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MATERNAL-FETAL TRANSMISSION OF THE ZIKA VIRUS: AN INTRIGUING INTERPLAY

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Running title: An update on congenital Zika virus transmission

Abstract

In this review, we give an overview of aspects related to the congenital transmission of the Zika virus (ZIKV). Although we acknowledge that important advances in research on ZIKV pathogenesis have come from studies using animal models, particularly non-human primates,

this review emphasizes studies using *ex-vivo* human cells and tissues as well as natural infections in pregnant women. The possible routes used by ZIKV to cross or breach the placental barrier and infect the fetal central nervous system are presented. Understanding the viral infection biology and ZIKV pathogenesis during pregnancy may guide the design of affordable antiviral strategies to benefit pregnant women in areas at risk.

Keywords: Zika virus, placental barriers, maternal-fetal transmission, Hofbauer cells, natural infections, *ex vivo* models.

Introduction

The Zika virus (ZIKV) is an emergent arbovirus of the genus *Flavivirus*, family *Flaviviridae*, that includes other viruses relevant to public health, such as dengue (DENV), yellow fever (YFV) and West Nile viruses (WNV). Historically, since it was first isolated from Rhesus monkey serum in 1947 in Uganda, the virus has only been associated with isolated cases of mild human infections in Africa and Asia.¹ In 2007, ZIKV circulation was detected on Yap Island causing mild disease in over 70% of Yap residents.² In 2013, an outbreak affecting approximately 29,000 people was recorded in French Polynesia,³ when cases of neurological manifestations such as Guillain-Barré syndrome were reported in patients infected by ZIKV.⁴⁻⁷ After its introduction in the Americas in 2014-2015, ZIKV infection has been associated with fetal malformations and death in an epidemic that has affected millions.⁸⁻¹⁰

In general, human ZIKV infections are self-limiting, and the most frequent clinical signs are low fever, myalgia, rash, arthralgia, headache and conjunctival hyperemia.^{2,11} However, ZIKV

infection during pregnancy has been associated with fetal malformations, particularly abnormalities in central nervous system (CNS) development and microcephaly in newborns.¹²⁻¹⁶

Studies on stillborn fetal samples from pregnant women diagnosed with ZIKV during pregnancy, as well as organoids and animal models, have demonstrated ZIKV's potential for replicating in CNS tissues and causing neurological disease.^{14,17-20} However, the mechanisms by which the virus crosses the placenta to infect the developing fetus are undefined.

Several approaches have been used in animal models to study the ZIKV mechanisms that produce congenital malformations.²¹ The most commonly used models include immunodeficient mice, such as the AG129 lineage that are deficient in key genes for type I IFN signaling.¹⁸ Although small animal models could contribute to our knowledge of ZIKV pathogenesis,²¹⁻²³ when studying the maternal-fetal transmission the differences between primate hemomonochorial and mouse hemotrichorial placental organization must be considered.²⁴ Many mouse models were established with laboratory-adapted ZIKV strains with an extensive passage history, and this raises concerns about their relationship with recent clinical isolates. Non-human primate (NHP) models appear to be more closely related to human infections.²⁵⁻²⁷ NHP pregnancies mimic human pregnancy in many ways, including uterine anatomy, single gestation and hemochorial placentation. The limitations include high cost, long experiment duration and small sample size.²⁸ Despite the importance of animal models on Zika's knowledge, this review emphasizes studies using *ex-vivo* human culture models and natural infections in pregnant women.

Placental architecture

The human placenta is a dynamic structure that begins to form 5/6 days after fertilization and is composed of fetal cells closely associated with maternal tissues. Here, we discuss the classic placental barrier with villous trophoblasts that form the tertiary villi as well as the broader non-classic placental tissue barrier. This wider definition also encompasses villous and non-villous structures during multiple stage of gestation, including primary and secondary villi, villous columns of extravillous trophoblasts, extravillous trophoblast islands, the chorionic plate, smooth chorion, septa, basal plate, placental bed, utero-placental arteries and veins, the fibrinous matrix and decidua.²⁹⁻³¹

At the third or fourth day post-conception, the totipotent cells of the blastocyst divide asymmetrically, giving rise to two distinct cell populations: trophoblastic cells and embryonic cells. The maternal-fetal interface consists of fetal-derived trophoblasts (TBs), progenitor cells that will differentiate into proliferative cytotrophoblasts (CTBs), and syncytiotrophoblasts (STBs) formed by the fusion of the CTBs (Figure 1 – 6 days). TBs form the villous and extravillous placental structures that mediate maternal fetal exchanges. At each stage of differentiation and growth, the CTBs and STBs, as well as their structures, are responsible for the different complexity levels of the placental tissue barrier.^{29,30}

Next, lacunas open in the mass of STBs (Figure 1 – 12 days), and primary villi are formed by the villous CTB proliferation (Figure 1 – 15 days). During these stages, CTBs and STBs interpose between the maternal blood and the embryonic tissues, functioning as a primordial placental barrier.^{29,30}

At the third week of gestation, the STBs form the continuous outermost cell layer, which extends over the villous tree surface and becomes the interface between the maternal and fetal blood. The chorionic villous core consists of Hofbauer cells (HBCs), fibroblasts, and endothelial cells that line fetal capillaries immersed in the extracellular matrix. The placental barrier now consists of STBs, CTBs, connective tissue and capillary endothelium (Figure 1 – 18 days).^{29–31}

HBCs are macrophages that appear in the chorionic villi on the 18th day of gestation and are present until term.³² HBCs are M2 macrophages located under the STB layer, adjacent to fetal capillaries, forming a critical site to protect the fetus against pathogens migrating from the mother. Multiple congenital pathogens such as bacteria, the Coxsackie virus, the human cytomegalovirus (HCMV) and the herpes simplex virus (HSV) are detected primarily in the HBCs and STBs.³³

By 10–13 weeks of gestation, the human placenta becomes hemochorial, indicating direct contact between maternal blood and placental chorionic villi.³⁰ The non-villous sections of the placenta are comprised of extravillous trophoblasts, the fibrinous matrix and decidua (specialized endometrium).³⁰ Therefore, the broader notion of the placental barrier is the interface for maternal-fetal exchanges and is involved in multiple stages of gestation until 12 weeks, when the tertiary villi are completely differentiated and the classical placental barrier reaches maximum differentiation.^{30,31} Due to placental formation dynamics, pathogenic vertical transmission mechanisms likely differ between earlier and later (>12 weeks) stages of human pregnancy.³⁴

Possible routes for maternal-fetal ZIKV transmission

Maternal-fetal infection transmission routes and their impacts on the fetus may vary depending on the gestational stage. Two basic routes exist for fetal access via the placental barriers during pregnancy: the transvaginal (or ascending route of infection) and the haematogenic (or transplacental transmission pathway) (Figure 2).^{29,34–36}

Ascending route

In addition to mosquito transmission, ZIKV can be transmitted through sexual contact.^{37,38} Men can shed viral particles in semen for weeks or months after the acute infection phase, acting as a long-term reservoir.^{39–42} The isolation of ZIKV from seminal plasma and spermatozoa from a volunteer with acute ZIKV infection by Joguet et al.⁴² reinforces the possibility of sexual transmission. ZIKV RNA has also been detected in the female reproductive tract,^{43–46} but its implication in sexual transmission remains unknown as there is only one case of female-to-male transmission in the literature.⁴⁷

ZIKV transmitted through sexual contact might be one of the ways by which the virus would reach the fetus via the ascending route. Although not yet proven in humans, Yockey et al.⁴⁸ demonstrated that vaginal infections in pregnant dams can lead to fetal brain infection in a mice model. In addition, when the vaginally acquired ZIKV infection occurred during early pregnancy, mice fetal growth restriction was observed.

Through the transvaginal pathway, infections can ascend directly from the vaginal canal, pass through the cervix and mucus plug, and reach the capsular decidua, the smooth chorion and the amnion/amniotic fluid (Figure 2). This results in inflammatory conditions including

chorioamnionitis, funisitis, and deciduitis. Microorganisms that use this pathway to infect the placenta and fetus include group B streptococcus, *E. coli*, ureaplasma, chlamydia and mycoplasma.^{29,34,36} However, chorioamnionitis and related abnormalities are not yet associated with natural intra-uterine ZIKV infections.^{17,29,34,49,50} It is worth mentioning that sexual transmission usually results in peripheral infection and disease signs, such as rash, fever, arthralgia, and conjunctivitis.³⁸ Consequently, fetal infection after sexual transmission may occur through the transplacental route from the virus in the maternal bloodstream (described below).

Transplacental transmission

In vertical transmission, microorganisms reach the placenta through maternal vessels and cross placental barriers by villous (from the most primitive villi to the classic tertiary villous structure) and non-villous structures, both of which are covered by maternal blood (Figure 2).^{29,35,36,51} Therefore, vertical transmission occurs when a virus passes through the villi and the STB layer and/or through the decidua and extravillous trophoblasts (Figure 1).^{34,35,51}

The histological hallmark of vertical transmission infections is villitis, an inflammatory process of the villi induced when microorganisms infect the placenta and fetus.^{29,34,36} The more frequent congenital infections are related to the pathogens known as TORCH, an acronym for *Toxoplasma gondii*, other (*Listeria monocytogenes*, *Treponema palladium*, parvovirus, HIV, and the varicella-zoster virus), rubella, cytomegalovirus (CMV) and herpesviruses 1 and 2 (HSV). The inclusion of ZIKV (TORCHZ) has been proposed due to its implication in fetal central

nervous system abnormalities.³⁴ Congenital Zika syndrome includes several fetal disorders including placental insufficiency and fetal growth restriction, ocular, and microcephaly and other CNS disorders.^{15,52}

To pass through the STB layer, pathogens can eventually cause cellular damage with inflammation process or villitis, or directly pass through ruptures that naturally occurs in the placental barrier throughout gestation (without causing inflammation or villitis).^{32,49,53} Thus, in vertical transmission villitis may or may not be observed.^{29,34,49} Some authors have reported first trimester abortion with villitis associated with ZIKV infection. On the other hand, they also reported congenital transmission of ZIKV without pathological evidence of villitis in second and third trimester placentas.^{14,20}

The STB layer continuously undergoes damage and repair throughout pregnancy. This damage to the STB layer can be implicated in the trans-syncytial movement of a virus from the intervillous space to the villous core, thereby exposing the more susceptible cells without villitis or replication within the STB cells.^{32,34,49,53} Second and third trimester placentas infected with ZIKV in mice, revealed the absence of villitis. Likewise, the human immunodeficiency virus (HIV) is another hematogenously transmitted virus that does not induce villitis.^{13,20,34,54,55} Other viruses, such as HSV (part of TORCH), can also breach the STB layer without villitis. This mechanism may be occurring with ZIKV infection.

Crossing the STB layer can also occur through the cell-associated transport pathway, either by passive diffusion or by an active transport mechanism known as transcytosis. This mechanism could involves non-neutralizing cross-reactive antibodies that bind ZIKV or by exosomes package.^{32,34,53,56,57}

An alternative method for pathogens to cross the placental barrier is to directly infect the STBs, which does not appear to occur with ZIKV.^{35,51,53,58,59} Primary STBs isolated from full-term placentas resist viral infection, whereas CTBs and other villous core cells are susceptible. Physical properties of the syncytium may also restrict microbial infections, such as the syncytial surface, comprised of dense, branched microvilli at the apical surface, and a complex cortical actin network that limits microbial invasion.^{29,35,36,60}

Extravillous trophoblasts are another means of ZIKV vertical transmission across the placenta, as has been described for *Toxoplasma gondii* and *Listeria monocytogenes*, infecting the decidua, crossing the extravillous trophoblasts, and subsequently to the villous core and fetal vasculature.^{29,35,36} Previous studies using *ex vivo* human placental tissues have demonstrated ZIKV replication in the maternal decidua, where fetal cells directly interact with maternal cells. This represents a possible mechanism whereby ZIKV from the mother can breach the placental barrier.^{35,51}

Putative alternative mechanisms for ZIKV to overcome the placental barrier

Trophoblast-derived exosomes

In natural infections, ZIKV in the amniotic fluid and fetal brain indicates that the virus crosses the placental barrier.⁶¹ Secretory autophagy or the exosome pathway may be implicated in the vertical transmission of ZIKV. It has been recently demonstrated that the inhibition of autophagy limits vertical transmission of ZIKV in pregnant mice.⁶² Furthermore, Delorme-Axford et al.⁶³ found that *ex vivo* human placental trophoblasts resisted infection by several viruses and transferred this resistance to other cells through exosomal flow containing specific microRNAs

(miRNAs). Those vesicles could be used by ZIKV to actively transport across the placental barrier as exosome cargo derived from the trophoblastic endoplasmic reticulum pathway.⁵⁷ Nevertheless, evidences of trophoblast-derived exosomes as an alternative mechanism for ZIKV to cross the placental barrier in natural infections are needed.

Immunoglobulin-mediated transcytosis

In addition to the potential viral spread through infected cells or exosomes to reach the placenta and the fetus, immune response and inflammation can also contribute to this mechanism.

The antibody-dependent enhancement (ADE) phenomenon postulates that pre-existing, sub-neutralizing levels of antibodies generated from a previous flavivirus infection (for example, dengue) would enhance the infection by ZIKV. The sub-neutralizing antibodies would complex with the ZIKV particle and the complex antibody-ZIKV would be more easily opsonized by Fc receptor-bearing cells.⁶⁴ Non-neutralizing IgG antibodies generated from a previous flavivirus infection could possibly complex with ZIKV particles in the maternal blood and cross the villous trees to reach the fetal capillaries by infecting the neonatal Fc receptor (FcRn)-bearing cells. Fc γ receptor II (CD32) expression on placenta/decidua cell surfaces could also be related to the increased viral load and tissue damage in severe cases.⁶⁴ However, ADE and ZIKV results are contradictory. ADE of ZIKV infections has been induced *in vitro*, *ex vivo* and in mouse models.^{63,65-69} Also, pre-existing DENV immunity does not result in a more severe ZIKV disease in NHP models or in patients who are pre-exposed to DENV infection.^{70,71} Thus, this enhancement is still undetermined in natural infections and physiological conditions.

Another possible indirect mechanism for congenital Zika syndrome is that placental infection deregulates immune system pathways and indirectly culminates in abnormal formation of fetal CNS tissues.⁵⁶ The placenta synthesizes and secretes essential molecules for normal fetal brain development; therefore, ZIKV-induced pro-inflammatory responses in the placenta may interfere with production and secretion of neuropeptides, non-coding RNAs and cytokines, consequently disrupting normal brain development.^{56,65} However, ZIKV viral RNA and antigens found in fetal and stillbirth brain tissue do not support this hypothesis.^{14,20}

***Ex vivo* models to study ZIKV congenital infection**

Several *ex vivo* models have been used to study ZIKV placental infection. The *ex vivo* models have followed four main approaches: embryonic stem cells (ESC), primary human placental cells, tissue explants, and 3D models.^{34,35,51,58}

Sheridan et al.⁵⁸ attempted to determine the most vulnerable gestational period for ZIKV infection for causing fetal abnormalities and targeting placental cells during ZIKV infection. They used human CTBs and STBs derived from placental villi at term and trophoblasts differentiated from embryonic stem cells (ESC). The authors claimed that the ESC-derived trophoblasts are analogous to primitive placenta formed during implantation. They demonstrated that the cells from term placenta are resistant to ZIKV infection probably due to the gene expression associated with antiviral defense and the absence of expression of molecules implicated in ZIKV entry. Conversely, the ESC-derived trophoblasts have several attachment factors for ZIKV entry and are unable to mount a robust antiviral response, thus making them susceptible to ZIKV infection. The authors hypothesized that the early gestational placenta are

more easily breached by ZIKV than the refractory cells of the mature placenta. Similarly, studies using *ex vivo* decidua tissues from pregnant women at the beginning and middle of gestation indicate that early-gestational cells are more susceptible to ZIKV infection.³⁵ However, ZIKV can induce congenital disease beyond the first trimester, and infection in later periods can result in adverse outcomes.^{13,14,66}

Weisemblum et al.³⁵ studied innate tissue response patterns using a 3D model of *ex vivo* placental tissues after parallel infection with ZIKV and human cytomegalovirus (HCMV). Gene expression analysis from placental tissues infected with ZIKV showed intense modulation of genes involved in cell death and apoptosis, whereas HCMV triggered the expression of genes related to immune cell activation and trafficking responses. This suggests that ZIKV induces more damage than protection to placental tissues, compared to HCMV. Moreover, immunohistochemistry of ZIKV-infected maternal-decidual and first trimester chorionic villus tissues revealed positive cells in the decidual tissue and fetal CTB cell layers in the villus, whereas the STB layer was consistently negative. The authors suggest that human maternal decidua is the probable ZIKV vertical transmission route.³⁵ Accordingly, Tabata et al.⁵¹ showed that ZIKV targets CTB cells in first-trimester chorionic villi as well as placental macrophages (HBCs). Furthermore, ZIKV infected primary amniotic epithelial cells, trophoblasts progenitor cells, placental fibroblasts, endothelial cells, HBCs and CTBs isolated from mid- and late-gestation placenta in *ex vivo* models.^{34,51,53}

In another study, Aagaard et al.⁶⁷ showed that PHPT from non-ZIKV exposed donors could be infected by clinical isolates of ZIKV, which is in contrast to DENV (another flavivirus). PHPT

express several putative ZIKV receptors, maintain cellular functions and preserve differentiation without host cell destruction, suggesting a reservoir role and a portal to fetal transmission leading to malformations.

Other studies suggest that STB cell layer refraction from term placentas to ZIKV infection may be due to their constitutive secretion of type III IFNs.^{35,51,68} Bayer et al.⁶⁹ used laboratory-adapted ZIKV strains to infect placental trophoblastic cell lines, but not primary human trophoblasts. They assumed that INF λ 1 secreted in the conditioned media from human trophoblasts could restrict viral replication in these cells. Conversely, Quicke et al.⁵³ uses *ex vivo* HBCs from full-term placentae to demonstrate their susceptibility to infection by a recent ZIKV clinical isolate and found that CTBs support ZIKV replication at later time points (72 to 96 h). However, INF λ 1 was undetected in the infected trophoblast cultures.

The *ex vivo* studies have contributed to a greater understanding of the placental infection by ZIKV and its consequence. However, all of them were performed by infecting tissues from healthy pregnant women. In addition, those models do not recapitulate the biological complexity of the human organism. Therefore, the studies on naturally infected women are essential for corroborate and complement those observations.

ZIKV infection in pregnant women

In naturally infected women, ZIKV antigens were detected in HBCs within chorionic villi from a first trimester placenta.⁵⁴ Likewise, Bhatnagar et al.¹⁷ analyzed placental/fetal tissues from women suspected of ZIKV infection during pregnancy, including cases of spontaneous abortion. In one case, the patient had a spontaneous abortion at 11 weeks of gestation and another at 8

weeks of gestation. Using in situ hybridization (ISH), replicative ZIKV genomic RNA was identified in HBCs from placental chorionic villi in both cases. Positivity in fetal endothelium and maternal leukocytes in first-trimester placentas have also been reported.⁷⁰ However, the results from our group demonstrated that HBCs seems to be the only placental cell persistently positive for ZIKV antigens regardless of the gestation period.¹⁴ Once in the villous core, ZIKV can access the fetal compartment.

HBCs in the chorionic villi are physically located near the umbilical blood vessels, and thus could be a source of viral dissemination through fetal blood. Furthermore, as M2 macrophages, HBCs can migrate and potentially be a bridge for infection and spread of ZIKV throughout fetal and placental cells by a mechanism known as a “Trojan horse”.^{32,71,72}

In human placenta from first trimester pregnancies, ZIKV positive HBCs are more abundant than in second and third trimester placentas. ZIKV has been identified by immunohistochemistry (IHC), ISH and PCR, in full-term placental HBCs in which the mothers become naturally infected in the first or second trimester. This indicates that the virus can persist in the placenta for long periods following the onset of maternal symptomatology and may provide a latent viral source of continued fetal infection,^{17,32,34,35,49–51,59,60} which could be related to the prolonged viremia in pregnant women and NHP models.^{19,25}

Final Considerations

Despite the increased knowledge of ZIKV biology and pathogenesis, the mechanism(s) of ZIKV intrauterine transmission and the cell types involved remain largely unknown. The current results from NHP models and human studies have shown variability among individuals, probably due to

the viral strain, individual genetic background, immunological status, co-morbidities and infection period. However, this could be an advantage, as it resembles the natural ZIKV infection scenario in the general population.

Natural infection results highlight that although the data obtained from *in vitro*, *ex vivo* and animal models provide knowledge of ZIKV pathogenesis, they do not completely recapitulate the complexity of a natural ZIKV infection. Studies on naturally infected pregnant women have consistently shown that HBCs are infected. It remains unclear, however, how ZIKV accesses the fetal compartment and infects HBCs, and which other cell types are also permissive to ZIKV. Many other open questions remain to be answered.

It has been demonstrated the influence of hormones in intravaginal (i.vg.) infection in animal models. Tang et al.⁷³ demonstrated that female mice in the diestrus-like phase are susceptible to ZIKV infection through the i.vg. route, whereas they are resistant during the estrus-like phase. Similarly, Carroll et al.⁷⁴ infected two Rhesus macaques with ZIKV, who initially resisted 8 i.vg. viral inoculations after treatment with artificial progesterone. Does this occur in humans as well? Sexual transmission resulting in fetal infection has not yet been documented in humans. Could it be attributed to hormonal interference?

Several studies have been conducted on mouse models using the transvaginal route to inoculate ZIKV and study its sexual transmission.^{48,73,75} However, specific seminal plasma peptides and proteins are signals for the female immune system, modulating sperm rejection and tolerance.⁷⁶ How does semen influence this transmission route? Could it modulate the immune response to the virus in natural infections?

Cases of prolonged viremia have been reported in pregnant women and monkeys.^{19,25} Does a change in the mother's immune system lead to this persistence? Is the mother reinfected with the infected fetal cells? Is there an interval delay between maternal and fetal infection? Could the placental infection also occur because of fetal infection? ZIKV-containing cells could cross the placenta as “Trojan horse”, enter the fetal blood stream, cause fetal infection and as a secondary event disseminates to the placenta?

Finally, it is important to better understand the mechanisms of ZIKV transmission, particularly in sexual and maternal-fetal transmission, to reduce infections during pregnancy. Further knowledge of the viral pathogenesis may help in the design of safer clinical trials for antiviral and vaccine strategies, thus allowing the inclusion of pregnant women.

Disclosure of potential conflict of interest

No potential conflicts of interest are disclosed.

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Figure Legends

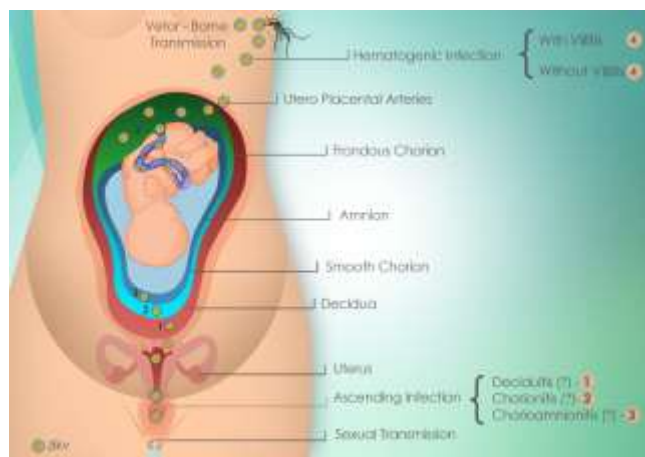


Figure 1 - Schematic representation of cytotrophoblast differentiation and the invasion of the syncytiotrophoblast in the endometrium, contributing to the blastocyst nidation, from the sixth day after conception (p.c.).

6 days p.c. - Prelacunar stage where a mass of syncytiotrophoblastic cells invades the endometrium to become the decidua. Maternal-fetal exchanges are made by the syncytiotrophoblastic cells.

12 days p.c. - Lacunar stage / primary villi showing lacunas with maternal red cells in the syncytiotrophoblastic cell mass and sprouts of primary villi composed of cytotrophoblasts, facilitating maternal-fetal exchanges.

15 days p.c. - Stage of secondary villi where cytotrophoblastic secondary villi become more specialized and penetrate deeper into the mass of syncytiotrophoblastic cells and the lacunas become larger, facilitating even more maternal-fetal exchanges.

18 days p.c. - Stage of tertiary villi where the tertiary villi present primitive fetal mesenchymal and fetal vessels with fetal red blood cells. These specialized villi are bathed in the intervillous

space, which is an expanse of the anterior lacunas, where the maternal blood facilitates maternal-fetal exchanges. The decidua is completely specialized, as is the placental bed.

Detail: Cross-section of a fully specialized tertiary villus demonstrating the outermost syncytiotrophoblast continuous layer, the innermost cytotrophoblast layer, the fetal mesenchyme and the fetal vessels coated by the fetal endothelial cells. Hofbauer cells are found in the fetal mesenchyme.

