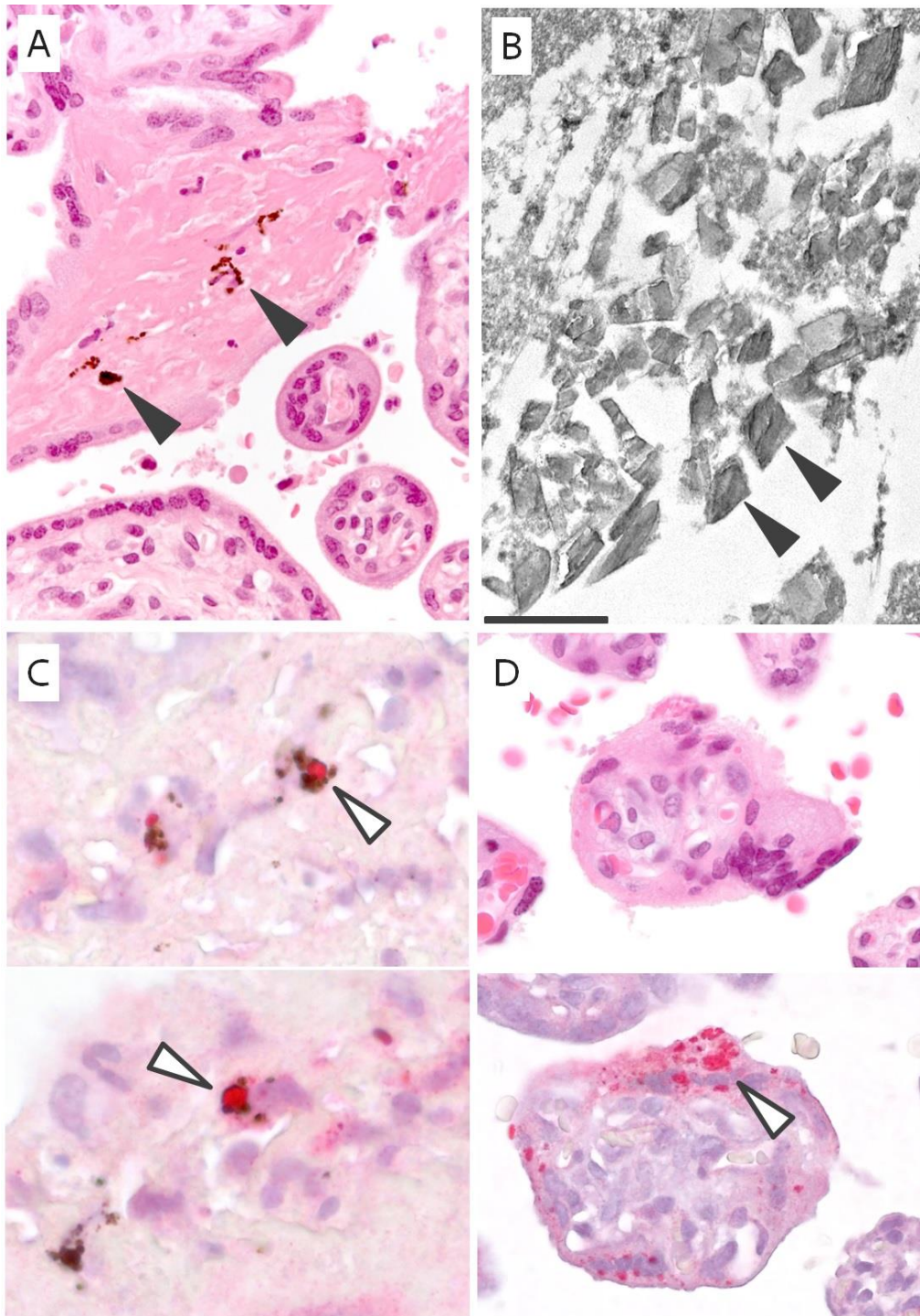
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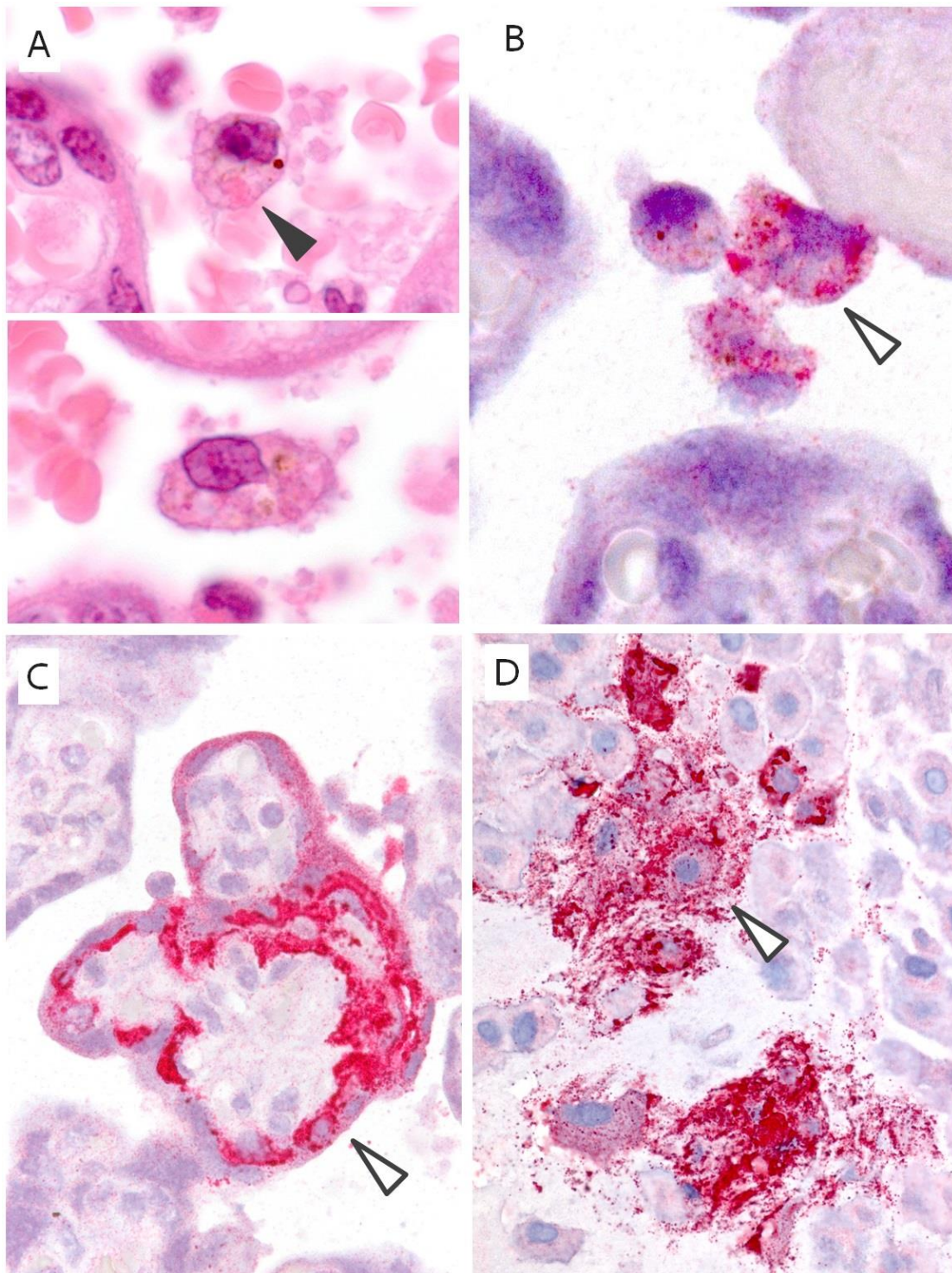
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- 325
- 326



327

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

333



334

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