Role of the C19MC miRNA cluster during viral congenital infections

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THÈSE

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Présentée et soutenue par

Yamileth CHIN

Le 13 décembre 2022

Effet du cluster de microARN C19MC au cours des infections virales congénitales

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Dedication

I dedicate this thesis to my beautiful and strong grandmother Elisa Santos who raised me with unconditional love. May God have her in his glory and hope one day we see each other again.

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Résumé

Les infections virales congénitales touchent les femmes du monde entier et sont associées à des troubles du développement néonatal. Par exemple, le virus Zika (ZIKV) a été associé à de graves anomalies congénitales, dont la microcéphalie. De même, le cytomégalovirus humain (HCMV) est l'infection virale la plus répandue chez le fœtus et entraîne des troubles neurodéveloppementaux. Le ZIKV et le HCMV infectent les cellules placentaires, affectant directement le développement du nouveau-né. Le placenta utilise des vésicules extracellulaires (EVs) pour interagir avec son environnement, et il a été démontré que leur contenu en microARN (miARN), notamment ceux du cluster C19MC, est dérégulé au cours des grossesses pathologiques. Le cluster de miARN C19MC, spécifique aux primates, présente un profil d'expression dynamique tout au long de la grossesse, avec des niveaux qui augmentent vers la fin du premier trimestre et chutent rapidement après la naissance. En utilisant un modèle innovant de cellules Knock-Out (KO) pour le cluster C19MC généré par la technique CRISPR/Cas9 par l'équipe de J. Cavaillé, nous avons étudié la fonction antivirale de ce cluster au cours des infections virales congénitales. Des expériences impliquant des techniques de PCR quantitative en temps réel, de western blotting, de cytométrie de flux et d'immunofluorescence ont montré que les cellules KO infectées par le HCMV et le ZIKV sont plus sensibles aux infections virales que leurs homologues sauvages (WT). De plus, des tests TCID50 et l'analyse par cytométrie de flux ont montré une infectivité virale significativement plus élevée des surnageants contenant des particules infectieuses libérées par les cellules KO infectées que par les cellules WT. Nous avons utilisé des dispositifs de RTqPCR^2 profiler pour

examiner les réponses immunes innées antivirales et d'autophagie des cellules KO et WT à l'infection par le ZIKV. Des différences génétiques distinctes dans l'expression suggèrent que le cluster C19MC influence la réaction à l'infection. Nous avons également exploré la possibilité d'un mécanisme d'action antiviral paracrine ; nous avons testé si cet effet antiviral pouvait être transmis à des cellules réceptrices (cellules Vero) via des EVs. Les données de cytométrie en flux ont montré un impact antiviral transférable dans les cellules Vero exposées au milieu conditionné des cellules WT par rapport aux cellules KO. Néanmoins, d'autres études sont nécessaires pour confirmer ces résultats. Notre recherche offre un modèle KO unique pour évaluer la fonction multidimensionnelle du cluster de miARN C19MC au cours d'infections virales congénitales et pour déterminer s'il peut ou non fonctionner en partie par le biais des EVs. Le développement de méthodes pronostiques et thérapeutiques améliorées et plus précises repose sur une compréhension approfondie des mécanismes d'action de ce cluster.

Abstract

Congenital viral infections caused by TORCH pathogens affect women worldwide and are associated with neonatal developmental disabilities. For instance, the Zika virus (ZIKV) has been linked to severe birth defects, including microcephaly. Similarly, the human Cytomegalovirus (HCMV) is the most prevalent viral infection acquired by the fetus, resulting in neurological damage. The ZIKV and the HCMV infect placental cells, directly affecting the neonate's development. In turn, the placenta employs extracellular vesicles (EVs) to interact with its environment, and it has been demonstrated that their C19MC microRNA (miRNA) cargo is dysregulated during pathological pregnancies. The primate-specific C19MC miRNA cluster shows a dynamic expression pattern throughout pregnancy, with levels rising toward the end of the first trimester and falling rapidly after parturition. Using an innovative Knock-Out (KO) model for the C19MC miRNA cluster generated by the CRISPR/Cas9 technique by J. Cavaillé's team, we investigated the antiviral function of this cluster during congenital viral infections. Experiments that involved real-time quantitative PCR, western blotting, flow cytometry, and immunofluorescence techniques showed that KO cells infected with the HCMV and the ZIKV are more susceptible to viral infections than their wild-type (WT) counterparts. Additionally, TCID50 assay and flow cytometry analysis showed a significantly higher viral infectivity of the supernatants containing infectious particles released from infected KO cells than from WT cells. Additionally, we used the RT^2 profiler qPCR arrays to examine KO and WT cells' immunological and autophagy responses to viral infections. Distinct genetic differences in expression suggest that the C19MC miRNA cluster

influences the reaction to ZIKV infections. We also explored the possibility of a paracrine antiviral mechanism of action; we tested whether or not this antiviral effect might be transmitted to recipient cells (Vero cells) via EVs. Flow cytometry data showed a transferable antiviral impact in Vero cells exposed to conditioned media from WT cells compared to KO cells. However, more studies are required to confirm these findings further. Our research offers a unique KO-model to evaluate the multidimensional function of the C19MC miRNA cluster during congenital viral infections and whether or not it might operate in part through EVs. The development of improved and more accurate prognostic and therapeutic methods relies on a thorough understanding of the antiviral significance of this cluster.

Conflict of Interest

The author declares no conflict of interest.

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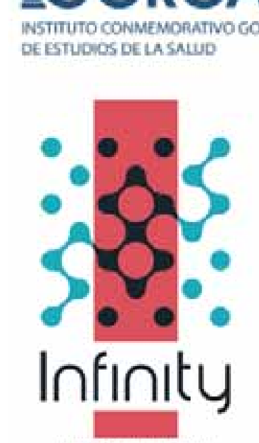

Acronyms and Abbreviation

AC Assembly Complex

APOBEC3A Apolipoprotein Polypeptide-like 3A

C19MC Chromosome 19 microRNA Cluster

CNS Central Nervous System

CRISPR/Cas9 Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9

DC-SIGN Dendritic Cell-Specific Intercellular adhesion molecule 3-Grabbing Nonintegrin

DENV Dengue Virus

DNA Deoxyribonucleic Acid

E Envelope

ELISA Enzyme-linked Immunosorbent Assay

EVs Extracellular Vesicles

EVT Extravillous Trophoblasts

GvHD Graft-versus-Host Disease

HCMV Human Cytomegalovirus

HIV Human Immunodeficiency Virus

HSV1 Herpe Simplex Virus 1

IDO Inhibition of Indoleamine 2,3-Dioxygenase

IE Immediate Early

JEV Japanese Encephalitis Virus

KO Knock-Out

MHC Major Histocompatibility Complex

miARN microRNA

MIEP Major Immediate Early Promoter

NS Non-Structural

NSPC Neural Stem Precursor Cell

ORFs Open Reading Frames

PPARγ Peroxisome Proliferator-Activated Receptor

RhCMV Rhesus Cytomegalovirus

RNA Ribonucleic Acid

RTqPCR Reverse Transcription Quantitative Polymerase Chain Reaction

SLEV St. Louis Encephalitis Virus

TAM Tyro3, Axl, Mer

TBEV Tick-Borne Encephalitis Virus

TIM T-cell Immunoglobulin and Mucin

TNF-α Tumor Necrosis Factor

TORCH Toxoplasmosis, Others, Rubella, Cytomegalovirus, and Herpes

TPSCs Trophoblast Progenitor Stem Cells

UL Unique Long

US Unique Short

Wnt Noncanonical and Canonical Wingless

WNV West Nile Virus

WT Wild Type

YFV Yellow Fever Virus

ZIKV Zika Virus

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Chapter 1

Introduction

1.1 The TORCH Complex

Pregnant women encounter many pathogens throughout pregnancy. Yet, some can place the fetus at a higher risk of acquiring several motor and neurological disorders, mainly if transmitted during the first trimester of pregnancy (Arora et al., 2017). Although vaccination has eradicated some of these congenital infections (e.g. rubella, varicella) in certain parts of the world, numerous diseases cannot presently be avoided by vaccines or efficiently treated with existing antimicrobial (Yadav et al., 2014). Furthermore, the 2015 Zika virus outbreak, the current Human Cytomegalovirus global burden, and the most recent Covid-19 pandemic remind us of the possibility of new viruses emerging and causing congenital diseases alongside existing infections. Governments and scientists must work together and be prepared to face these new challenges. Congenital diseases result from the transmission of pathogens from the mother to the fetus through the placenta. These pathogens are collectively known as the TORCH complex (Hennessey et al., 2016).

The acronym TORCH, which is also occasionally referred to as TORCHZ, stands for a group of infections caused by pathogens that are known to bypass the placenta and infect the fetus or infect the neonate during childbirth, causing congenital diseases. The

acronym stands for the following TORCH infections (Fitzpatrick et al., 2022): toxoplasmosis (T), "Others" that include infections like syphilis, varicella-zoster, hepatitis B, parvovirus B19 (O), rubella (R), cytomegalovirus (C), and herpes simplex virus (H). Most recently, new viruses have emerged, such as the Zika virus (ZIKV) (Sharma et al., 2020) and the SARS-Cov-2 virus (Haque & Pant, 2022), along with other well-known viruses like the human immunodeficiency virus (HIV) and the lymphocytic choriomeningitis virus, that have been added to the list of TORCH infections (Neu et al., 2015; Schwartz, 2017). This research study focuses on the Human Cytomegalovirus and the ZIKV, detailed in the following sections, and concerning congenital infections.

1.2 The Human Cytomegalovirus

1.2.1 History

The ubiquitous human cytomegalovirus (HCMV) is a virus that infects most people worldwide without causing any noticeable symptoms in the general population (Landolfo et al., 2003; Semmes et al., 2022; Stern-Ginossar et al., 2012). Notwithstanding, HCMV has become an exceedingly significant pathogen due to its ability to cause congenital and acquired infections, affecting the fetal brain and the immunocompromised. The virus traces its beginnings in the late 1800s. Ribbert (1904), was the first to demonstrate cell enlargement and intranuclear inclusions in the kidneys of a stillborn infant in 1881. Later, Goodpasture and Talbert (Riley Jr, 1997) were the first to postulate in 1921 that the symptoms denominated "cytomegalia" may be caused by a viral agent. The cytomegalia term refers to the expansion of the infected cell, and its characteristic inclusion bodies, from where the disease acquires its name (Goodpasture & Talbot, 1921, Figure 1.1). In 1950, Smith and Vellios demonstrated that infection might occur in a pregnant woman's uterus. The development of the exfoliative cytology approach made it possible to identify distinctive cells in the urine of newborns infected with the virus (Ho, 2008). Later, in 1956, three separate research groups isolated the HCMV viral strain for the first time. After isolating HCMV from the urine of children suffering from widespread sickness, Weller and his colleagues came up with the actual complete name "cytomegalovirus" in 1960 (M. G. Smith, 1956; Wallace et al., 1956; Weller et al., 1957).

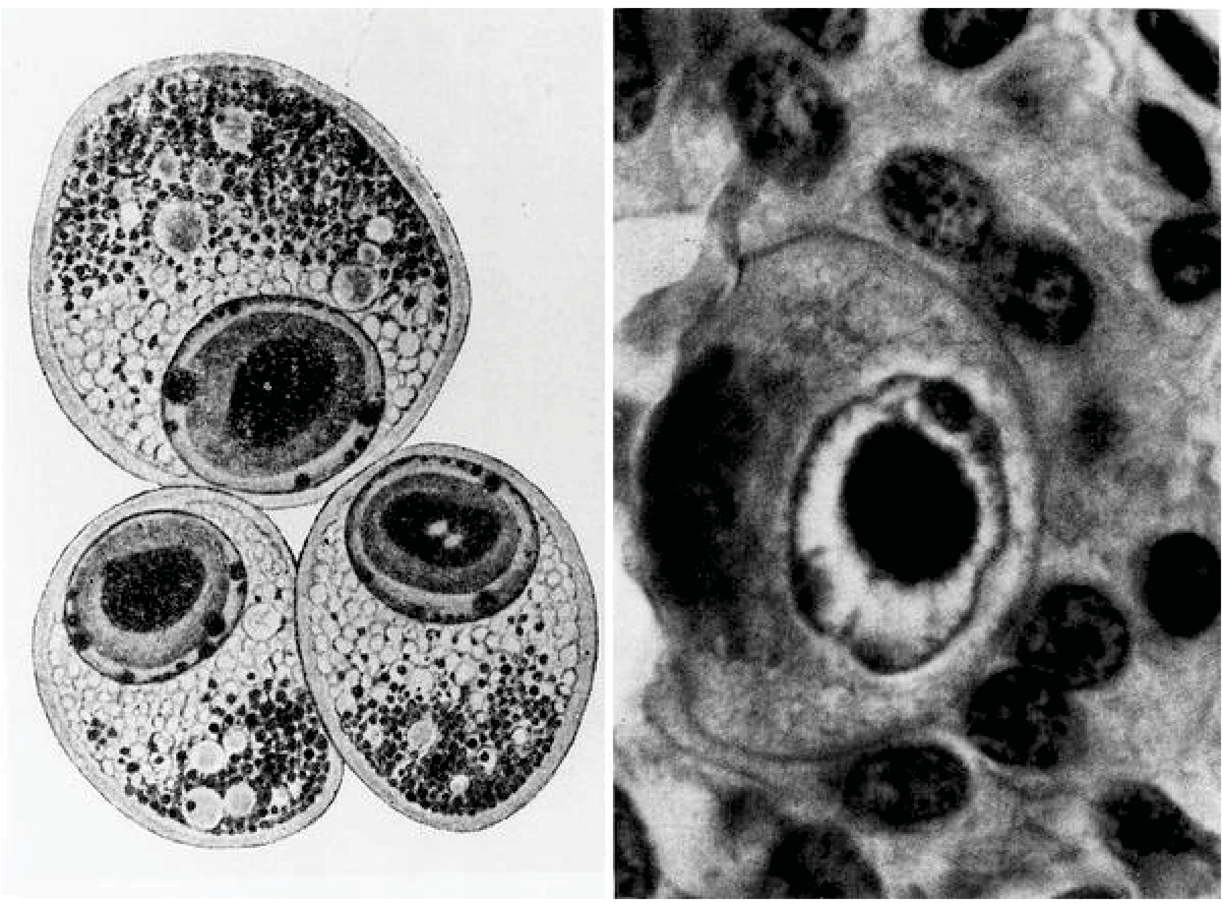

Figure 1.1: HCMV characteristic intranuclear inclusion bodies or owl's eyes in infected cells. Cells infected with HCMV contain intranuclear and cytoplasmic inclusion bodies with a halo (Ho, 2008).

1.2.2 Taxonomy

HCMV is also referred to as the human herpesvirus 5 (HHV-5) and is a member of the *Herpesviridae* family (Figure 1.2), under the *Betaherpesvirinae* subfamily of the Cytomegalovirus genus (ICTV, 2021, Figure 1.2).

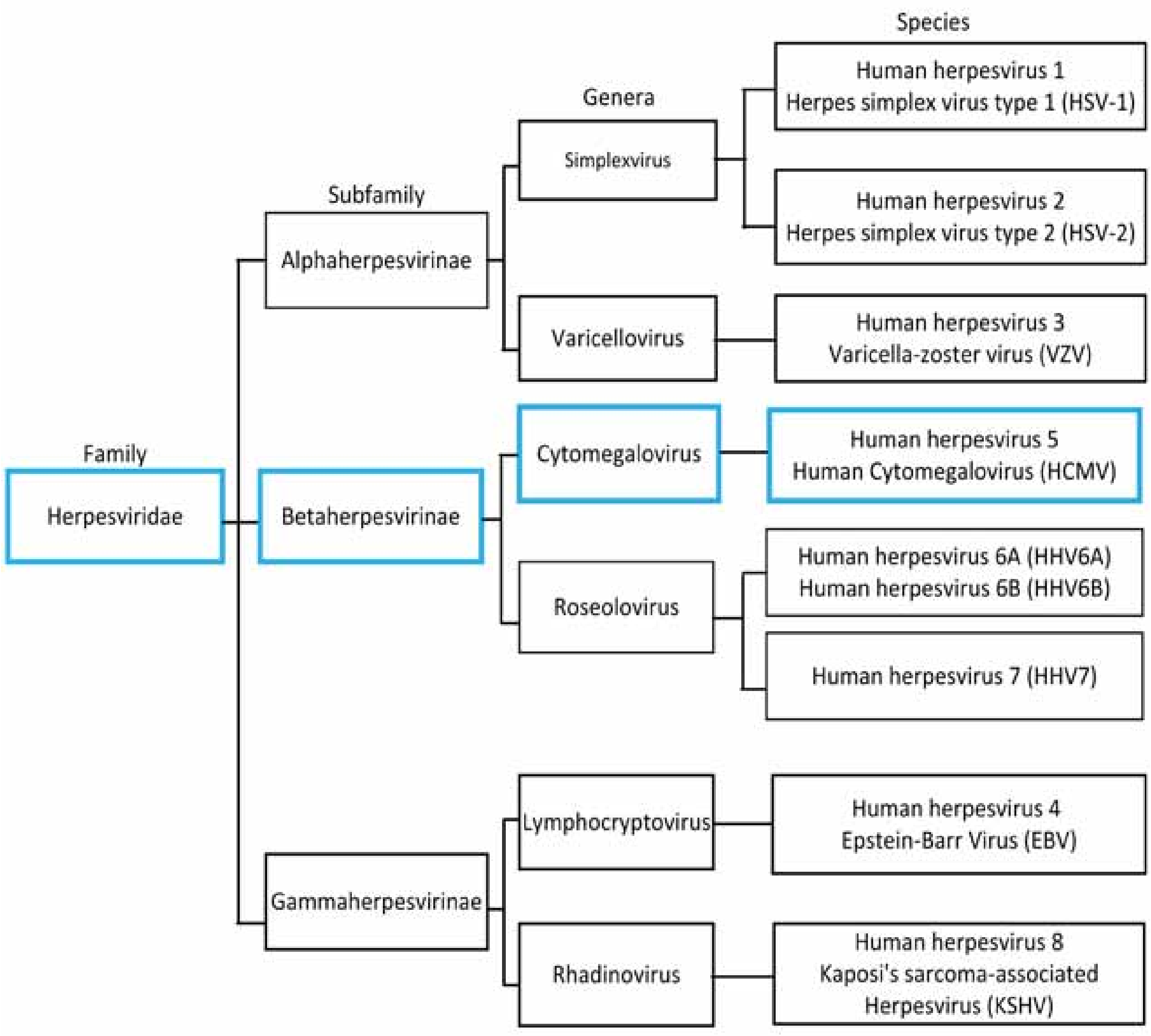

Figure 1.2: Phylogenetic tree of the taxonomic classification of the human cytomegalovirus (marked in light blue) and other members of the Herpesviridae family (Chodosh & Ung, 2020).

1.2.3 Viral Genome and Molecular Structure

The human cytomegalovirus genome is a linear double-stranded DNA that contains around 230 kbp, with a high number of G+C pairs that encode approximately 167 gene products, of which more than 40 are destined for the host immune regulation (Patro, 2019). This viral genome is the largest of all the herpesviruses and human infecting viruses. It has an arrangement of 2 inverted domains with unique core regions: unique long (UL), unique short (US), and two repeated regions (similar to the herpes simplex virus-1). The two repeated regions (positions) in the genome are as follows; one at the terminal end and the other at the intersection with the different unique domain (corresponding to TRL/IRL and TRS/IRS) (Ye et al., 2020). This genomic order results in the following organization: TRL–UL–IRL–IRS–US–TRS, as refered in Figure 1.3.

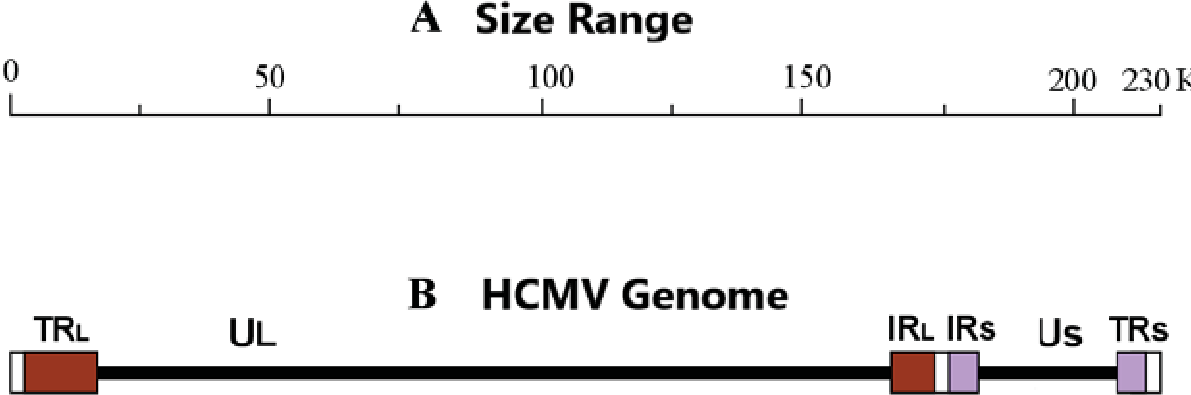

Figure 1.3: Genomic organization of HCMV. The diagram shows at a size range scale the viral genome of 230 kbp. HCMV genome is divided into unique long (UL) and unique short (US) regions. These two regions are flanked by the terminal and internal inverted repeats (Zhao, 2001).

As each long and short region can be positioned in either direction, viral progeny can make four genome isomers (type E genome structure). On the contrary, the genomes of

the animal CMV and other betaherpesviruses are linear and do not have any repeat regions (type F genome structure). All four genomic isomers are equally represented in areas of the world where the disease is endemic. In addition, HCMV DNA encodes approximately 700 suspected open-reading frames (ORFs) that have the potential to generate functional proteins (Mocarski Jr, 2007).

An icosahedral nucleocapsid measuring 100 nm in diameter and consisting of 162 capsomers encapsulates the genome. The virion's outer covering, the envelope, consists of a lipid bilayer containing several viral and host glycoproteins that have a role in viral attachment. Additionally, there is a layer of protein known as the tegument (or matrix) between the viral nucleocapsid and the viral envelope (Z. Li et al., 2021, Figure 1.4).

The envelope's lipid component is derived from the host cell's nuclear membrane. Within this lipid bilayer are at least eight distinct glycoproteins generated by the virus (gB, gH/gL/gO, or pentameric gH/gL/UL128/130/131). Among them, gB is an integral surface glycoprotein that impacts viral binding, cell entrance, and cell-to-cell transmission (X. Yu et al., 2017).

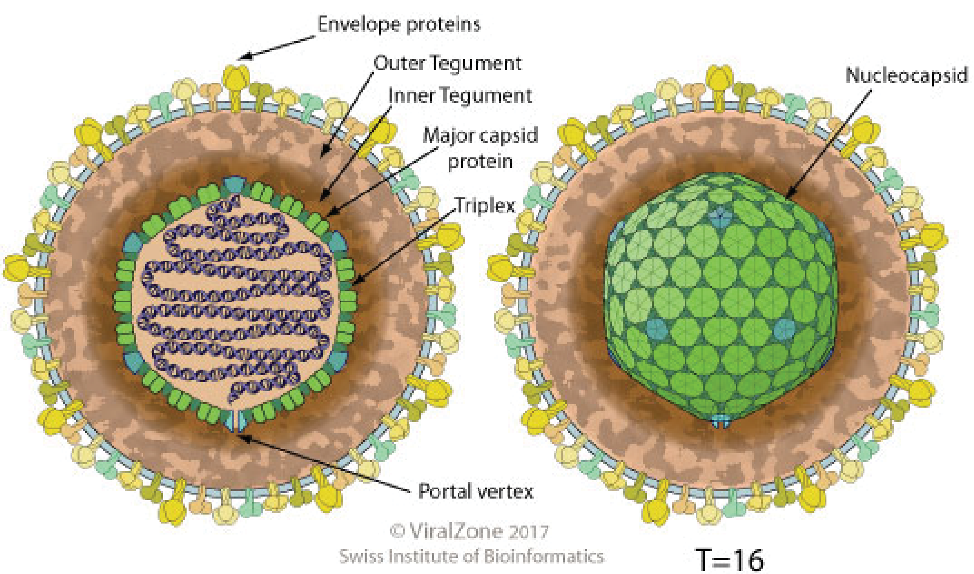

Figure 1.4: HCMV mature virion structure. `https://viralzone.expasy.org/180?outline=all_by_species`. (accessed on 06 August 2022).

1.2.4 HCMV Replication Cycle

HCMV infects host cells by membrane fusion and endocytosis, both dependent on distinct glycoprotein complexes of the viral envelope (gB, gH/gL/gO trimeric complex, and the gH/gL/UL128–131 pentameric complex). During the viral entry, these envelope glycoproteins come in contact with the cell receptors (e.g., PDGFR alpha, and Nrp2, depending on the cell type) to facilitate either fusion or endocytosis. After the capsid has entered the cytosol, the viral tegument proteins bound to the capsid interact with the host microtubule machinery to transport viral capsids to the nuclear envelope and into the nucleus (Isaacson et al., 2008; Kalejta, 2008; C. C. Nguyen & Kamil, 2018). Some important tegument proteins involved in viral replication are pp65/ppUL83, pp71/ppUL82, pp150/pUL32, and pp28/pUL99 (Vanarsdall & Johnson, 2012; Varnum et al., 2004).

Proteins from the HCMV capsid and tegument are initially transported to the cytosol after interactions with the cell's membrane receptors. The capsid now reaches the nucleus, where the genome gets circularized. The nucleus is where viral transcription, genome replication, and encapsidation occur. In the nucleus, the virus utilizes the host cell machinery for the production of viral immediate early (IE) genes, which in turn will initiate a temporal cascade of events that also includes the expression of delayed early (DE) genes and finally late (L) genes. IE gene products are essential for replication since they act as transcription factors that control E and L gene expression. The host cell creates IE proteins 1–4 hours after infection, which function as trans-activators for viral and cellular gene expression and are essential for E and L protein synthesis. The expression of these viral proteins will modulate the different stages of the infectious cycle regulating the host's immune and stress responses (Fortunato & Spector, 1999; Moorman et al., 2010).

In general, despite the linear orientation of the HCMV genome, it undergoes a circularization process during replication, initially by theta-like replication and then through rolling circle amplification, producing many copies attached simultaneously. Then, the genome is cleaved and linearized, and finally packaged into the nucleocapsid (Anders et al., 2011). Ex-

pression of the late gene starts the assembly of the capsid in the nucleus, followed by nuclear egress of the capsids into the cytoplasm through the nuclear double membrane. Once the capsids have entered the cytoplasm, the virions' assembly and transport occur through the integration of several different cellular trafficking routes (Figure 1.5).

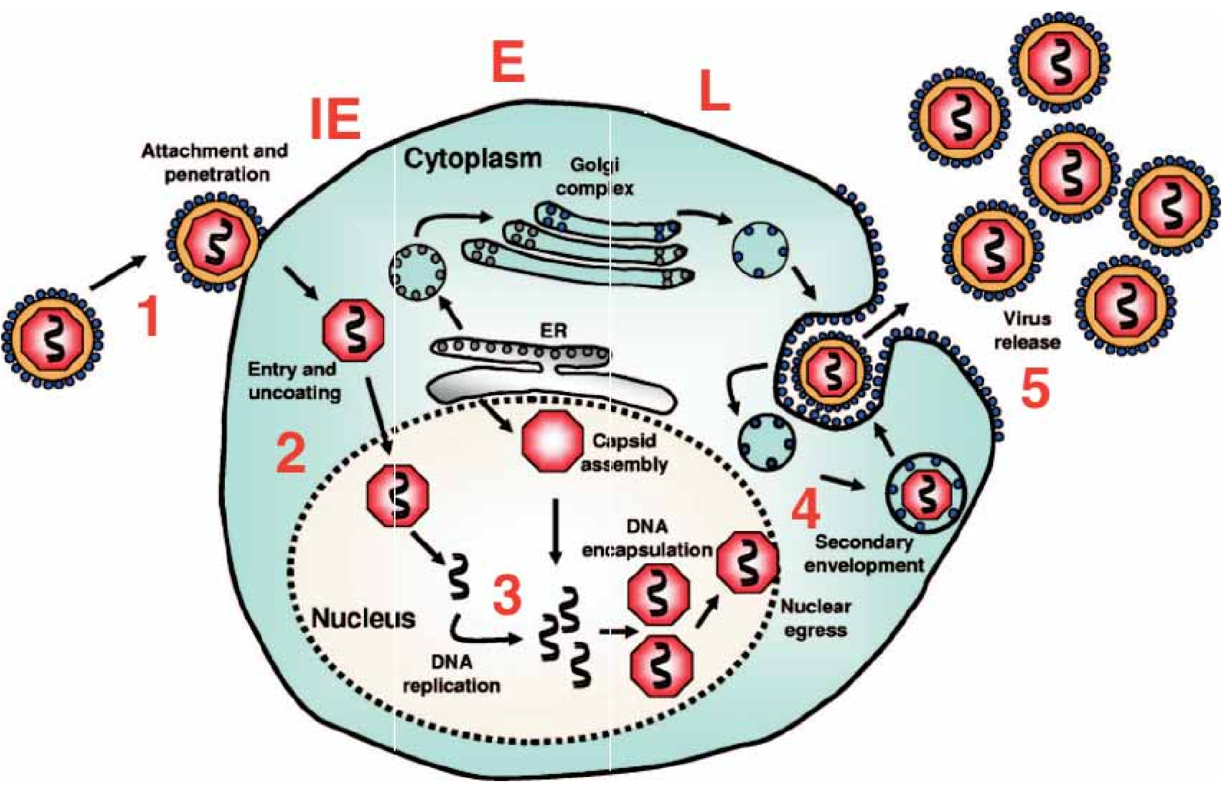

Figure 1.5: Viral replication cycle of HCMV. Viral cycle phases and corresponding protein production (IE=immediate early; E=early; L=late). Adapted and modified from (Crough & Khanna, 2009)

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For the formation of a cytoplasmic viral assembly complex (AC), the cellular secretory machinery, which includes the endoplasmic reticulum, Golgi apparatus, and endosomal machinery, is taken over and utilized by the virus. Intracellular vesicles provide the capsids with their tegument layer and viral envelope, both of which are acquired at the assembly complex (Gibson, 2008; Ogawa-Goto et al., 2003). After the production of the infectious mature particles and other non-infectious particles (dense bodies), the next step is the discharge into the extracellular space (Alwine, 2012).

1.2.5 Natural History of HCMV Infection

There are three distinct sub-types of HCMV infection; when a person without immunity to this virus becomes infected for the first time, they are said to have a primary infection. The next type is when the virus enters a dormant state called latency, from which it might become active again, leading to the second type of infection referred to as reactivation. The third category of illness is known as reinfection. It occurs when an individual who has previously been infected comes in contact with an infectious person (and a new strain), leading to the individual becoming superinfected despite having natural immunity (Britt, 2015).

1.2.6 Viral Latency and Reactivation

Human herpesviruses may cause persistent latent infection. The alphaherpesviruses have a neuronal tropism, whereas the beta and gamma herpesviruses favor haematopoietic cells to establish latency with no infectious viral production. The most notable reservoirs of HCMV are the CD34+ haematopoietic cells, bone marrow-derived CD34+ myeloid progenitor cells, CD14+ monocytes, and neural progenitor cells. Research studies demonstrate that either adherent monocytes or macrophages maintain HCMV latent infection, not B-cells and T-cells (Reeves et al., 2005). Latency at a molecular level is characterized by overall suppression of viral lytic gene expression due to epigenetic regulation via histone reconfiguration machinery, a very confined but specific transcriptional profile during latency, and the ability to respond to the host signals to exit latency and re-enter the lytic life cycle, regardless of the cell type and when the cell environment is favorable.

Similar to other herpesviruses, such as herpes simplex virus type 1 (HSV1), it seems that the expression of viral IE genes determines reactivation, while the IE expression is suppressed during latency. According to Goodrum et al. (2002), IE transcripts such as IE1/IE72 and IE2/IE86 are absent during latency. The IE gene expression during latency and reactivation is

regulated by the chromatin structure at the major immediate early promoter (MIEP, Groves et al. (2009)). The repressive chromatin state, repressive cellular factors like HP1, KAP1, ERF, and YY1, and latency viral factors (UL138 and US28) all contribute to the suppression of MIEP and consequently; the expression of the IE viral protein. According to Cristea et al. (2010) and Hong et al. (2022) the MIEP may not be completely silent and low levels of viral transcripts are produced during the latency phase. In fibroblasts, the expression of IE1 and IE2 may also trigger reactivation. Studies like Collins-McMillen et al. (2018) have shown the activity of RNA polymerase II and the presence of transcripts from major immediate early (MIE) genes during latency. This remains an inconclusive topic because of the differences in the latency programs of the diverse haematopoietic cells.

Reeves et al. (2005), have shown that monocytes must develop into macrophages or dendritic cells to reactivate HCMV, which then can enter a lytic phase meaning that a change in the cellular environment (cellular differentiation) causes this reactivation effect. In this case, a change in the MIEP from oppressed to active promoter must occur to permit the expression of the viral IE protein. In fibroblasts, the expression of IE1 and IE2 may also trigger reactivation. Notwithstanding, further research is needed to determine the specific mechanism necessary for the cell to exit a state of latency and enter viral reactivation. Reactivation of the latent virus is linked to a greater risk for multiorgan disease, decreased graft survival, graft-versus-host disease, infection with other pathogens, and post-transplant lymphoproliferative disorders under immunosuppressive conditions (Forte et al., 2020).

1.2.7 HCMV Cell Tropism

A wide range of host cells are susceptible to HCMV infection and replication including, epithelial, vascular endothelial, fibroblasts, and smooth muscles as their main target cells. HCMV has also been found to infect monocytes/macrophages, dendritic cells, and hepatocytes. This broad cell tropism makes it easier for the virus to spread throughout the body

and for inter-host transmission. Infecting mucosal epithelium or placental trophoblasts (congenital infection) are considered presumably the approach of HCMV to enter new host cells. Upon entrance, HCMV infects organs such as the spleen, liver, and lungs through myeloid cells that serve as viral transit carriers for dissemination. Additionally, HCMV enters a state of latency in myeloid cells of the bone marrow, likely leading to a lifelong infection with intermittent reactivation. In the blood, HCMV virions are coupled with the cells of the immune system, particularly granulocytes and macrophages (Belzile et al., 2014; C. C. Nguyen & Kamil, 2018; Sinzger et al., 2008).

1.2.8 HCMV and Immune Evasion

HCMV encodes defenses against various immunological responses (La Rosa & Diamond, 2012; Park et al., 2018; Patro, 2019; E. C. Wang et al., 2018). For this reason, various tegument proteins are deposited in infected cells by incoming virions. These proteins are then targeted to different subcellular regions to block the earliest phases of the immune response and control viral gene expression (Cristea et al., 2010; Feng et al., 2006; Mitchell et al., 2009). For example, the tegument protein UL82 (pp71) binds with stimulators of interferon genes to inhibit the host immune response. Additionally, virally encoded proteins are known to modulate cell-signaling pathways and cellular metabolism to maintain viral replication and immune evasion (Bagga & Bouchard, 2014). For example, the protein UL83 (pp65) is involved in the inhibition of proteasome complex and impede the antiviral gene expression of the cell. Another example is UL37 which is involved in the inhibition of apoptosis. The production of these viral proteins happens as a skilfull series of events. The process is broken down into many temporal phases, with each protein responsible for controlling a distinct phase of the infectious cycle, for example, the IE, DE, and L gene products (Yurochko, 2008).

Recapitulating, HCMV proteins such as US2, US3, US6, US11, and IE target antigen presentation by the major histocompatibility complex (MHC) class I and class II molecules;

it also employs cytokine homology to exert intercellular functions against immune cells and generate proteins that suppress the host innate immune responses to the virus (Manandhar et al., 2019).

1.2.9 Transmission

HCMV spreads by smear infection, which happens when infected bodily fluids, such as tears, nasal secretions, saliva, vaginal secretions, semen, urine, or breast milk come in contact with mucous membranes (Kurath et al., 2010). Therefore, sexual contact, placental transfer, breastfeeding, blood transfusion, solid-organ transplantation, and hematopoietic stem cell transplantation are all potential ways to spread HCMV from one host to the next. Following infection, the virus first replicates in the epithelial cells at the point of entry and then spreads via the bloodstream to various organs and cell types. Typically, young children acquire the virus through breastmilk and saliva.

1.2.10 Pathogenesis

HCMV remains a primary opportunistic infection in patients living with HIV, organ transplant recipients, and fetuses or newborns. Moreover, HCMV may be a factor in immunosenescence in the elderly (Griffiths & Reeves, 2021; Steininger et al., 2006). Since the beginning of the AIDS epidemic (the 1980s), the range of diseases caused by HCMV has dramatically incremented. At that time, before highly active antiretroviral medications were developed, HCMV significantly contributed to these patients' morbidity and death rate (Boeckh et al., 2003). Furthermore, HCMV can cause direct damage to organs of the cardiovascular system concerning organ transplantation. HCMV is the most pervasive and dangerous infection during solid organ and hematopoietic stem cell transplantation. Statistically, HCMV is related to indirect effects post-transplantation (organ rejection and mononucleosis) and is a

precursor to certain cancers (Hill Jr et al., 1964; A. Smith & Weidman, 1910). For example, in hematopoietic stem cell transplantation, HCMV is a significant risk factor for death and transplant-related complications.

In addition, there is a link between HCMV and graft-versus-host disease (GvHD). According to Hill et al. (2018) GvHD and its related treatment can enhance the potential of acquiring HCMV, and in parallel, HCMV contributes to GvHD complications in transplant patients. Although existing antiviral drugs (ganciclovir, forscarnet, and cidofovir) have reduced death caused by early HCMV disease in immunocompromised patients, some direct consequences of HCMV infection include pneumonia, gastrointestinal illnesses, retinitis, and problems of the central nervous system (CNS) and indirect manifestations like atherosclerosis and bacterial or fungal superinfections (Campos et al., 2016). In most cases, the immune system can manage a latent HCMV infection in a healthy person without developing the disease. However, approximately 10% of infected healthy adults will develop either HCMV mononucleosis or hepatitis in their lifetime (Griffiths & Reeves, 2021).

Congenital infection by HCMV also represents a significant cause of illnesses worldwide and is the leading cause of neurological disabilities in newborns. Notably, the chronic effects of this virus that hide from an efficient cell-mediated and humoral immune response increase mortality in the general population. This suggests that this virus is not as harmless as it would appear at first glance (Leruez-Ville et al., 2020).

1.2.11 Congenital HCMV Infection

1.2.11.1 Generalities and Epidemiology

HCMV is ubiquitously widespread and infects roughly 60% of individuals in industrialized nations and almost 90% in developing countries (Zuhair et al., 2019, Figure 1.6). Factors such as lifestyle, childcare ideology, and cleanliness standards contribute to the pathogenesis and infectivity of the virus. Children acquiring HCMV at birth or during breastfeeding that

at times do not presents evident symptoms, continue to spread the virus asymptomatically for years via their saliva, tears, and urine, causing the virus to remain endemic in a specific region (Leruez-Ville et al., 2020).

As mentioned earlier, HCMV can be pathogenic to a fetus during a woman's pregnancy, people with impaired immune systems, and organ transplant donors/recipients. Based on epidemiological statistics, the HCMV seroprevalence worldwide in women of reproductive age is of 86%, a percentage that appears to increase with age (Navti et al., 2021). The three kinds of HCMV infections (primary, latency/reactivation, and reinfection) cause complications during pregnancy, resulting in HCMV being the most prevalent cause of congenital disease and fetal neurological damage worldwide. The risk of HCMV transmission through the placenta to the fetus is 40% for a primary infection and <0.05% after reactivation, causing the majority of severe clinical cases (Fowler et al., 1992).

Congenital HCMV infection has a broad range of clinical manifestations, from asymptomatic infection (about 85–90%) to severe fetal swelling, miscarriage, and postnatal mortality (0.5%). The clinical complications in symptomatic congenital HCMV that fall between these extremes include hearing loss, motor and cognitive developmental delay, microcephaly with intracranial calcifications and damage to the CNS, bone marrow, and other internal organs. When primary HCMV infection occurs during the early gestation phase, children are severely afflicted; the severity of the illness lessens as gestational age increases. Overall, congenital HCMV infection affects more children than the more general conditions of trisomy 21 or fetal alcohol syndrome, and it is one of the primary causes of hearing loss in children without a history of hearing loss in their families.

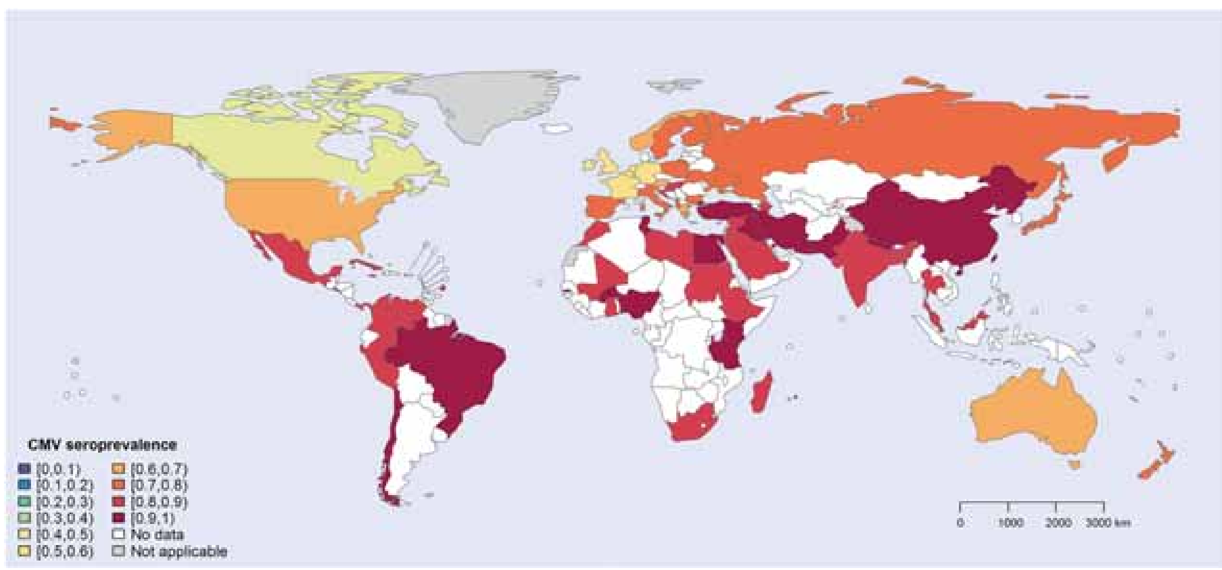


Figure 1.6: Map representing the global human HCMV percentage of seroprevalences in women of reproductive age (Zuhair et al., 2019).

1.2.11.2 Vertical Transmission of HCMV

The pathways of vertical transmission used by HCMV to infect the developing embryo remain unclear. In part this is because HCMV doesn't infect rodents efficiently, and although in vivo studies like Schleiss and McVoy (2010) describe guinea pig models, they do not replicate effectively the vertical transmission profile of CMV in humans. The Rhesus macaque CMV (RhCMV) model has been utilized for the study of congenital HCMV. However, this model has considerable limitations, including a lack of RhCMV-seronegative macaques and costly animal and setup costs (Powers & Früh, 2008). Therefore, in vivo experimentation is mostly restricted to human placental primary cell cultures and/or explant models.

In congenital HCMV, the virus can infect the cytotrophoblast cells of the placenta, allowing for vertical transmission to the fetus. Changes in the placenta throughout gestation may influence the processes of HCMV vertical infection in trophoblasts. This is suggested by Enders et al. (2011) due to the differences in gestational age-specific infectivity and fetal outcomes observed during an HCMV infection. Studies by Uenaka et al. (2019) show that during a congenital HCMV infection, the placenta histology might vary from normal to acute and chronic intervillous state. Additionally, Satosar et al. (2004) observed a higher incidence

of the typical owl's eye viral inclusion bodies in the placenta throughout the first and second trimesters of pregnancy than in full-term placenta.

According to Pereira and Maidji (2008) and Weisblum et al. (2011) and their studies in human tissue, HCMV may replicate actively in the mother's decidua, creating a viral repository that increases the possibility of HCMV passing the fetal barrier in the context of primary infection. The replication of HCMV in the maternal decidua has been found to be regulated by the apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3A (APOBEC3A), restricting HCMV replication (Weisblum et al., 2017). On the contrary, APOBEC3G doesn't show to directly affect HCMV replication (Pautasso et al., 2018).

Furthermore, there is evidence that in the presence of the virus in the maternal circulation, HCMV may primarily infect placental pericytes to reach the fetus. Even though their function at the mother-child interface is mainly unknown, they may be a key location through which HCMV may cross the placenta and induce congenital infection, particularly in late gestation (Aronoff et al., 2017).

1.2.11.3 HCMV and Placental Infection

Congenital HCMV infection can induce fetal impairment in two ways: direct harm to the fetus and indirectly through placental malfunction caused by disease or by the host immune system without knowledge of the virus infecting the fetus. The ability of trophoblasts cells to differentiate, migrate and invade surrounding tissue is necessary for implantation, as well as vascular reorganization and fetal development.

HCMV replicates in trophoblast progenitor stem cells (TPSCs) located in the chorionic villi of the placenta, interfering with the subsequent differentiation into cytotrophoblasts, and multinucleated syncytiotrophoblasts. This viral infection affects the villous differentiation and growth, significant to the normal development and function of the placenta. Additionally, infection of the TPSCs may result in the facilitation of the spread of the virus

to other placental cell types, dysregulating proteins required for pluripotency of TPSCs, further obstructing the maturation of the placenta during early gestation (Tabata et al., 2015; Zydek et al., 2014).

HCMV replicates in extravillous trophoblasts (EVT), interfering with their invasiveness and differentiation capabilities by changing the expression of critical molecules such as MMP2/MMP9 enzyme, c-erb-2 oncogene, $\alpha1\beta1$ ahesion protein, and the cytokine IL-10 suggested by a variety of in vitro studies (Fisher et al., 2000; Pereira et al., 2005; Tao et al., 2011; Yamamoto-Tabata et al., 2004). In addition, studies by Leghmar et al. (2015) and Rauwel et al. (2010) show that HCMV infection induces the activation of Peroxisome proliferator-activated receptor γ (PPARγ). Furthermore, the noncanonical and canonical Wingless (Wnt) signaling pathways are inhibited by HCMV. Both of these interferences by HCMV dysregulate the migration and differentiation of EVTs and CTBs respectively (Knöfler & Pollheimer, 2013).

Another HCMV mechanism that decreases the number of trophoblasts and the integrity of the placenta is the induction of an irregular trophoblast apoptosis mediated by tumor necrosis factor α (TNF-α) cell secretion (Chan et al., 2002). Studies by Hamilton et al. (2012) have observed that HCMV alters placental cytokine patterns. Cytokines (TNF-α, MCP-1) of the matenal-fetal interface are imperative for the proper function of the placenta, the growth of the villous involved in the transport of oxygen and nutrients to the fetus, and the immune response among others.

Inhibition of Indoleamine 2,3-dioxygenase (IDO) is another way HCMV dysrupts the adequate function of the placenta. IDO regulates the acceptance of the fetus by maternal tissue in part by the suppression of T cells (Lopez et al., 2011). On the same note, HCMV also downregulate the expression of the major histocompatibility complex molecules HLA-C and HLA-G, involved in protecting the fetus from rejection (Jun et al., 2000). The various mechanisms presented, are the result of several in vitro studies, yet further research is necessary to completely understand HCMV congenital pathology.

1.2.11.4 HCMV and the Developing Brain

The HCMV viral transfer to the fetus begins at the uterine–placental interface by infecting uterine smooth muscles and endothelial cells in the decidua. Moreover, interactions between the virus and trophoblast cell receptors facilitate perinatal transmission, allowing HCMV to enter the fetal circulation and cause infection. In light of the low permeability of the placenta, it is presumed that the virus reaches the fetal bloodstream in a cell-free state. In part because the placenta prevents maternal cells from entering the fetal blood flow. When HCMV enters the circulatory system of the fetus after passing through the placenta, it begins the process of replicating itself in a variety of fetal organs. However, the distinct dissemination pathway from the placenta to organs of the developing fetus, including the brain, is not yet fully understood (Gabrielli et al., 2012; León-Juárez et al., 2017; Pereira et al., 2013).

Histopathological and epidemiological studies represent the limited methodologies employed when researching the mechanism of HCMV infection in the fetal brain. In addition, there are inconsistencies in the time of fetal viral transmission and load and vast variances in histopathological alterations, which, along with a lack of non-invasive approaches, make it difficult to conduct direct studies of HCMV in the brain. As a result, the mouse model has partly helped define viral transmission, immunological response, and pathology (Van Den Pol et al., 2002).

Studies in mice suggest that fetal brains are more susceptible to HCMV than adult brains. Furthermore, some speculate that HCMV enters the CNS and crosses the blood-brain barrier by infecting mononuclear cells, although the mechanism remains unclear. Studies in vitro with primary cell cultures have found that HCMV successfully infects all brain cell types, lacking a specific cell tropism in this organ (Cheeran et al., 2009).

Scheld et al. (2014) and Teissier et al. (2014) histological observations show that astrocytes and neural stem precursor cell (NSPC), a common neuroepithelial precursor, both resident

cells of the CNS, appear to be the main targets of HCMV infection. On the other hand, microglia (immune cells of the CNS) do not seem to be the main target of congenital HCMV, demonstrated by histological observations of damaged fetal brains showing a 10% of infected cells of this type. Furthermore, according to histological evaluation, neuron infection and elevated apoptotic levels in the brains of neonates with severe gestational HCMV infection were detected. However, only a minute percentage of differentiated neurons were HCMV-positive, suggesting that the infection is less widespread in neurons than in other glial cells (Teissier et al., 2014). However, several discordant in vitro results show a range from no infection to complete permissiveness of HCMV in neurons (Cheeran et al., 2005; M. H. Luo et al., 2008). Our team also demonstrated that PPARγ is activated during congenital HCMV infection, and this activation inhibits the differentiation of human neural stem cells into neurons (Rolland et al., 2016).

1.2.12 Diagnosis, Treatment and Vaccine Perspective

Primary HCMV infection is identified with serological testing. The immediate condition may be determined by seroconversion. Diagnosis is based on a testing pattern for IgG and IgM when seroconversion cannot be established. The IgG and IgM test performance is crucial to the sensitivity and specificity of the diagnosis of primary maternal infection. In addition, prenatal diagnosis relies on the detection of HCMV DNA in the amniotic fluid (amniocentesis) since infected fetuses excrete the virus in their urine.

There are just five approved antiviral medications available today to treat HCMV: ganciclovir, valganciclovir, foscarnet, cidofovir, and fomivirsen. All medications except for formivirsen target viral DNA polymerase UL54. These antivirals are known to cause adverse effects and drug resistance; therefore, they can't be given to pregnant women or congenitally infected patients (S.-J. Chen et al., 2019; Mercorelli et al., 2011). However, on some occasions, patients have been treated with acyclovir along with ganciclovir for congenital

infection in utero.

As of today, there is no approved vaccine to prevent infection in pregnant women, transmission to a fetus, or illness in infected babies. However, several pharmaceutical firms are working to create an effective mode of vaccination. Some potential forms of vaccines like attenuated live-virus vaccines and adjuvanted viral proteins have offered some understanding into vaccine development but not definite proof of effectiveness (Plotkin et al., 2020). There are several diagnostic methodologies to detect an HCMV infection in humans. Some of these diagnotic techniques are ELISA test, antigenemia assay, HCMV culture, and PCR-based methods (Razonable et al., 2020).

1.3 The Zika Virus

1.3.1 History and Viral Lineages

The Zika virus (ZIKV) was initially discovered in 1947 in a Rhesus monkey (Dick & Haddow, 1952), then in 1948, it was found in mosquitoes collected for viral studies in Africa (Weaver et al., 2016). Human infections with ZIKV remained rare for the next half-century until they reappeared in the Pacific and the Americas. Later, in 1952 the virus was spotted in humans (L. Wang et al., 2016) and by 1964, was connected to Zika virus transmission (Gubler et al., 2017). Nonetheless, the virus remained unnoticed until an epidemic similar to dengue was reported in the Yap State of Micronesia in 2007. Reports showed that 73% of the population of the Yap State had ZIKV (L. Wang et al., 2016).

In 2013, a new epidemic broke out in French Polynesia and spread to neighboring islands in the Pacific. Subsequently, by August 2016, an estimated 165,000 ZIKV infections were first suspected and later confirmed in Brazil and spread quickly into South and Central America and the Caribbeans, making it the worst case reported and classified as an epidemic (De Carvalho et al., 2016). L. Wang et al. (2016) performed a phylogenetic analysis of circulating viruses which confirmed the existence of two lineages (Figure 1.7), an African lineage

and an Asian-American lineage comprising all the strains that have contributed to the pandemic outbreaks (Beaver et al., 2018). Consequently, ZIKV infections have been associated with significant neurological conditions such as Guillain-Barré syndrome and microcephaly in newborns when acquired during pregnancy. It's important to remember that ZIKV "old Asian lineage" and the "American or emerging lineage" have been linked to cases of microcephaly. Currently, there is no therapy or vaccine for ZIKV, so it continues to be a public health problem and potential danger (Johansson et al., 2016).

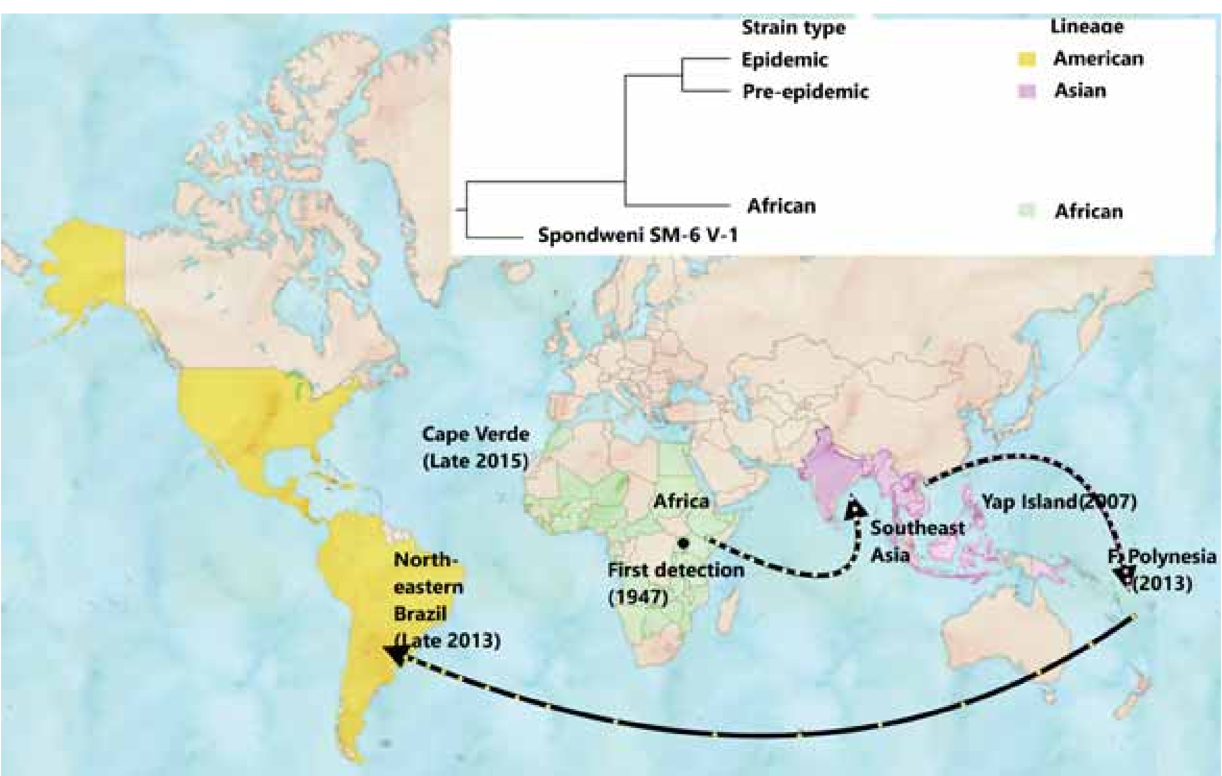


Figure 1.7: World map representing the evolution of ZIKV since its first appearance in 1947 and its lineages. The top-right corner represents the color-coded conventional phylogenetic tree of ZIKV viral lineages. Dotted arrows represent the transitional lineage from one region to another. Raster computationally sketched by Yamileth Chin using QGIS. Adapted from the original version presented by L. Wang et al. (2016).

1.3.2 Geographic Distribution of ZIKV

According to the World Health Organization (WHO, Figure 1.8), approximately 87 nations and territories have evidence of endemic mosquito-borne Zika virus (ZIKV) circulation as of July 2019 (African Region, Region of the Americas, South-East Asia Region, and Western Pacific Region). Reports of an epidemiological analysis in 2014 added Ethiopia to the list of nations with evidence of mosquito-borne transmission. In the Americas, infections with ZIKV increased in 2016 and dropped in 2017 and 2018. In addition, all countries in the Americas except for Chile, Uruguay, and Canada continue to have Zika virus transmission.

Epidemiologic data from Africa, South-East Asia, and the Western Pacific are scarce, but scientific evidence continues to emerge and increase our knowledge of worldwide ZIKV transmission and related problems. Therefore, the virus is prevalent in numerous places, and rare outbreaks have been recorded, such as in Jaipur, India, in 2018. Consequently, additional viral emergencies may cause occasional outbreaks in specific high-introduction-risk locations (World Health Organization, 2022). Even though more than 2,300 imported cases of Zika were documented between 2015 and 2017, there has never been a report of endemic vector-borne transmission of the virus in Europe before 2019. Surprisingly, the first locally acquired cases were reported in the fall of 2019 in southern France (Giron et al., 2019), where *Aedes aegypti* is not present, but *Aedes albopictus* is the likely mosquito vector because of its adaptation to colder climates. These new results imply that the Zika virus can arise in all nations with potential mosquito vectors (Aedes species) and the potential to re-emerge in countries where the disease has previously been afflicted (Parola & Musso, 2020).

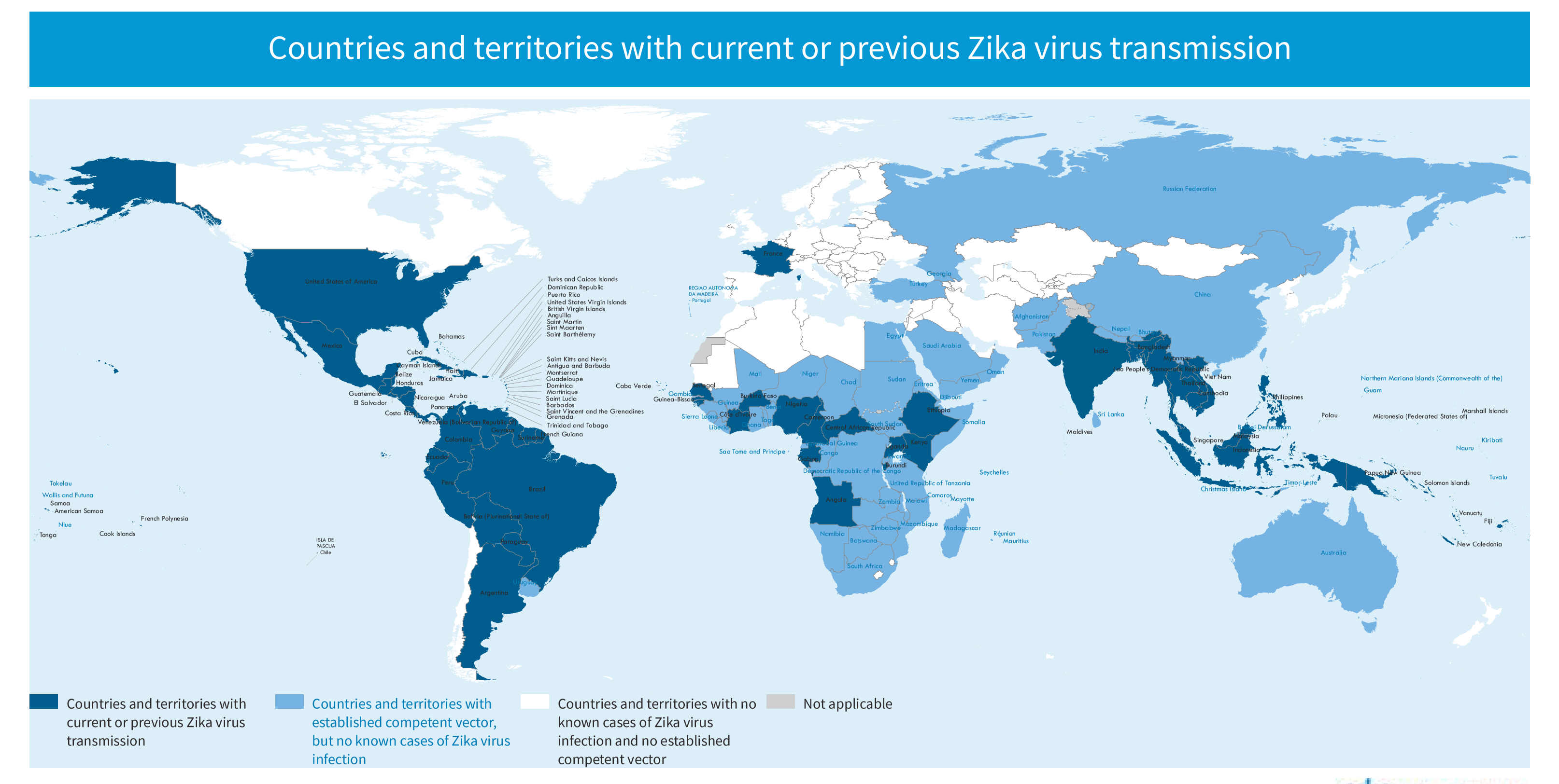


Figure 1.8: Geographic distribution of ZIKV (World Health Organization, 2022).

1.3.3 Taxonomy

According to the International Committee on Taxonomy of Viruses (ICTV, 2021) the Zika virus (ZIKV) is an arthropod-transmitted virus (arbovirus) belonging to the Flavivirus genus and *Flaviviridae* family. Other examples of flaviviruses are yellow fever virus (YFV), dengue virus (DENV), West Nile virus (WNV), St. Louis encephalitis virus (SLEV), Japanese encephalitis virus (JEV), and the tick-borne encephalitis virus (TBEV).

1.3.4 Viral Genome and Molecular Structure

The ZIKV genome of the African strain (MR 766) is a single-stranded, 11 kb, positive-sense RNA molecule. The genome has a $5'$ untranslated region (UTR) of about 100 nucleotides, one ORF of 10 kb, and a $3'$ UTR of approximately 420 nucleotides. After processing, the single ORF encodes the following viral structural proteins: the capsid (C), the precursor membrane (prM), an envelope protein (E), and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) for a total of 10 proteins. According to Pierson and Diamond (2018), nonstructural proteins aid in genome replication, packaging, and modulation of the host response (Figure 1.9). Structurally, the E protein of ZIKV is similar to other flaviviruses such as DENV, WNV, and JEV. However, ZIKV has a more compact overall structure, which may contribute to its stability across different temperatures and physiological fluids, also enabling sexual transmission (Kostyuchenko et al., 2016). Even though ZIKV is most similar to the African Spondweni flavivirus, it has sequence similarities with other flaviviruses. For example, between ZIKV and DENV virus strains, about 50% of the E protein sequence is the same. This characteristic hinders the development of virus-specific diagnostics and increases the likelihood of severe immunological responses in persons sequentially exposed to ZIKV and DENV (Hasan et al., 2018).

The ZIKV virion consists of a nucleocapsid surrounded by an envelope composed of a lipid membrane containing structural proteins (prM/M and E). Inside the 25-30 nm nucleocapsid resides the viral RNA genome. The virion is small, spherical, and approximately 40-50 nm in size (Figure 1.9). Mature flavivirus particles have an icosahedral arrangement of 90 E dimers that adequately support the viral surface. They also exhibit 180 copies of the E protein and membrane (M) protein on the envelope (Sirohi & Kuhn, 2017).

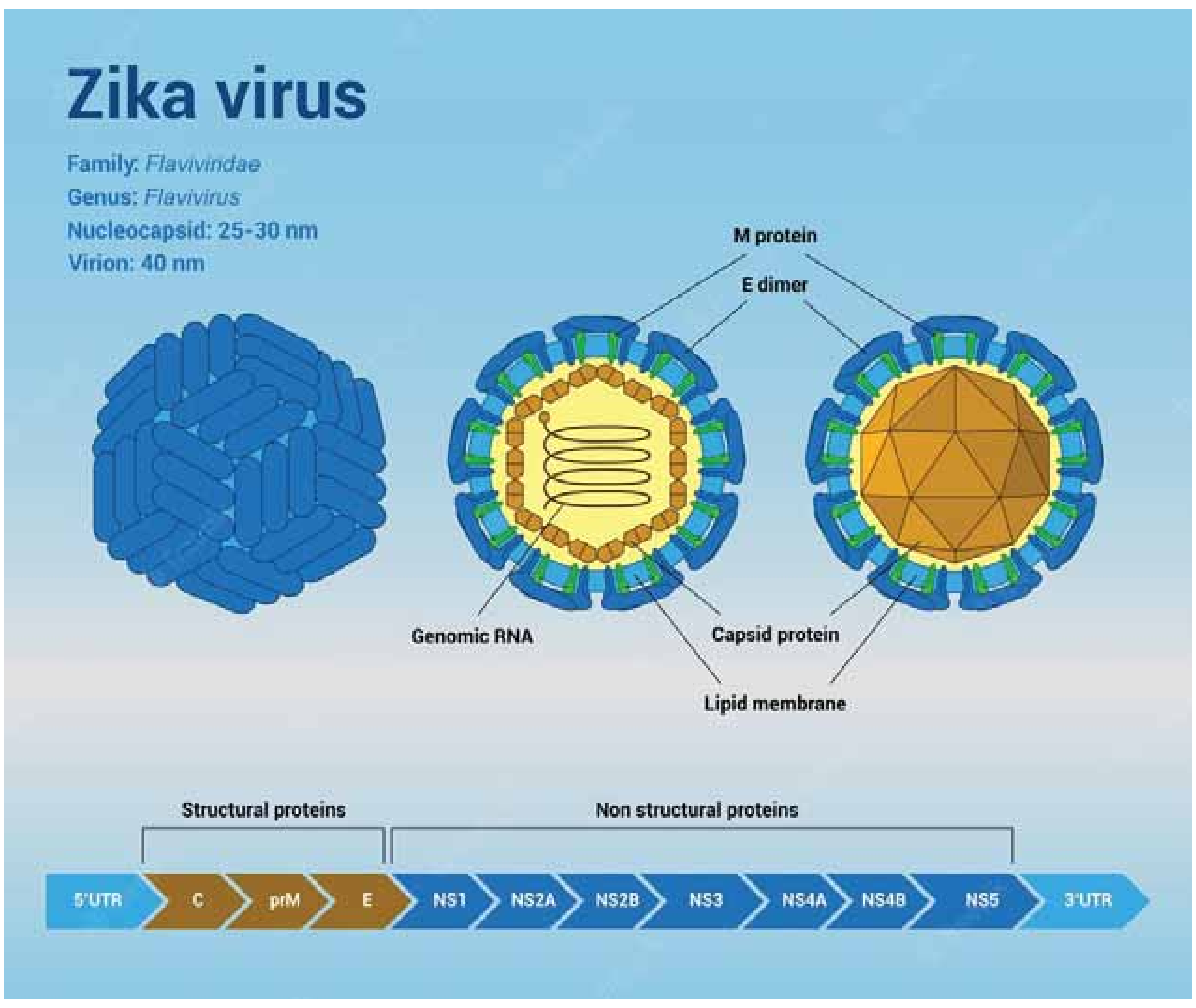


Figure 1.9: ZIKV structure and genomic organization. https://www.vectorstock.com/royalty-free-vector/zika-virus-structure-vector-15714136. (accessed on 06 August 2022).

1.3.5 Replication Cycle

This subsection describes the different steps involved in the replication cycle of most flaviviruses with a focus on the ZIKV (Figure 1.10).

1. Binding and entry: First, the ZIKV utilizes its envelope (E) protein to identify and bind to the cell receptor. Members of the Tyro3, Axl, Mer (TAM) family of receptor tyrosine kinases, T-cell immunoglobulin and mucin domain (TIM), dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) have been postulated as ZIKV receptors (Agrelli et al., 2019, although controversial).

2. Endosomal fusion: The ZIKV is internalized by clathrin-mediated endocytosis by binding to the viral E protein. Microtubules and actin transport the ZIKV inside the endosome. The acidic environment in the endosome causes the irreversible trimerization of the E protein exposing its fusion loop (Alcon et al., 2002), which leads to the fusion of the viral and the endosomal membranes, resulting in the ejection of the nucleocapsid and the release of the genome into the cytoplasm (the fusogenic state, Stiasny et al., 2011). The ideal pH for conformational rearrangements and viral fusion is 6.3–6.4, and both processes likely rely on cholesterol and particular lipids in the cellular membrane (Martin-Acebes et al., 2016).

3. Translation and polyprotein processing: At this stage, the viral genome release into the host cell's cytoplasm also kickstarts the translation of the viral polyprotein. This viral polyprotein is subsequently fragmented into structural and nonstructural (NS) proteins by viral and host proteases. Non-structural proteins (NS) are essential for formation of the viral replication machinery to further replicate, translate, and for the capping of the ZIKV genome (e.g. NS5, Esteves et al., 2017).

4. RNA Replication: The ZIKV-RNA that has been transferred from the endosome into the cytoplasm, is followed by the initiation of its replication by NS proteins as the viral

positive-sense RNA serves as mRNA to construct the negative-strand (Esteves et al., 2017).

5. Immature virus assembly: The prM and E proteins are responsible for packaging newly generated RNA and capsid protein to create “spiky” immature viral particles. These immature virus particles then bud into the endoplasmic reticulum (ER), where they are glycosylated.

6. Virus transport and maturation: The immature virion subsequently travels to the Golgi and trans-Golgi network (TGN), where the prM protein is cleaved (pr peptide and M protein) by the host’s furin protease, and the trimeric prME spikes are reconfigured to form dimeric ME heterodimers, resulting in the formation a mature virus (lacking spikes). The endoplasmic reticulum (ER) and the Golgi apparatus (GA) are the main sittes for replication and propagation of the virus: Initially, flaviviruses utilize and modulate autophagic flux, a cellular process, to promote their proliferation and to begin infection. Lastly, virions are assembled in the ER and travel via the Golgi network for the virion’s maturation before thea are released from the infected cell (Carneiro & Travassos, 2016). Therefore, the ER-Golgi intermediate compartment (ERGIC) and the Golgi apparatus are responsible for the virus assembly, budding, and maturation although not well-understood processes (Rombi et al., 2020).

7. Mature virus release: Exocytosis is the last step in the process, after which the mature virion is ejected, and the subsequent life cycle may begin (Agrelli et al., 2019).

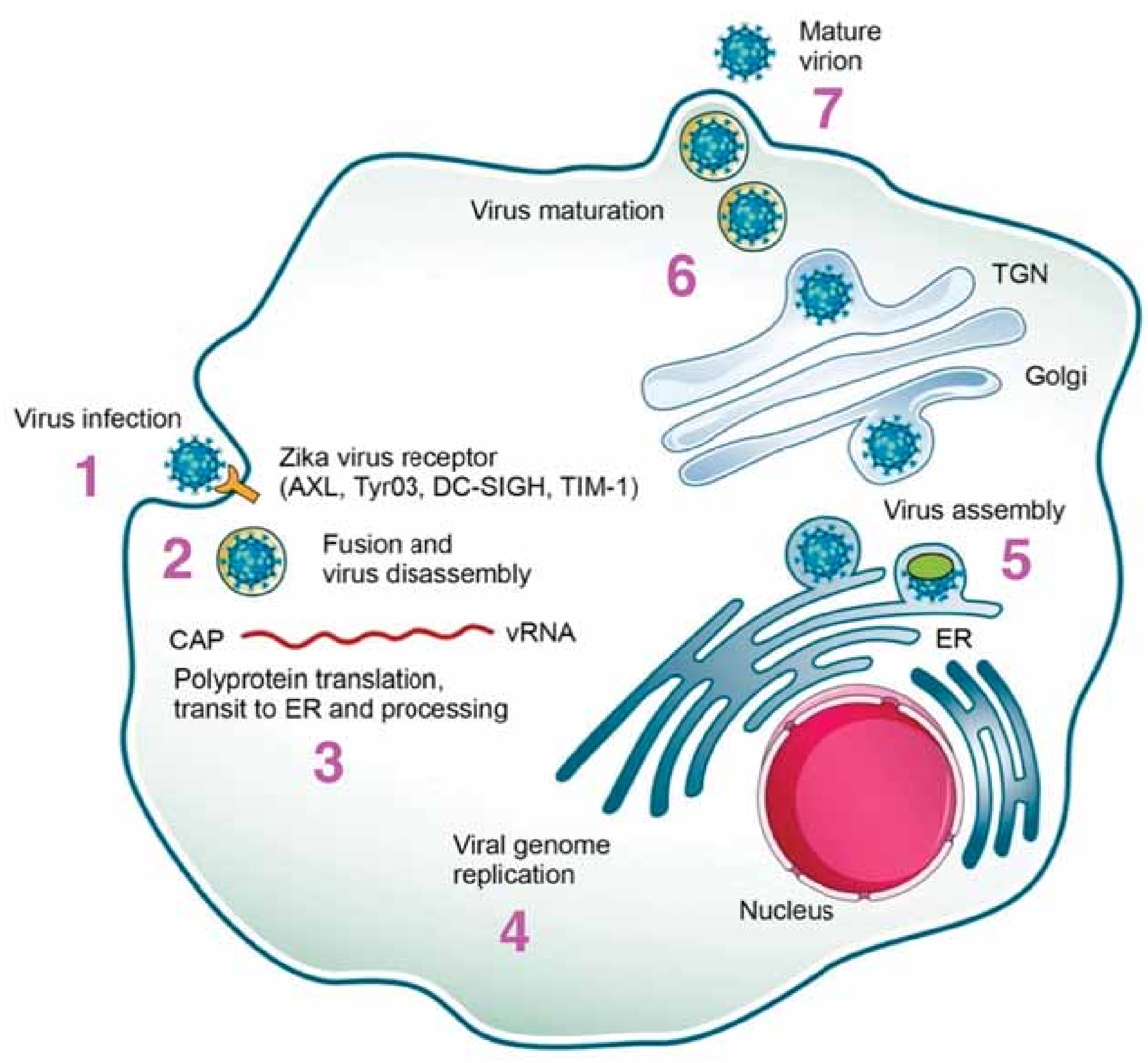


Figure 1.10: ZIKV replication cycle representation. Adapted from the original version presented by Baz and Boivin (2019). (**1**) Attachment and entry. (**2**) Endosomal fusion. (**3**) Translation/Transcription. (**4**) Replication. (**5**) Virion Assembly. (**6**) Maturation of the virion. (**7**) Release of the mature virion.

1.3.6 Tissue and Cell Tropism

The ZIKV has a wide range of tissue and cell tropism. It has been found that the virus can infect several cell types, including Hofbauer cells (HBCs), trophoblasts, endothelial cells of the placenta, cells of the reproductive tract (male or female), neuronal progenitors and mature neurons, oligodendrocytes, astrocytic cells, hepatocytes and various epithelial and fibroblast cells (Elong Ngono & Shresta, 2018). In vivo studies by Miner and Diamond (2017) has shown that ZIKV infection can lead to the virus and viral nucleic acid residing in body fluids such as saliva, tears, blood, and cervical mucus, urine, semen, cerebrospinal fluid, and vaginal secretions. The presence of ZIKV impairs the function of several mammalian organs, notably those called "immune-privileged" organs, including the brain, eye, testes, and placenta (Kalkeri & Murthy, 2017). These organs not only allow for the infection and subsequent viral proliferation, but they also have the potential to act as repositories for the virus contributing to chronic infections.

1.3.7 Viral Transmission

Like other flaviviruses, the ZIKV primary mode of infection is vector-borne (Figure 1.11). ZIKV is mainly spread by mosquitoes of the genus *Aedes*, notably *Aedes aegypti* and *Aedes albopictus*, species that are present in all tropical parts of the world. Unlike other flaviviruses, ZIKC is characterized by human-to-human transmission. Additional ways the ZIKV is transmitted include blood and platelet transfusion, sexual transmission (female-to-male, male-to-female, and male-to-male sexual), contact with infected body fluids, and vertical transfer from mother to fetus.

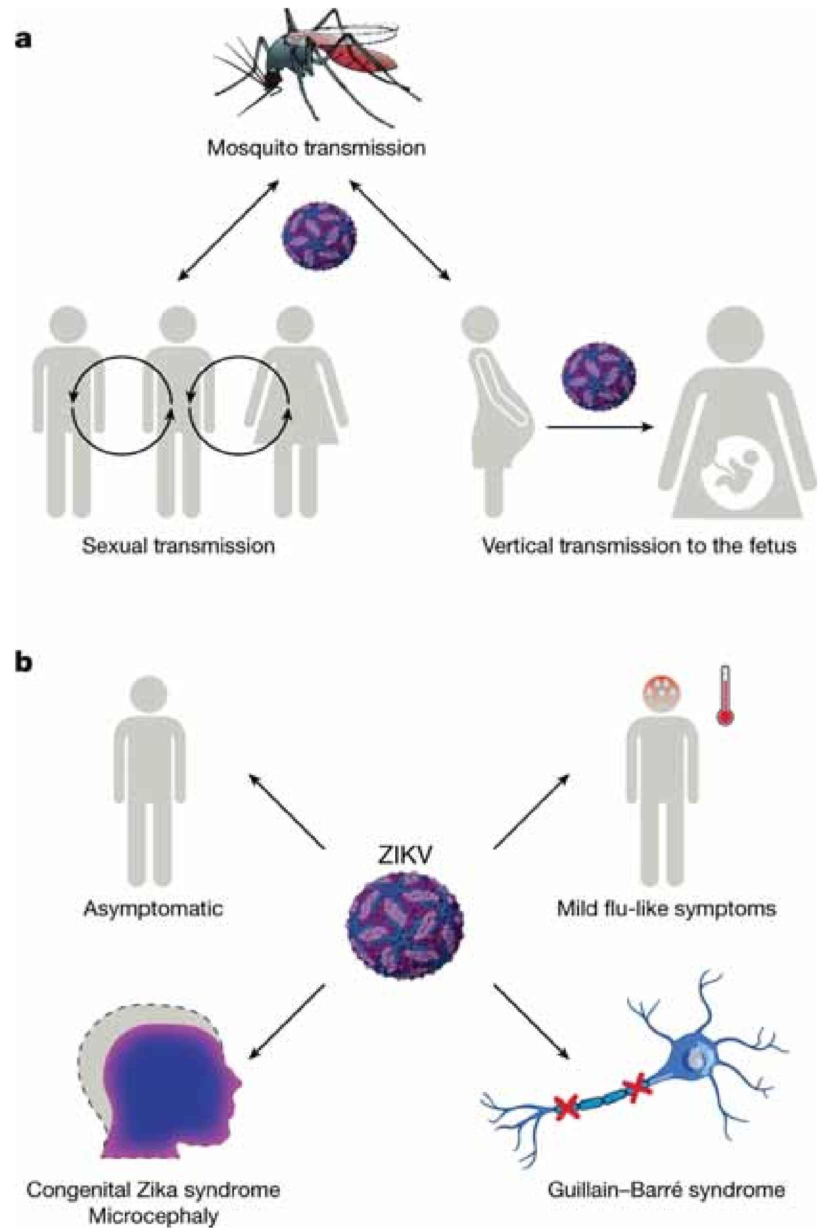


Figure 1.11: Schematic of the transmission routes and consequences of ZIKV infection.(**a**) Transmission vector: mosquito. ZIKV may be transmitted sexually and from the mother to the fetus. (**b**) ZIKV infection may be asymptomatic, cause mild flu-like symptoms, congenital ZIKV syndrome in newborns or affect neurons in Guillian-Barre syndrome (Pierson & Diamond, 2018).

1.3.8 Pathogenesis

Around 20% of those cases who contracted ZIKV had severe symptoms, with most experiencing a moderate flu-like fever that went away on its own after a few days although in most cases the acute infection is asymptomatic. Typical clinical manifestations of several arboviruses are fever, headache, a maculopapular rash, joint stiffness, and conjunctivitis in some cases (Duffy et al., 2009). In adults, ZIKV may cause an eye infection called uveitis, an inflammatory illness that can lead to blindness (Furtado et al., 2016).

During the recent ZIKV epidemic in French Polynesia and Latin America, reports of Guillain-Barré syndrome cases were confirmed (Jasti et al., 2016). The syndrome is characterized by ascending paralysis and polyneuropathy. Furthermore, clinical features such as severe illnesses including multi-organ failure, neuropathy, and low platelet count were also reported. Perhaps, the most relevant discovery has been that ZIKV was able to cause microcephaly, congenital abnormalities, and fetal death, recognizing its potential for transplacental transmission. For example, in 42% of symptomatic ZIKV-infected fetuses in Brazil during the 2015 epidemic, ultrasonography abnormalities were identified. Retrospective analysis of the outbreak in French Polynesia indicated an elevated incidence of microcephaly as well, with 95 instances per 10,000 infected first-trimester women (Carteaux et al., 2016).

1.3.9 ZIKV Infection and Pregnancy

1.3.9.1 Generalities

Although flaviviruses are not often related with vertical transmission and fetal brain injury, research have revealed that this association happens more frequently than may be detectable. For example, no genetic microcephaly instances have been identified from millions of Dengue infections every year. It was previously assumed that congenital West Nile Virus infection only presented complications to the fetus in a small number of cases, however

new data indicate that this is not the case. Despite all of these, the ZIKV RNA has been identified in maternal and fetal tissues, such as cord blood, various placental cells, amniotic fluid, and the developing fetal and newborn brain, indicating that the virus can cross the maternal-fetal protective interface. In addition, the ZIKV genome was found in the brain and placenta of spontaneous abortions (El Costa et al., 2016). The virus RNA may survive in vaginal secretions for weeks and in sperm and semen for many months (Mansuy et al., 2016). ZIKV infection during pregnancy has been associated with spontaneous abortions, stillbirth, and neonate's congenital malformations (microcephaly) as mentioned earlier.

1.3.9.2 Congenital ZIKV Syndrome

More than 3,000 Congenital Zika Syndrome (CZS) cases have been documented in 31 countries, including travel-related cases in Europe, Canada, and the United States. The term CZS refers to a collection of symptoms and manifestations in infants whose mothers had a verified ZIKV infection while pregnant (Freitas et al., 2020). Common clinical manifestations of CZS infection are brain calcifications, congenital malformations (microcephaly, cerebral atrophy), intrauterine growth restriction, and fetal death (Figure 1.12). Neuroimaging of newborns with suspected or confirmed ZIKV infection reflects those of congenital HCMV. Intracranial calcifications are subcortical in congenital ZIKV infection and periventricular in HCMV. These brain calcifications are the result of cell's apoptosis or necrosis (Fujita et al., 2014; Martin et al., 2022; Parmar & Ibrahim, 2012). Severe microcephaly is associated with fetal brain disruption sequence (FBDS) in which certain characteristics such as overlapping cranial sutures, protruding occipital bone, and excessive scalp skin, and severe brain damage are observed (Brasil et al., 2016). Sensorineural hearing loss, ocular abnormalities, and blindness are other possible consequences of CZS infection. The pathophysiology of the posterior eye lesions is unclear, which may be caused by ZIKV-induced cellular damage or an inflammatory effect. Other manifestations are hypertonia or hypotonia, hyperreflexia,

irritability and seizures, brainstem dysfunction and dysphagia, and congenital contractures.

The symptoms may vary from minor developmental delay to severe, as is the case of microcephaly, and depends on the condition of the patient. Viral transfer to the fetus usually occurs in 20% to 30% of pregnant women infected with ZIKV. Up to now, it is challenging to determine the timing of infection (especially in asymptomatic pregnant women) and how it affects fetal transmission. However, a greater risk of acquiring CZS is linked to infections occurring during the first trimester of pregnancy. Unfortunately, the complete range of signs of CZS may not be apparent immediately at birth; therefore, afflicted children must be assessed at an older age (Moore et al., 2017).

Figure 1.12: Microcephaly in children with CZS ("Chapter 9 - Post-Zika Virus Infection Survival", 2018)

1.3.9.3 Vertical Transmission

The underlying processes required for ZIKV to infect the placenta and cross into the neonate's compartment are poorly understood. According to Tabata et al. (2016) two potential vertical transmission mechanisms exist: in a placental and paraplacental manner. ZIKV may spread in the bloodstream, infecting cells in regions in the uterus that are highly vas-

cularized, such as the decidua and the placenta, and by amniotic fluid, which engulfs the amniotic membrane and thus the fetus.

Tabata et al. (2016) in vitro studies showed that primary human placental cells and explants, are susceptible to infection by ZIKV and produce viral progeny. These cells include cytotrophoblasts, endothelial cells, fibroblasts, and Hofbauer cells (chorionic villi). Additionally, ZIKV infects the amniotic epithelial cells and the amniochorionic membrane's trophoblast progenitor cells. The in vitro studies were done with mid and late gestational age placenta and first trimester explants.

As noted previously, flaviviruses engage with a variety of entrance receptors in host cells, in the case of ZIKV: DC-SIGN, Axl, Tyro3, and TIM1. The group also showed that the cells' susceptibility to ZIKV was in part due to the high concentration of these different receptors. Furthermore, the expression of these receptors differed depending on the cell type, donor, gestational age, and differentiation state. For example, TIM1 was highly expressed in the decidua, chorionic villi, and amniochorionic membranes around the fetus and in amniotic endothelial cells.

1.3.9.4 Damage to the Placenta

Gestational age and genetic differences in the placenta's host factors (expression of viral attachment protein or immune restriction factors) may affect how vulnerable different placental cells are to ZIKV infection (Tabata et al., 2016).

For instance, ZIKV replicates effectively in placental cells expressing viral entry molecules Axl and TIM1, such as extravillous trophoblasts and placenta-specific Hofbauer macrophages, but not in syncytiotrophoblasts (Tabata et al., 2016).

ZIKV replication may cause the expression of antiviral genes, as shown by in vitro infection in placental cells damaging the placenta (Tang et al., 2016). ZIKV can damage the placenta mainly by three courses of action: ZIKV can enter cells through the AXL and Gas6

receptors and destroy trophoblasts and fetal endothelial cells, affecting the placenta and infecting the fetus. Second, when ZIKV infects the placenta, it disrupts the lipid metabolic pathway, essential to support fetal development, resulting in miscarriage and growth limitation in the uterus. And thirdly, the placental lipidome is reprogrammed after ZIKV infection during pregnancy, favoring viral replication, mitochondrial dysfunction, and a dysregulated inflammatory response.

1.3.9.5 Damage to the Developing Brain

The ZIKV may pass the fetal-placental barrier, infecting the developing brain and nervous system. ZIKV has been found in the brain tissue of newborns with microcephaly. According to Tang et al. (2016) ZIKV directly infects and induces apoptosis in neural progenitor cells and radial glia in the fetal brain, although the virus may infect mature neurons to a lesser extent. Their group demonstrated that human neural progenitor cells are particularly sensitive to ZIKV infection in vitro, with infection between 65–90% of cells after 3 days of infection. Microscopic findings revealed that postmigratory neurons, largely prematurely differentiated, were dead in a fetal brain infected with ZIKV (Garcez et al., 2016; Mlakar et al., 2016).

It has been proposed that after the ZIKV crosses the placenta, the virus reaches the fetal brain via the bloodstream or the cerebrospinal fluid and reaches the glial cells with the highest concentration of the AXL receptor (Nowakowski et al., 2016). Additionally, the ZIKV NS proteins (NS4A, NS4B) have been shown to inhibits the Akt-mTOR signalling pathway which is crucial for the regulation of different biochemical processes of neural progenitor cells. The mTOR signalling pathway is involved in modulating apoptosis, autophagy, and replication and is downregulated by ZIKV although it has been shown that other flaviviruses upregulate the pathway (Q. Liang et al., 2016; J. S. Yu & Cui, 2016).

Studies have shown that ZIKV infection downregulates genes involved in cell cycle pathways (e.g. CENPF) and genes associated with neural stem cells, neuronal cell types, and

oligodendrocytes. Downregulation of cell cycles genes are associated with hereditary microcephaly (C. Li et al., 2016; Thornton & Woods, 2009). ZIKV may also upregulate the expression of astrocyte related genes (inducing differentiation) and antiviral genes (Toll-Like Receptor 3 (TLR3), Viperin and CCL2; Lossia et al., 2018).

1.3.10 Diagnosis, Treatment and Vaccine perspective

RT-PCR in the blood (or urine) can identify ZIKV infection in the first week following the appearance of symptoms. Approximately towards the end of the first week of sickness, ZIKV-specific IgM antibodies emerge. PCR and IgM/IgG antibody tests (ELISA) are available to detect the infection and positive results are validated by using plaque reduction neutralization (PRNT). However, the high cost and cross-reaction with other flaviviruses (especially DENV) restrict the laboratory diagnosis of ZIKV infection. Hence guidelines for clinical diagnosis must be adopted in the setting of concurrent infection by other arboviruses to characterize ZIKV infection in pregnant women (Rodríguez-Morales et al., 2016; Ximenes et al., 2019).

In addition to mosquito bite prevention, creating Zika treatments and vaccinations is crucial. At the moment, we have neither. Anti-ZIKV medications must consider the virus's transit through immune-privileged areas (e.g., CNS, placenta, and gonads) and adapted to the immunocompromised pregnant woman or those intending to become pregnant. Promising results have been observed in pre-clinical trials of vaccines such as DNA, mRNA, ZIKV purified inactivated virus vaccine (ZPIV), and viral-vector-based vaccines. However, in light of the recent decline in Zika virus transmission, a phase III clinical compelling study could be difficult to carry out. In addition, the development of animal models that can address the efficacy and protection of vaccines during pregnancy are essential (Abbink et al., 2018).

One way to control infection is to combat the virus particle or a specific stage of the viral cycle (entry, replication). Unfortunately, despite several promising in-vitro studies, no recognized therapy has been enhanced. Alternative methods rely on repurposing different active medicinal compounds to boost host antiviral responses. For example, Bortezomib (protea-

some inhibitor) in-vivo trials showed some antiviral efficacy. Consequently, targeting host responses reduces the probability of treatment-resistant viral variations. Other repurposed drugs like clofazimine, daptomycin, and ivermectin, also show an antiviral effect against ZIKV, although they present adverse effects on the fetus (Devillers, 2018).

1.4 The Human Placenta

1.4.1 Generalities

The human placenta supports fetal development, growth, and protection throughout pregnancy. During its short life, the fetus relies on the placenta as its lungs, gut, liver, and kidney. As a fetal organ, the mature placenta possesses predominantly fetal characteristics and maternal aspects like the basal plate and maternal blood in the intervillous region (Burton & Fowden, 2015). During pregnancy, specific pathogens can cross the placenta resulting in vertical transmission to the fetus. However, the placenta has evolved powerful defense mechanisms against many pathogens. Consequently, many microorganisms have, in turn, developed various ways to circumvent these placental defenses (Benirschke et al., 2012; Gude et al., 2004). In order to understand the mechanisms of vertical transmission and the role of the placenta during a viral infection, it is essential to look into its structure and function at the maternal-fetal interface.

1.4.2 Development and Structure of the Placenta

The following events are involved in the development of the human placenta. During implantation, the blastocyst adheres to the endometrium dividing trophoblast cells into an inner cytotrophoblast (CT) layer and an outer syncytiotrophoblast (ST) layer (Figure 1.13). Soon, the CT will proliferate, migrate to the outer ST layer, and lose its cell membrane, forming a rapidly growing multinucleated mass.

The CT secretes proteolytic enzymes, and the ST sends finger-like projections allowing the blastocyst to embed in the endometrium. As a result, spaces (lacunae) begin to form within the ST. These spaces start to fill with maternal blood and glandular secretions. The ST layer erodes endometrium blood vessels and glands as the area establishes an early feto-maternal circulation by forming a maternal vessel network where maternal blood will flow. At the end of the second week, small projections of the CT begin to expand into the ST, forming the primary chorionic villi. In the third week, the extraembryonic mesoderm grows into this villi, becoming a core of loose connective tissue (secondary chorionic villi).

In the third week, embryonic blood vessels have begun to form in the extraembryonic mesoderm of the secondary chorionic villi, which becomes the tertiary chorionic villi. CT from the tertiary villi grows towards the decidua basalis and spreads to form the cytotrophoblastic shell. The villi connected to the decidua basalis through the sides of the CT shell are the anchoring villi and the branch villi. The branch villi are surrounded by the intervillous space serving as the primary site of exchange between the mother and the fetus. By the 4th week, fetal blood flow is established, and poorly oxygenated blood is transported to the placenta from the fetus via paired umbilical arteries. Oxygenated fetal blood then travels back to veins converging to form a single umbilical vein that goes to the fetus (Turco & Moffett, 2019).

1.4.3 Placental Cells

1.4.3.1 Trophoblast

The trophoblast is the primary placental cell type that develops into a trophectoderm to form the placenta. Most of the placenta's functions are carried out by trophoblast cells. Therefore, the appearance of trophoblasts was a crucial evolutionary step essential for the survival of placental mammals. The term "trophoblast" is termed by Hubrecht referring to the specialized cells that line the placenta, responsible for transporting nutrition to the

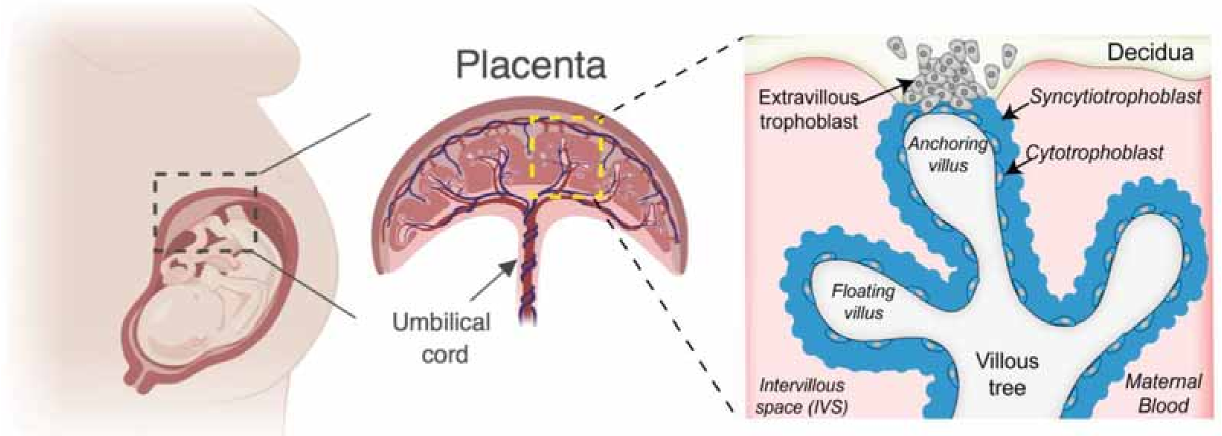


Figure 1.13: The human placenta structure and cellular components (Wells and Coyne 2018)
.

fetus from the mother. In addition, he concluded that the trophoblast is inherently extremely invasive and needs the decidua for its development. Trophoblast stem cells eventually become cytotrophoblasts (CT), mononucleated cells, and syncytiotrophoblasts (STBs) (Hubrecht, 1908; Pijnenborg & Vercruysse, 2013).

In the context of congenital infections Pereira (2018) shows that placental cell proteins and glycosphingolipids may act as cofactors for viral entrance into the placenta and perinatal transmission of many viruses. For instance, the PVB19 virus that causes hydrops fetalis may enter cells through the glycosphingolipid globoside coreceptor, which is highly expressed in first and second-trimester human placenta. Another example is the transmembrane TMPRSS2 serine protease which is necessary for preparing the SARS-CoV-2 viral surface to enter cells employing the ACE2 receptor also expressed in placental trophoblasts (Hoffmann et al., 2020; M. Li et al., 2020).

1.4.3.2 Other placental cells

In addition to trophoblast cells, the placenta's stromal core contains fibroblasts, vascular, and immune cells. Single-cell RNA-seq (scRNA-seq) done on first-trimester villi indicated two

fibroblast populations. The imprinted gene DLK1 distinguishes the populations. The DLK1+ cells resemble pericytes and may help build blood arteries. Also, placental macrophages, or Hofbauer cells, exist in the placenta and are the only placental immune cells known (Boss et al., 2018).

1.4.4 The Placenta Innate Immunity

1.4.4.1 Interferons

The innate immune system is vital for detecting pathogens such as viruses. In cells, pattern recognition receptors stimulate the innate immune system by distinguishing foreign invaders from the cell's antigens. Identifying a foreign material triggers a complicated series of signaling steps needed for initiating an immune response and, if necessary, inducing adaptive immunity. Interferons (IFNs) are important cytokines generated during innate immune detection of viral infections. IFNs are fundamental to the barrier function and integrity against viral infections (Hardy et al., 2004). IFNs are a varied group of cytokines with significant antiviral action. There are three IFN groups: type I, II, and III IFNs. The type I and III IFNs′ are known for their antiviral activity at the barrier interfaces. Isaacs and Lindenmann discovered and termed type I IFN because it "interfered" with viral replication (Duncan et al., 2021). The Type I IFN group comprises 13 IFN- α and 1 IFN-β sub-type.

Type I IFNs signal via the IFNAR1/2 receptor complex (Figure 1.14) to trigger hundreds of antiviral genes (interferon-stimulated genes or ISGs). All nucleated cells express IFNAR, allowing type I IFNs to establish a robust antiviral response in the tissue. Type III IFNs is the most recently discovered group. This family of cytokines includes IFN-λ1, IFN-λ2, and IFN-λ3, also referred to as interleukin (IL)-29, IL-28A, and IL-28B, respectively. In addition, the IFN-λ4 is also part of the type III IFN group and was discovered in 2013. Type III IFNs activates signaling pathways by the IFNLR1 and IL-10Rβ receptor subunits (Figure 1.14). The type II interferon group is composed of only 1 member, the IFN-γ, with pro-inflammatory and immunomodulatory functions that differ from type I and III IFNs groups

(Lazear et al., 2019).

IFNs from types, I and III are triggered when pattern recognition receptors or PRRs (like TLRs) identify either pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). Toll-like receptors (TLRs) and other PRRs identify a wide variety of infections by recognizing common microbial characteristics. PRRs identify PAMPs, which trigger an intracellular signaling pathway that alters the cell's transcriptional processes and the upregulation of transcription factors like IRFs and NF-kappaB by IFNs (Mesev et al., 2019).

1.4.4.2 Type III Interferons and Viral Infections

There is mounting evidence that type III IFNs are critical for human placental defense against viral infections. Studies by Bayer et al. (2016) show that type III IFN (IFNγ) protects primary human trophoblasts against viral infection. Interestingly, human trophoblasts produce type III IFNs even in the absence of viral infections, in contrast to other tissue barrier types, which need PAMP-mediated IFN induction. Viruses like ZIKV, rubella virus, HCMV, and herpes simplex virus-1 are viruses that can be inhibited by the medium of uninfected term human trophoblast cells or from chorionic villi from human mid-gestation placentas based on in vitro studies. Further studies also show that antiviral components, such as placental-specific chromosome 19 miRNAs (C19MC cluster), were also found in this medium, along with IFN-s1–3. This antiviral effect doesn't apply to non-viral pathogens like Toxoplasma gondii (Schoggins, 2018; Wells & Coyne, 2018).

The syncitiotrophoblast layer seems responsible for IFN-λ release, based on studies in which the cytotrophoblasts to syncitiotrophobast fusion was inhibited with DMSO. This appears to lower the ISG production in placental conditioned medium-exposed cells compared to controls. Furthermore, human trophoblast cell lines, including BeWo, JEG-3, and JAR, do not produce type III IFNs like placental trophoblasts. However, when treated with synthetic RNA ligands or infected with ZIKV, these cell lines may produce type III IFNs (Bayer et al.,

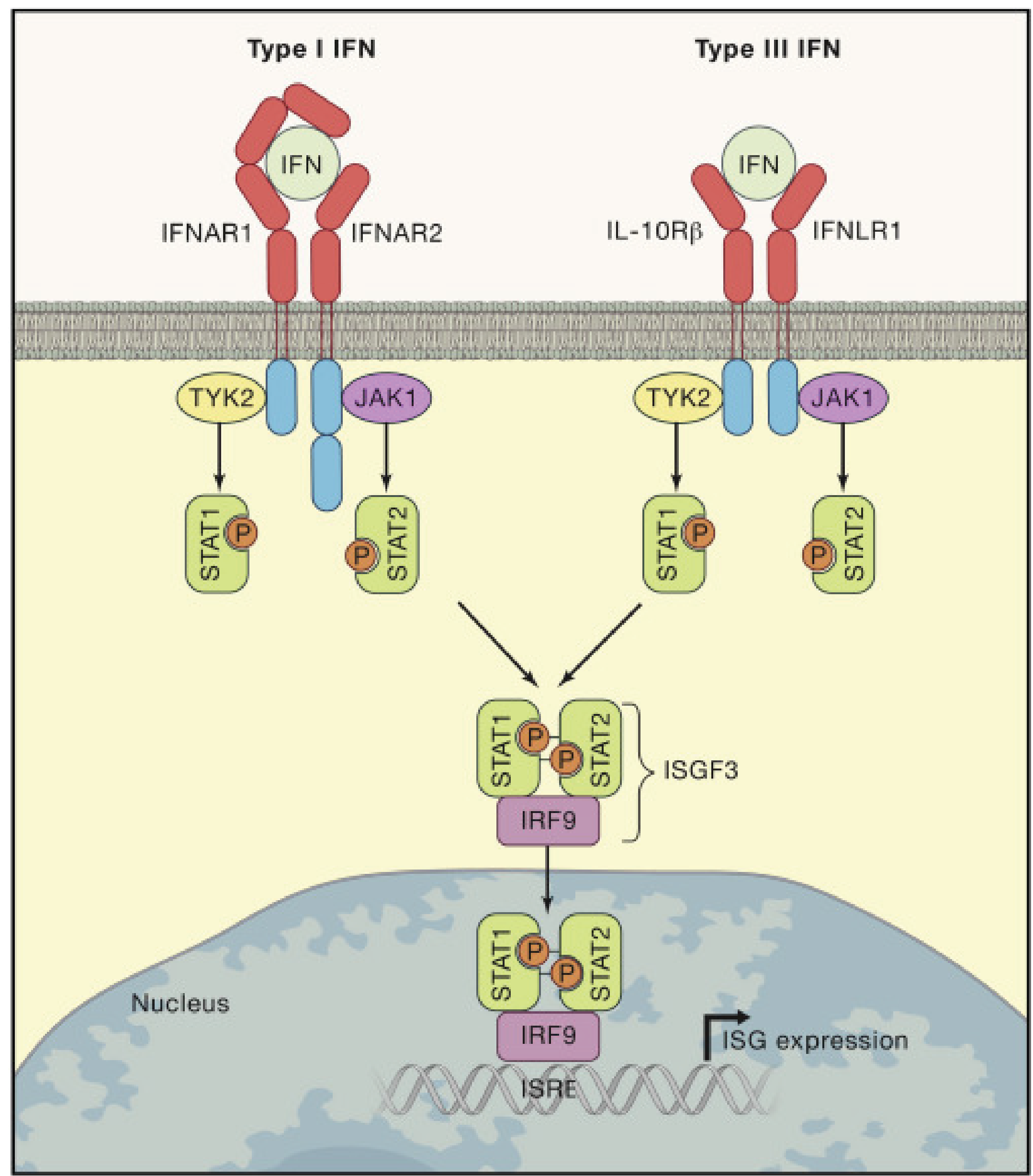


Figure 1.14: Type I and type III interferon signaling pathways. Despite that type I and type III receptors are different, both cytokines activate STAT phosphorylation via Jak kinases associated with the corresponding receptor subunits. (Lazear et al., 2019).

2016). Wells and Coyne (2018), showed that while type III IFNs boost antiviral responses that may protect the baby and placenta from viral infections, type I IFNs seem to damage placental structure and function. Pregnant women with interferonopathies (abnormal production of type I IFNs) developed gestational complications, and their babies were born with congenital disorders in the absence of any pathogenic infection (Meuwissen et al., 2016).

1.5 The Large Human C19MC microRNA Cluster

1.5.1 Generalities

Gene regulation beyond RNA transcription has resulted in considerable interest in the field of human reproduction. The study of gene expression and variation that is not caused by shifts in the DNA sequence is termed epigenetics. There are four main epigenetic processes: DNA methylation, imprinting, histone modification, and small RNA-mediated control, specifically through microRNAs (miRNAs) (Maccani & Marsit, 2009; Wilkins-Haug, 2009).

MiRNAs are molecules made up of a single strand of RNA (around 19-21 nt) that behave as antisense RNAs by base-pairing partly to complementary sequences in mRNA 3′–UTRs. When bound to mRNA, they act as translational repressors by either degrading or inhibiting the translation of their mRNA targets (Bartel, 2018). These short molecules frequently have a moderate effect on the suppression of their targets due to the several regulations concerning the architecture of the miRNA binding sites, and up to now, it is not entirely understood the aspects that determine the specificity of the interactions of miRNA-to-mRNA in vivo. However, perfect binding interaction is required in the nucleotide positions 2-7 from the 5′-end of the miRNA, also called the seed region, to induce a gene silencing effect. Therefore, this minimum requirement for a "relationship" suggests numerous targets for miRNAs and, at the same time, a variety of effectors targetting mRNAs (Jonas & Izaurralde, 2015; Lewis et al., 2005).

MiRNAs are in practically all cell types, miRNAs control a wide variety of biological

activities, including metabolism, cell proliferation, apoptosis, and differentiation (Bueno et al., 2008; H.-X. Chen et al., 2010; Cheng et al., 2005; Seitz et al., 2004). For example, it is well established that miRNAs play a role in the processes associated with initiating and maintaining pregnancy. These processes include the preparation of the endometrium for implantation (Pan & Chegini, 2008), controlling genes associated with inflammatory responses (Chakrabarty et al., 2007), and regulating immune tolerance-associated genes, such as HLA-G (Veit & Chies, 2009). Epigenetic alteration of genes may control embryogenesis, such as gamete development, pre-implantation embryonic development, and placenta formation (Wilkins-Haug, 2009).

1.5.2 The miRNA Processing Pathway

The processing of miRNAs is a complex endeavor that combines the work of two proteins, Drosha and Dicer. These proteins are members of the ribonuclease (RNase) III family acting successively in the cytoplasm and the nucleus. Some cofactors are known to regulate the miRNAs biogenesis steps such as the epidermal growth factor receptor (EGFR) (Shen et al., 2013). This process consists of the following events stated in chronological order (Bortolin-Cavaille et al., 2009, Figure 1.15).

1. Synthesis of the miRNA gene by the RNA polymerase II into the primmary transcript occurs at the nucleus. The endonuclease Drosha-DGCR8 complex (also called the Microprocessor) converts the pri-miRNAs into precursor miRNAs (pre-miRNAs) that will fold into short hairpin-like structures of about 70 nucleotides in size.

2. The pre-miRNAs are transferred to the cytoplasm through the exportin-5 pathway, where the endonuclease Dicer protein (complexed with TRBP) cuts the miRNA into duplicate strands of 19-24 nucleotides (the mature guide miRNA and passenger miRNA). Both miRNA strands are stacked onto an Argonaute protein (AGO2) to generate a

RNA-induced silencing complex (RISC) precursor.

3. The complex unwinds the miRNAs strand and discards the passenger RNA strand, leaving a mature RISC with the mature miRNA

4. MiRNAs combine with ribonucleoprotein (RNP)complexes during the final processing stages (miRNPs or miRISC) where the silencing of target genes (mRNAs) occurs in a sequence specific fashion (seed region). Other supression regulatory controls include stability of the mRNA duplex and the presence of ALU sequences (Ouyang et al., 2014).

5. The miRNA partially base-pair with protein-coding mRNA 3′-UTRs, silencing genes by inhibiting translation and/or accelerating degradation (Bortolin-Cavaille et al., 2009).

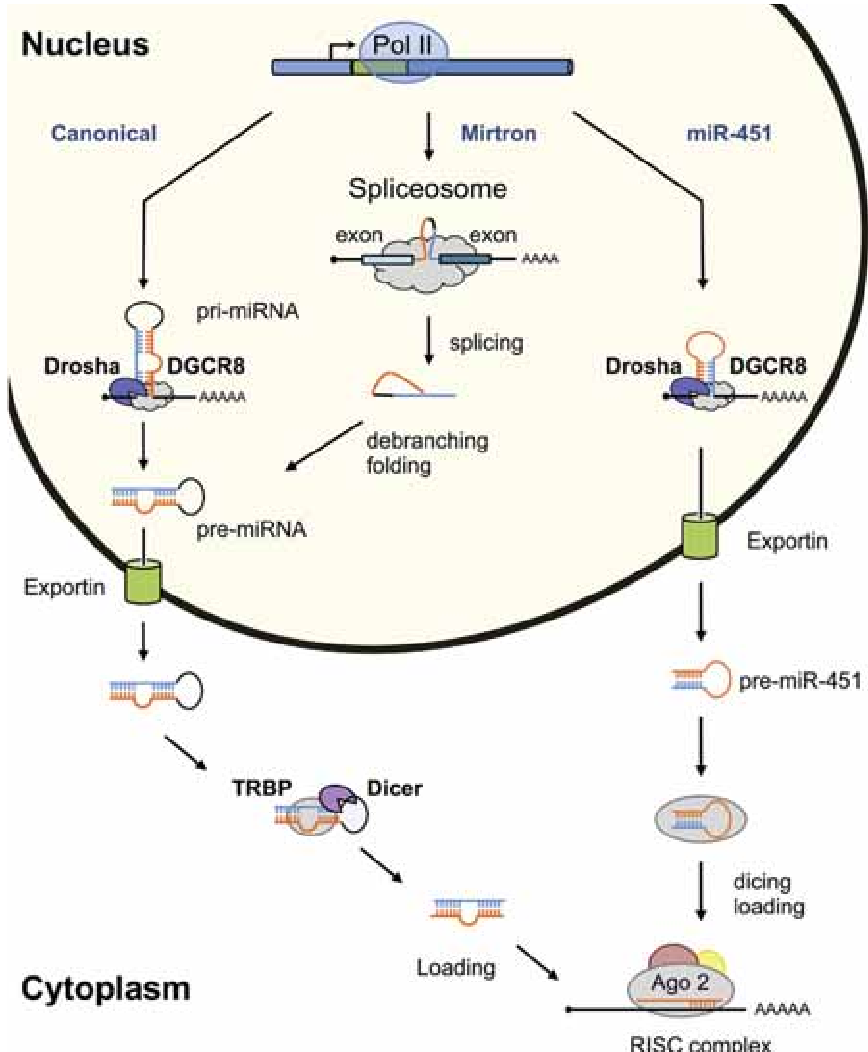


Figure 1.15: Schematic of the processing pathway of miRNAs. The Dicer-dependent pathway processes the majority of miRNAs. The pri-miRNA transcripts are trimmed by Drosha in the nucleus after transcription, resulting in a 60-70 nt pre-miRNA. Exportin 5 exports pre-miRNA to the cytoplasm, where Dicer dices the hairpin into a 22-nt miRNA duplex and then separates the duplex into a guide and passenger strand. Typically, the guide strand is loaded into Ago proteins to create RISC (Peng & Croce, 2016).

1.5.3 MiRNAs and Pregnancy

Studies by Barad et al. (2004) have shown a placenta-specific miRNA profile. The tissue-specific profile is dominated by the expression of trophoblast miRNAs, also observed in maternal plasma, and includes the expression of numerous miRNAs from three main miRNA clusters: chromosome 14 miRNA cluster (C14MC), Chromosome 19 miRNA cluster (C19MC), and the miR-371-3 cluster (Figure 1.16). The miRNA expression from these 3 clusters fluctuates throughout pregnancy and between preterm and term placentas. These clusters are generally located in imprinted genes, vital for embryonic development, cell differentiation, and function. Imprinted genes affect the development of the placenta, suggesting great importance to the miRNA placental profile (Malnou et al., 2019).

Placental miRNAs that seem to impart unique evolutionary traits on the placenta of primates and humans may also have a role in pregnancy abnormalities. Many miRNAs, including those in the C14MC, C19MC, and miR-371-3 clusters, are dysregulated in diseased placentas. However, it is unclear whether or not miRNAs, and which of them, are engaged in the pathogenesis of illnesses or if they constitute protective mechanisms in the stability of the placenta (Y. Liang et al., 2007; Miura et al., 2010).

Trophoblast biological processes such as syncytialization, invasion, proliferation, and autophagy may be altered by miRNAs, resulting in an abnormal structure and functions of the placenta, causing pregnancy complications. miRNAs associated with placental abnormalities usually are involved in the regulation of placentation-related genes (Hayder et al., 2018).

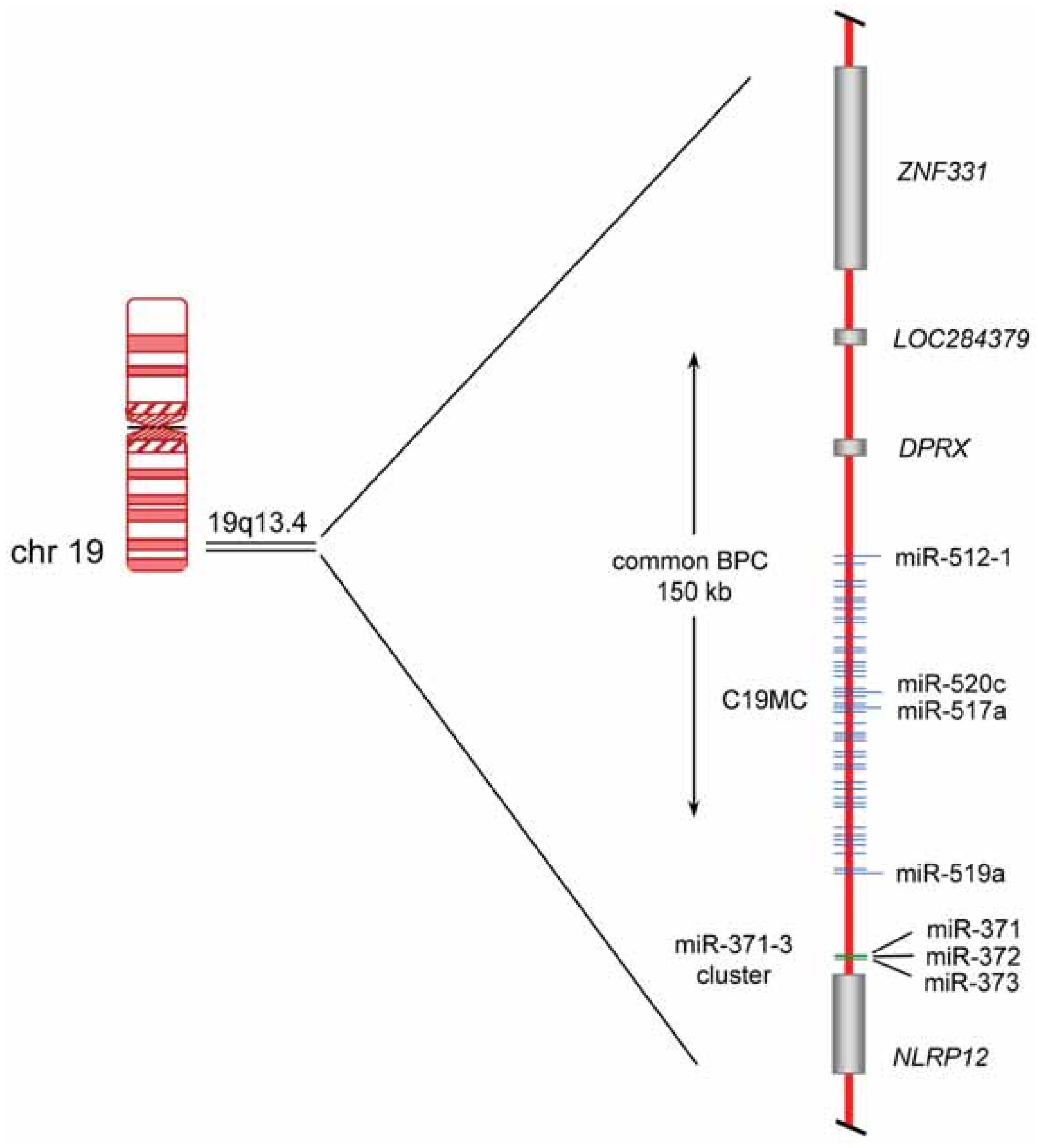


Figure 1.16: Scheme of the chromosome 19 region 19q13.4 depicting the C19MC miRNA and mir-371-3 clusters. Other genes located in chromosome 19 are also showed (Rippe et al., 2010).

1.5.4 Description of the C19MC miRNA Cluster

The chromosome 19 miRNA cluster (C19MC) gene is located on chromosome 19 q13.42 and extends for over 100 kb in length; it is one of the largest miRNA gene cluster in the human genome. This cluster was first identified by Bentwich et al. (2005) of Rosetta Genomics, and is only found in primates (successfully preserved in human cells). Up to now, no miRNA cluster homologous have been discovered in rats, mice, or dogs, demonstrating its primate-specificity. Supporting this notion, a hypothesis has been developed suggesting that C19MC may have some connection to the evolution of human primates and play a key role in embryo development, being essential in pregnancy (Tsai et al., 2009; R. Zhang et al., 2008).

The C19MC cluster consists of 46 intronic pre-miRNAs processed from a RNA Pol-II-transcribed non-protein-coding C19MC-HG (host-gene) pri-miRNA transcripts, flanked by 37 non-coding DNA exons of unknown function, generating 58 mature miRNA species (Bortolin-Cavaille et al., 2009; S. Lin et al., 2010). The members of the C19MC cluster share common seed base pair sequences, suggesting that they are descended from a single common ancestor. It is suspected that a member of the miR-371-3 cluster is this common ancestor (R. Zhang et al., 2008). Figure 1.17 shows chromosome 19, the region q13.42, and all of the mature miRNAs sequence members of the C19MC cluster.

Remarkably, C19MC miRNA genes are enriched by numerous dispersed Alu elements (AluJ and AluS), approximately 10x more than in other regions, suggesting a co-evolution of Alu sequences and miRNAs (R. Zhang et al., 2008). However, there is still a lack of clarity on the roles of Alu repeat elements and their interactions with miRNAs. However, Lehnert et al. (2009) have indicated the possibility of these sequences having a role in the control of the structure of the genome, the expression of genes, and the production of miRNAs (Gu et al., 2009).

The C19MC miRNAs have a quite selective expression pattern, and the placenta is the primary location. They are the most common kind of miRNA found in human trophoblasts, to the point that the literature may referred them as trophomiRs. As a result of its high

expression, these trophomiRs are now regarded as one of the most reliable indicators to characterize trophoblastic cells (Lee et al., 2016). Notwithstanding, the expression of miR-498 and other C19MC members has been reported in the fetal brain (Flor & Bullerdiek, 2012), the testis, and in human (and chimpanzee) embryonic stem cells (ESCs, Y. Liang et al., 2007; Sadovsky et al., 2015).

In cell lines, C19MC miRNAs appear to be solely expressed in cells derived from trophoblasts, including choriocarcinoma cell lines JEG3, JAR, and BeWo; however, the extravillous trophoblast line HTR8/SVneo is an important exception to this rule (Donker et al., 2012; Morales-Prieto et al., 2012). Treatment with 5-aza-2-deoxycytidine (5-Aza-CdR), a DNA methylation inhibitor, can reactivate the expression of these miRNAs in cell lines, suggesting that C19MC miRNAs expression is epigenetically controlled, partly by mechanisms involving methylation. Cells that did not produce these miRNAs had an abnormally high level of methylation in a CpG-rich area situated around 17 kb upstream of the first miRNA gene. This fact also helps support the theory that C19MC miRNAs are regulated epigenetically (Noguer-Dance et al., 2010; Tsai et al., 2009).

As observed with C14MC, C19MC miRNAs are found inside imprinted genes and the cluster can only be expressed if located in the chromosome inherited from the father. The expression of the C19MC cluster is directed by the Polymerase-II promoter region that spans over the differentially methylated region (DMR). The DMR acts as an imprinting control area determining monoallelic expression. Despite an uncertain cause, the C19MC-HG transcript attracts an increased amount of the Drosha-DGCR8 (Microprocessor) complex to transcription sites where gene processing of the C19MC pri-miRNA transcript (and other pri-miRNAs elsewhere) may occur (Bellemer et al., 2012; Noguer-Dance et al., 2010; Stelzer et al., 2013). This suggests that local pri-miRNA processing may increase near the C19MC site, or in contrast, C19MC transcripts may saturate the Microprocessors, allowing only for C19MC pri-miRNA processing.

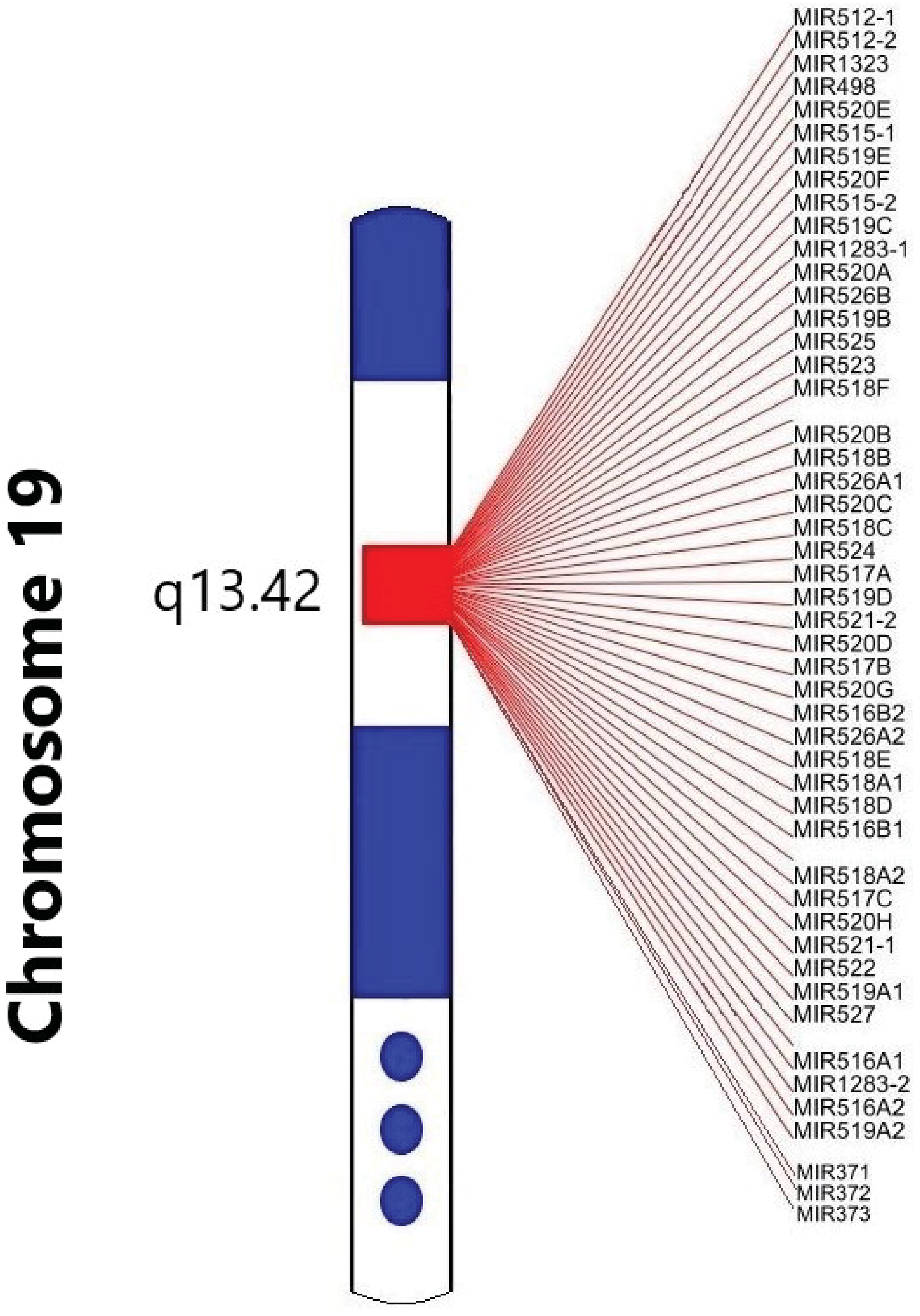


Figure 1.17: Zoom of composite chromosome 19 genomic organization of homo sapiens (human) in the q 13.42 region. C19MC miRNA cluster from miR-512-1 to miR-373 created by Yamileth Chin using biopython (`http://biopython.org/DIST/docs/tutorial/Tutorial.html`). Sequence and annotation from GenBank (National Library of Medicine, USA). Data retrieved from `https://www.ncbi.nlm.nih.gov/genome?LinkName=nuccore_genome&from_uid=568815579` (Accessed on 21 June 2022).

1.5.5 The C19MC miRNA Cluster and Pregnancy

1.5.5.1 C19MC miRNAs in Normal Placental Physiology

The C19MC cluster has the most elevated miRNA expression among human placental miRNAs. In addition, the trophoblast layer releases C19MC miRNAs into maternal circulation in exosomes (EXs), which regulate gene expression to alter maternal physiology. Research reveals the many functions of trophoblastic C19MC miRNAs during pregnancy (Lv et al., 2019; Mouillet et al., 2014).

Over-expression of some C19MC miRNAs reduces extravillous trophoblast migration since, in these villi, low levels of miRNAs are found (B. Xie et al., 2022; L. Xie et al., 2014) suggesting a regulatory effect of C19MC miRNAs in the migration and invasion of the human placental cells. In vitro studies by M. Zhang et al. (2016) show that overexpression of the miRNA-515-5p suppresses the differentiation of placental CT into STBs during villous formation. This may occur by miRNAs actively targetting hCYP19A1/aromatase P450, GCM1, and FZD5 genes. Another study by L. Xie et al. (2014) utilized C19MC non-expressing cell line HTR-8/SVneo (extravillous human trophoblast) and observed that the impairment of trophoblasts cells' migration occurred when expressing the C19MC cluster forcefully. Several C19MC miRNAs (miR-519d-3p, miR-520g, miR-517a/b, and miR-520c-3p) have been established as affecting migration and invasion of differentiated trophoblasts by several research groups (Anton et al., 2015; Ding et al., 2015; Jiang et al., 2017; Takahashi et al., 2017).

Another area in which C19MC miRNAs may be essential is in regulating the placenta metabolism. The placenta is a dynamic organ that requires the adaptation to different depleted oxygen conditions during pregnancy. Studies demonstrate the importance of the miRNA-520c-3p in the adaptation to gestational hypoxia Donker et al. (2012). In vitro, treating trophoblast cells with the hypoxia mimicking agent Cobalt Chloride increased the expression of miRNA-520c-3p (Anton et al., 2015).

Furthermore, another functional role attributed to C19MC miRNAs is the conservation

of the pluripotency of embryonic stem cells (ESCs). Approximately 16 out of 46 miRNAs from the C19MC cluster show the seed sequence "AAGUGC" characteristic of miRNA-302 , an ES-identified miRNA contributor to the maintenance of pluripotency (P. N. N. Nguyen, Choo, et al., 2017; P. N. N. Nguyen, Huang, et al., 2017). Nonetheless, evidence correlating the expression of C19MC miRNAs to ES development hasn't been obtained. However, high expression of C19MC miRNAs has been observed in human trophoblast stem cells (Okae et al., 2018); perhaps their expression may relate to trophoblast stem cell growth.

1.5.5.2 Antiviral Role of C19MC miRNAs

The placenta's syncytialized cells function as a physical barrier, preventing the risk of viral infection from the maternal circulation to some extent. Therefore, term primary human trophoblast cells manifest in vitro a relevant degree of viral resistance compared to other cell types. This viral resistance, in part, has been attributed to high baseline autophagy levels in primary human trophoblasts and not due to any viral replication obstruction (Delorme-Axford et al., 2013).

Furthermore, the Sadovsky and Coyne research groups have demonstrated that primary human villous trophoblasts resist various DNA and RNA viruses (HSV, HCMV, HIV-1, and ZIKV). In vitro studies by Delorme-Axford et al. (2013) demonstrate the antiviral effect of the C19MC miRNAs against a VSV infection: First, by inserting into virally permissive non-placental cells a bacterial artificial chromosome containing the C19MC miRNA cluster, resulting in an increment in viral resistance to VSV. Second, the overexpression of three miRNAs encoded by C19MC (miR-517-3p, miR-516-5p, and miR-512-3p) as miRNA mimics mirrored similar antiviral results. Lastly, this effect is transferred to non-placental cells by exposing them to a conditioned medium (or EXs isolates) containing an abundance of C19MC miRNAs (Donker et al., 2012; S.-S. Luo et al., 2009; Ouyang et al., 2016). Of note, this anti-pathogenic effect of C19MC miRNAs is not observed in intracellular bacteria or protozoa

(Delorme-Axford et al., 2013; Dumont et al., 2017).

Neighboring non-trophoblastic cells may also benefit from C19MC-contained sEVs antiviral activity, including adjacent placental, maternal, and fetal cells. Two primary human cells that do not express C19MC miRNAs, human placental fibroblasts and human uterine microvascular endothelial cells, demonstrate a consistent level of viral resistance following absorption of C19MC miRNAs contained in EXs (Chang et al., 2017; H. Li et al., 2020). Overall, it is then speculated that C19MC miRNAs are secreted into EXs, transported to the placenta, and integrated by targeted cells of either maternal or fetal origin, where they promote autophagy mechanisms and trigger an antiviral response. These results reveal novel methods used by human trophoblasts on a systemic scale throughout pregnancy.

In a clinical context, it is crucial to consider that the concentration of C19MC miRNAs in the mother's blood rises from the second part of the first trimester (although detectable from 2 weeks of pregnancy) until term and then falls precipitously after delivery. Therefore, early viral infections may be detrimental to the fetus since C19MC miRNAs' potential antiviral effect is observed only after the first-trimester of gestation (Delorme-Axford et al., 2013).

Notably, the C19MC miRNAs carried in EXs do not influence INF or interferon-stimulated genes (IFI44L). This was observed in EXs from pregnant women's plasma compared to non-pregnant women. Additional studies show that C19MC miRNAs, independent from IFNs, protect primary human trophoblasts against viral infection. Therefore, it suggests that C19MC miRNAs and IFN regulate two distinct antiviral defense pathways in primary human trophoblasts. Furthermore, concurrent exposure of target cells to IFN and EXs may have an additive antiviral impact (Bayer et al., 2016, 2018).

Notwithstanding, the exact biochemical mechanisms by which C19MC miRNAs convey an antiviral protective effect to placental and non-placental cells or the relationship with the immune system is unknown. Some essential discoveries are relevant to further examining the C19MC miRNA cluster antiviral effect and its modus operandi. In vitro and in vivo studies by Dumont et al. (2017) demonstrated that viral infections do not alter the expression levels

of C19MC miRNAs early in gestation. Delorme-Axford et al. (2013) shows that the paracrine actions mediated by C19MCs are presumably virus-specific since they do not inhibit or even promote CMV infection. Exosomal C19MC miRNAs have been linked in other research studies by Kambe et al. (2014) and Ishida et al. (2015) to cell-to-cell interaction, prominently between the placenta and immune cells. Human choriocarcinoma (BeWo) cells release miR-517a-3, which may be transferred in vitro to immortalized T lymphocyte (Jurkat) cells, where it reduces PRKG1 mRNA expression. In addition, natural killer cells isolated from the blood of pregnant women in their third trimester of gestation express miR-517a-3 and miR-518b, which decline after parturition. In the context of our research, the next section will describe the world of extracellular vesicles and their protective antiviral role as C19MC miRNA protective mode of transport.

1.6 The Diverse Extracellular Vesicles

1.6.1 Generalities

Some specialized cells in the body are able to release secretory vesicles that carry substances like hormones or neurotransmitters. However, all cells (prokaryotes and eukaryotes) can release different membrane vesicles, called extracellular vesicles (EVs). In a type of intercellular communication, a specific cargo is carried in EVs, representing its cell of origin, and is transported to a target cell where it may cause an effect. Moreover, throughout evolution, the process seems to be conserved from bacteria to humans and plants (Gurung et al., 2021).

A decade ago, scientists believed that releasing extracellular vesicles was simply a way for cells to excrete cell debris. However, rather than just carrying waste, extracellular vesicles serve other functions. Recently, the main interest in this relatively new field is in EVs ability to exchange their cargo (nucleic acids, lipids, and proteins) between cells and represent a signaling mechanism present in normal cells for their equilibrium purposes or as a result

of pathological changes. (Hessvik & Llorente, 2018). Although the "extracellular vesicles" terminology refers to all cell-secreted membrane vesicles, they are highly heterogeneous, significantly hindering efforts to characterize and manipulate their functional properties. Nevertheless, transmission and immunoelectron microscopy, as well as biochemical methods, have helped discover insights into these membrane vesicles' formation, characteristics, and functions (Raposo & Stoorvogel, 2013).

1.6.2 Categorization of Extracellular Vesicles

Extracellular vesicles (EVs) are broadly classified into: exosomes (EXs), microvesicles, and apoptotic bodies based on their biogenesis, size, composition, and surface molecules, even though their categorization is a continuous work in progress (Théry et al., 2018; Tkach & Théry, 2016). Microvesicles and apoptotic bodies are vesicles that pinch off the plasma membrane by outward budding. They vary from $\sim$ 50 nm to $\sim$ 1mm in diameter and include oncosomes. They are also referred as large extracellular vesicles (lEVs) (Cocucci & Meldolesi, 2015). EXs are 40 to 160 nm (average 100 nm) in diameter and endosomal in origin. They were discovered in late 1980 by Johnstone et al. (1989). EXs are formed by double invagination of the plasma membrane and the production of intracellular multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs). The ILVs are released as EXs through MVB fusion with the cell membrane (Van Niel et al., 2018). Recently, other "non-classical" EVs have been discovered, such as exomeres of 35-50 nm in size and of unknown biogenesis; and supermeres of which little is known (Anand et al., 2021; Clancy et al., 2021).

For the remainder of the section, I will describe most of the main aspects of the formation, characteristics, and functions of one type of EV: EXs, as they represent one of the focal points of my research study. However, it is essential to mention that although the term "exosomes" will be used in this section, it implies accordingly "small extracellular vesicles", interchangeably. I will use the term "exosomes" particularly to describe the category, although for this

study experimentations , I will refer to small extracellular vesicles (sEVs).

Considering that the sizes of EVs are just approximations, this is not completely accurate characteristic for classification; instead, EVs are traditionally classed based on their biogenesis, with the estimated sizes that go along with that classification. Therefore, EVs may be separated according to their size, after which writers may refer to large and small EVs, which would comprise a combination of the many EV types produced via biogenesis.

This is partly because of the technical limitations in the complete characterization (biomarkers, biogenesis, and content) of the EVs subtypes. This required evolution in the semantics was proposed by the International Society of Extracellular Vesicles (ISEV) especially when sub-cellular markers are not identified as sometimes is the case (Tieu et al., 2021; H. Zhang et al., 2018). The ISEV recommend referring to vesicles that range in size less than 200 nm as sEVs and more than 200 nm as large EVs. Eventhough, the ISEV recommends the use of sEVs, I will continue to use the term “EX” (interchangeably) as is referred to in the literature for the purpose of simplicity and to avoid confusion.

1.6.3 Biogenesis of EXs

As mentioned before, the classification of an EV subtype depends also on how it is generated by the cell. EXs are formed by the cellular endocytic pathways and the following events describe how they are generated (Figure 1.18):

1. The formation of EXs starts within the endosomal system. Initially, the plasma membrane invagination generates a concave structure containing cell-surface and soluble proteins.

2. This causes the new development of an early-sorting endosome (ESE), which can sometimes combine with a previously formed ESE. The trans-Golgi network and endoplasmic reticulum may contribute to ESE production and composition.

3. The ESEs grow into late-sorting endosomes (LSEs) and MVBs. These MVBs arise through invagination of the endosomal limiting membrane (double invagination of the plasma membrane), creating MVBs with several ILVs (future EXs).

4. Now, the MVB may merge with lysosomes or autophagosomes for product degradation or may merge with the plasma membrane to release ILVs as EXs by exocytosis (Gurung et al., 2021).

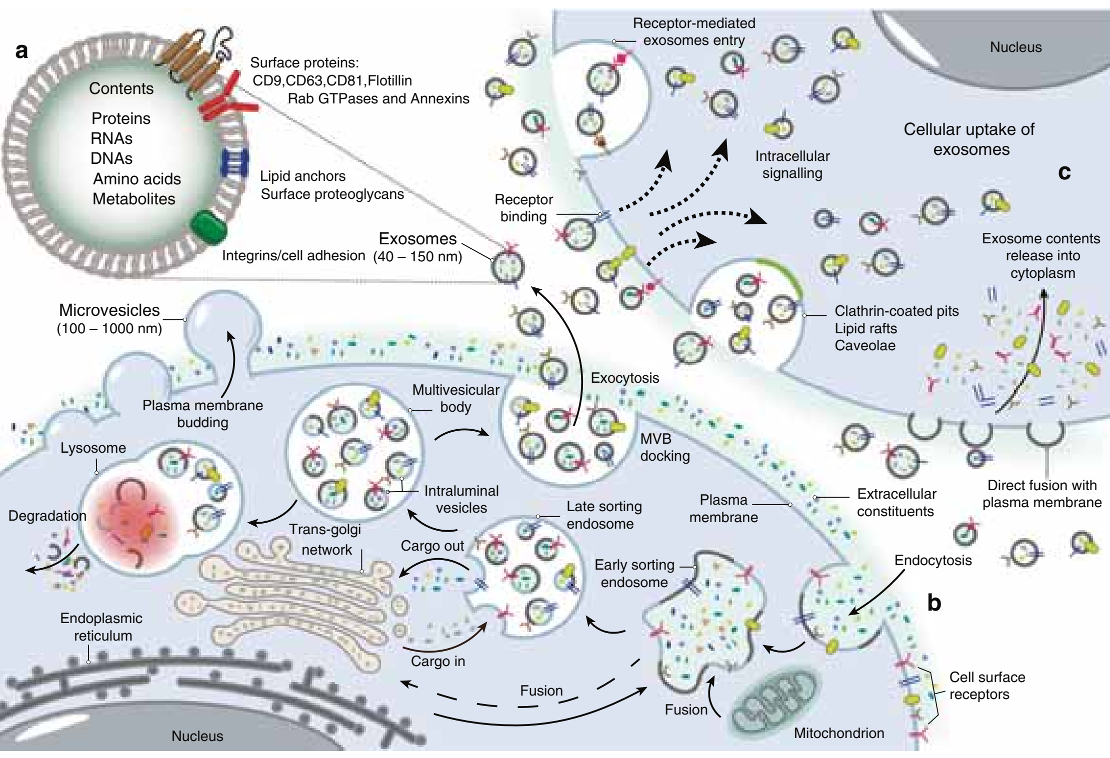


Figure 1.18: Schematic of exosome's biogenesis, cargo sorting, and release. (**a**) EX protein markers and diverse cargo. (**b**) Beginning with the endocytosis-mediated internalization of membrane by cell's receptors, endocytotic vesicles are conveyed to early endosomes, which merge to create late endosomes (cargo sorting) to multivesicular bodies (MVB). MVBs either release EXs by fusing with the cell membrane or destroy their contents by fusing with lysosomes. (**c**) EXs enter cells by different types of pathways (Sidhom et al., 2020).

It is important to mention that the ESCRT (endosomal sorting complex required for transport) machinery plays an important role in the biogenesis process of EXs. The ESCRT complex consists of the ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III protein groups, which work with the vacuolar protein sorting gene 4 (VPS4) protein AAA ATPase in MVB formation, cytokinesis, nuclear envelope resealing, autophagy, and viral budding (Colombo et al., 2013). These many functions have influenced the hypothesis that the ESCRT pathway is responsible for EX release, especially since EXs contain proteins from ESCRT-0 and ESCRT-1. Nonetheless, some studies have shown no effect on EX biogenesis when inhibiting the cell's ESCRT pathway. Thus, ILV formation and EX biogenesis may also be influenced by ESCRT-independent pathways, including those involving lipids, tetraspanin, and heat shock proteins. Noteworthy is that ESCRT-deficient mammalian cells still generate MVBs (Stuffers et al., 2009).

Regarding the rate of EX production, the dynamic processes connected with the biogenesis and absorption of external EX by any particular cell type make it difficult to calculate its production rate. For example, some studies show contradictory results about the production of EXs by some cancers, some arguing that cancerous cells produce more EXs than non-cancerous healthy cells, while others show quite the opposite (Bebelman et al., 2018; Chiu et al., 2016; Kalluri, 2016).

1.6.4 EXs Diversity

EXs vary in size, composition, functional influence on recipient cells, and biological origin. For example, size variations may be the result of the uneven invagination of the MVB membrane, resulting in different fluid and solid contents or separation procedures that incorporate other EVs. Nevertheless, enhanced EXs separation techniques showed size-defined subpopulations (Ciardiello et al., 2016). Size may affect exosomal content. Additionally, microenvironment and cell biology may affect the content of EXs and their biological markers.

Studies show that EXs carry membrane proteins, cytosolic and nuclear proteins, extracellular matrix proteins, metabolites, lipids, and nucleic acids (mRNA, non-coding RNA, and DNA). However, not all EXs have the same amount of a particular cargo, as shown by differences in miRNA exosomal cargo in cells (Pathan et al., 2019).

EVs proteomics has also highlighted the variability of protein exosome markers, limiting their usefulness in the validation of purification methods. EXs may also induce different types of effects such as cell survival, death, and immuno-modulation in diverse target cells depending on the type of cell surface receptors that is expressed. This observed diversity in EXs may also depend on the organ and tissue of origin, including EXs from cancerous cells, giving them traits a distict faith to a particular organ and absorption by specific cell types. All of these factors might increase EXs' complex nature and assortment (Kowal et al., 2016; Wen et al., 2019).

1.6.5 Proteins and EXs

EXs are associated with many types of membrane proteins including trans-membrane, lipid-anchored, peripheral, and soluble proteins. In this section I will describe briefly some of the most important proteins for my research study, especially those involved in characterization. Several proteins are involved in the origination and biogenesis process of EXs. These proteins are Sytenin-1, the Ras-related protein GTPase Rab, ALIX (apoptosis-linked gene 2-interacting protein X), TSG101 (tumor susceptibility gene 101), syndecan-1, SNARE [soluble N-ethylmaleimide–sensitive factor (NSF) attachment protein receptor] complex proteins, ESC proteins, tetraspanins, MHC class II molecules, flotillin, and sphingomyelinases (Bebelman et al., 2018; Mathieu et al., 2019).

Some of the tetraspanin proteins (CD81, CD82, CD37, CD9 and CD63) but not plasma or lysosomal membrane proteins are significantly concentrated in EXs. Since the early beginnings of EXs studies, CD81 and CD63 have joined other exosomal tetraspanins, particularly

CD9, as widely-used proteins for the characterization of EXs. Despite lacking enzymatic functions, tetraspanins are essential for many different proteins to ease their transport, stability, and oligomerization processes. Also, the scaffold protein ALIX and TSG101, which are part of the ESCRT complex, have emerged as commonly used markers for EXs characterization (Escola et al., 1998; Hemler, 2003).

Studying the proteins mentioned above is essential to explore their restrictive functions during the EX biogenesis process, especially *in-vivo*. In addition, Rab and ESCRT loss- or gain-of-function studies may interfere with autophagy, lysosomal pathways, and Golgi apparatus–derived vesicle trafficking, which may affect EXs formation (Kenific et al., 2021). Different cell types, growth media, and overall cell genetic health may promote or disfavor some of the suspected central regulatory proteins of EXs formation in vivo. Finally, inconsistencies in identifying exosome biogenesis regulatory proteins may emerge from varied manufacturing, enrichment, and concentration procedures (Willms et al., 2018).

1.6.6 Lipids and EXs

The EXs' outer surface comprises a polysaccharide layer connected to surface proteins and lipids. Under this polysaccharide layer, the EXs membrane includes phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositols, phosphatidic acid, cholesterol, ceramides, gangliosides, sphingolipids, and lipid rafts (a combination of glycosphingolipids, cholesterol and glycosylphosphatidylinositol (GPI) - anchored protein). Studies show that the concentration of lipids in pure EXs varies from that of plasma and other cellular membranes, which may give insights into EXs biogenesis (Skotland et al., 2017).

1.6.7 Nucleic Acids and EXs

EXs may carry different types of DNAs. Exs may be composed of single-stranded DNA,

double-stranded DNA, genomic DNA, mitochondrial DNA, and also EXs may contain reverse-transcribed complementary DNAs. It's not apparent whether EXs sort DNA selectively, unlike other cargo. Some studies have shown that sequencing exosome-associated DNA may reveal the whole genome sequence of the parental cell. EXs-based DNA release may help monitor DNA quality, regulate inflammation, and be a helpful biomarker of disease, viral infection, or chemotherapy response (Kalluri & LeBleu, 2016; Thakur et al., 2014).

While EXs mostly do not seem to transport many RNA molecules, the RNA they do contain is a highly biased subset of total cellular RNA. Small non-coding RNAs (ncRNAs) such as small nuclear RNAs (snRNAs), miRNAs (miRNAs), transfer RNAs (tRNAs), Y RNAs, vault RNAs, repetitive element RNAs, and fragmented RNAs, such as 3 mRNA fragments, are abundant in EXs, according to analyses of total RNA in these organelles. In contrast, EX's RNA profile is enriched in certain RNA species compared to microvesicles with a similar cell RNA composition (Shurtleff et al., 2017; Wei et al., 2017).

1.6.8 Sorting of Exosome Cargo

EXs carry various cargo, which I have mentioned up to now. Nonetheless, EXs from specific cell lines are likely diverse in the type of cargo they contain. Their cargo makeup depends on the cell type and the state of the cell. Several studies define EXs' protein, lipid, and RNA cargo, but little is known about their proper sorting (Villarroya-Beltri et al., 2014). Furthermore, exosomal mRNAs contain higher amounts of 3' UTR segments, which may aid in mRNA sorting. Additionally, EXs include ubiquitinated proteins, which might target proteins as cargo. Lipids may help in the sorting of proteins also since they contain more cholesterol, sphingomyelin, and glycosphingolipids than the cell of origin (Kowal et al., 2016).

miRNAs can be found in the human system after their release from cells, linked to proteins, and as cargo of microvesicles and EXs/sEVs (Arroyo et al., 2011; Mori et al., 2019). Previous studies show that specific miRNAs are more abundant in EXs than in the original

cell, suggesting that they can be sorted. Yet, the exact mechanism that explains the sorting of miRNAs into EXs, and the uptake by the target cell is mostly unclear. According to Garcia-Martin et al. (2022) special gene sequences in miRNAs may determine their sorting into EXs and their preservation in cells (EXOmotifs and CELLmotifs). The introduction or deletion of these motifs result in the increment or decrease of miRNAs in EXs or their conservation. Also, knock-down experiments of the proteins Alyref and FUS (RNA binding proteins) reduced by 50% CGGGAG-containing miRNA, suggesting that these proteins (among maybe others) participate in the sorting process. The study demonstrated that the miRNA composition released in EXs for each cell type (adipocytes, endothelium, liver, and muscle) differs from the population present in the parent cell and the EX-miRNA profile is different depending on the cell type.

1.6.9 Exosome Release

Based on different studies, the Rab proteins control exosome synthesis through endosomes and their release from the cell. Specifically, the Rab27 proteins may promote MVB maturation and further trafficking to the plasma membrane. Also, the Ral family of small GTPases is involved in the accumulation and transport of MVBs during endosomal biogenesis to the cell membrane (Ostrowski et al., 2010). Baietti et al. (2012) found that the proteins syndecan-syntenin associates with ALIX and contribute to exosomal cell membrane release.

The cell's actin and microtubule cytoskeleton are essential for MVB endosomal transport to the cell membrane and as is the ESCRT complex. In addition, the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), tethering factors, Rab proteins, and some Ras GTPases are involved directly in cell membrane fusion (Bonifacino & Glick, 2004; Pfeffer, 2007). Last but not least, studies by Savina et al. (2003) have shown the importance of calcium in regulating the exosomal release. An increment in intracellular calcium has been shown to increase EX secretion. All of these findings conclude that

a complex system of regulatory molecules controls the production and release of EXs.

Furthermore, studies by several research groups indicated that EXs may protect cells from intracellular stress and that the cell's equilibrium is a factor in determining whether MVBs are degraded or secreted. Also, that stress may, after all, increase the secretion of EXs (Baixauli et al., 2014; Kanemoto et al., 2016; Lehmann et al., 2008). Lastly, autophagy pathways may have an effect on secretion. Guo et al. (2017) showed that in the absence of Atg5 (autophagy-related protein), cancer cells reduce their EXs production, while in contrast, Abdulrahman et al. (2018) found that the release of EXs was increased in neuronal cells.

1.6.10 Challenges Related to the Purification of Extracellular Vesicles

Currently, numerous procedures are utilized to purify the varied EV types from a determined sample (Figure 1.19). The most widely used laboratory purification methods are precipitation, differential ultracentrifugation (with or without a gradient), and size exclusion chromatography techniques that rely on the EV's varied sizes and densities for their separation. It has been suggested that the most effective approach for isolating of EVs involves using several distinct procedures to achieve the desired level of purity and recovery (Brennan et al., 2020).

Around the world, research groups select the method of purification based on factors such as experimental design, EV type being studied, the sample where the EV originates, and the EV's final use. The comparability of EV data measured across studies and labs presents limitations as a consequence of the diversity of both the sources and the methodology used. Therefore, there is still a need for standardized methods for the separation of EVs that may allow for the proper comparison of data among labs. As a result, the ISEV was released in 2018 in the Journal of Extracellular vesicles an updated compilation of guidelines for the standardization of protocols (purification and characterization) and communication in the EV

field, the MISEV (Minimal Information for Studies of Extracellular Vesicles, Théry et al., 2018). Another useful initiative is the creation of EV track to help researchers to record their data, receive knowledgeable feedback, and improve their EV research methodology (evtrack, 2022; Van Deun et al., 2017).

In the context of our study, the isolation method of choice is differential ultracentrifugation (dUC). It is worth acknowledging the main limitations and advantages of choosing this technique. A complete description of the EV isolation technique used in this study is found in chapter 2, section 2.8. dUC is a widely used method of purification by clinicians and researchers. However, some limitations should be considered, such as time and labor intensive, and resulting in medium EV product purity and yield. Nonetheless, the methodology is by far the best option among other techniques (chromatography, precipitation) for the finality of the EVs in functional assays and in terms of homogeneity of experimental designs in the laboratory.

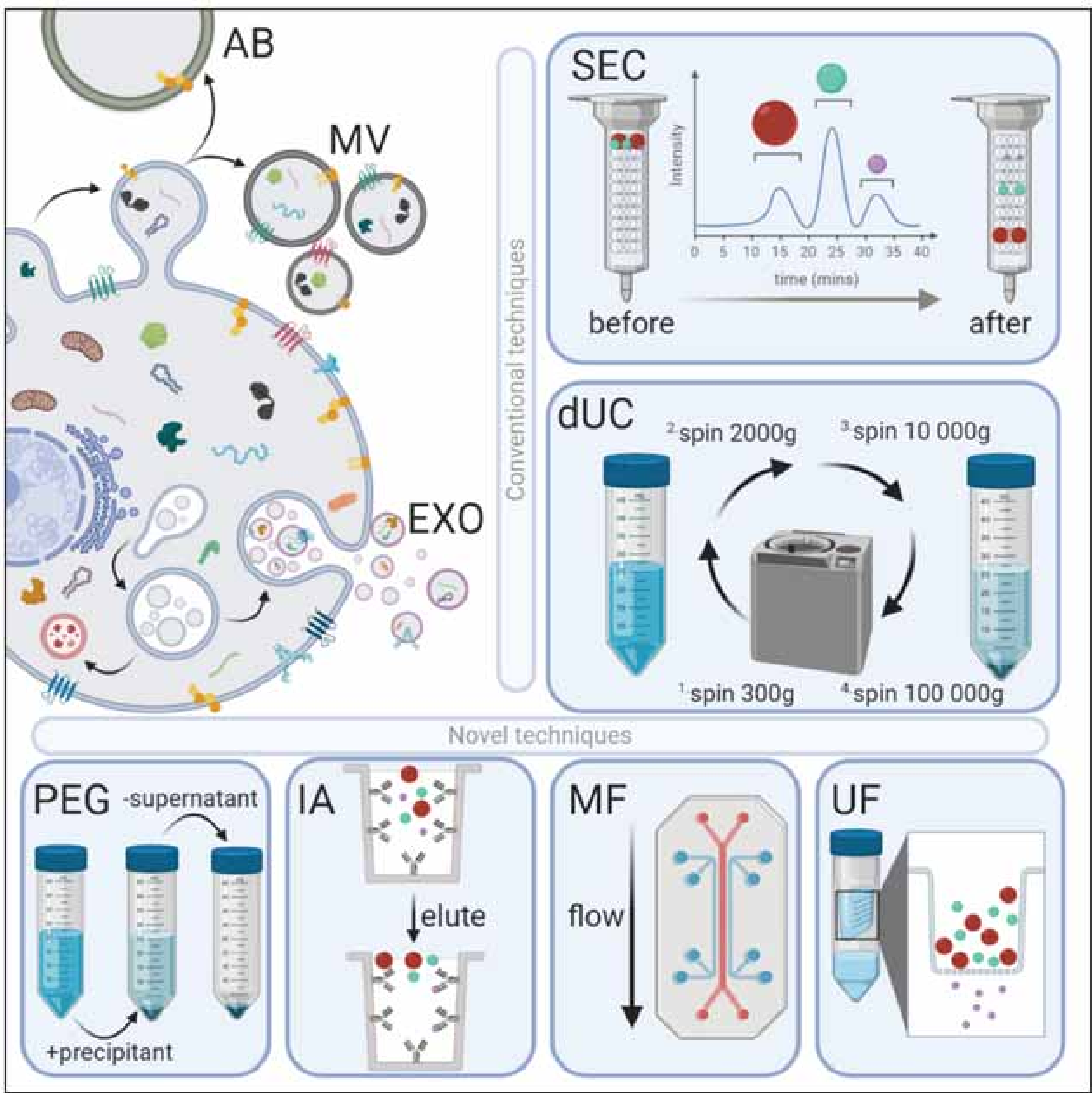


Figure 1.19: EV purification techniques. Schematic showing EV biogenesis of microvesicles, apoptotic bodies, and exosome. Conventional EV purification techniques such as size-exclusion chromatography (SEC), and differential ultracentrifugation (dUC); novel techniques including size exclusion chromatography, Poly-ethylene glycol (PEG)-based precipitation, Immunoaffinity (IA), Microfluidics (MF) technology, and Ultrafiltration (UF) are shown (Sidhom et al., 2020).

1.7 Extracellular vesicles in Pregnancy

1.7.1 EVs in Healthy and Pathological Pregnancies

Human placental EXs derived from trophoblasts and non-trophoblastic cells (placental mesenchymal progenitor cells) are associated in cellular adaptation during normal pregnancy, including immuno-regulation and tolerance, cell migration, and proliferation and invasion of extravillous trophoblasts Burnett & Nowak, 2016; Jin & Menon, 2018.

In a healthy pregnancy, the exosomal cargo (miRNA and proteins) varies depending on gestational age. In vivo studies have shown that EXs in murine plasma changes with gestational age and seem to be involved in labor and delivery. In particular, EXs from plasma produced near the end of pregnancy can cause preterm birth compared to EXs produced in early gestation. EXs in breast milk were found to regulate the infant's immune response and inflammation. It has been suggested that EXs in breast milk contain miRNAs that can increase the number of T-regulatory cells in the blood, potentially modulating immunological tolerance (Foster et al., 2016).

The irregular formation, trafficking, and activity of EVs foster many non-gestational disorders, like neurological diseases, cancer, and infectious diseases as described by Shah et al. (2018). Similarly, abnormal placental EVs' synthesis, content, activity, and signaling may promote pregnancy disorders that have been extensively studied (James-Allan et al., 2020; Salomon et al., 2015, 2016)).

The literature confirms the many pathophysiological effects that placental EVs have on pregnancy-related disorders such as hyperglycemia (Salomon & Rice, 2017), gestational diabetes mellitus (Gillet et al., 2019; James-Allan et al., 2020), fetal growth restriction (FGR)/intrauterine growth restriction (IUGR, Miranda et al., 2018), preterm birth (Rubens et al., 2014), and preeclampsia. For example, preeclamptic women have a higher trophoblastic EV production than in normal pregnancies. Several proteins, including sFlt-1, endoglin,

tissue factor, and PAI, are also irregularly found in trophoblastic EVs, suggesting the potential for EVs to contribute to the pathophysiology of preeclampsia in pregnancy (Palma et al., 2021).

Therefore, because of their presence in the maternal circulation and varied functions at the feto-maternal interface, both in healthy and pathological pregnancies, placental EVs, especially EXs, have been of great interest in recent years as possible biomarkers for many pregnancy-related pathologies. They are also considered a therapy tool to combat diseases for their innate cargo or as "vehicles" to transport healing agents (Salomon & Rice, 2017). I am particularly interested in their miRNAs cargo as a potential biomarker or as a therapeutic tool as it compels with my thesis study, and for their role in containing C19MC miRNAs (Figure 1.20).

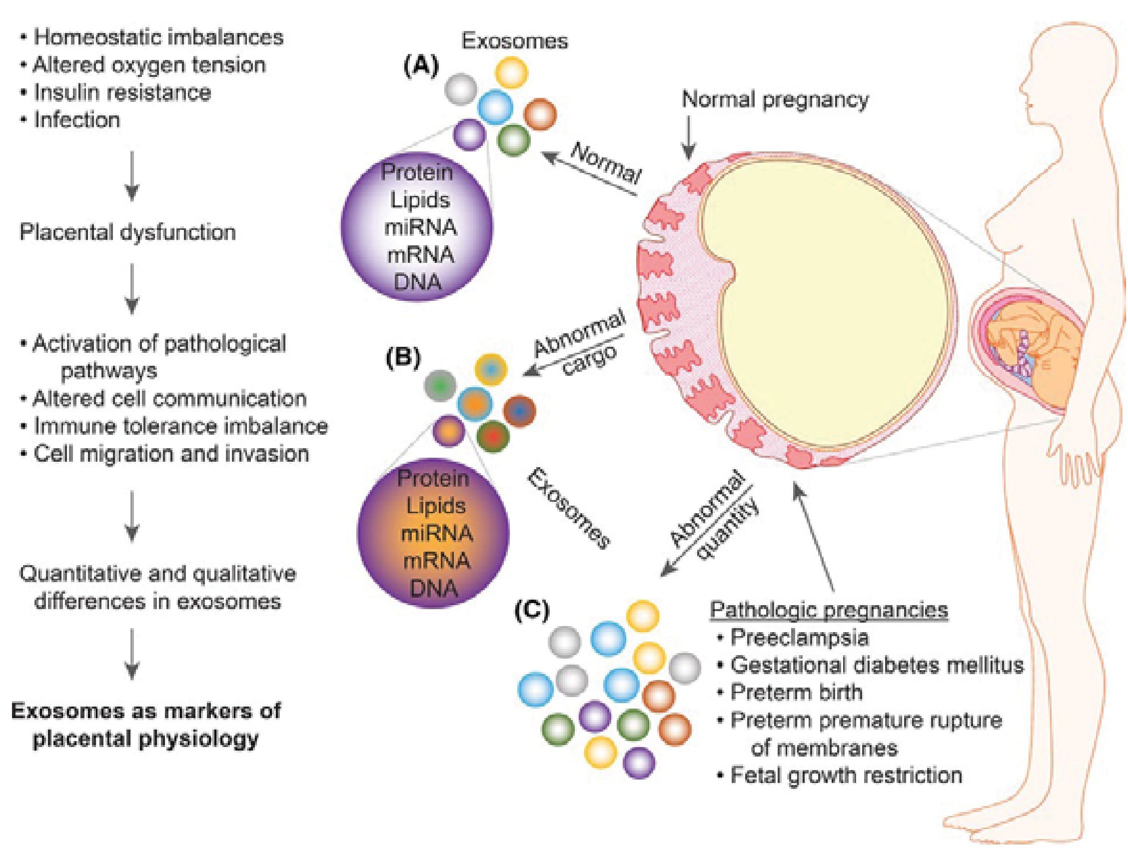


Figure 1.20: EVs in normal and pathological pregnancy. Many of the consequences of the abnormal quantities and cargo of EVs in pathological pregnancies (Jin & Menon, 2018).

1.7.2 EVs and Viruses

Although EVs and viruses such as retroviruses may have evolved separately, they have some structural and functional traits comparable to one another. These similarities may include biogenesis routes, size distribution, cargo, and cell-targeting mechanisms. This section will mention some of these similarities between viruses and EVs to be able to describe their interplay later.

Most viruses' sizes are between 20-500 nm, which overlaps with the range in size of EVs. Additionally, EVs and viruses are comprised of a lipid membrane. Some viruses like the HIV and vesicular stomatitis virus (VSV) may have a similar spherical shape found in EVs because of an extra lipid layer with viral envelope proteins (Colombo et al., 2014).

Moreover, viruses can be non-enveloped or enveloped, enclosing the nucleocapsid in a lipid layer. Enveloped viruses mirror EVs and suggest that viruses and EVs may employ similar entry pathways. Furthermore, EVs produced from virus-infected cells may contain viral miRNAs, thereby blurring the distinction between EVS from cells and EVs from newly replicated virions (Pegtel et al., 2010).

EVs and viruses use the endosomal system and MVBs for their formation/replication in cells along with essential proteins (ex. Rab27a/b50, SNAP, SNARE, ALIX1, TSG101) involved in exocytosis. However, evidence suggests that EVs are fundamentally different since they do not replicate in a host cell (Colombo et al., 2014; Jahn & Südhof, 1999).

Regarding cargo, viruses contain DNA or RNA that translates into essential viral proteins required for their propagation in the host cell. For instance, HCMV and HIV-1 may hijack the host's machinery to produce viral miRNAs that inhibit the cell's mRNA transcription and enhance viral replication, which is always to the detriment of the cell. Similarly, the miRNA cargo of EVs may dampen the host's gene expression promoting either pathological states (senescence, tumor growth, or edema) or, depending on which pathway is taken, may cause beneficial effects (Stern-Ginossar et al., 2007).

Viruses with their genomes in nucleocapsids transcribe miRNAs after entering the host.

On the other hand, EVs contain miRNAs upon egress of the cell of origin. Still, the specific pathway involved in the processing and delivery of the miRNA cargo to Argonaute proteins in RISC is unknown. They also share similar lipid content because of their biogenesis but differ in their protein content since EV contain cellular proteins and viruses produce only viral proteins that allow for cell entry and replication (Bartel, 2018; Kincaid & Sullivan, 2012).

EVs enter recipient cells to perform their functions, whereas viruses must do so to replicate. Therefore, EVs and viruses may employ similar cell identification, adhesion, and endosomal pathways for entry into cells. More is known of viruses and their uptake and subsequent processing in the host than EVs processing (Mathieu et al., 2019).

It has been suggested that fusogenic proteins such as syncytin1/2 (ERVW-1 and ERVFRD-1) regulate cell entry of sEVs and some enveloped viruses (HSV). Interestingly, these proteins are derived from human endogenous retroviruses and be highly expressed by trophoblastic cells and sEVs (de Lima Kaminski et al., 2019).

1.7.3 Interactions between EVs and Viruses

EVs generated during viral infection may perform pro- or antiviral functions in a system.

1. Antiviral effect of EVs:

   As a combat strategy, donor cells may provide surrounding and/or remote recipient cells RNAs and proteins contained in EVs that can disrupt viral replication and the production and release of viral progeny. For example, some EX-derived proteins like IFITM3 and APOBEC3G suppress viral replication of viruses such as DEN, ZIKV, HIV-1, Influenza, and Sars-covid. Nonetheless, HIV-1 expresses the viral infectivity factor (Vif) that counteracts the antiviral effect of APOBEC3G (Brass et al., 2009; Miyagi et al., 2007).

EXs may rebuild antiviral defense systems at the organ level, which involves the work effort of many cells. In essence, packaging antiviral factors to be transported in EXs to recipient cells can enhance the effect of suppressing the infection. For instance, Type I IFN (innate immune) limits viral infection until an adaptive immune response is formed. IFN may induce the packing of antiviral components into EXs and spread its "IFN" antiviral activity to different cell types by usurping these vesicles to enhance the immune activity. Exosomes from liver macrophages treated with IFN α, for example, showed an altered protein profile that elicited an antiviral response in hepatitis B virus-replicating hepatocytes, according to Yao et al. (2018). Additionally, miRNAs such as miR-423-5p are produced and sorted into EXs of MRC-5 cells infected by the rabies virus to decrease viral replication (J. Wang et al., 2019). Antiviral responses from EXs may occur within and across species. For instance, EXs from healthy human semen inhibit HIV-1 proviral transcription although the mechanism is unclear. Bacterial lactobacillus-derived EXs may also protect women against HIV-1 (Ñahui Palomino et al., 2019; Welch et al., 2018).

2. Proviral effects of EVs:

   Certain viruses, like retroviruses, employ the EV biogenesis pathway to assemble offspring virions, suggesting EVs may increase viral infectivity. As a result, virions are shrouded with host cell-derived EVs and egress from infected cells through the usual EV exocytosis route, evading immune monitoring. Viruses like hepatitis A and C may disguise themselves with cell-derived lipid membranes, gaining improved viral entry into cells and preventing antibody inactivation. EVs carrying numerous virion copies may overwhelm the host's defenses and accelerate viral propagation (Santiana et al., 2018; Schwab et al., 2015).

   In certain medical conditions, the composition of EXs, including vesicular protein and miRNA content, may be changed by integrating substantial quantities of viral compounds, thus increasing the viral permissivity of recipient cells (Ahsan et al., 2016;

Pegtel et al., 2010).

1.7.4 EVs antiviral role during Viral Congenital Infections

In the human placenta, EVs may confer protection against viral infections acquired in pregnancy. According to Delorme-Axford et al. (2013) EXs (not large vesicles), from primary human trophoblasts may induce an antiviral response that can be transferable to non-placental cells. Notable, ectosomes and EXs although similar in their miRNA content, both differ in their protein and phospholipid cargo. In line with this notion, between ectosomes and EX, the latter has been shown to provide the highest antiviral effect to non-trophoblastic cells (Ouyang et al., 2016).

The in vitro study utilized term placental cell conditioned media containing EXs that elicit resistance against different types of viruses like VSV, Coxsackievirus B, and Hepatitis C in non-placental cells. To some degree, this effect is accredited to the placental sEVs cargo, the C19MC cluster of miRNAs, that, as mentioned earlier, are primate and placenta specific (Donker et al., 2012; Ouyang et al., 2014).

Trophoblastic EXs may prevent placental viral infection by delivering miRNAs from the C19MC miRNA cluster such as miR-517a-3p to neighboring and non-placental cells by activating autophagy. The autophagy process activates PRRs signaling in response to PAMPs of invading viruses, directing them to autolysosomes for degradation, preventing viral replication. The syncytiotrophoblast's ability to release many EVs into the mother's circulatory system also contributes to the antiviral effect (Zaga-Clavellina et al., 2021).

1.7.5 Clinical Applications of EVs during Pregnancy

As mentioned before, EXs have been considered potential tools for diagnostics because their complex cargo can help detect and monitor diseases; and treatment, because of their intrinsic functional property in delivering their cargo to diseased cells. In recent years, EXs

have been found in almost all bodily fluids such as blood, urine, saliva, breast milk, cerebrospinal fluid, semen, amniotic fluid, and ascites. EXs from these fluids may represent the donor cell's physiological condition and identity as a "fingerprint" or "signature" Clinically, EXs can generally be taken from a patient's tissue or body fluids without major complications making them a practical method for prognosis (Jansen et al., 2017; Keller et al., 2011).

Therefore, EXs seem to be an applicable diagnostic tool because of the high accessibility of bodily fluids and subsequently their high EXs content; unfortunately, not a reality yet, although if accomplished, it will absolutely change how we perceive clinical diagnostics (J. Lin et al., 2015). Also, EXs as nanosized membrane vesicles have the unique ability to target specific cells or tissues. Their interaction with cells may facilitate the horizontal transfer of genetic material, which changes how recipient cells function (Conigliaro et al., 2017). In addition, EXs appear to have good biodistribution and biocompatibility with the host, making the macrophage system less likely to interfere with them. This is an advantage since the problem of immunogenicity can be avoided, and therapeutic agents can be delivered without the fast clearance and toxicity that would happen otherwise (Y. Zhang et al., 2019).

Chapter 2

Problematic and Objectives

2.1 Problematic

As mentioned earlier, the TORCH pathogens can cross the placenta, reach the fetal brain, and cause severe neurological damage. Specifically, viruses such as the HCMV and ZIKV can infect trophoblast cells in the placenta and disseminate to fetal brain cells. On the other hand, the literature has extensively described that the expression of the C19MC miRNAs by trophoblast cells confers viral resistance. These C19MC microRNAs may be contained in sEVs and may transfer this viral resistance to remote cells. However, specific experimental gaps in the in vitro studies can be identified in the literature. First, experimental results were based on the artificially forced expression or the overexpression of the C19MC miRNA cluster, or the introduction of specific C19MC miRNA mimics. These experimental methods may not reflect what actually occurs in a physiologically infected cell with "normal" quantities of C19MC miRNAs. Second, the various studies of the antiviral function of C19MC miRNAs were limited to term placentas. As discussed previously, the expression levels of C19MC miRNAs are at their highest near the end of gestation. Thus, these findings do not account for what actually unfolds during a congenital viral infection in the early stages of pregnancy, when C19MC miRNAs are less abundant, and when the risk of congenital infection by these

viruses is higher. Thirdly, findings were studied mostly from cells that were infected with the vesicular stomatitis virus (VSV). Considering that the antiviral impact may be virus-specific, the VSV viral model is of less relevance in congenital infections than other viruses like ZIKV and HCMV. In accordance with this rationale, our work employs a unique Knock-out (KO) model for the C19MC miRNA cluster to evaluate the antiviral function played by C19MC miRNAs.

Our collaborators at the University of Toulouse, led by Jérôme Cavaillé, utilized the human JAR choriocarcinoma cell line and the CRISPR-CAS9 technology to create three knock-out clones and three wild-type control cell lines. Traditionally, the JAR cell lines are employed as a model for invasive trophoblast cells, reflecting the early stages of pregnancy. Additionally, by utilizing the CRISPR-CAS9 system, the whole C19MC cluster gene was entirely deleted. This approach may compare C19MC-KO cells to cells that express natural physiological quantities of miRNAs (WT JAR cells), as opposed to cells that express an excessive amount or are compelled to express C19MC miRNAs. Furthermore, beside replicating the early phases of pregnancy, this study includes viral models (HCMV and ZIKV) that are known to severely affect the developing embryo. Thus, our research seeks to further validate (or not) by the use of a C19MC KO-model prior statements made by numerous research groups (such as the Sadovsky team) on the antiviral impact of the C19MC miRNA cluster during pregnancy.

Moreover, the precise method through which the C19MC cluster exerts its antiviral activity is yet unknown. Nonetheless, the large body of data implies that it is mediated in part by EXs and includes the host's autophagy response. Hence, this study explores the antiviral impact of the cluster C19MC during a congenital viral infection, and for the first time, a new C19MC-KO model is utilized to evaluate this effect.

2.2 General objective

This PhD study investigates the antiviral activity of the C19MC miRNA cluster during fetal viral infection, as well as the possibility that this action might be transferred to other cells via EXs.

- Examine how the miRNAs from the C19MC cluster exert an antiviral protective effect on cells, intracellularly or transitting in small extracellular vesicles, during congenital viral HCMV and ZIKV infections.

Specific objectives

- Understand the role of the C19MC miRNA cluster during viral infection by HCMV and ZIKV by examining the permissivity of the C19MC-KO JAR cells compared with WT JAR control cells.
- Examine the antiviral role of C19MC miRNAs by analyzing if this effect may be transferable to other cell types by EXs, hypothesizing that it may be done in a paracrine manner by the uptake by recipient cells of EXs containing C19MC miRNAs.
- Characterize in part the immune response of the C19MC-KO JAR and WT JAR cells during hCMV and ZIKV infection.

Chapter 3

Methodology

3.1 Cell Lines

JAR immortalized choriocarcinoma cells (ATCC HTB-144), Vero cells (ATCC CCL-81), and MRC5 human immortalized fetal lung fibroblast cells (ATCC CCL171) were cultured at 37 °C and 5% CO2 in Dulbecco's Modified Eagle Medium (DMEM with GlutaMAX, Gibco), supplemented with 10% fetal bovine serum (FBS, heat-inactivated, Sigma-Aldrich), 100 U/mL penicillin—100 $\mu g/mL$ streptomycin (Gibco), and 100 $\mu g/mL$ normocin (InvivoGen). The JAR cell line (3 JAR WT clones and 3 C19MC-KO clones) were obtained from Dr. Jérôme Cavaillé (CBI, Toulouse).

3.2 Viral production, titration, and infection

The ZIKV strain PF/2013 from the French Polynesian outbreak was propagated in Vero cells and viral stock was concentrated by centrifugation and filtration (0.22 μm filter). The VHL/E strain of the human cytomegalovirus used in the study was a gift from Dr. C. Sinzger from the University of Ulm in Germany (Stegmann et al., 2019). The VHL/E strain was propagated in MRC5 cells before the viral stock was concentrated by ultracentrifugation (Rolland et al., 2016). For both viruses, viral titration was performed by indirect immunoflu-

orescence against the Immediate Early (IE) antigen of hCMV and the ENV antigen for ZIKV following infection of MCR5 and Vero cells, respectively, by serial dilution of the viral stock (Rolland et al., 2016). In some cases, viral titration was achieved by the TCID50 assay from the cell culture supernatant. Cells were infected with HCMV or ZIKV for 2 hours at indicated multiplicity of infection (MOI) before removing the viral inoculum and replacing it with fresh media for some experiments.

3.3 Immunofluorescence

Cells were cultured on coverslips, fixed in 4% paraformaldehyde PFA (Electron microscopy sciences) for 20 min and washed with PBS; for permeabilization, cells were incubated in 0.3% Triton X-100 (Thermo Fisher Scientific) for 15 min at room temperature, and blocked with PBS supplemented with 5% FBS for 1 hour. Primary antibodies were diluted in blocking buffer, and cells were allowed to incubate overnight at 4 °C. Antibodies to the immediate early protein (IE, 1 $\mu g/ml$; Abcam CH160 ab53495) and the flavivirus group antigen 4G2 (Env, 1:400 dilution, Novus Biologicals, D1-4G2-4-15) were utilized. Secondary antibodies (goat anti-mouse or rabbit, Alexa-fluor 488, 2 $\mu g/ml$, Thermo Fisher Scientific) were diluted in blocking buffer, and incubation was done for 1 h at room temperature. After washing the cells with blocking buffer (3x) and with PBS (3x), cells were counterstained with 1 $\mu g/ml$ DAPI (Sigma) and rewashed three times with PBS before mounting the coverslips onto glass slides. Coverslip mounting was done using ProLong Gold without Dapi (Thermo Fisher Scientific). Coverslips widefield acquisitions were performed using an Apotome microscope (Zeiss) and image processing using ImageJ.

3.4 Western Blot

Culured JAR cells (infected or non-infected, WT and KO cells) and sEVs preparations were lysed in non-reducing conditions in Laemmli buffer and heated for 5 min at 95°C. Lysed

samples were then loaded into a mini protean TGX precast 4-20% gradient gels (Biorad) in Tris-glycine buffer. Electrophoresis was then performed for 2h at 70 V, followed by the electro-transfer of protein onto a nitrocellulose membrane by the use of the trans-blot turbo transfer system (Biorad). The membrane was blocked with the Odyssey blocking buffer (LI-COR Biosciences) for approximately 1h, and with different primary antibodies including mouse anti-CD81(200 ng/ml, Santa Cruz), mouse anti-CD63 (500 ng/ml, BD Pharmingen), mouse anti-CD9 (100 ng/ml, Millipore), rabbit anti-TSG101 (1 μg/ml, Abcam), rabbit anti-Alix (1 μg/ml, Abcam), rabbit anti-Env (1 μg/ml, GeneTex), mouse anti-IE (1 μg/ml, Abcam CH160 ab53495), mouse anti-actin (1 μg/ml, Abcam) then incubated overnight at 4°C in Odyssey blocking buffer, followed by incubation with secondary antibodies IRDye 700 anti-mouse IgG or IRDye 800 anti-rabbit IgG (LI-COR Biosciences) for 1 hour at room temperature. Membranes were washed with TBS 0.1% Tween 20 and incubated for 10 min, 3 times; then visualized with the Odyssey Infrared Imaging System (LI-COR Biosciences).

3.5 RNA extractions

According to the manufacturer's recommendations, total RNA was isolated from cells using chloroform extraction step for the TRIzol reagent protocol (Thermo Fisher Scientific) and RNeasy Plus Mini Kit (Qiagen). Using a NanoDrop spectrophotometer, RNA concentration and quality were carefully assessed following RNA extractions. Absorbance ratios of 260/280 and 260/230 were employed to determine the purity of the extraction. For both absorbance ratios a value of approximately 1.8 was considered "pure" for RNA.

3.6 RTqPCR Analysis

After RNA extraction, approximately 1μg of RNA was exposed to reverse transcription reaction using the LunaScript RT SuperMix Kit (New England Biolabs), following manufacturer's protocol. The acquired cDNA was then used as template in a qPCR reaction

using Sybr Green I Master Mix (Roche) and the following primers: hCMV-Forward: TGAG-GCTGGGAAGCTGACAT; hCMV-Reverse: TGGGCGAGGACAACGAA; ZIKV-Forward: TTGGTCATGATACTGCTGATTGC; ZIKV-Reverse: CCTTCCACAAAGTCCCTATTGC; β-actin-Forward: GTGCTGTCCCTGTACGCCTCT; and β-actin-Reverse: GGCCGTGGTG-GTGAAGCTGTA. The qPCR reactions were performed with a Roche LightCycler 480 apparatus using the following program: 95°C for 5 min, then 40 cycles of 95°C for 15s, and 60°C for 10s. The fold induction in viral transcript expression was quantified by calculating the $2^{-\Delta\Delta CT}$ value, with β-actin mRNA as internal control.

3.7 Intracellular Staining and Flow Cytometry

Fixation and permeabilization of the infected JAR WT and KO cells were performed using the BD Cytofix/ Cytoperm Plus kit, following the directions provided by the manufacturer. The envelope antigen was detected utilizing a dilution at a ratio of 1:400 of the Alexa fluor 488-conjugated mouse monoclonal antibody 4G2 (Novus Biologicals) after incubating for 25 minutes at 4°C. Cells and PKH67 stained-sEVs (previously described) were diluted 1:200 in filtered PBS. Their fluorescence was visualized using a Macsquant VYB Flow Cytometer (Miltenyi Biotec) by employing FCS and FITC fluorescence parameters and deducting the background of cell autofluorescence. To standardize sEV measurements, the Macsquant VYB was calibrated using the Megamix-plus SSC FITC (Biocytex Stago) beads. Using SSC side scatter, Megamix-plus SSC beads of varying sizes (160 nm, 200 nm, 240 nm, and 500 nm) were sorted by size. A gating approach was established for the 160 nm and 200 nm bead populations to examine events less than 200 nm in size. The sEVs' preparations were then measured according to the calibration beads parameters (gating on < 200 nm events), and their concentrations were established. Readings for each sample were counted twice. The FlowJo (BD) software was used to analyze the resulting flow cytometry data.

3.8 TCID50 Viral Dissemination Assay

C19MC-KO JAR and WT JAR cells were cultured and infected with ZIKV at indicated MOIs and times. The supernatants from both types of infected cells were collected and frozen at -80 ° C. Then, Vero cells were cultured in a 96-well plate and infected with the supernatant from C19MC-KO JAR and WT JAR cells (collected previously) at various dilutions (10^{-1} to 10^{-8}) and incubated for 4 days. After the incubation period, cytopathic effect and cell death were detected under the microscope. The Reed and Muench method was utilized to calculate the relative TCID50 under varying settings.

3.9 RT^2 Profiler PCR Arrays

Following RNA extraction, approximately 500 ng of RNA was reverse-transcribed to cDNA using the RT2 First Strand kit (Qiagen) according to the supplier's recommendations. The derived cDNA was used for RT2 profiler PCR arrays (Human Antiviral Response PAHS-122Z, and Autophagy Pathway PAXX-084Y, Qiagen), profiling 84 genes specific for innate immune response and for autophagy pathways; with the inclusion of five housekeeping genes as internal controls (ACTB, B2M, GAPDH, HPRT1, and RPLP0). Quantitative PCR reactions were performed using the Roche LightCycler 480 instrument with the following program: 95 ° C for 10 min, followed by 45 cycles of 95 ° C for 15 sec and 60 ° C for 1 min, and then a melting curve acquisition step. In addition, the RT2 profiler RT-PCR array data analysis software, version 5.1, carried out automated calculations to determine the fold change in gene expression and the Student's t-test.

3.10 Functional Assays

Two types of functional assays were performed in this study. First, Vero cells were cultured and exposed for 2h to conditioned or ultracentrifuged supernatant (during 16h at

100,000 g to deplete from extracellular vesicles) harvested from C19MC-KO JAR and WT JAR cells for 2h prior to ZIKV infection. Alternatively, Vero cells were also exposed to purified sEVs (approximately 1 million sEVs per well, e.g. 10 sEVs per cell) from C19MC-KO JAR and WT JAR cell preparations at the time of ZIKV infection. Infection was monitored, and after 24h and 48h post-infection, cells were stained and analyzed by flow cytometry for Env-positive cells. Data were then further analyzed by the FlowJo software.

3.11 Purification of Extracellular Vesicles

Serial differential ultracentrifugation was the technique of choice to purify EVs. To isolate sEVs from JAR KO and WT cells, 4 million cells of each cell type were seeded in 150 cm2 flask, with 3 flasks per cell type (3 flasks for WT and KO cells). Cells were seeded directly in "Exofree" media, and the conditioned culture medium was collected at 24 and 48h. Exofree media is culture medium that has been previously depleted from EVs from FBS. To prepare this EVs-free media, DMEM supplemented with 20% FBS, 100 U/mL penicillin—100 $\mu g/mL$ streptomycin (Gibco), and 100 $\mu g/mL$ normocin (InvivoGen) was ultracentrifuged at 100,000g for 16 hours at 4°C (rotor SW32Ti, with maximal acceleration and brake) and filtered with a 0.22 μm filter. The final medium was diluted 1:1 with DMEM to reach 10% FBS. The collected medium from JAR WT and KO cells was pooled accordingly and centrifuged at 1,200 g for 30 min to remove dead cells or large debris, followed by an ultracentrifugation at 12,000 g for 30 min (rotor SW32Ti, with maximal acceleration and brake) to discard large EVs (microvesicles mostly). One more ultracentrifugation step followed at 100,000 g (rotor SW32Ti, with maximal acceleration and brake) to obtain the sEVs pellet. The aggregate was then resuspended in 100 μl of PBS or diluent C (Sigma) to stain the vesicles with the lipophilic dye PKH67 (Sigma) per the manufacturer's instructions (1:1000 dilution, 5 min incubation time) followed by the last ultracentrifugation at 100,000 g (rotor SW32Ti, with maximal acceleration and brake) for 1h to wash the sample. Lastly, the sEV

pellet was resuspended in 100 μl of PBS and stored at -80°C. All previous ultracentrifugation steps were performed at 4°C, and PBS solution was filtered with a 0.22 μm syringe filter. The purification process of sEVs was performed according to the ISEV guidelines (Théry et al., 2018).

3.12 Nanoparticle Tracking Analysis (NTA)

Preparations of small extracellular vesicles were diluted 1:100 in filtered PBS using a 0.22 μm filter. Dilutions of sEVs were visualized and analyzed in the NanoSight LM10 (Malvern Panalytical) equipped with a 405 nm laser. Measurements were repeated three times. Three videos were recorded for each sample at a constant temperature of 22 °C for 60s. An installed Nanoparticle Tracking Analysis software 2.0 (Malvern instruments Ltd) was used to analyze all frames containing particles captured by the camera.

3.13 Statistics

Experiments were performed at least three times as indicated in the figure legends. GraphPad Prism (v8) software was used to perform data statistical analysis. One-way or two-way ANOVA tests were carried out, followed by Tukey's multiple comparison test for two-way ANOVA. p-value of less than 0.05 was considered to be statistically significant.

Chapter 4

Results

4.1 JAR C19MC-KO cells are more permissive to HCMV infection than JAR WT cells

As previously mentioned, C19MC miRNAs are expressed by trophoblast cells during pregnancy, and among their many functions, these microRNAs may confer an antiviral effect. To study the functional role of the C19MC miRNAs during a congenital viral infection, we used an immortalized placenta-derived choriocarcinoma cell line: the JAR cells. These cells are able to differentiate into syncytiotrophoblastic cells in vitro and produce gonadotropic hormones, similar to early placental trophoblasts, making them suitable for studying viral infections (Gru et al., 1994; Ruddon et al., 1980. Since other studies have relied mainly in C19MC miRNAs overexpression models (Delorme-Axford et al., 2013) we generated C19MC-KO JAR cells that do not express the C19MC miRNA cluster using CRISP-Cas9 technology and specific guide RNA (collaboration with Dr. Jérôme Cavaillé from the Centre de Biologie Integrative CBI, Toulouse). To minimize the off-target effects of the Cas9 enzyme, three different C19MC-KO and WT clones were created and selected to perform in vitro experiments (Boyle et al., 2017). The desired deletion was done on the maternal (silent) and paternal (active) alleles to generate WT JAR and C19MC-KO JAR cell lines, respectively. Despite their

viability, C19MC-KO JAR cells exhibit a decrease in cell proliferation rate and an increase in the proportion of cells in the G1 phase of the cell cycle (Figure 4.1).

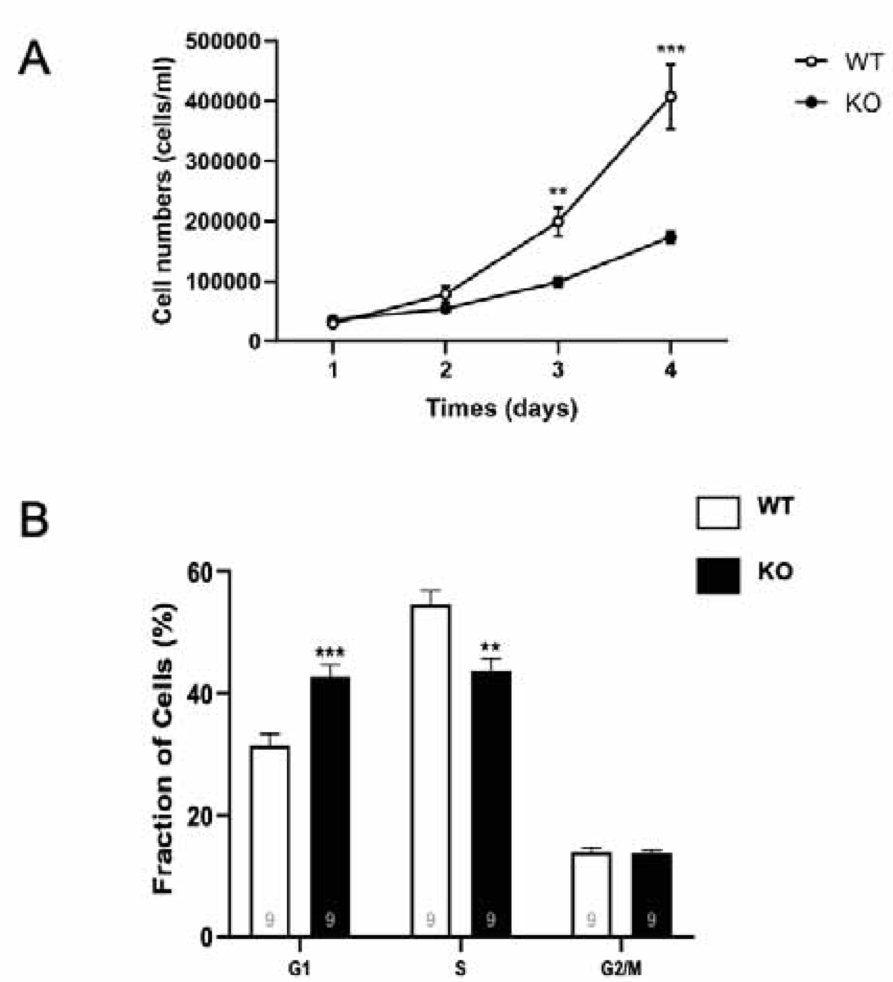


Figure 4.1: **C19MC-KO JAR cells show altered growth and cell cycle.** (**A**) C19MC-KO cells growth is decreased compared to WT JAR cells. N = 3 independent cell counting experiments including 3 WT JAR and C19MC-KO JAR clones. (**B**) C19MC-KO JAR cells have altered cell cycle profiles shown by FACS analysis of DNA content (DAPI staining) and EdU incorporation (Alexa Fluor 647 staining). N = 3 independent cell counting experiments including 3 WT JAR and C19MC-KO JAR clones. WT = white bars, KO = black bars. Data from J. Cavaillé's lab.

Using this cell model of the C19MC-KO JAR and WT JAR cells, we aimed to examine the permissiveness of cells lacking the C19MC miRNA cluster to HCMV infection. We infected the C19MC-KO JAR and JAR WT cells with HCMV at a multiplicity of infection (MOI) of 3 for 48 hours. All of the 3 clones for C19MC-KO JAR and for WT JAR cells were infected with HCMV in 3 individual replicate experiments. After infection, immunofluorescence assay (IF) was done against the HCMV immediate early protein (IE) (Figure 4.2A). The IE viral protein is generated as a nuclear-localized, multifunctional viral transcription regulator early during the HCMV replication cycle. Interestingly, we observed that after 48 hours post-infection, expression levels of the IE protein were higher for C19MC-KO JAR cells (infection shown by the presence of the IE protein stained in green) and that in contrast WT JAR cells were nearly resistant to HCMV since a negligible number of cells expressed the IE. These results were consistent with results obtained by western blot (Figure 4.2B), which revealed that expression levels of the IE protein were higher for C19MC-KO JAR cells and reduced for WT JAR cells. Additionally, quantitative western blot analysis showed that expression of the HCMV IE protein was 15-fold higher for C19MC-KO JAR cells than for WT JAR cells at 48hpi.

In order to validate these results at the transcription level, we next performed RTqPCR (Figure 4.2C). We confirmed the previous findings, demonstrating that the IE and the viral major envelope glycoprotein B (UL55) mRNAs were over-expressed for C19MC-KO JAR cells compared to WT JAR cells. Expression of the IE mRNA was 6.42-fold greater in C19MC-KO JAR cells than in WT JAR cells at 24hpi. The viral glycoprotein B is expressed in the last stages of HCMV cycle, after viral genome replication, and is involved in viral particle formation. For UL55, mRNA expression levels were 8.71 times greater in C19MC-KO JAR cells than in WT JAR cells at 48hpi. Collectively, our data indicate that C19MC-KO JAR cells are significantly more permissive to HCMV compared to WT JAR cells which appear to be nearly resistant to HCMV infection.

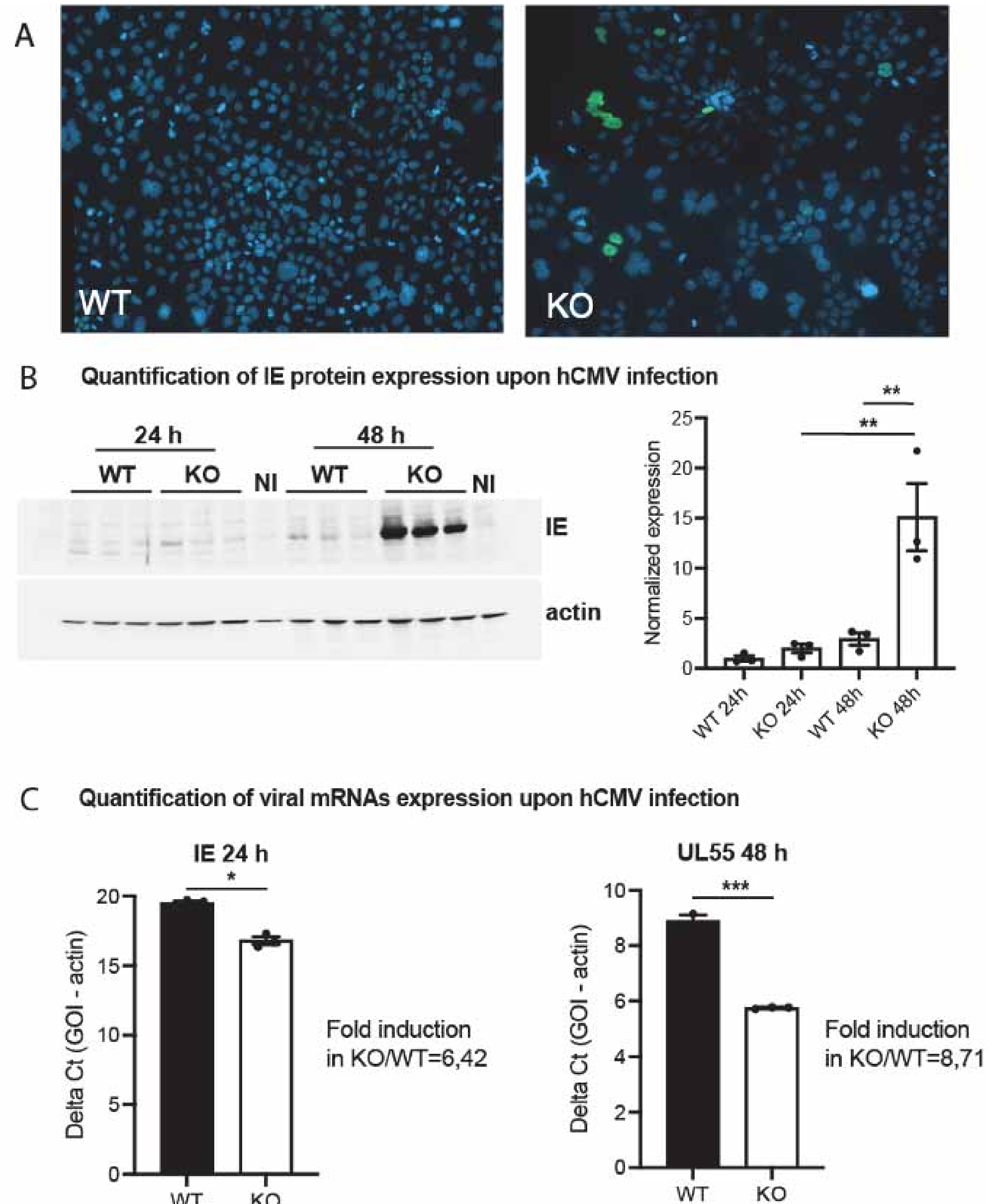


Figure 4.2: **C19MC-KO JAR cells are more permissive to HCMV infection than WT JAR cells.** (**A**) Immunofluorescence against IE antigen is done at 48h post-infection at MOI 3 (green: IE, blue: DAPI). Scale bar = 20 μm. Image representative of at least 3 independent experiments. (**B**) Left panel: Western-blot experiment done on WT JAR and KO-C19MC JAR cells' extracts at indicated times post-infection by HCMV at MOI 3 (NI: non-infected). The WT and KO wells correspond to 3 independent clones for each. Image representative of 3 independent experiments. Right panel: Quantification of the western-blot experiments for 3 independent replicates performed each time on 3 independent clones. Histograms represent the mean ± SEM. (**, $p<0.01$ by Tukey's multiple comparisons test). (**C**) Left panel: Quantification of IE mRNA expression at 24h post-infection at MOI 3 (normalized by actin) for 3 independent replicates realized each time on 3 independent clones. Right panel: Quantification of UL55 mRNA expression at 48h post-infection at MOI 3 (normalized by actin) for 3 independent experiments performed each time on 3 independent clones. Histograms represent the mean ± SEM. (*, $p<0.05$; ***, $p<0.001$ by Tukey's multiple comparisons test).

4.2 C19MC-KO JAR cells are more permissive to ZIKV infection than WT JAR cells

To continue further, we infected C19MC-KO JAR and WT JAR cells with ZIKV for 24h at MOI of 1. We fixed and stained the cells against the flavivirus envelope-protein antigen and conducted an immunofluorescence experiment (Figure 4.3A). The structural envelope protein (Env) is present during the ZIKV replication cycle in the cytoplasm, indicating viral dissemination in cells. Microscopy analysis revealed that C19MC-KO JAR cells expression of the Env protein was considerably higher showing a large proportion of cells, approximately 75% infected. In contrast, only 20% of JAR WT cells show an active ZIKV infection. These results suggest that altogether JAR KO cells are considerably more permissive to ZIKV infection than WT JAR cells .

Next, flow cytometry analysis on C19MC-KO JAR and WT JAR cells infected at MOI 1 and 3 at different time frames (6, 16, and 24 hours) showed consistent results (Figure 4.3B). C19MC-KO JAR cells were highly positive for ZIKV Env antigen by flow cytometry at 16 and 24hpi compared to WT JAR cells. Nonetheless, for both cell types, infection increased as time progressed, except at 6hpi which showed inconclusive results. We suspect that 6h wasn't sufficient time for the virus to complete a replication cycle in JAR cells. In addition, the ZIKV show to successfully achieved replication in JAR cells based on the presence of infectious particles in the cell's supernatant (Figure 4.3C). C19MC-KO JAR cells show a higher release of ZIKV virions into the supernatant at 24hpi than WT cells (MOI 1 and 3). At MOI 1, JAR KO cells exhibit a 10-fold increase relative to WT JAR cells, while at MOI 3, KO cells display a 10^3-fold increase relative to WT cells. Overall, by immunofluorescence, flow cytometry, and TCID50, we were able to demonstrate that C19MC-KO JAR cells appear to be more permissive to ZIKV infection.

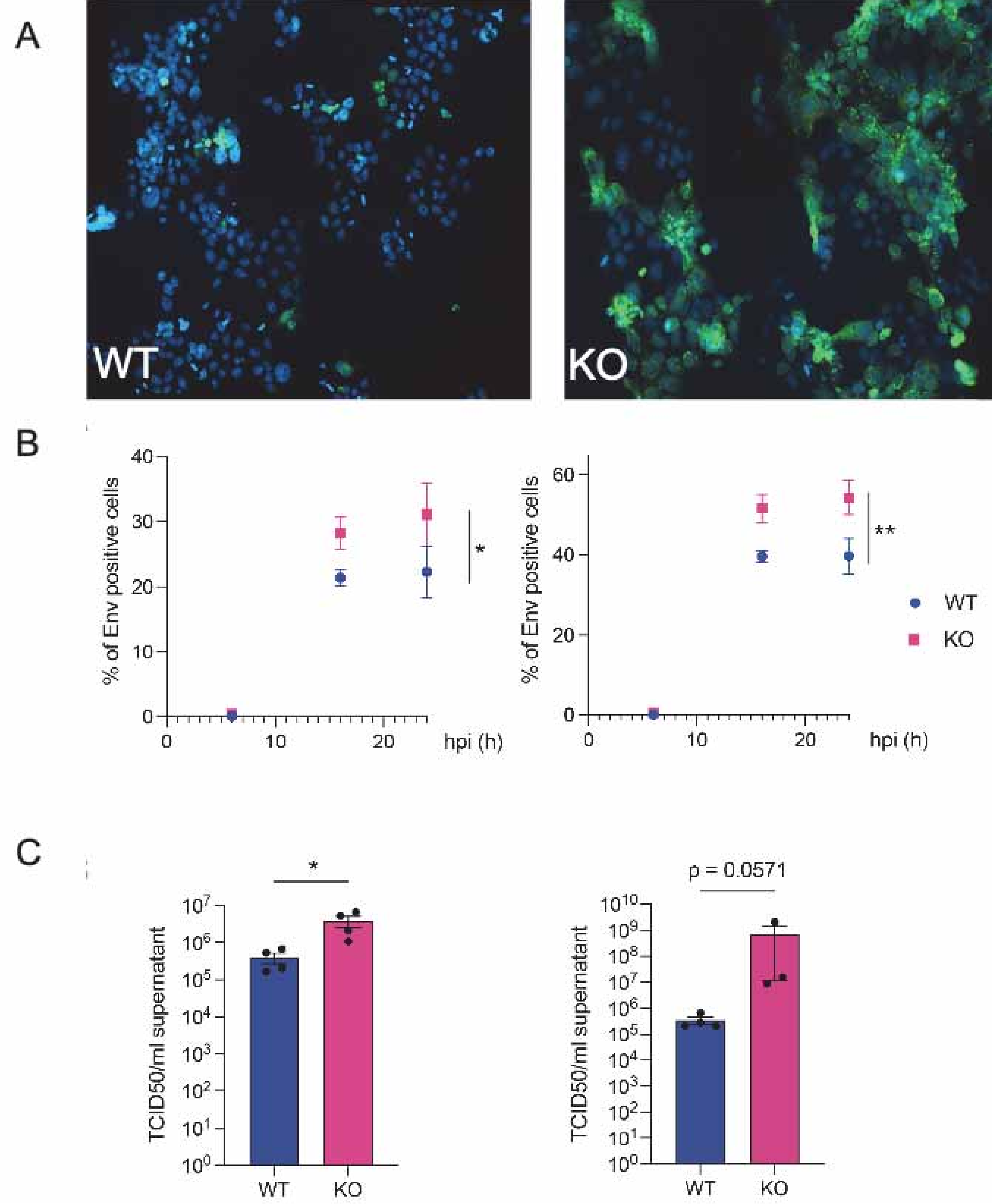


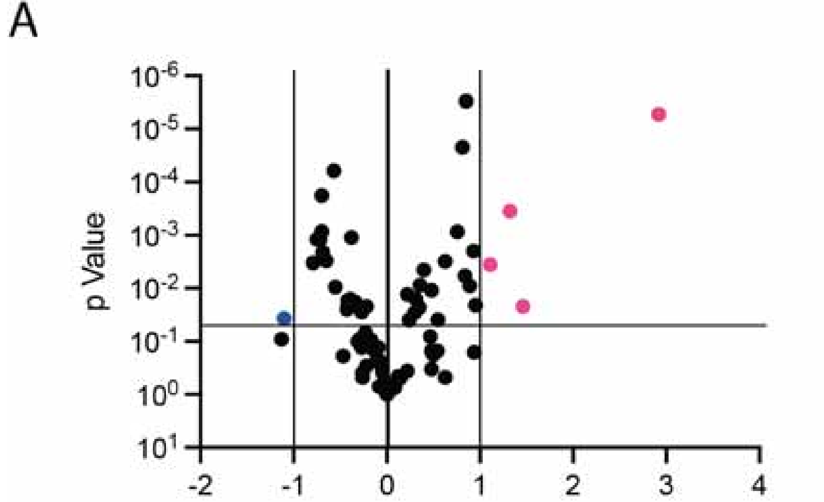
Figure 4.3: **C19MC-KO JAR cells are more permissive to ZIKV infection than WT JAR cells.** (**A**) Immunofluorescence against Env antigen is done at 24h post-infection at MOI of 1 (green: Env, blue: DAPI). Scale bar = 20 μm. Image representative of at least 3 independent experiments.(**B**) Quantification of the percentage of ZIKV infected cells by flow cytometry experiments, during different times post-infection. Cells were inoculated with ZIKV at MOI of 1 (left panel) or 3 (right panel). Dots and error bars represent the mean ± SEM of 4 independent experiments. (*, $p<0.05$; **, $p<0.01$; 2-way ANOVA statistical test for "C19MC" factor). (**C**) Quantification of viral infectious particle release at 24h post-infection by TCID50 titration on the cell's supernatant. Cells were infected at MOI of 1 (left panel) and MOI of 3 (right panel). Histograms represent the mean ± SEM of 3 to 4 independent experiments. (*, $p<0.05$; by Mann Whitney test).

4.3 C19MC-KO JAR and WT JAR cells show differences in antiviral pathways upon ZIKV infection

Furthermore, the expression levels of 84 genes involved in the innate antiviral response were analyzed in both infected C19MC-KO JAR and WT JAR cells by means of the Qiagen RT^2 profiler array by the "Antiviral pathway" device (Figure 4.4a). As expected, we observed that after 24h of infection at MOI of 10, several genes' mRNA expression dramatically increased and some decreased, in C19MC-KO JAR and WT JAR cells in response to viral infection (data not shown). However, the levels of induction of some genes were different in C19MC-KO JAR cells compared to WT JAR cells upon ZIKV infection.

Infection of the cells by ZIKV significantly induced the over-expression of several genes in C19MC-KO JAR cells including APOBEC3G (apolipoprotein B mRNA editing enzyme catalytic subunit 3G, involved in innate antiviral immunity), the cytokine CXCL8 (also known as Interleukin 8), CARD9 (Caspase Recruitment Domain Family Member 9), and PSTPIP1 (Proline-serine-threonine phosphatase-interacting protein 1) in comparison to WT JAR cells. For example, APOBEC3G was up-regulated approximately by 8-fold in C19MC-KO JAR cells compared to WT JAR cells. On the contrary, the down-regulation of the mRNA encoding the NLRP3 protein was elicited upon ZIKV infection in WT JAR cells compared to C19MC-KO cells by 2-fold (Figure 4.4b).

We compared the expression of the mRNA encoding these proteins (APOBEC3G, CXCL8, CARD9, PSTPIP1, and NLRP3) across both cell types upon ZIKV infection (Figure 4.5). In contrast to C19MC-KO JAR cells in which expression of several genes including APOBEC3G and CARD9 was up-regulated, their expression was considerably down-regulated in WT JAR cells. Moreover, similar expression levels of PSTPIP1 and NLRP3 were observed, being both genes also down-regulated by both cell types. Expression levels of CXCL8 were up-regulated in both cell types.



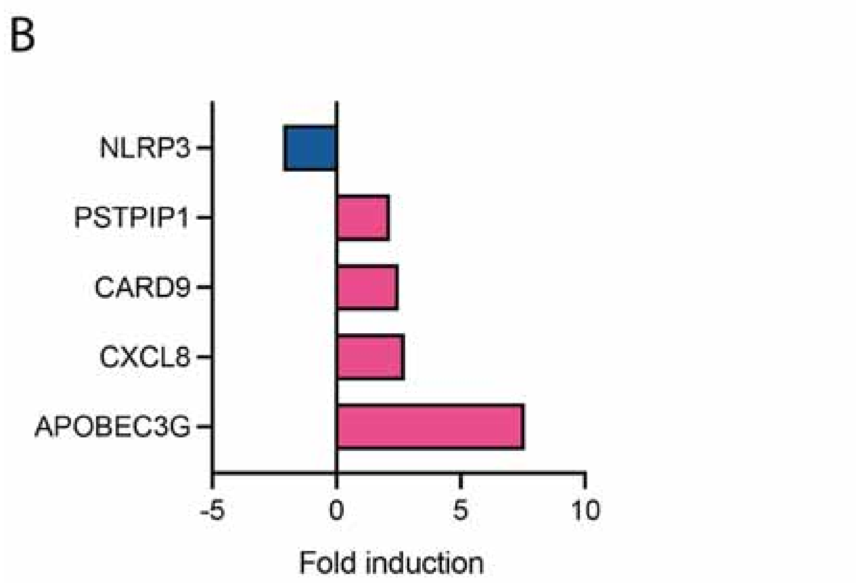


Figure 4.4: **WT JAR and C19MC-KO JAR cells show differences in antiviral pathways upon ZIKV infection.** (**A**) Volcano-plot representing differences in normalized mean mRNA expression at 24h upon ZIKV infection in C19MC-KO JAR cells compared to WT JAR cells, from three independent experiments. mRNAs exhibiting significant differences upon infection are represented by colored circles (Student T-test p-value <=0.05 and log2 ratio >=2 or <=-2). (**B**) Detail of the under- and over-expressed mRNAs upon ZIKV infection in C19MC-KO JAR vs WT JAR cells.

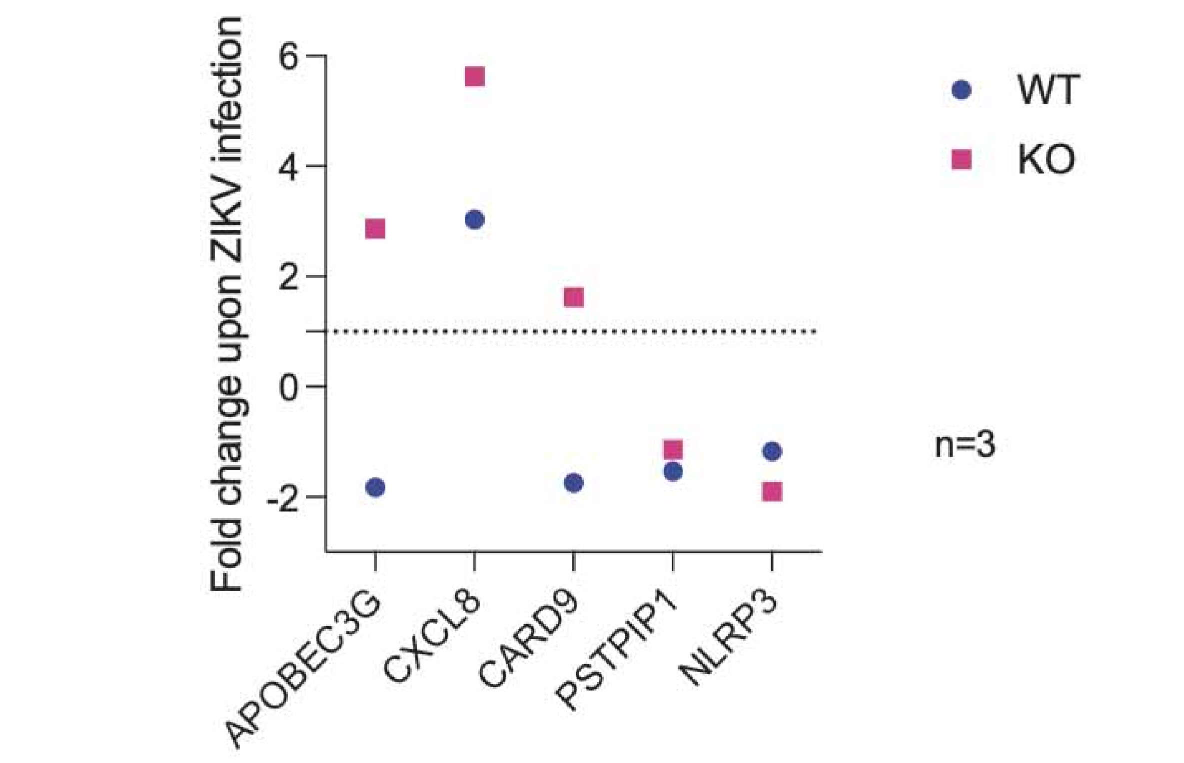


Figure 4.5: **RT^2 profiler analysis of antiviral pathways upon ZIKV infection of WT JAR and C19MC-KO JAR cells.** Graphical representation of the fold change in the gene expression implicated in the innate immune response upon ZIKV infection, shown independently for WT JAR and C19MC-KO JAR cells.

Similarly, we also examine the expression levels of 84 genes involved in autophagy upon a ZIKV infection in both cell types, using the Qiagen RT^2 profiler array "Autophagy pathway" device (Figure 4.6). Among the 84 mRNAs examined, few had their expression modified upon ZIKV infection (data not shown). The mRNA encoding FAS (Cell Surface Death Receptor, apoptosis-inducing receptor), GABARAPL1 (Gaba type A receptor-associated protein-like 1), and ATG9B (a lipid scramblase that mediates autophagosomal membrane expansion) were found to be slightly upregulated and down-regulated, respectively, upon ZIKV infection in both C19MC-KO JAR and WT JAR cells. However, no differences in expression levels between the two cell types were observed (Figure 4.6).

Alternately, other genes such as IFNG (Interferon gamma, from the interferon family of cytokines II) involved in the innate and acquired defenses against pathogens was considerably down-regulated for C19MC-KO JAR cells compared to WT JAR cells (Figure 4.6). We also found a slight difference in gene expression for TMEM74 (transmembrane protein 74), known to play an essential role in autophagy. Its expression was down-regulated for both cell types, but more so for C19MC-KO JAR cells (approximately 3 folds). Additionally, the TNF (Tumor Necrosis Factor), a soluble proinflammatory cytokine known to induce caspase-dependent apoptosis and autophagy, was significantly upregulated for WT JAR cells by 100-fold compared to C19MC-KO JAR cells (50-fold). These data suggest distinct antiviral pathways in relation to autophagy upon ZIKV infection in C19MC-KO JAR cells and WT JAR cells but they have to be interpreted with caution since they are the result of two independent experiments only.

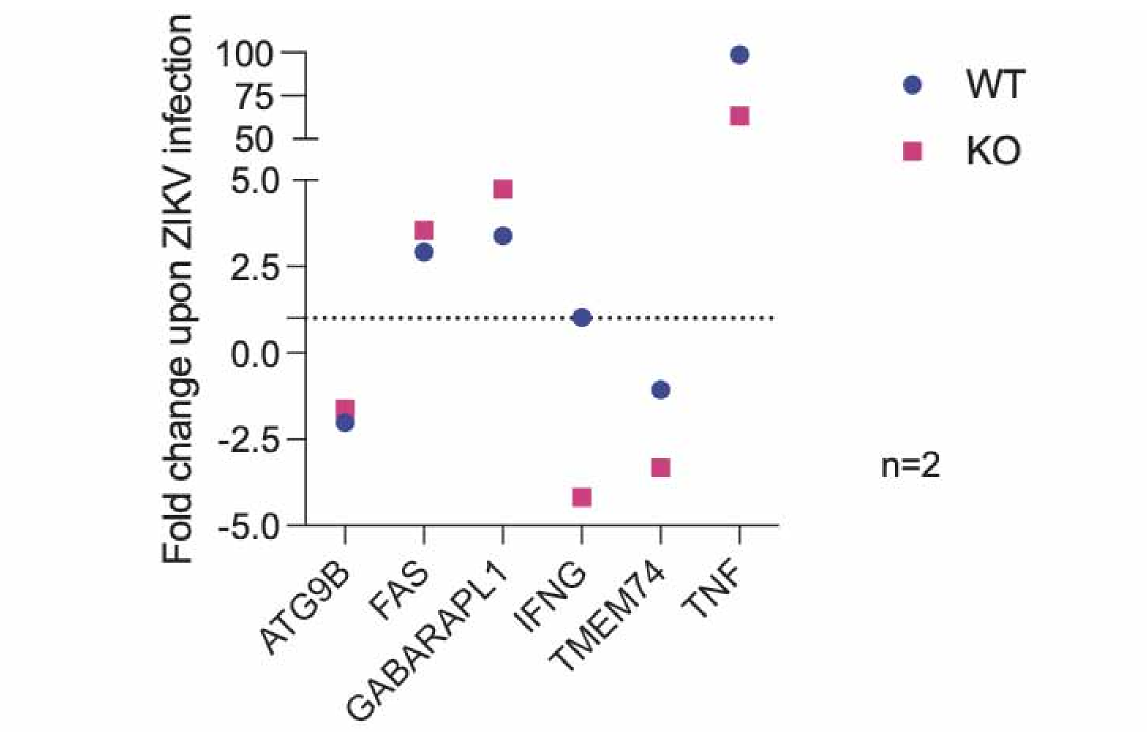


Figure 4.6: **RT^2 profiler analysis of autophagy pathways upon ZIKV infection of WT JAR and C19MC-KO JAR cells.** Graphical representation of the fold change in the gene expression implicated in the autophagy pathway upon ZIKV infection, shown independently for WT JAR and C19MC-KO JAR cells.

4.4 Supernatant from WT JAR cells exert an antiviral effect against ZIKV infection in recipient cells, in contrast to C19MC-KO JAR cells

To investigate whether the antiviral resistance of C19MC miRNAs in JAR WT cells could be transferred to cells which do not typically express these miRNAs, we used the non-placental Vero cells. First, we pre-exposed Vero cells to conditioned supernatant from WT JAR cells and C19MC-KO JAR cells for 2 hours. Then, we infected with ZIKV at MOI of 1 for 24h and 48h. The identical procedure was conducted but Vero cells were instead subjected to conditioned medium from WT JAR cells and C19MC-KO cells that had been previously ultracentrifuged for 16h at 100,000 g to deplete it from EVs. Vero cells were also infected with ZIKV only without exposing them to conditioned supernatant as control cells (Figure 4.7A).

We discovered that exposing recipient Vero cells for 2h prior to infection to WT JAR conditioned supernatant (derived from WT JAR cells 48h after seeding) significantly reduced ZIKV replication in Vero cells (Figure 4.7B). This impact was found 24 hours post-inoculation in both WT JAR supernatant (SN WT) and ultracentrifuged WT JAR supernatant (SN WT UC) depleted of EVs. In contrast, C19MC-KO JAR cell-conditioned media (conditioned and supernatant devoid of EVs) had no effect on ZIKV infection in Vero cells. After 48h of infection, we observed that the antiviral effect reported at 24hpi in Vero cells exposed to SN WT UC was no longer present. However, an antiviral effect continue to be observed for cells exposed to SN WT (Figure 4.7C).

Our observations demonstrate that the supernatant of WT JAR cells (SN WT and SN WT UC) provides antiviral protection against ZIKV infection in recipient Vero cells. These results

further imply that WT JAR cells release specific components (IFN III, free-range C19MC miRNAs) into the supernatant that may initially (24h) cause a transferable antiviral action in recipient cells independent of the presence of EVs. However, these unstable compounds disappeared upon the onset of viral infection (48h). Notwithstanding, we see an antiviral effect at 48h for SN WT, suggesting that the more stable EVs might be also implicated in ZIKV viral resistance in Vero cells.

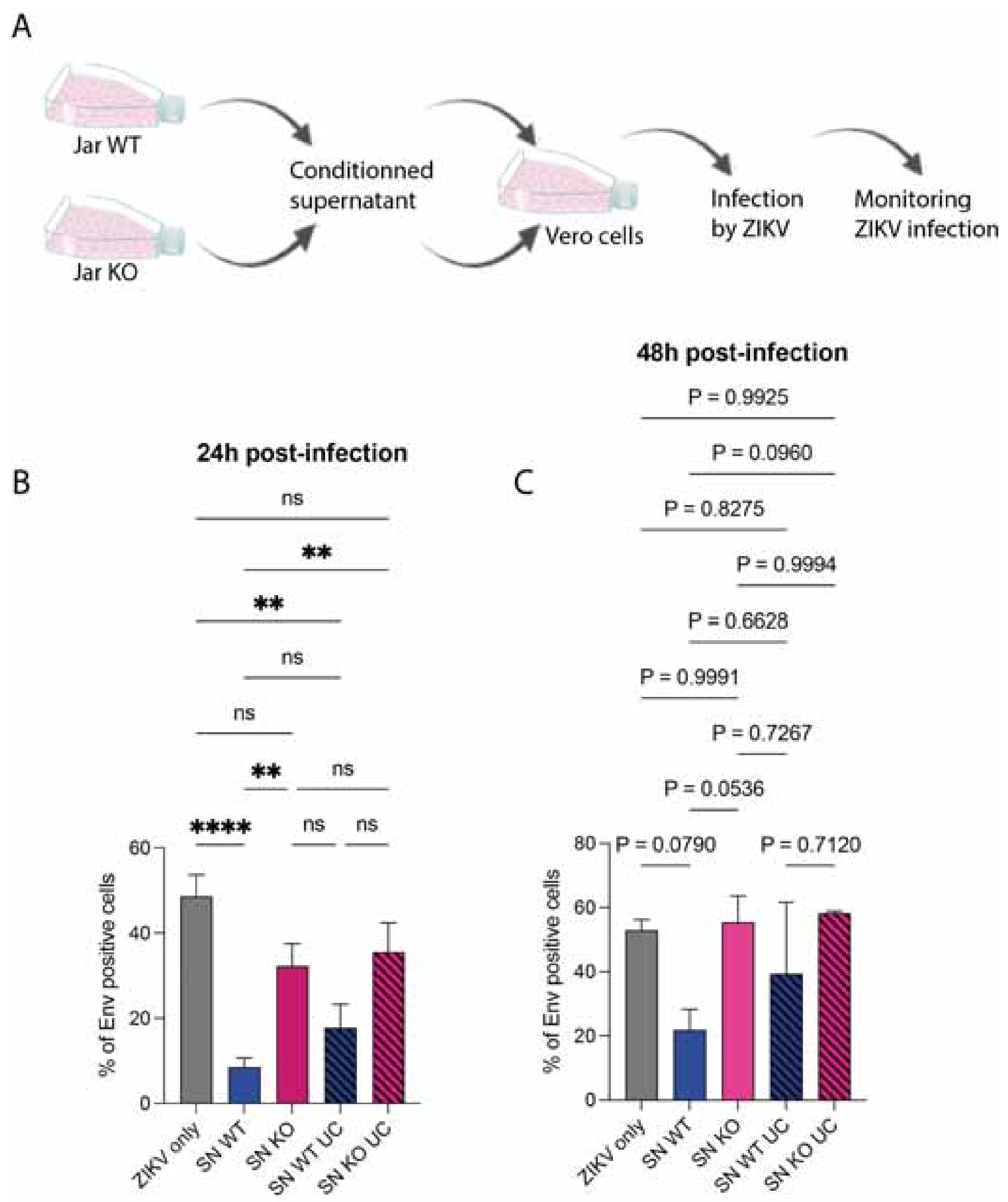


Figure 4.7: **Supernatant from WT JAR cells exert an antiviral effect upon ZIKV infection, in contrast to C19MC-KO JAR cells' supernatant.** (**A**) Experimental pipeline of the supernatant transfer experiments. (**B**) and (**C**) Percentage of Vero cells infected by ZIKV at MOI of 1 during 24h (B) or 48h (C) post-infection. Vero cells were either infected by ZIKV only or upon 2h of pre-incubation with clarified supernatant from either WT JAR (SN-WT) or C19MC-KO JAR (SN-KO) cells or supernatant that has been ultracentrifuged during 16h at 100,000 g to deplete from extracellular vesicles (SN WT UC or SN KO UC respectively) prior to cell culture. Histograms represent the mean ± SEM from 7 independent experiments for B, and 5 independent experiments for C. (ns, non-significant; **, $p<0.01$; ****, $p<0.0001$ by Tukey's multiple comparisons test).

4.5 Small extracellular vesicles from C19MC-KO JAR and WT JAR cells initial characterization for functional assays

Then, to evaluate the effect of sEVs from the JAR model using functional tests, we attempt to isolate and initially characterize sEVs from WT JAR and C19MC-KO JAR cells. Based on ISEV guidelines, we evaluated several protein markers of purified sEVs preparations from WT JAR and C19MC-KO JAR cells by western blot and assessed their size and concentration by Nanoparticle Tracking Analysis (NTA). Western blot results have shown that sEVs' preparations expressed the specific vesicular markers Alix, TSG101, CD9, and CD63 (Figure 4.8), which partly confirms the quality of the sEVs' purification technique and preparation (Théry et al., 2018). This western blot assessment didn't identify the presence of the CD81 protein marker. These findings correlate to the endosomal origin of sEVs confirmed by the expression of transmembrane proteins CD63 and CD9 (Mathieu et al., 2021). However, we did not assess the potential endoplasmic reticulum or mitochondrial vesicle contamination of the sEVs' preparations from WT JAR and C19MC-KO JAR cells which we plan to do in the future. (Théry et al., 2018). NTA was used to acquire the yield and the relative size distribution for the sEVs preparations isolated from WT JAR cells and C19MC-KO JAR cells. C19MC-KO JAR cells appear to have produced lower quantities of sEVs than WT JAR cells, the concentrations of sEV nanoparticles was higher for WT JAR cells at 2-3^{10} sEV nanoparticles per ml. This lower secretion of sEVs by C19MC-KO cells may be due to the delayed growth rate and altered cell cycle of C19MC-KO cells. We also calculated the mean and the mode for the sEV preparations by NTA (Figure 4.9 A). Since our size data contain some outlier values, we found it most informative to examine the mode sizes of sEVs from WT JAR cells and C19MC-KO cells, which appear to be significantly comparable, showing that most of the sEVs fall in sizes smaller than 200 nm (Figure 4.9 B-C).

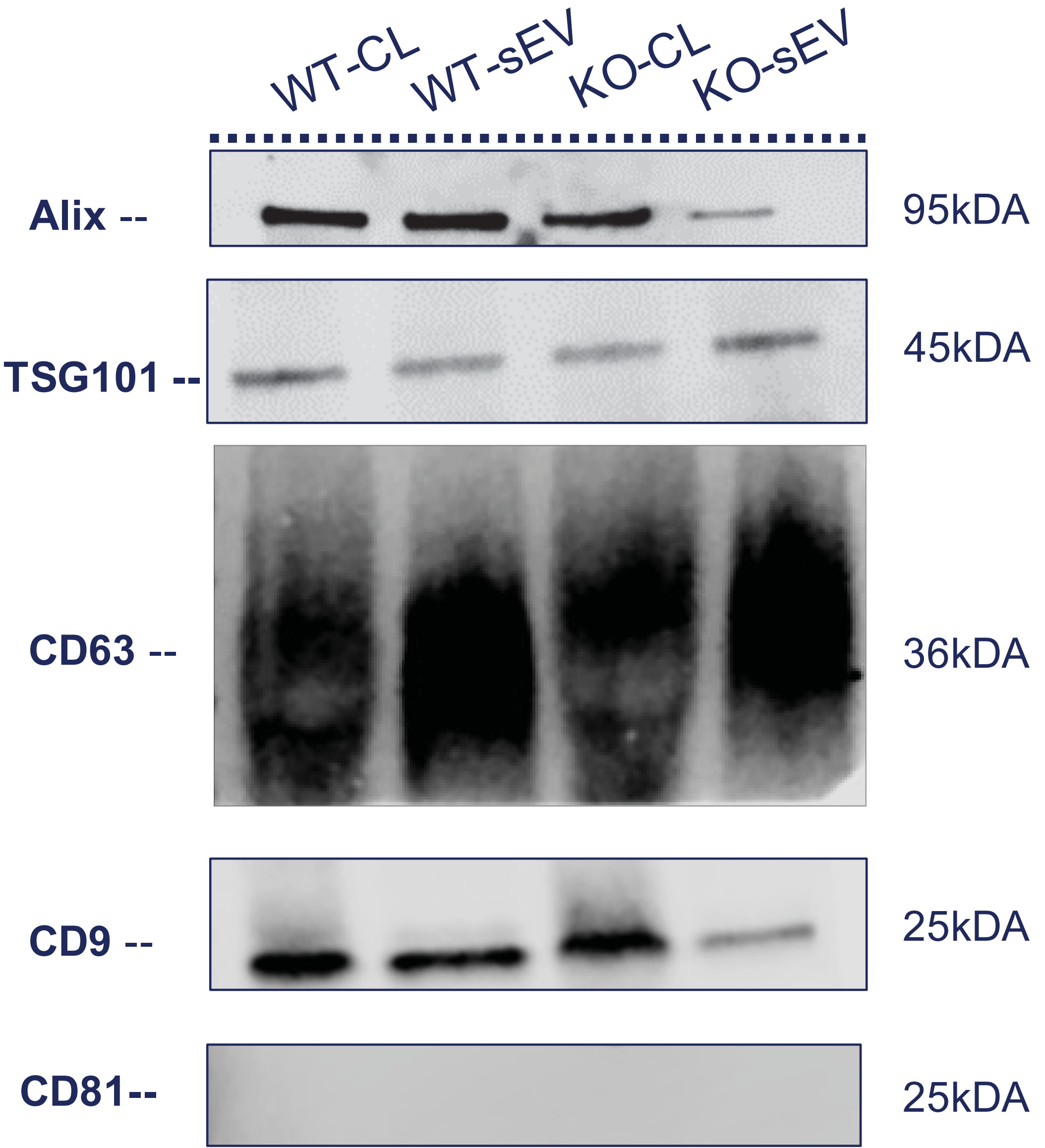


Figure 4.8: **Protein markers characterization of sEVs from C19MC-KO JAR and WT JAR cells.** Western blot analysis of whole cell lysate (WT-CL, KO-CL) or purified sEVs from WT JAR and C19MC-KO JAR cells (WT-sEV, KO-sEV). The protein of interest is indicated on the left side, and their corresponding molecular weights on the right side of the Figure. A smear (36-75kDA) is shown for the transmembrane protein CD63 because of the western blot non-reducing conditions, in this way preserving its intensified glycosylated pattern.

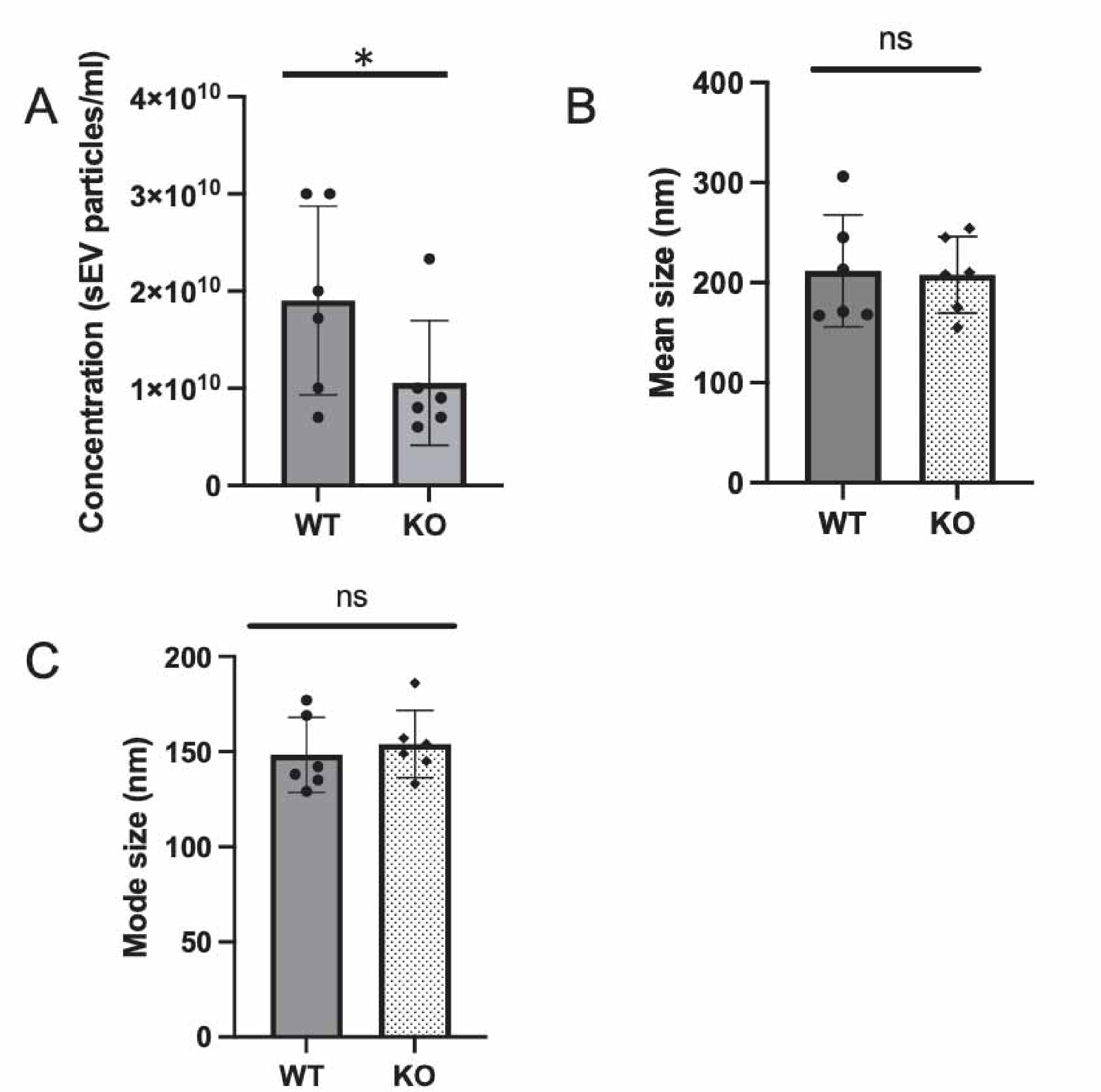


Figure 4.9: **Nanoparticle Tracking Analysis of sEVs from WT JAR and C19MC-KO JAR cells.** (**A**) Concentrations from sEVs' preparations from WT JAR and C19MC-KO JAR cells calculated by NTA experiments. *, p = 0.0312 by Mann Whitney test for 6 independent experiments and (**B**) Mean (**C**) and Mode size comparison between sEVs prepared from WT JAR and C19MC-KO JAR cells by NTA experiments. Histograms represent the mean ±SEM for 6 independent experiments. ns: non-significant by Mann Whitney test.

4.6 Small extracellular vesicles from WT JAR and C19MC-KO JAR cells' impact on Vero cells upon ZIKV infection

As discussed in the introduction, miRNAs can be conveyed via sEVs as a means of intercellular communication. It has been shown that the C19MC miRNAs are the most prevalent miRNAs species in trophoblastic cells and in placental EXs (Donker et al., 2012; Noguer-Dance et al., 2010; Y. Zhang et al., 2019). Since results in the previous section indicated a transferable antiviral effect from WT JAR supernatant in Vero cells and that this effect is not seen upon ultracentrifugation of the supernatant, we seek to determine if it is associated with the uptake of sEVs (containing C19MC miRNAs) released from WT JAR cells. We purified sEVs from WT JAR and C19MC-KO JAR cells' culture medium by serial differential ultracentrifugation. The previous section described the protein markers assessed by western blot and the size measurements, partly confirming that preparations of sEVs were obtained. However, not all of the controls suggested by ISEV to certify their purity have been obtained for the moment. Then, we exposed Vero cells to sEVs from WT JAR cells and C19MC-KO cells for 2h prior to infection with ZIKV at MOI of 5 for 24h. We assesed this experiment by flow cytometry and our results (n=1) revealed that infection by ZIKV was attenuated in Vero cells exposed to sEVs from WT JAR cells. A decreased number of Env positive Vero cells exposed to WT JAR sEVs were observed. In contrast, sEVs from C19MC-KO JAR cells had no effect on ZIKV replication in Vero cells, indicating the role of C19MC miRNAs in conferring resistance to non-placental cells. Additional experiments are needed to confirm these data.

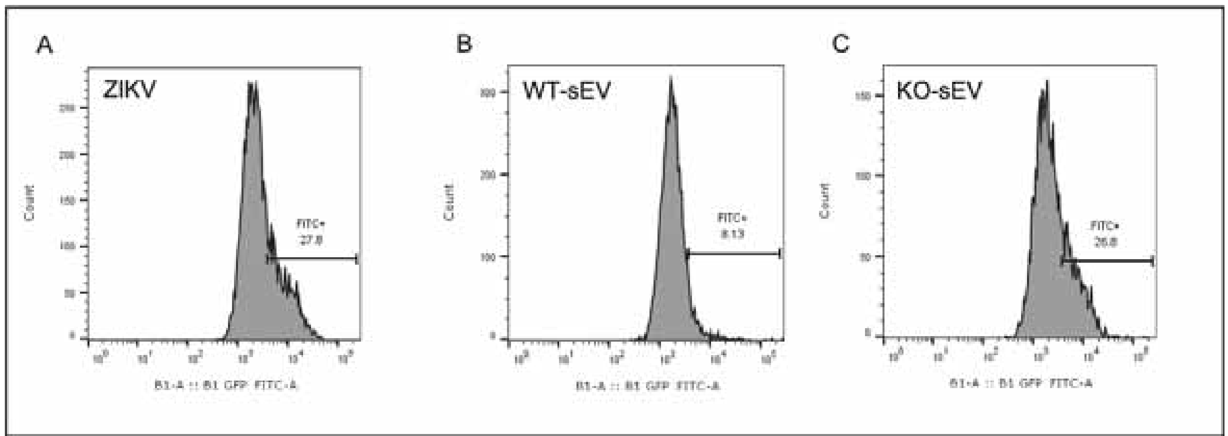


Figure 4.10: **Small extracellular vesicles from WT JAR and C19MC-KO JAR cells' effect on Vero cells upon ZIKV infection.** Representative flow cytometry graphs for Vero cells labeled with FITC-tagged Env coupled 488 antibody showing percentage of positive cells upon ZIKV infection. (**A**) Vero cells infected with ZIKV and (**B**) incubated with sEVs from WT JAR cells and (**C**) incubated with sEVs from C19MC-KO JAR cells upon ZIKV infection at MOI of 5 for 24h. N=1 experiment.

Chapter 5

Discussion and Future Perspectives

The placenta guards the developing embryo from pathogens. However, certain microorganisms are able to pass the placenta and infect the embryo, resulting in debilitating impairments. Placental trophoblast cells release microRNAs (in EVs) that may exert viral resistance in recipient cells (Bayer et al., 2015; Delorme-Axford et al., 2013). These miRNAs have also been involved in proliferation, migration, and invasiveness of placental cells during the development of pregnancy. The purpose of this work is to investigate whether or not C19MC miRNAs provide an antiviral defense to cells. These numerous miRNAs from the C19MC cluster are unique to primates and are only expressed in placental cells. It has been demonstrated that C19MC miRNAs circulate in EVs and are taken up by distant cells in order to alter their biological functions (H. Li et al., 2020). Our objective is to determine if EVs and their cargo of C19MC miRNAs can provide viral resistance to cells. Currently, it is uncertain how C19MC miRNAs exert their antiviral properties. This study also intends to partially comprehend some of the immunological components involved in the host response to viral infection in relation to these miRNAs.

In this context, we employ a novel C19MC-KO JAR model. This C19MC-KO JAR model intends to experimentally validate (or not) prior in vitro overexpression experiments

and attempt to investigate the impact of C19MC miRNAs in early pregnancy rather than term placental cells (Bayer et al., 2015; Delorme-Axford et al., 2013), as shown before; as the level of expression of the C19MC miRNA cluster varies depending on time of pregnancy and because the risk of congenital transmission of viruses increases in early pregnancy (Dumont et al., 2017). C19MC-KO JAR cells and WT JAR cells were examined to determine if congenital disease-relevant viruses HCMV and ZIKV induced an antiviral response. Our findings indicate that C19MC-KO JAR cells (lacking the C19MC cluster gene fully) are more prone to infection by HCMV and ZIKV. In addition, our data demonstrate that the supernatant of WT JAR cells has an antiviral effect on ZIKV-infected recipient cells that do not genetically express the C19MC miRNA cluster. On the basis of a single experiment, we also noticed that sEVs from JAR WT cells may impart viral resistance, but additional experiments are required and are planned for the future. These findings are consistent with recent findings by other research groups (Sadovsky and Coyne), which confirmed the antiviral action of the members of the C19MC miRNA cluster found in EVs (Bayer et al., 2018; Delorme-Axford et al., 2013; Krawczynski et al., 2021). Moreover, our results indicate that the innate immune and autophagy response of C19MC-KO JAR cells and WT JAR cells differ during ZIKV infection, suggesting that both antiviral pathways could be activated by the C19MC miRNA cluster. This is similar to what was demonstrated by (Delorme-Axford et al., 2013) that prior to the activation of viral replication, C19MC miRNAs increased autophagy in VSV-infected cells. Overall, the objective of this Ph.D. thesis study is to contribute to a greater understanding of the role of C19MC miRNAs during prenatal viral infections. In addition, long-term examination of sEVs expressing C19MC miRNAs and their paracrine mode of action should aid in the development of non-toxic treatments that can be provided to immunocompromised persons, such as pregnant women. To uncover the molecular processes, mediated by C19MC miRNAs, that lead to organ, tissue, and cellular resistance against viral infections, additional study needs to be conducted, and a higher amount of resources needs to be allocated to this effort.

1. Do C19MC microRNAs provide protection against ZIKV and HCMV based on the study's results? Do C19MC miRNAs antiviral activity is transferred by sEVs in cells?

   Our studies with the C19MC-KO model show that upon ZIKV and HCMV infection, cells lacking the C19MC miRNA cluster are more permissive to infection, suggesting that this cluster has a direct effect in cells that express it. Our results validates previous results on other viruses suggesting that the C19MC miRNA cluster may have a general antiviral effect on RNA and DNA viruses. We observed that the antiviral effect was stronger in cells infected with HCMV. This may suggest that the degree of antiviral effect may depend on the virus. Our functional assay results with supernatant and sEV preparations from C19MC-KO JAR and WT JAR cells imply that sEVs mediate the antiviral protection against ZIKV in recipient cells, conferred by C19MC miRNAs. This correlates with previous studies (Delorme-Axford et al., 2013; Ouyang et al., 2014) in which antiviral protection from sEVs' preparations from placental trophoblasts was observed cells during viral infection with different viruses such as VSV. Trophoblast cells release high numbers of sEVs which contain C19MC miRNAs constituting most of the miRNA cargo. Our studies with a C19MC-KO model validate further previous studies. We propose for future experiments, functional assays (supernatant and sEVs from C19MC-KO JAR and WT JAR cells) with HCMV, other viruses involved in congenital infections, and even emergent viruses as in the case of Sars-CoV-2 to assess further the role of C19MC miRNAs. To further explore the role of C19MC miRNAs mediated by sEVs, a complete characterization of the sEVs will be performed. More sEV functional assays are necessary to examine the antiviral function of C19MC miRNAs further. Something that will be relevant for future studies is to determine which are the specific miRNAs from the C19MC cluster contained in sEVs that may elicit the antiviral-effect observed.

2. What effect does the difference in gene expression of the immune response between WT JAR and C19MC-KO cells have on viral infection?

The mechanism pathway employ by C19MC miRNAs during viral infections continues to be an enigma. This study reveals variations in the immune response gene expression between JAR WT and C19MC-KO cells following ZIKV infection. PSTPIP1 and NLRP3 were discovered to be down-regulated in both JAR WT and C19MC-KO cells, despite the lack of a discernible difference between the two cell types. In addition, several other genes, including APOBEC3G, CXCL8, and CARD9, were differentially expressed in JAR WT and C19MC-KO cells. The APOBEC3G gene was significantly overexpressed in C19MC-KO cells. This suggests that in the absence of the C19MC miRNA cluster, the protein APOBEC3G, known to suppress viral replication of RNA or DNA viruses by acting directly on the viral genome (R. S. Harris & Dudley, 2015; Salter et al., 2019) may have a significant immunity role upon ZIKV infection. Meanwhile, the presence of the C19MC cluster in WT JAR cells down plays the role of APOBEC3G, therefore relying in a different immune response. These data may indicate that C19MC miRNAs drive distinct immune antiviral responses upon ZIKV infection that in this absence in C19MC-KO JAR cells, this antiviral response seams modified and other antiviral responses like APOBEC3G could be increased. The CARD9 gene participates in both the innate and adaptive immune responses to infection (fungi, bacteria, and viruses). It activates immune cells such as macrophages and plays a crucial role in the production of pro-inflammatory cytokines (Hsu et al., 2007). Upon infection with ZIKV, the CARD9 and CXCL8 (Interleukin 8) genes were differentially expressed in both cell types. These results may required additional testing in order to specify specific antiviral mechanisms induced by ZIKV infection in which C19MC miRNA cluster play an important role. It may be suggested for future experiments to test for the immune response of the JAR WT and C19MC-KO cells upon HCMV infection by em-

ploying the autophagy and immune response RT2 profilers. This may give a clue as of whether the antiviral immune response in reference to the C19MC miRNA cluster, is virus-dependent or general for all RNA and DNA viruses.

3. What may be said about the difference in gene expression of the autophagy pathway upon ZIKV infection in WT JAR and C19MC-KO JAR cells?

The antiviral mechanism initiated by C19MC is associated with the stimulation of autophagy (Delorme-Axford et al., 2013) via an autophagosome/lysosomal pathway in virus-infected non-placental recipient cells, thereby inhibiting viral replication. Furthermore, in Ouyang et al. (2014) the impact is also detected in the EX extracts from the plasma of pregnant women as opposed to women who are not pregnant. Therefore we aim to explore further the autophagy response of C19MC-KO JAR cells and WT JAR cells upon ZIKV infection. We observe specific genes to be expressed differently in each cell type, such as: Interferon gamma (IFNG), the Tumor Necrosis Factor (TNF), and Transmembrane Protein 74 (TMEM74). The protein coding gene TMEM74 is involved in macroautophagy, and it is speculated that TMEM74 may be found in the membrane of autophagosomes, cytoplasmic vesicles, and lysosomal membranes. However, our study shows that expression levels of TMEM74 were down-regulated in C19MC-KO JAR and WT JAR cells (more in C19MC-KO cells). Additionally, IFNG and TNF genes which encode cytokines and are part of the innate and adaptive immune response, were expressed diferentially in both cell types. IFNG gene is responsible for the production of a soluble cytokine that belongs to the type II interferon class. It has been found to stimulate Natural Killer cells (NK) and neutrophiles. In addition, studies by Chaiwangyen et al., 2020; Ishida et al., 2015; Kambe et al., 2014 suggest that members of the C19MC miRNA cluster contained in sEV are uptaken by NK cells resulting to decrease immunity to allow for fetal tolerance or an increase in NK cells proliferation

which acts to protect against viruses. The observed down-regulation of the INFG gene in C19MC-KO JAR cells may correlate with these studies as we speculate that IFNG is necessary to stimulate NK cells which in turn may uptake sEVs containing C19MC miRNAs, in this way protecting cells against viral infection. Additionally, IFNG is known to activate macrophages which in turn induce autophagy. Additionally, levels of IFNG and TNF along with NK cells appear to be increased in patients infected with some types flaviviruses (Blom et al., 2016; Marquardt et al., 2015; Zimmer et al., 2019). The TNF gene was significantly up-regulated in WT JAR cells. This cytokine encoding gene induces apoptosis and autophagy in cells and is secreted by macrophages (J. Harris, 2011), suggesting that C19MC miRNAs may induce autophagy, apoptosis and the activation of macrophages during a viral infection. However, our results are based on 2 experiments on ZIKV infection. More experimentation must be done to refine the interpretation of our results and perhaps test the immune response with other viral models. Experiments that may directly assess the autophagic activity of cells like the LDH sequestration or western blot based assays for LC3 lipidation may assess further the role of autophagy in the antiviral effect of C19MC miRNAs.

4. Other than C19MC miRNAs, what molecular components may also contribute to the antiviral response reported after viral infection in WT JAR?

Our results indicate that in early ZIKV viral replication, the supernatant from WT JAR cells (conditioned and ultracentrifuged-EV-depleted media) attenuates ZIKV infection in Vero cells. Eventually, this effect continues for conditioned WT supernatant but dissapears for ultracentrifuged WT supernatant. It may be apparent that other molecular components contribute to antiviral resistance in recipient cells other than C19MC miRNAs. According to Delorme-Axford et al. (2013) and Bayer et al. (2015), miRNAs from the C19MC cluster expressed by human placental trophoblast cells are

part of the cells' antiviral repertoire, and their effects may be transferred through sEVS. Alternatively, research such as Bayer et al. (2016) and Bayer et al. (2018) demonstrate the antiviral effect of INF III during congenital viral infections, although it has been proposed to act independently from C19MC miRNAs. Our data may suggest that IFN III may be another molecular antiviral factor in the attenuation of ZIKV in Vero cells initially. However, the IFN III effect may be transient due to their lack of molecular stability after the initial replicative phases; hence, sEVs expressing C19MC miRNAs may continue to exert their antiviral impact. Therefore, we suspect that these two powerful antiviral pathways (C19MC miRNAs and IFN III) work in combination to give many options for protecting placental cells and other cell types at the maternal-fetal interface against ZIKV and other viruses. Additionally, it is also possible that placental cells possess other antiviral defense mechanisms that have not yet been identified but are unique to placental cells. For future experiments, the supernatants from C19MC-KO JAR and WT JAR cells could be assessed by the IFN and receptors RT^2 profiler device and also by next generation sequencing.

5. How might this study contribute to the scientific community? What weaknesses and strengths may be found?

This is the first study to use a new JAR Knock-out model for the C19MC miRNA cluster to examine the effects of these miRNAs during congenital viral infections. The majority of in vitro research to date has utilized overexpression of the C19MC miRNAs (Delorme-Axford et al., 2013), which may result in mutant phenotypes; therefore, it is crucial to look into alternative experimental methods to assess and validate further earlier C19MC miRNA antiviral claims. By using the JAR human choriocarcinoma cell line (syncytiotrophoblast-like), this model also provides the possibility to explore the impact of C19MC miRNAs in the early stages of pregnancy during viral infection. The

majority of research has utilized term placental cells, which express a larger amount of C19MC miRNAs when the most serious infections occur in early pregnancy when C19MC miRNAs are at their lowest. Moreover, the viral models utilized in this study, ZIKV and HCMV, are viruses that are known to infect the placenta, which facilitates the study of these miRNAs in the context of pregnancy. This model allows for further mechanistic studies to examine the immune response (interferon pathway, APOBEC3G) and autophagy response upon viral infections in relationship to the effect of C19MC miRNAs. Other pathogen models that are known to cause congenital infections may be assess also with this C19MC-KO model.

6. What impact this research have on a global scale?

The recent SARS-Covid-2 pandemic has sparked significant interest and investment in Covid virus research. Other critical infections, such as viruses that affect pregnancy and fetal health, have been neglected despite the value of studying these pathologies, given that they are still a problem in our society. HCMV infections account for 1% of children born in France (higher in underdeveloped countries) and are the leading infectious cause of sensorineural impairments and brain deformity, a major public health concern. The ZIKV caused a recent outbreak in Latin America that had devastating consequences for newborns. Due to the lack of immunity in the population and the endemic colonization of its vector *Aedes albopictus* in European countries, there is still a considerable danger of re-emergence, particularly in more temperate regions of the world. Currently, there is a lack of diagnostic testing for pregnant women infected with HCMV and ZIKV. Clinical diagnosis relies on ultrasound and invasive amniocentesis, which can only detect severe developmental impairments, preventing early treatment. Furthermore, no therapeutic options are available following the diagnosis of the infection. Most of the time, infection consequences vary among children and are rarely

foreseeable, providing a significant challenge for impacted families and the health care system. Therefore, we must advance our understanding of these congenital illnesses, representing a fairly overlooked study area, and this study aims for just that. Recently, the evolutive emergence of abundant placenta-specific C19MC cluster miRNAs in primates revealed their importance throughout pregnancy. Besides their role in inducing the adaptation of the fetus to the mother, among other functions, these miRNAs may act in a cell-autonomous or paracrine mode to attenuate viral infections like HCMV and ZIKV. Therefore, this study may have biomedical implications, as systemic C19MC miRNAs may serve as a unique, non-invasive, prognostic tool for fetal health following congenital viral infections. It is important to also consider and continue investigations on other viruses such as the Usutu flavivirus that was found to infect placental cells as depicted by our team's scientific recent paper. In this publication I demonstrated by immunofluorescence imaging that Usutu infects placental cells. The following section contains our recent publication on Usutu.

Appendices

Article

Usutu Virus Infects Human Placental Explants and Induces Congenital Defects in Mice

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Abstract: Usutu virus (USUV) is a neurotropic mosquito-borne flavivirus that has dispersed quickly in Europe these past years. This arbovirus mainly follows an enzootic cycle involving mosquitoes and birds, but can also infect other mammals, causing notably sporadic cases in humans. Although it is mainly asymptomatic or responsible for mild clinical symptoms, USUV has been associated with neurological disorders, such as encephalitis and meningoencephalitis, highlighting the potential health threat of this virus. Among the different transmission routes described for other flaviviruses, the capacity for some of them to be transmitted vertically has been demonstrated, notably for Zika virus or West Nile virus, which are closely related to USUV. To evaluate the ability of USUV to replicate in the placenta and gain access to the fetus, we combined the use of several trophoblast model cell lines, ex vivo human placental explant cultures from first and third trimester of pregnancy, and in vivo USUV-infected pregnant mice. Our data demonstrate that human placental cells and tissues are permissive to USUV replication, and suggest that viral transmission can occur in mice during gestation. Hence, our observations suggest that USUV could be efficiently transmitted by the vertical route.

Keywords: arbovirus; flavivirus; Usutu virus; placenta; vertical transmission

1. Introduction

The arboviral risk is globally rising on the European continent and more generally worldwide, and it is already well established in various regions, particularly in Africa and Latin America. Among potential emerging viruses, Usutu virus (USUV) has drawn the attention of the scientific community in recent years, since this virus has massively dispersed out of Africa, mainly into Europe [1]. USUV is an icosahedral-enveloped virus of approximately 40–60 nm in diameter. Its genome is a single-stranded positive-sense RNA of 11 kb in length, with a 5′ cap structure and one large open reading frame encoding a unique polyprotein of 3434 amino acids. This polyprotein is post-translationally processed into three structural proteins: capsid, pre-membrane/membrane, and envelope (Env), and eight non-structural proteins (NS1, NS2a, NS2b, NS3, 2K, NS4a, NS4b, and NS5) [2]. USUV

strains are classified into eight genetic lineages divided in two major African or European groups: Africa 1, 2, and 3, and Europe 1, 2, 3, and 4, that have all been detected in Europe, except for Africa 1 [3].

USUV was first identified in 1959 in South Africa from a mosquito of the *Culex neavei* species, and isolated by intracerebral inoculation of newborn mice [4–7]. Similar to Zika virus (ZIKV) and West Nile virus (WNV), with which it shares many common features, USUV belongs to the genus *Flavivirus* from the *Flaviviridae* family. It is maintained through an enzootic cycle involving birds (mainly passerine and Strigiformes) acting as amplifying hosts, and ornithophilic mosquitoes, such as *Culex pipens*, as vectors [8]. Humans and other mammals, such as horses, dogs, rodents, and wild boars, are considered as accidental hosts [8]. In humans, USUV infection was first described in Africa, in Central African Republic (1981), and in Burkina Faso (2004) [9]. Major USUV epizootics affecting avifauna, associated with a large epidemic of West Nile virus (WNV), were demonstrated in Europe in 2016 and in 2018 [8]. Molecular and serologic evidence of USUV infection in European blood donors suggests a silent spread of this virus among asymptomatic humans, which could thus be a concern for blood transfusions or organ transplants [8]. In Europe, epizootics were accompanied by several descriptions of human neurological disorders, including facial paralysis, encephalitis, meningitis, and meningoencephalitis, in immunocompromised and immunocompetent patients [10–22]. USUV infection had been reported in a dozen of European countries, and to date, more than one hundred cases of acute human infection have been described, mainly in Europe [8].

Despite the fact that USUV is an emerging pathogen dispersed quickly in Europe, very little is known about its pathogenicity and transmission routes. Among the different transmission routes described for arboviruses, it is known that some flaviviruses, such as ZIKV and WNV, have the ability to be vertically transmitted [23–25]. Vertical transmission of ZIKV was at the heart of concerns during the major epidemic of 2015–2016 in Brazil and Latin America, with the discovery of congenital Zika syndrome (CZS) observed in newborns of mothers who contracted the virus during their pregnancy. CZS is mainly characterized by microcephaly, associated with severe cerebral malformations and ocular alterations, among others [26,27]. In recent years, the maternal-fetal transmission of ZIKV has been the subject of numerous studies seeking to characterize the cells and mechanisms allowing the transplacental passage of the virus towards the fetal compartment [25,28]. ZIKV has been shown to infect a wide variety of cell types at the maternal–fetal interface, including decidual cells, endothelial cells, cytotrophoblasts, or macrophage Hofbauer cells [29–33]. Moreover, placental cell permissiveness has been shown to differ among cell type and gestational age, with modulation of viral receptor expression and a lower innate immune response in first-trimester versus third-trimester placenta [30–33]. This correlated with the degree of severity of the sequelae observed during ZIKV infection of animal models [34,35] or upon natural infection in human population, the earliest infection being the most severe [36].

In the present study, we examined whether USUV may be vertically transmitted via replication in the placenta. To this end, we combined the use of several trophoblast cell lines, ex vivo human placental histocultures from the first and third trimester of pregnancy, and in vivo USUV-infected pregnant mice, to assess the ability of USUV to replicate in the placenta and gain access to the fetus. Our data demonstrate that placental cells and tissues are permissive for USUV replication, and suggest that viral transmission can occur in offspring during pregnancy.

2. Materials and Methods

2.1. Ethics Statement

The biological resource center Germethèque (BB-0033-00081; declaration: DC-2014-2202; authorization: AC-2015-2350) obtained the written consent from each patient (CPP.2.15.27) for the use of human samples and their associated data. The steering committee of Germethèque gave its approval for the realization of this study on 5 February 2019. The hosting

request made to Germethèque bears the number 20,190,201 and its contract is referenced under the number 19 155C.

Mice were bred and maintained according to the French Ministry of Agriculture and European institutional guidelines (appendix A STE n° 123). Experiments were performed according to national regulations and approved by the regional ethics committee of Languedoc-Roussillon (Comité Régional d'Ethique sur l'Expérimentation Animale-Languedoc-Roussillon), France (approval n° 6773-201609161356607).

2.2. Cell Lines

JAR choriocarcinoma cells (ATCC HTB-144), deriving from a trophoblastic tumor of the placenta, and Vero cells (ATCC CCL-81) were cultured at 37 °C and 5% CO_2 in DMEM (Gibco), supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 100 U/mL penicillin—100 µg/mL streptomycin (Gibco), and 100 µg/mL normocin (Invivogen). HIPECs (human invasive proliferative extravillous cytotrophoblast) [37], an immortalized cell line deriving from first-trimester primary cytotrophoblasts, obtained from Dr T. Fournier (Inserm, Paris; Transfer agreement n° 170448), were cultured in DMEM/F12 medium (Gibco) at 50/50 ratio (*v*/*v*) and supplemented as above.

2.3. USUV Strains, Viral Stock Production and Cell Infection

USUV (Africa 2 strain, Rhône 2705/France/2015-KX601692) was provided by Anses (Agence nationale de sécurité sanitaire de l'alimentation, de l'environnement et du travail) and was not propagated more than three times on Vero cells. Viral stocks were prepared by infecting sub-confluent Vero cells at a multiplicity of infection (MOI) of 0.01 in DMEM with 2% heat-inactivated FBS. Cell supernatant was then collected between 5 to 7 days post-infection and harvested after centrifugation at 300× *g* to remove cellular debris. Viral titers were determined by the 50% tissue culture infective dose (TCID50) using the Spearman–Kärber method, and were expressed as TCID50 per mL [38].

For infection, cells at 60–70% confluence were rinsed once with phosphate-buffered saline (PBS), and USUV diluted to the required MOI were added to the cells in a low medium volume. Cells were incubated for 2 h at 37 °C and then culture medium was added to each well. As a control, cells were incubated with the culture supernatant from Vero cells (mock condition).

2.4. Placental Histocultures

Placental histocultures were realized as described [39] on first-trimester placentas (6 placentas; mean = 10.65 ± 0.35 (SEM) weeks of amenorrhea, i.e., 9.65 ± 0.35 weeks of pregnancy; age of the women: mean = 27 ± 2.3 years old) or term placentas (3 placentas; mean = 40.95 ± 0.05 weeks of amenorrhea, i.e., 38.95 ± 0.05 weeks of pregnancy; age of the women: mean = 32.33 ± 4.9 years old). Trophoblastic villi were dissected in small explants and infected or not by USUV viral stock diluted 1:1 with DMEM medium in 1 mL final volume overnight before deposition on gelatin sponges (Gelfoam, Pfizer) upon intensive washes in PBS. Culture medium was collected and renewed every 3 to 4 days. At 15 days of culture, placental explants were fixed or flash frozen for future use.

2.5. Mouse Experiments

C57BL/6 WT pregnant mice were purchased from Janvier Laboratories (Saint-Berthevin Cedex, France). Mice were inoculated with USUV by subcutaneous (footpad) route with 10^4 TCID50 in 50 µL of PBS on embryonic days 6 (E6) or E12 and sacrificed on E13 or after the birth, respectively. Mice were infected at ECE (Etablissement Confiné d'Expérimentation), a level 3 animal facility of the University of Montpellier. USUV-infected mice and control mice were euthanized by cervical dislocation or with a lethal dose of pentobarbital (Sigma-Aldrich, Darmstadt, Germany) at indicated day post-infection, depending on the experimental design. Organs and tissues were snap frozen with liquid nitrogen for viral burden.

2.6. Immunofluorescence

Immunofluorescence against anti-flavivirus group antigen were realized as described [40] using mouse 4G2 primary antibody (Novus Biologicals) diluted 1:400. Widefield acquisitions were realized using Apotome microscope (Zeiss) and image processing was performed using ImageJ.

2.7. Intracellular Staining and Flow Cytometry Analysis

Cells were fixed and permeabilized with BD Cytofix/Cytoperm fixation/permeabilization buffer according to the manufacturer's instructions. Envelope antigen was then detected with a 1:400 dilution of Alexa fluor 488-conjugated mouse monoclonal antibody 4G2 (Novus Biologicals) for 25 min at 4 °C. Cell fluorescence was then analyzed on a Macsquant VYB Flow Cytometer (Miltenyi Biotec), by using FCS and FITC fluorescence parameters, and by subtracting cell autofluorescence background. Data were analyzed with FlowJo (BD) software.

2.8. Analysis of Cell Growth and Viability

10^5 cells were initially seeded in 12-well plates and infected or not by USUV at MOI 3 during 2 h, before medium change for removing of viral inoculum. At different times post-infection, supernatants were collected, and cells were trypsinized. Number of viable cells was determined upon trypan blue staining after trypsinization. Number of dead cells was determined by addition of previously determined adherent dead cells and floating dead cells, collected in supernatant in a given time point, and expressed as percentage of the total cell number.

2.9. TUNEL Assay

TUNEL assay was done using Click-iT Plus TUNEL Assay for In Situ Apoptosis Detection Kit (Life Technologies), following manufacturer's instructions, as previously described [39]. Image acquisition was performed on a Zeiss Axiovert 200 microscope.

2.10. RNA Extraction

Total RNA was extracted from cells using RNeasy Plus Mini Kit (Qiagen), and from placental explants using miRNeasy Mini Kit (Qiagen), according to the manufacturer's instructions. When extracted from cell or tissue supernatant, RNA extraction was realized using QiaAmp Viral RNA Mini Kit (Qiagen) from 220 µL supernatant. Upon extraction, RNA concentration and quality were systematically determined using a NanoDrop spectrophotometer.

2.11. RT-qPCR Analysis

Upon extraction, 500 ng RNA were subjected to reverse transcription reaction using LunaScript RT SuperMix Kit (New England Biolabs), following manufacturer's protocol. cDNA was then used as matrix in qPCR reaction using Sybr Green I Master Mix (Roche) and the following primers: USUV-Forward: AACAGACGGTGATGCGAACT; USUV-Reverse: TACAGCTTCGGAAACGGCTT; β-actin-Forward: GTGCTGTCCCTGTACGCCTCT; and β-actin-Reverse: GGCCGTGGTGGTGAAGCTGTA. qPCR reactions were carried out with a Roche LightCycler 480 apparatus using the following program: 95 °C for 5 min, then 40 cycles of 95 °C for 15 s, and 60 °C for 10 sec. The fold induction in viral transcript expression was quantified by calculating the $2^{-\Delta\Delta CT}$ value, with β-actin mRNA as internal control. For quantification of viral transcript release in cell or tissue supernatant, qPCR reactions were performed with primers targeting USUV transcripts only. Results were compared to those obtained with a standard curve realized using a plasmid containing the USUV cDNA with known concentration and genome copy correspondence.

2.12. RT2 Profiler PCR Arrays

Upon RNA extraction, 500 ng of RNA were subjected to reverse transcription using RT2 First Strand kit (Qiagen), following manufacturer's protocol. The resulting cDNAs were

then used for RT2 profiler PCR arrays (Human Antiviral Response PAHS-122Z, Qiagen), for profiling 84 genes specific for innate immune response, with five housekeeping genes as internal controls (ACTB, B2M, GAPDH, HPRT1, and RPLP0), following the manufacturer's instructions. qPCR reactions were carried out with a Roche LightCycler 480 apparatus using the following program: 95 °C for 10 min, then 45 cycles of 95 °C for 15 s, and 60 °C for 1 min, followed by a melting curve acquisition step. The fold change of gene expression and Student's *t*-test were automatically calculated by the RT2 profiler RT-PCR array data analysis software, version 5.1.

2.13. Histology and Immunohistochemistry

For histology studies of placental tissues, explants were fixed 24 h in 10% formalin, dehydrated, embedded in paraffin, sectioned (5 µm), and de-waxed. Upon epitope retrieval, immunohistochemistry was carried out as described using mouse anti-placental alkaline phosphatase (Biolegend; 1 µg/mL) as primary antibody [39]. Image acquisition was performed on a Panoramic 250 scanner (3DHISTECH).

Alternatively, for detection of USUV Env antigen, immunohistochemistry was performed on the Discovery Ultra Automated IHC staining system using the Ventana DAB Map detection kit. For viral antigen immunostaining, antigen retrieval was performed for 4 min at 37 °C with Protease 1 solution from Ventana, which is an endopeptidase (alkaline protease) of the serine protease family. Endogenous peroxidase was blocked with Discovery Inhibitor CM for 8 min at 37 °C. The slides were incubated after rinsing at 37 °C for 32 min with a mouse anti-flavivirus group antigen monoclonal antibody (Millipore, MAB10216, 1:800 in Dako antibody diluent with background reducing components). Signal enhancement was performed using Rabbit monoclonal to mouse IgG1 + IgG2a + IgG3 as the secondary antibody (Abcam, ab133469, 1/8000) and the Discovery DAB Rabbit HQ Kit. Slides were then counterstained with hematoxylin for 8 min and manually dehydrated before coverslips were added. Slides were treated with a Hamamatsu NanoZoomer 2.0-HT scanner by MRI platform, and images were visualized with the NDP.view 1.2.47 software.

2.14. Measurement of Viral Burden In Vivo

Mouse organs (brain, placenta, spleen, and mammary glands) were weighed and homogenized with zirconia beads in a Fastprep 24 apparatus (MP Biomedicals) in 250 µL PBS, and RNA was extracted using the RNeasy Mini Kit (Qiagen). Blood RNAs were extracted from 100 µL of samples, with the EZ1 apparatus running the EZ1 DSP virus kit (Qiagen). Viral USUV RNA levels were measured by a one-step quantitative reverse transcriptase PCR assay on the Light Cycler 480 (Roche) with primers, probe, and cycling conditions previously described [41]. Viral burden was expressed on a log10 scale as TCID50 equivalents per gram or ml after comparison with a standard curve produced using serial 10-fold dilutions of USUV with known viral titers.

2.15. Statistical Analyses

GraphPad Prism (v8) software was used to perform data statistical analysis. One-way or two-way ANOVA tests were carried out, followed by Tukey's multiple comparison test for two-way ANOVA.

3. Results

3.1. Human Placental Cell Lines Exhibit Variable Permissiveness to USUV Replication

To assess the ability of USUV to replicate in human placental cells, we used two cellular models: the JAR cells, which share many characteristics of early placental trophoblasts such as the ability to differentiate into syncytiotrophoblastic cells in vitro and secrete gonadotropic hormone [42,43]; and HIPECs, deriving from first-trimester cytotrophoblasts with invasive capacities [37]. USUV was able to disseminate in both cell types (Figure 1a,b), however with different efficiencies and impact on cell growth and survival. Globally, a significant difference was observed in the level of infection between JAR cells and HIPECs.

In JAR cells, a high proportion of cells, nearly 60%, were infected as soon as 16 h post-infection. In contrast, only 17% of HIPECs were infected at the same time (Figure 1b). A maximum level of infection was reached for both cell lines at 24 h before a decrease. At that time, a decrease in cell growth was observed for both HIPECs and JAR cells (Figure 1c). However, HIPECs were then able to resume their growth, while the JAR cell growth remained highly affected by USUV infection. By evaluating the mortality rate in both cell lines upon infection, we observed that the percentage of cell death was significantly higher in JAR cells than in HIPECs, reaching more than 50% comparing to less than 25%, respectively (Figure 1d). Taken together, these results indicated a difference in susceptibility for USUV infection between the two cell lines, explaining the higher infection rate and mortality observed in JAR cells compared to HIPECs.

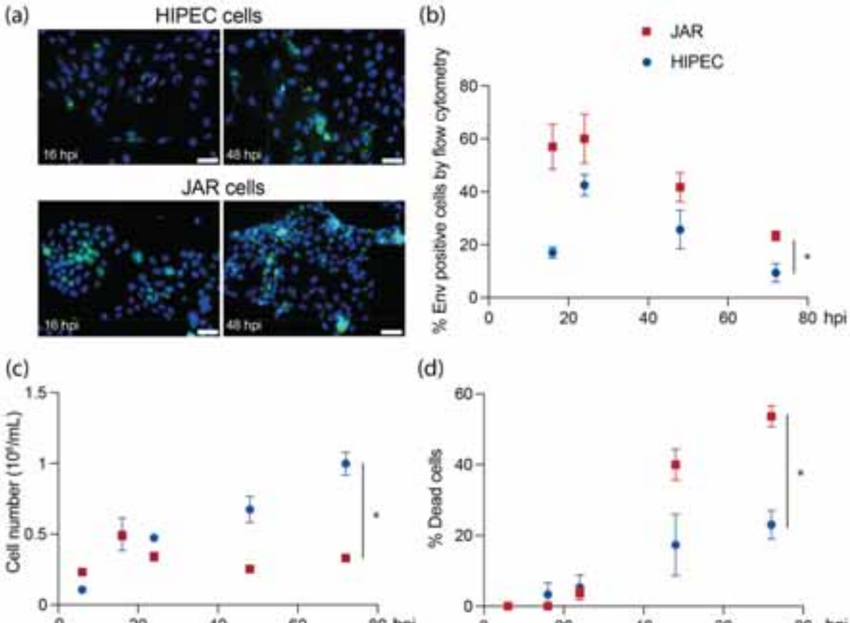

Figure 1. Kinetics of USUV infection in placental cell lines and effect on cell growth and viability. JAR cells or HIPECs have been infected by USUV at MOI 3 at different times before proceeding to analyses. (**a**) Indirect immunofluorescence realized against USUV Env antigen (green: Env; blue: DAPI) at 16 or 48 h post-infection (hpi). Images are representative of at least three independent experiments, and three independent fields each time. Scale bar = 50 µm. (**b**) Quantification of the percentage of infected cells determined by flow cytometry, via intracellular staining of Env antigen. (**c**) Quantification of cell growth between JAR cells and HIPECs upon USUV infection, by counting viable cell number at different time points. (**d**) Cell mortality was evaluated by trypan blue staining for JAR cells and HIPECs at different times upon USUV infection. In (**b**–**d**), symbols represent mean ± SEM for three independent experiments. In each experiment, a two-way ANOVA statistical test was performed and indicated a significant difference between cell lines (*, $p < 0.05$).

To address whether trophoblast cell lines may achieve productive infections, we examined different parameters during the first 24 h post-infection (Figure 2). Results obtained for infected-JAR cells confirmed their high permissiveness for USUV. Cells were highly positive for USUV Env by flow cytometry and showed high induction of USUV viral RNA (vRNA) expression (Figure 2a,b, right panels). Moreover, USUV was able to perform a productive cycle in JAR cells, as assessed by the high level of vRNA released in supernatant (Figure 2c, right panel), and by the presence of infectious particles with around 5×10^5 TCID50/mL in supernatant upon 24 h post-infection (Figure 2d, right panel). Interestingly, except for flow cytometry experiments, there was no significant difference

between MOI 1 or 10 in JAR cells, indicating that the maximum level of infection or viral production was already reached for MOI 1. Moreover, infection was very fast in these cells, since for most of the experiments carried out, a plateau was reached at 16 h post-infection.

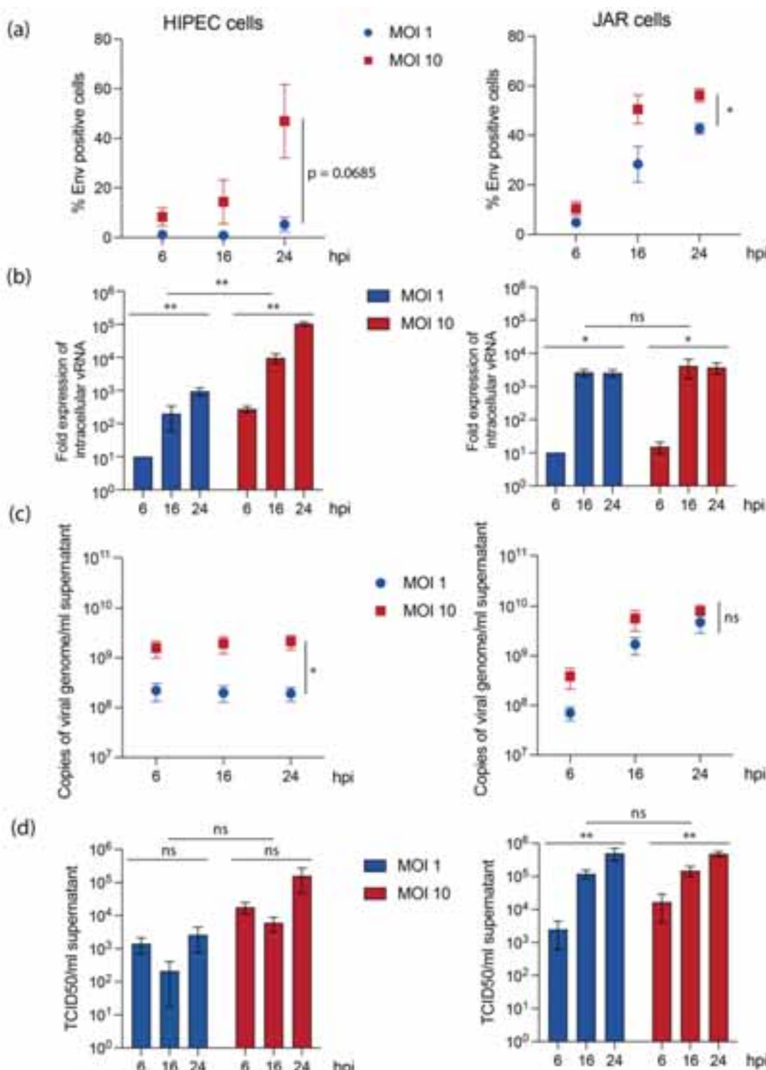

Figure 2. JAR cells and HIPEC allow USUV productive replication cycle with different efficiencies. HIPECs (left column) or JAR cells (right column) have been infected by USUV at MOI 1 or 10 during indicated times (hpi: hours post-infection) before proceeding to analyses. (**a**) Quantification of the proportion of infected cells determined by flow cytometry, via intracellular staining of Env antigen. Symbols represent the mean ± SEM for three independent experiments for HIPECs and four independent experiments for JAR cells. (**b**) Quantification of viral RNA extracted from infected cells, upon normalization by actin mRNA and by the level of viral RNA at 6 hpi for MOI 1. Histograms represent mean ± SEM for three independent experiments. (**c**) Quantification of viral RNA released in cell supernatant. Symbols represent the mean ± SEM for five independent experiments. (**d**) Quantification of infectious particles released in cell supernatant. Histograms represent mean ± SEM for five independent experiments. In (**a**–**d**), a two-way ANOVA statistical test was performed to evaluate a statistical difference between cell lines and MOI (ns, non-significant; *, $p < 0.05$; **, $p < 0.005$).

Our results showed that HIPECs were also permissive for USUV replication. However, compared to JAR cells, USUV infection of HIPECs was slower and more dependent on the MOI used (Figure 2a,b, left panels). vRNA and infectious particle release in supernatant was also less efficient (Figure 2c,d, left panels), with no significant increase with time, reaching a maximum of 1.6×10^5 TCID50/mL upon 24 h post-infection for MOI 10. However, despite this decrease in permissiveness compared to JAR cells, USUV was also able to productively infect HIPECs.

3.2. USUV Elicits a Strong Antiviral Response in JAR Cell Line

To better describe the effect of USUV infection on cytotrophoblasts, the level of expression of 84 genes involved in innate antiviral response was monitored in both cell lines (Figure 3). At 16 h upon infection at MOI 10, mRNA level of numerous genes was significantly enhanced in infected JAR cells compared to non-infected cells (Figure 3a). USUV infection induced the expression of the pattern recognition receptors (PRRs) IFIH1, DDX58, and DHX58 (also known as MDA 5, RIG-I, and RIG-I-like receptor 3 (RLR-3), respectively; Figure 3b). In accordance with the stimulation of PRRs, induction of type I interferon pathway upon USUV infection, with expression of IRF7, IFNA1, and IFNB1, was also observed. In parallel with the induction of STAT1 expression, transcription of some IFN stimulated genes (ISGs) was also noticed upon USUV infection of JAR cells, such as OAS2 and ISG15. Finally, a high number of pro-inflammatory cytokines were up-regulated upon infection, such as CXCL10, CCL5, CXCL11, TNF, CCL3, CXCL8, and IL6.

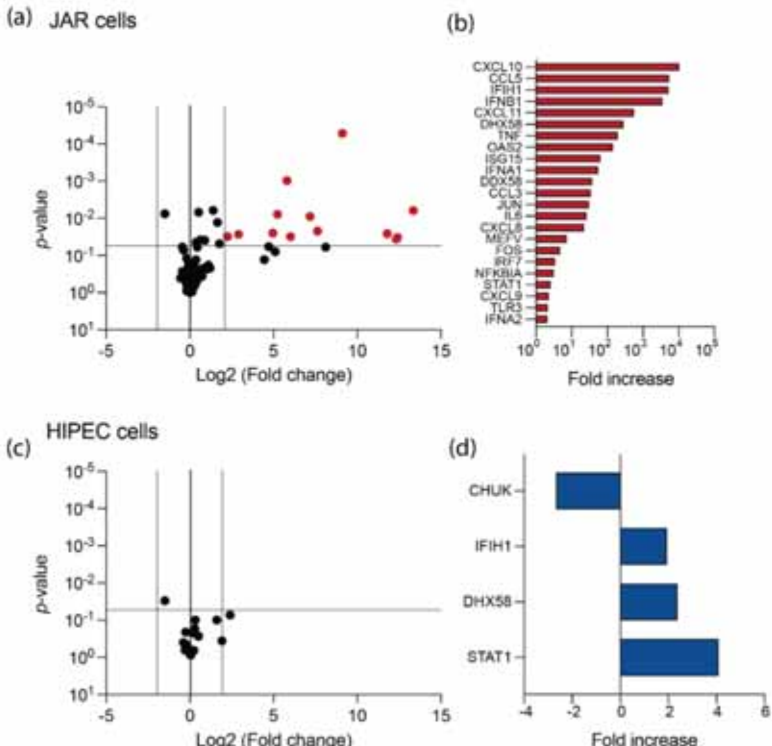

Figure 3. Characterization of innate immune responses of HIPEC and JAR cells upon USUV infection. (**a**) And (**c**) volcano plot representing differences in normalized mean mRNA expression in JAR cells (**a**) or HIPECs (**c**) 16 h upon USUV infection compared to non-infected cells, from three independent experiments. mRNAs exhibiting significant differences upon infection are represented by colored circles (Student's *t*-test *p*-value ≤ 0.05 and log2 ratio ≥ 2 or ≤ -2). (**b**–**d**) Detail of the under- and over-expressed mRNAs upon USUV infection in JAR cells (**b**) or HIPECs (**d**).

In contrast to what was observed in JAR cells, innate antiviral immune response in HIPECs was quite inexistent, with few induced genes and very low amplitude of induction at the same time post-infection (Figure 3c,d). Induction of antiviral genes was no more evidenced at 24 h post-infection (data not shown), albeit the percentage of infected cells reached its maximum with around 50% of infected cells at MOI 10 (Figure 2a), indicating that this cell line was not able to elicit an efficient antiviral immune response against USUV.

3.3. Human Placental Tissues Are Permissive to USUV Replication

To go further in the evaluation of USUV placental infection, we next used a model of human tissue explants of first-trimester or term placenta (see pipeline Figure 4a) [39,44]. First, placenta cyto-architecture and viability upon 15 days of culture were assessed by performing an anti-placental alkaline phosphatase (PLAP) immuno-histochemistry and a TUNEL assay (Figure 4b). As expected, PLAP expression was evidenced in the syncytiotrophoblast layer of the villi, with no visible alteration of the tissue architecture upon USUV infection upon 15 days of infection. Moreover, no overt cell death was observed upon infection by TUNEL assay. At 15 days post-infection, the presence of vRNAs in placental tissue was evaluated both in first-trimester and term placentas (Figure 4c). The level of vRNA in placental tissues, normalized by actin, was represented by a double gradient color heat-map, with red color indicating high amplification, and blue indicating no amplification. In both cases, presence of significant amount of vRNAs was evidenced in USUV-infected placental tissues, as indicated with red colored boxes, in comparison to non-infected tissues, or tissue infected with UV-inactivated virus, indicating that the presence of vRNAs was due to an active viral transcription process and not the remaining viral inoculum. Thus, both first- and third-trimester placental tissues allowed active transcription of USUV. Moreover, the presence of USUV Env antigen was also detected in a few cells in some, but not all, term placental explants by immuno-histochemistry (Figure 4d). We next examined vRNAs and infectious particles released in supernatant of placental histocultures in collected media along the different times post-infection. vRNAs were detected in first-trimester and term placental explant supernatant along the duration of the culture, albeit with lower quantities for first-trimester placental explants (Figure 4e, red and blue dots). Release of infectious viral particles was also monitored by TCID50 titration on histoculture supernatants. No infectious particles could be detected in supernatant of first-trimester explants (data not shown) in contrast to term explants, in which infected cells were sometimes detected (Figure 4e, grey dots). Finally, the different histoculture supernatants at day 15 post-infection were incubated with Vero cells during 24 h, and an indirect immunofluorescence against USUV Env was realized (Figure 4f). Infected Vero cells were observed with supernatants from first-trimester and term placenta histocultures, and high productive infection was recorded in some of them, confirming the results obtained in Figure 4e and the intrinsic variability of the placenta permissiveness.

3.4. USUV Can Achieve Congenital Infection in Immunocompetent Mice and Causes Occasional Fetal Demise

To the best of our knowledge, the capacity of USUV to infect fetuses has not been studied in any experimental model. We chose to study the potential of USUV to be transmitted vertically in vivo using immunocompetent mice. Individual fetuses were evaluated morphologically for size and appearance and for the presence of virus in the brain and/or blood. A low intrauterine transmission rate was observed in pups born to immunocompetent mice infected in the first week of gestation whereas no transmission was detected when mice were infected in the second week (Table 1).

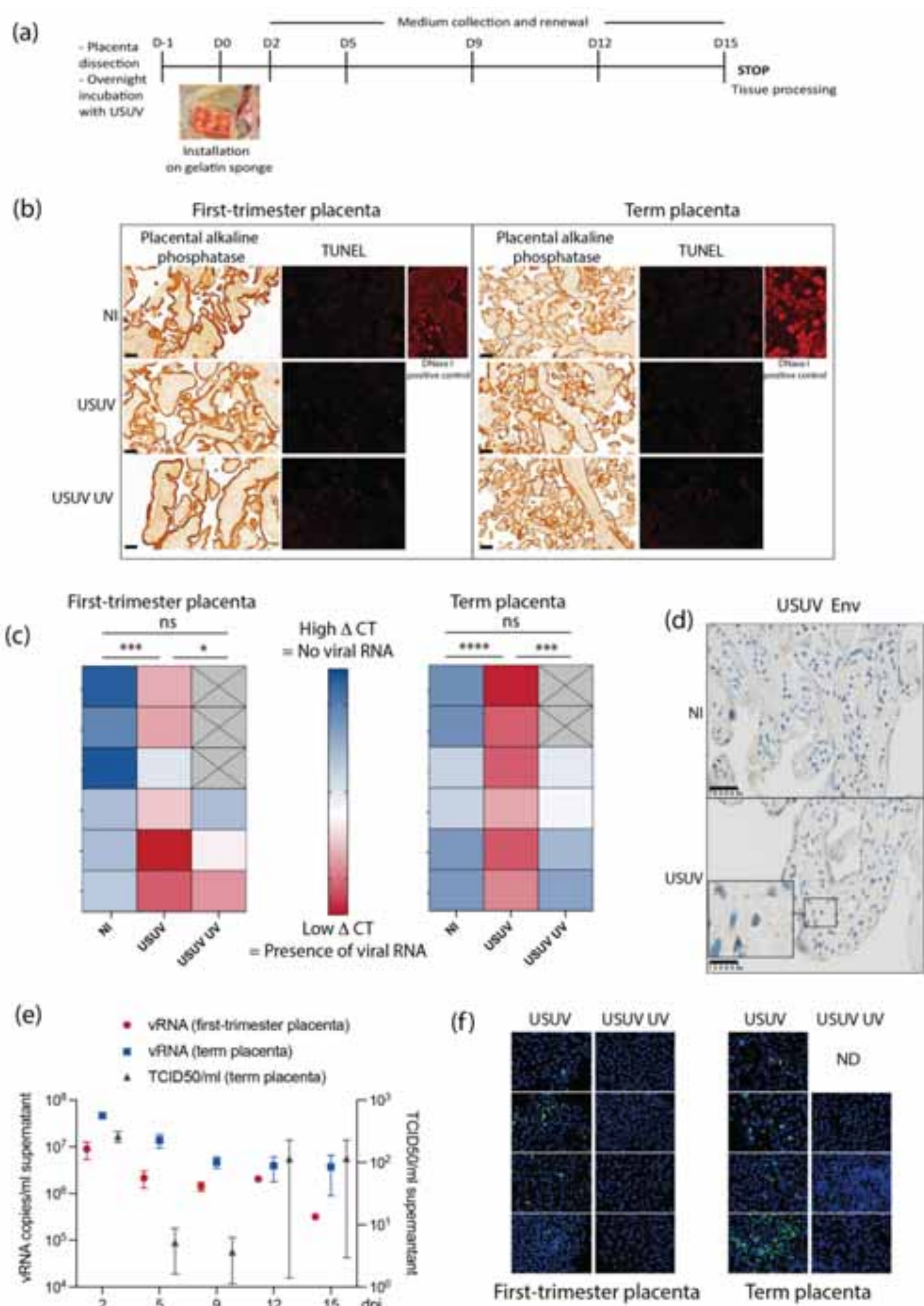

Figure 4. Human first- and third-trimester placentas are permissive for USUV replication. (**a**) Pipeline of placental explant infection and histoculture. (**b**) Left columns: cross sections of placental alkaline phosphatase immunohistochemistry and hematoxylin staining of placental villi from histoculture at day 15, observed by bright field microscope. Image representative from at least three independent experiments. Scale bar = 100 μm. Right columns: fluorescence-based TUNEL assay done on cross section of placental villi from histoculture at day 15. Image representative from three independent experiments (NI: non-infected; USUV UV: infection by UV-inactivated USUV). (**c**) Heat-map representing the level of viral RNA expression (normalized by actin) in placental tissues upon infection by USUV or by UV-inactivated virus (USUV UV), for six independent experiments (NI: non-infected). The ΔCT values are represented by a double gradient color map (blue: high ΔCT = no amplification

of viral RNA; red: low ΔCT = amplification. of viral RNA; and crossed gray: no data). A one-way ANOVA statistical test was performed to evaluate a statistical difference between conditions, followed by Tukey's multiple comparison test (ns, non-significant; *, $p < 0.05$; ***, $p < 0.0005$; ****, $p < 0.0001$). (**d**) Anti-USUV Env immunohistochemistry done on term placental villi from histoculture at day 15. Blue staining corresponds to hematoxylin coloration of the nucleus and brown staining correspond to the USUV Env detection. Scale bar = 50 µm (NI: non-infected). (**e**) Quantification of viral RNAs (red circles: first-trimester placenta; blue squares: term placenta; and left Y-axis) and infectious particles (blue triangles, term placenta, and right Y-axis) released in supernatant by placenta histocultures at different times upon USUV infection (dpi: days post-infection). Symbols represent the mean ± SEM for three to six independent experiments. (**f**) Results of anti Env immunofluorescence realized upon reinfection of Vero cells incubated with supernatant of placental histocultures, either from first-trimester (left panels) or term (right panels) placentas, done with supernatants collected at day 15 after infection by USUV or UV inactivated USUV (ND: not determined). Blue: DAPI, green: USUV Env antigen.

Table 1. USUV-positive brains and blood from babies born from experimentally infected mothers. Viral RNA was detected by quantitative RT-qPCR targeting the USUV NS5 gene.

Week of Pregnancy	Type of Birth	% of RT-PCR Positive Brain	% of RT-PCR Positive Blood	% of Death/Birth Defect
First	Natural delivery	12%	16% (2 weeks after delivery)	15% (11/73) *
	Cesarean (2° week)	6%	ND	3% (2/52) #
Second	Natural delivery	0%	0%	0% (0/41)

* Death/birth defect observed in 3 different littermates among the 9 dams infected. # Birth defect observed in 1 littermate among 6 dams infected. ND: Not determined.

The percentage of USUV RNA-positive pups' brains was not significantly different between natural delivery (12%) and cesarean section (6%), while around 16% of naturally delivered pups presented USUV RNA-positive blood two weeks after delivery (Table 1; Figure 5a). Fetal demise was observed in 15% of births after natural delivery, and in 3% of births after cesarean section one week after infection (Table 1; Figure 5b). Differential vertical transmission during the first or second week of gestation could be due to variation in maternal infection. For this reason, we assessed the levels of USUV in the spleen of the pregnant dams one week after infection, in the two conditions of infection. Maternal viral burden in the spleen was not substantially different seven days after inoculation at E6 or E12, suggesting that differences in systemic infection of the pregnant mice are not responsible for the observed phenotype (Figure 5c).

To determine whether direct infection of the placenta occurred, we measured USUV vRNA in the placenta of infected pregnant mice on E6 and E12, and sacrificed them seven days post-infection. We detected viral infection in about half of the placentas tested at E6 and E12. USUV appeared to replicate at higher titers at seven days post-infection (98-fold, $p < 0.005$) in placentas from dams infected at E6 compared to those infected at E12 (Figure 5d). To determine the possibility of USUV transmission during breastfeeding, because we still detected virus in the blood of pups two weeks after delivery, we sampled the mammary glands of the dams for the presence of the virus in these organs, and found no positive results (Figure 5e).

Overall, these results in mice confirm the data obtained on cells and human placenta, and indicate that USUV, similar to other flaviviruses, can be potentially transmitted by intrauterine route and can induce fetal demise and central nervous system infection at low-level rates. In addition, the gestational stage of the fetus has an impact on the extent of USUV replication in the fetus.

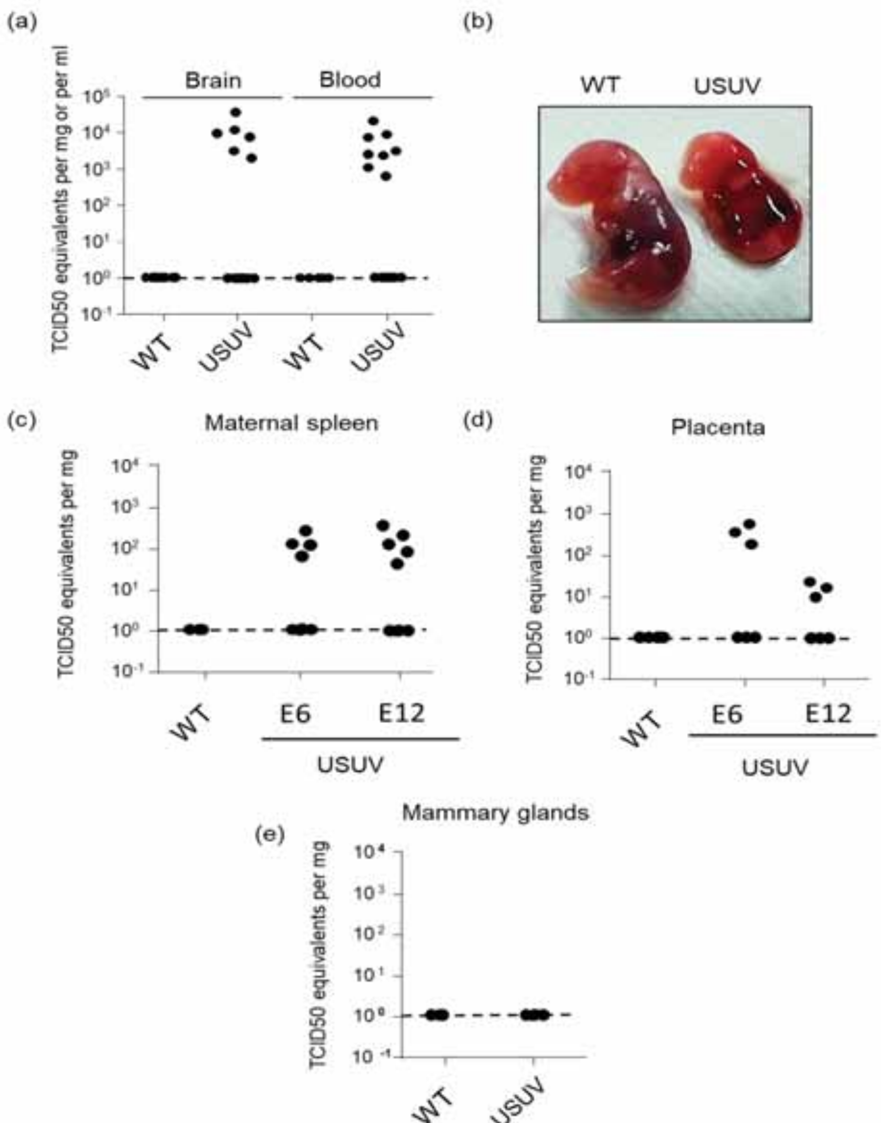

Figure 5. USUV can achieve congenital infection of immunocompetent mice and causes occasional fetal demise. C57BL/6 pregnant mice were inoculated with USUV by subcutaneous (footpad) route with 104 TCID50 in 50 μL of PBS. (**a**) Brain and blood of suckling mice were collected after the birth for the brain and 2 weeks later for blood. Viral burden was measured by RT-qPCR assay and indicated by TCID50 equivalent per g or per ml. (**b**) Fetal demise in USUV-infected mice after cesarean section in the second third of gestation. Spleen (**c**), placenta (**d**), and mammary glands (**e**) of infected pregnant mice were collected and viral burden was measured by RT-qPCR assay. Organs were harvested at E6 and E12 stages of gestation for spleen and placenta, and 2 weeks after delivery for mammary glands.

4. Discussion

Several studies suggest that several neurotropic flaviviruses, including ZIKV, Japanese encephalitis virus (JEV), St. Louis encephalitis virus (SLEV), and WNV, can cause fetal disease, with congenital infection, and could be involved in pregnancy complications and congenital malformation more frequently than is actually detected [45–57]. For this reason, we chose to study the ability of the USUV, closely related to WNV, to be transmitted vertically, which had never been investigated until now.

Because of the limited availability of fresh primary placental tissues, human immortalized placental cell lines provide tools for first-intention functional studies. We therefore chose to first examine the permissiveness of USUV in two different placental cell lines: the JAR choriocarcinoma cells [42,43], and the HIPECs [37]. Both cell lines display various key characteristics of human placental trophoblasts [58,59], but with some differences, the JAR possessing the ability to differentiate into syncytiotrophoblastic cells in vitro, and the HIPECs corresponding to extravillous cytotrophoblasts with invasive capacities. Both JAR cells and HIPECs were permissive for USUV, albeit with different efficiencies. JAR cells were more efficiently infected than HIPECs, with higher mortality rate, and higher production of infectious viral particles than HIPECs. Using a RT-qPCR multi-array device, we observed a strong antiviral response in JAR cells, characterized by the induction of some PRRs, interferon pathway molecules, ISGs, and chemokines such as CXCL10, CCL5, and CCL11. This pattern of gene induction was similar as what was observed in other cell types upon USUV infection, such as astrocytes [60]. In contrast, HIPECs were not able to elicit a strong antiviral immune response against USUV, with few induced genes and a very low level of induction. Hence, these data may reflect the variety of virus–cell interactions in a multicellular organ such as placenta, where closely related cell types organized in a defined cytoarchitecture, may be differentially permissive to USUV and elicit variable innate antiviral responses.

In a second experiment, we used primary explants of human placenta, from first- and third-trimester, to examine whether human placenta could allow productive replication of USUV, depending on gestational age. At the end of the two-week histoculture, placental tissues were positive for USUV vRNA (and for Env protein for some of them), indicating that USUV was able to express viral transcripts along the duration of the culture. Moreover, even if the quantities were low and variable between placentas, infectious particles were released from tissues explants, albeit with an apparent slightly better efficiency for third-trimester rather than first-trimester placenta. These quantities, although less important than what can be observed from ZIKV infected placental histocultures [32,61], nevertheless suggest that human placenta may be permissive for USUV viral replication. However, it should be kept in mind that JAR and HIPEC cell lines as well as placental histocultures do not perfectly reflect the pathophysiology of the infection, in particular because they lack the adaptive immunity component, which cannot be explored in these models.

Finally, our experiments suggest that USUV can be potentially transmitted by the intrauterine route in immunocompetent mice and induce fetal demise when infection occurred at the first week of pregnancy. With regard to *Flaviviruses*, the most striking effect of congenital infection has been described for ZIKV. The congenital Zika syndrome, consisting in microcephaly and other neurodevelopmental defects, is probably linked to the ability of ZIKV to replicate in the placenta and cross the blood–placental barrier [62]. ZIKV infection in mice during early pregnancy resulted in placental insufficiency and fetal demise whereas infections at late pregnancy caused no apparent fetal disease [36,45]. Vertical transmission also has been demonstrated for WNV in a mice model, showing that the virus is efficiently transmitted by vertical routes (intrauterine and lactation) even at the third week of pregnancy [24]. For both ZIKV and WNV, the vertical transmission rates are much more important than for USUV. For example, after natural delivery, 80% of fetuses from dams infected by WNV at the second week of pregnancy exhibit RNA virus in their brains [24], whereas for USUV, this figure is only 12% after the first week, and none after the second week. Moreover, fetal demise was detected in approximately 50% of WNV-infected animals [23]. Since we still detect viral load in blood of suckling mice two weeks after delivery it would be interesting to study the long-term impact on surviving pups after infection of pregnant females.

In our study, we observed a quite similar permissiveness of the human placenta to infection in first- and third-trimester placenta ex vivo, but vertical transmission in mice was observed following infection in the first tier of gestation only. During the ZIKV epidemic, observational data showed that ZIKV-associated congenital microcephaly was

most common when pregnant women were infected during the first or early second trimesters of pregnancy [63]. Moreover, studies in mice described that the placenta and fetus were more susceptible to ZIKV infection at earlier gestational stages [36]. In Ifnar1$^{-/-}$ mice, ZIKV infection at embryonic day six (E6) resulted in fetal demise, infections at mid-stage (E9) resulted in fetal morphologic abnormalities, and infection later in pregnancy (E12) caused no apparent fetal disease [36]. The placenta is a physical and immunological barrier that undergoes important changes during gestation, particularly between the beginning (first trimester) and the end (second and third trimesters) of human pregnancy [64]. The mechanisms underlying the gestational-stage-dependent variation in fetal injury following ZIKV infection have not been fully elucidated. The reduced susceptibility to ZIKV or USUV infection at later stages of gestation could result from differential spatio-temporal expression patterns of putative entry receptors, as suggested for ZIKV [31,32]. Alternatively, and additionally, early and late placenta could be distinguished by their innate immune profiles, notably by their type I and III interferon response [34,36,65,66] or by the expression level of the primate- and placenta-specific C19MC miRNA cluster, known to exert an antiviral activity, and the expression of which are temporally regulated during pregnancy [67–69]. Moreover, the fact that human and murine gestation and immune system are very different has to be taken into consideration, and results obtained in mice cannot be directly translated to humans.

In conclusion, our observations suggest that the emergence of the USUV in the human population could potentially represent a subject of concern in pregnant women, since the virus could be vertically transmitted to the fetus in a sporadic manner. Further studies are needed to better characterize the potential for vertical transmission of USUV, and to elucidate the capacity of this virus to cross the blood–placental barrier.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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TITRE: Effet du cluster de microARN C19MC au cours des infections virales congénital
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LIEU ET DATE DE SOUTENANCE: 13 décembre 2022, Toulouse

RÉSUMÉ:

Les infections virales congénitales touchent les femmes du monde entier et sont associées à des troubles du développement néonatal. Par exemple, le virus Zika (ZIKV) a été associé à de graves anomalies congénitales, dont la microcéphalie. De même, le cytomégalovirus humain (HCMV) est l'infection virale la plus répandue chez le fœtus et entraîne des troubles neurodéveloppementaux. Le ZIKV et le HCMV infectent les cellules placentaires, affectant directement le développement du nouveau-né. Le placenta utilise des vésicules extracellulaires (EVs) pour interagir avec son environnement, et il a été démontré que leur contenu en microARN (miARN), notamment ceux du cluster C19MC, est dérégulé au cours des grossesses pathologiques. Le cluster de miARN C19MC, spécifique aux primates, présente un profil d'expression dynamique tout au long de la grossesse, avec des niveaux qui augmentent vers la fin du premier trimestre et chutent rapidement après la naissance. En utilisant un modèle innovant de cellules Knock-Out (KO) pour le cluster C19MC généré par la technique CRISPR/Cas9 par l'équipe de J. Cavaillé, nous avons étudié la fonction antivirale de ce cluster au cours des infections virales congénitales. Des expériences impliquant des techniques de PCR quantitative en temps réel, de western blotting, de cytométrie de flux et d'immunofluorescence ont montré que les cellules KO infectées par le HCMV et le ZIKV sont plus sensibles aux infections virales que leurs homologues sauvages (WT). De plus, des tests TCID50 et l'analyse par cytométrie de flux ont montré une infectivité virale significativement plus élevée des surnageants contenant des particules infectieuses libérées par les cellules KO infectées que par les cellules WT. Nous avons utilisé des dispositifs de $RTqPCR^2$ profiler pour examiner les réponses immunes innées antivirales et d'autophagie des cellules KO et WT à l'infection par le ZIKV. Des différences génétiques distinctes dans l'expression suggèrent que le cluster C19MC influence la réaction à l'infection. Nous avons également exploré la possibilité d'un mécanisme d'action antiviral paracrine ; nous avons testé si cet effet antiviral pouvait être transmis à des cellules réceptrices (cellules Vero) via des EVs. Les données de cytométrie en flux ont montré un impact antiviral transférable dans les cellules Vero exposées au milieu conditionné des cellules WT par rapport aux cellules KO. Néanmoins, d'autres études sont nécessaires pour confirmer ces résultats. Notre recherche offre un modèle KO unique pour évaluer la fonction multidimensionnelle du cluster de miARN C19MC au cours d'infections virales congénitales et pour déterminer s'il peut ou non fonctionner en partie par le biais des EVs. Le développement de méthodes pronostiques et thérapeutiques améliorées et plus précises repose sur une compréhension approfondie des mécanismes d'action de ce cluster.

MOTS CLES: Zika, infection congénitale, C19MC microARN
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