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When viruses meet the placenta

Human herpesvirus-6 in pregnancy

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Abstract

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Preeclampsia is a major cause of maternal and perinatal morbidity, but its underlying mechanisms are not fully understood. The objective of this thesis was to investigate whether viral infection, specifically Human herpesvirus-6A/B (HHV-6A/B), may contribute to the development of preeclampsia, using a combination of population-based epidemiology and experimental studies in primary human trophoblast cells.

In **paper I**, with a Swedish register-based cohort of 618,814 primiparous women we showed that antiviral medication prescription during pregnancy was associated with a lower likelihood of preeclampsia (3.8% vs. 4.4%), an association that remained after adjustment (aOR 0.88; 95% CI 0.81–0.96). The reduction was most pronounced when antiviral therapy was initiated in the third trimester, supporting the hypothesis that viral reactivation may influence preeclampsia risk.

In **paper II**, we demonstrated that HHV-6A and HHV-6B can infect primary trophoblast cells and induce distinct DNA methylation changes. HHV-6A infection resulted in 37 differentially methylated regions linked to genes involved in angiogenesis and placental development, whereas HHV-6B affected a single but biologically important region in *PEG10*, a gene important for placental development. These findings provide the first evidence of targeted epigenetic effects of HHV-6A/B in trophoblasts.

In **paper III**, transcriptomic profiling revealed substantial changes in bulk RNA and miRNA expression following infection, including activation of interferon-mediated antiviral pathways and downregulation of genes involved in angiogenesis and placental function. These results suggest that HHV-6A/B may impair placental development through combined epigenetic and transcriptional mechanisms.

In **paper IV**, functional analyses showed that both viral variants increased secretion of anti-angiogenic factors such as Soluble fms-like tyrosine kinase-1 and Angiopoietin-2, while HHV-6B induced broader anti-angiogenic shifts, with a reduction in Placental growth factors and increased Vasohibin-1 and Thrombospondin-1. HHV-6A selectively activated the PERK-mediated endoplasmic reticulum stress pathway. Together, these findings show that HHV-6A/B differentially affect trophoblast function at the protein level.

In summary, this thesis supports a broader role for viral infection in preeclampsia and identifies mechanisms by which HHV-6A/B may contribute to disease. We show that HHV-6A/B can infect primary trophoblasts, alter epigenetic and transcriptomic profiles, and disrupt angiogenic and stress-response pathways, offering new insight into viral and molecular drivers of preeclampsia and related pregnancy complications.

Keywords: Preeclampsia, viral infections, herpesvirus-6, antivirals, pregnancy

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To Michael

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. **A. Zancanaro**, S. Hesselman, R. Hastie, A. Fogdell-Hahn, T. Kunovac Kallak, A.K. Wikström, S. Lager. Use of antiviral medications during pregnancy and the likelihood of preeclampsia in a population-based register study. *Sci Rep 15, 20809 (2025). doi:10.1038/s41598-025-09283-6*

II. **A. Zancanaro**, S. Dekker, A. Gravel, L. Flamand, A.K. Wikström, A. Fogdell-Hahn, T. Kunovac Kallak, S. Lager. Herpesvirus-6A and 6B infection induces significant differential methylation in primary human trophoblast cells. *Manuscript.*

III. **A. Zancanaro**, T. Kunovac Kallak, A. Gravel, L. Flamand, A.K. Wikström, A. Fogdell-Hahn, S. Lager. Human herpesvirus-6A/B induces antiviral responses and suppresses placental development pathways in human trophoblasts. *Manuscript.*

IV. **A. Zancanaro**, S. Dekker, A. Gravel, L. Flamand, A. Fogdell-Hahn, T. Kunovac Kallak, A.K. Wikström, S. Lager. Human herpesvirus 6A and 6B differentially disrupt placental function through angiogenic pathways and ER stress. *Manuscript.*

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Contents

Abbreviations

Ang-2	Angiopoietin-2
AT1-AA	Agonistic autoantibody to angiotensin II type I receptor
ATC	Anatomical Therapeutic Chemical
ATF6	Activating transcription factor 6
cDNA	Complementary DNA
CD46	Cluster of differentiation 46
BMI	Body mass index
CMV	Cytomegalovirus
DAG	Directed acyclic graph
DAMPs	Damage associated molecular patterns
DMEM	Dulbecco´s Modified Eagle Medium
DMP	Differentially methylated probe
DMR	Differentially methylated region
DMSO	Dimethyl sulfoxide
eHHV-6	Endogenous HHV-6
ER	Endoplasmic reticulum
EV	Extracellular vesicles
EVT	Extravillous trophoblast
F12	Ham´s F-12 Nutrient mixture
FBS	Fetal bovine serum
FDR	False discovery rate
FMF	Fetal Medicine Foundation
GO	Gene ontology
GSEA	Gene set enrichment analysis
hCG	Human chorionic gonadotropin
HHV	Human herpesvirus
HI	Heat inactivated
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HSV	Herpes simplex virus
iciHHV-6	Inherited chromosomally integrated herpesvirus 6
IFN	Interferon
IL	Interleukin
IRE1	Inositol-requiring enzyme 1
ISSHP	International Society for the Study of Hypertension in Pregnancy

LDH	Lactate dehydrogenase
MBR	Medical Birth Register
miRNA	MicroRNA
MOI	Multiplicity of infection
MS	Multiple sclerosis
NBCS	Newborn calf serum
NPDR	National Prescribed Drug Register
NGI	National Genomic Infrastructure
NLRP3	Nucleotide binding domain, leucine-rich repeat, pyrin domain containing 3
NPR	National Patient Register
NTC	No template control
ORA	Overrepresentation analysis
PAPP-A	Pregnancy associated plasma protein A
PERK	Protein kinase R-like endoplasmic reticulum kinase
PlGF	Placental growth factor
PMM	Predictive mean matching
R-region	Repeat region
RIN	RNA integrity number
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
SCB	Statistics Sweden
sFlt-1	Soluble fms-like tyrosine kinase-1
SFOG	Swedish Society of Obstetrics and Gynecology
sEng	Soluble endoglin
TGF	Transforming growth factor
Th1	Type 1 helper cells
Th2	Type 2 helper cells
Th17	Type 17 helper cells
Thbs-1	Thrombospondin-1
TLR	Toll like receptor
Treg	Regulatory T cell
TNF-α	Tumor necrosis factor α
U-region	Unique region
UPR	Unfolded protein response
Vash-1	Vasohibin-1
VEGF	Vascular endothelial growth factor

Introduction

Preeclampsia is a major contributor to maternal and neonatal morbidity and mortality. Although it affects millions of pregnancies each year, the underlying causes remain incompletely understood. Beyond pregnancy, women who develop preeclampsia face a significantly increased risk of future cardiovascular and renal disease, underscoring the urgent need to understand and prevent this condition. Increasing evidence suggests that inflammation, immune dysregulation, and environmental or infectious triggers may play important roles. One such potential factor is latent viral infections and reactivations. Human herpesvirus 6 (HHV-6) is highly prevalent, capable of integrating into human DNA, and can reactivate at any time, and more frequently under times of psychological or physiological stress, including during pregnancy. Yet, its role in placental biology and pregnancy complications has been largely unexplored.

This thesis investigates whether viral activity, particularly HHV-6A and HHV-6B, may contribute to placental dysfunction and the development of preeclampsia. By combining large-scale population data with mechanistic studies in primary human trophoblasts, this work aims to shed light on a possible infectious component of preeclampsia and to advance our understanding of how viral reactivation may influence maternal and fetal health.

Preeclampsia

Preeclampsia is a multisystem pregnancy disorder that affects between 2 and 8% of all pregnancies worldwide.[1] If untreated, preeclampsia may progress to eclampsia, a condition characterized by the development of seizures, HELLP syndrome (a serious blood clotting disorder), stroke and organ damage such as liver rupture, kidney failure and pulmonary edema.[2,3] These are all life-threatening complications. Preeclampsia and its associated complications contribute to approximately 10 to 15% of maternal mortalities worldwide and 25% of fetal and neonatal deaths, with the great majority of these being in low-resource settings.[4,5]

According to the 2022 International Society for the Study of Hypertension in Pregnancy (ISSHP) guidelines, preeclampsia is defined by the new-onset of gestational hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥ 90 mmHg on two occasions at least four hours apart) occurring after 20 weeks of gestation and accompanied by proteinuria, maternal organ dysfunction, and/or uteroplacental dysfunction.[6]

A large meta-analysis including over 25 million pregnancies has identified a number of risk factors for preeclampsia. Some maternal characteristics are nulliparity, maternal age over 35, high body mass index (BMI), multifetal pregnancy, and ethnicity. Health conditions linked to a higher likelihood of developing preeclampsia include antiphospholipid antibody syndrome, chronic hypertension, chronic kidney disease, pre-gestational diabetes, systemic lupus erythematosus, and a previous pregnancy complicated by preeclampsia.[7]

The mechanisms behind the pathogenesis of preeclampsia are complex and remain incompletely understood. What is known is that the disease is closely linked to the presence of the placenta, and that its delivery is the only cure. Preeclampsia presents an immediate threat to both mother and child, with potential long-term health ramifications. For mothers, long lasting consequences that are associated with preeclampsia include an elevated risk of developing preeclampsia in future pregnancies, increased risk of chronic hypertension, ischemic heart disease and other cardiovascular events, stroke, and the development of kidney disorders.[2] Additionally, preeclampsia can have an impact on long-term cognitive function.[8] For the child, long-lasting health effects are mainly related to preterm birth and intrauterine growth restriction predisposing them to an increased risk of hypertension and cardiovascular disease later in life, as well as cognitive impairment.[2]

The Placenta

Preeclampsia is a placental disorder, therefore it is important to understand the structure and development of the placenta. The placenta is an organ of both fetal and maternal origin that develops after implantation from the outer trophoblast layer of the blastocyst. The trophoblasts proliferate and form two stacked layers: an underlying cytotrophoblast layer and a multinucleated syncytiotrophoblast layer. Beneath this layer, cytotrophoblasts also give rise to extravillous trophoblasts (EVTs), a specialized invasive population of trophoblasts responsible for anchoring the placenta and remodelling maternal spiral arteries.[9] Defects in EVT invasion are a hallmark of early-onset preeclampsia.[10,11] During the early stages of development the placenta has relatively low oxygen levels.[12] However, around gestational week 6-10, trophoblast cells will invade the maternal uterine wall and induce spiral artery remodelling.[13] The process of spiral artery remodelling widens the uterine arteries allowing for increased blood supply to the placenta and the fetus.

Structurally, the placenta is made up of tissues of both fetal (chorionic plate) and maternal (basal plate) origin, connected to facilitate the exchange of nutrients, gases and waste products between mother and fetus. Maternal–fetal exchange occurs where maternal blood in the intervillous space comes into close proximity with the fetal capillaries inside the chorionic villi. Although the two circulations do not mix, this close proximity allows efficient transfer across the trophoblast barrier.

The placenta also serves as an endocrine organ, secreting hormones that are fundamental to maintaining pregnancy and supporting placental development, angiogenesis, immunotolerance and fetal growth. These hormones also prepare the mother for childbirth and lactation. Among the most important hormones produced by the placenta are human chorionic gonadotropin (hCG, measured in pregnancy tests), progesterone, estrogens, and leptin.[14,15]

Subtypes of preeclampsia

Preeclampsia is a complex and heterogenous syndrome that can be classified into subtypes. Recognizing the subtype is important for providing appropriate supportive treatment.[16] These subtypes are commonly defined by the gestational age at disease onset and the presence or absence of fetal growth restriction. Because gestational age at onset is commonly unknown, gestational age at delivery is often used as a proxy in epidemiological studies.[16]

The two main subtypes are:

- Early-onset preeclampsia: with onset or delivery before week 34.
- Late-onset preeclampsia: with onset or delivery after week 34.[17,18]

In addition to these categories, preeclampsia is also frequently described as:

- Preterm preeclampsia: with delivery before 37 weeks of gestation.
- Term preeclampsia: with delivery at or after 37 weeks of gestation.[17,18]

The early-onset subtype, often referred to as the placental type,[19] is characterized by abnormal placentation due to inadequate EVT invasion and spiral artery remodelling.[10,11] In contrast, the late-onset or term preeclampsia subtype is characterized by a later decline of placental function that is thought to be caused by premature placental ageing and/or villous overcrowding.[20,21] Preeclampsia can also manifest postpartum, most frequently within the first week after delivery, though there are known cases of it manifesting up to six weeks postpartum.[6]

It is agreed upon that angiogenic dysfunction, endoplasmic reticulum (ER) stress, inflammation, and oxidative stress are involved in the pathogenesis of preeclampsia.[16] While the two main subtypes of preeclampsia are characterized by different early manifestations, the downstream consequence is the same: placental stress.

Screening, diagnosis and treatment

Preeclampsia is a dangerous condition that can progress rapidly and pose immediate risks to both mother and child. Early identification of individuals at increased risk is therefore essential. In this context, screening refers primarily to efforts made early in pregnancy to distinguish between women at high and low risk of developing preeclampsia, so that those at elevated risk can be offered prophylactic low-dose aspirin. In Sweden, the most commonly used screening approach is based on individual maternal risk factors, such as medical history and characteristics recorded at the first antenatal visit. The Swedish Society of Obstetrics and Gynecology (SFOG) recommends routine blood pressure and urine testing throughout pregnancy. Blood pressure is measured at every antenatal visit, and all pregnant women are screened for proteinuria at their first visit. Thereafter, proteinuria testing is repeated at subsequent visits in women with risk factors or in the presence of rising blood pressure.[22]

Internationally, risk assessment is increasingly being expanded beyond maternal factors to include biochemical markers and ultrasound assessment of uterine artery blood flow.[23] These parameters can be combined using algorithm-based models to generate an individualized risk estimate, the most commonly used being the model developed by the Fetal Medicine Foundation (FMF).[24] However, routine use of biochemical markers or prediction models for preeclampsia screening is currently not recommended by Swedish national guidelines.[22]

There is no definitive treatment for preeclampsia aside from delivery of the placenta. Preventive strategies such as administration of aspirin can be employed in at risk pregnancies to prevent early-onset preeclampsia.[25] If severe features of preeclampsia manifest after week 37 delivery is typically recommended. When severe features develop before week 34, the administration of corticosteroids to promote fetal lung maturity is recommended to prepare the preterm fetus for delivery.[26]

Biomarkers

Although the use of biochemical methods for preeclampsia screening is not currently recommended for clinical use in Sweden, several studies have explored the utility of different biomarkers. The growing availability of automated laboratory assays may further support their clinical implementation at lower costs. Different algorithms that include biomarkers can be used to predict preeclampsia. First trimester screening models, such as the FMF model, include placental growth factor (PlGF) and pregnancy-associated plasma protein A (PAPP-A). Low levels of both PlGF and PAPP-A have been associated with preeclampsia.[27,28]

Other biomarkers, such as soluble fms-like tyrosine kinase-1 (sFlt-1) and soluble endoglin (sEng), have limited predictive value in the first trimester but can be used in the second and third trimesters, particularly when preeclampsia is suspected. These angiogenic markers are commonly evaluated as a ratio.[29,30] Both the sFlt-1/PlGF and PlGF/sEng ratios have proven effective in predicting early-onset preeclampsia.[31,32]

Inflammatory and placental dysfunction biomarkers have also been investigated. Low levels of placental protein 13 have been observed in pregnancies complicated by preeclampsia and may contribute to impaired endothelial dysfunction.[33]

Pathophysiology of Preeclampsia

The pathophysiology of preeclampsia is complex and multifactorial, involving several mechanisms that reinforce and amplify each other (Summarized in Figure 1). Impaired placentation, oxidative stress, ER stress, inflammation, and angiogenic imbalance are all factors contributing to the disease.

Impaired placentation, hypoxia and oxidative stress

Shallow trophoblast invasion and inadequate spiral artery remodeling can lead to reduced uteroplacental perfusion, characteristic of early-onset preeclampsia.[12] This impaired remodeling results in intermittent and turbulent blood flow. These fluctuations generate oxidative stress in the placenta through increased production of reactive oxygen species (ROS) by the mitochondria and the ER. In preeclampsia there is a shift from an anti-oxidative to a pro-oxidative environment, contributing to further production of ROS.[34] This oxidative stress contributes to further increase in production of pro-inflammatory cytokines[35] as well as a decrease in nitric oxide (NO), which in normal pregnancies has a vasodilating effect.[36]

Endoplasmic reticulum stress

The ER has a central role in the post-translational modification of proteins, and is also involved in the coordination of a number of pathways regulating cell metabolism, proliferation, and death. When the ER cannot keep up with protein folding, misfolded proteins can accumulate in the lumen and lead to ER stress. In women with preeclampsia the ER is enlarged due to the accumulation of proteins, which can be observed microscopically in placental pathology specimens.[37]

ER stress activates the Unfolded Protein Response (UPR), a protective mechanism aimed at restoring ER function. If the UPR fails, the apoptotic cascade is activated to destroy damaged cells.[38] The UPR is made up of three main cellular pathways: protein kinase R-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6).[39] The accumulation of proteins in the ER leads to the binding of unfolded proteins to PERK, IRE1 and ATF6 activating each respective pathway.[39] When it comes to preeclampsia, placental ER stress has been observed in the placentas of women with early-onset preeclampsia[40] and severe preeclampsia.[41]

Inflammation, immune activation and endothelial dysfunction

A local increase in inflammation levels is fundamental for a successful implantation[42] and for establishing an immunotolerant environment that facilitates maternal acceptance to the semi-allogeneic fetus.[43] In preeclampsia, this immunotolerant state is not achieved, and a shift towards a pro-inflammatory state is observed.[44]

In women with preeclampsia, an excess of pro-inflammatory cytokines is released by Type 1 helper cells (Th1) and Type 17 helper cells (Th17),[44] while the release of anti-inflammatory cytokines by regulatory T cells (Tregs) and Type 2 helper cells (Th2) is reduced.[45] In pregnancies complicated by preeclampsia, this immune imbalance begins in early pregnancy, and becomes more pronounced in the second and third trimesters as placental ischemia and oxidative stress develop. Elevated levels of interleukin (IL)-6, IL-17, tumor necrosis factor α (TNF-α) and interferon-γ (IFN-γ) are found in the maternal circulation of preeclamptic women,[46,47] and increased IL-1β has also been reported.[48,49] At the same time, levels of anti-inflammatory cytokines such as IL-10 and IL-4 are decreased,[44] further contributing to immune imbalance. In healthy pregnancies IL-10 is upregulated and contributes to the suppression of the maternal immune system to accept the fetus, but in serum samples of women with preeclampsia IL-10 levels are reduced.[50]

The release of these pro-inflammatory factors has multiple consequences. By activating endothelial cells, TNF-α increases the production of endothelin-1, a vasoconstrictor that further induces an increase in blood pressure.[44,51] TNF-α, IL-6 and IL-17 stimulate the production of agonistic autoantibody to the angiotensin II type 1 receptor (AT1-AA). AT1-AA, in turn, stimulates the production of ROS, which further stimulates the production of TNF-α, and has a direct effect on vasoconstriction,[52] again causing an increase in blood pressure. Additionally, the release of damage associated molecular patterns (DAMPs), such as uric acid, cholesterol crystals, extracellular DNA and extracellular vesicles (EV) debris that are released in excess in women with preeclampsia activate the nucleotide-binding oligomerization domain, leucine-rich repeat-, and pyrin domain containing 3 (NLRP3) inflammasome causing additional release of IL-1β.[53] Complement activation, another component of innate immunity, is also enhanced in preeclampsia and contributes to endothelial injury and inflammation.[54,55]

Angiogenesis

Angiogenesis, the formation of new blood vessels, is essential for placental development. In early-onset preeclampsia, abnormal placentation results in more pronounced angiogenic dysregulation, while in late-onset preeclampsia it is present to a lesser degree. Overall, preeclampsia is characterized by an

imbalance between angiogenic and anti-angiogenic factors, resulting in endothelial dysfunction and placental insufficiency.

A known anti-angiogenic factor that has higher levels in preeclampsia is sFlt-1 (also known as sVEGFR1).[56,57] An increase in sFlt-1 causes a decrease in PlGF and vascular endothelial growth factor (VEGF) by binding to and neutralizing them.[57] sEng also has anti-angiogenic properties and its levels are increased in women with preeclampsia.[58] sEng is released into the maternal circulation and binds to the transforming growth factor β1 (TGF-β) preventing it from giving a pro-angiogenic signal to endothelial cells.[59] Inflammatory cytokines and NLRP3 activation can further drive sFlt-1 and sEng release.[53]

In addition to these well-characterized factors that are already used for preeclampsia screening, other regulators of angiogenesis are emerging. Thrombospondin-1 (Thbs-1), a matricellular glycoprotein with anti-angiogenic properties, is altered in pregnancies complicated by preeclampsia, and has been reported as both elevated and downregulated.[60–62] Vasohibin-1 (Vash-1), is an endothelial derived negative feedback regulator of angiogenesis, is also altered in preeclampsia.[63] Angiopoietin-2 (Ang-2), which destabilizes blood vessels, is similarly elevated in preeclampsia.[64–66]

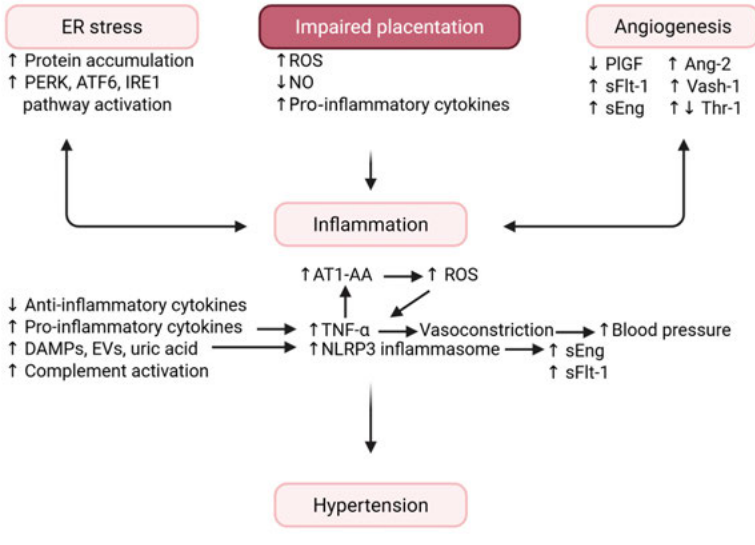

Figure 1. **Elements participating in the pathophysiology of preeclampsia**. These mechanisms are interlinked through feedback loops, with oxidative stress, pro-inflammatory cytokines, and altered angiogenic balance reinforcing each other ultimately leading to hypertension.

Differential gene expression

The placenta is a highly dynamic organ that undergoes continuous molecular and structural adaptation throughout pregnancy to support the developing fetus. These processes depend on precise regulation of gene expression within pathways that direct trophoblast invasion, responses to oxidative stress, immune interactions, and angiogenesis. In preeclampsia, this regulatory balance is disrupted, leading to characteristic abnormalities in placental development and function. Transcriptomic studies consistently demonstrate altered activity in key pathways and have identified several dysregulated genes, yet the specific genes implicated vary considerably across studies. This variation reflects both the biological heterogeneity of preeclampsia, with emerging evidence from single-cell RNA sequencing revealing that early-onset and late-onset preeclampsia represent molecularly distinct syndromes,[67] and methodological differences between studies. Furthermore, confounding factors such as gestational age, fetal sex, and delivery method all influence placental gene expression.[68]

Epigenetic modulation

Placental development is regulated by epigenetic mechanisms, processes that modify gene activity without changing the underlying DNA sequence. These mechanisms, such as DNA methylations and microRNAs (miRNAs), play a critical role in fine-tuning gene expression, with methylations generally being associated with gene silencing, whereas miRNAs regulate gene expression post-transcriptionally. Several studies have shown that these regulatory patterns differ significantly in women with preeclampsia compared to those with healthy pregnancies.[69]

Significant differences in methylation patterns of CpG islands have been found in placental samples of women with preeclampsia compared to women with healthy pregnancies,[70] and abnormal DNA methylation has been found to be associated with accelerated placental aging in early-onset preeclampsia.[71] These altered DNA methylation patterns (hypomethylations or hypermethylations) of specific genes can lead to unregulated expression of proteins involved in angiogenesis, immune response, and inflammation.

MiRNAs are non-coding small RNAs that regulate gene expression at the post-transcriptional level.[72] A number of studies reviewed by Ashraf et al. have shown the upregulation or downregulation of several miRNAs in women with preeclampsia that can be linked to decreased trophoblast viability, impaired vascular development, and decreased levels of pro-angiogenic factors.[73]

Infections of the placenta

One of the many functions of the placenta is to protect the fetus from pathogens. The protection occurs through multiple levels of defence. To reach the fetus, pathogens need to pass the syncytiotrophoblast layer, which lacks intercellular junctions and offers a physical defence barrier. Under the syncytiotrophoblast, pathogens need to pass the chorionic villi that contain Hofbauer cells (fetus derived macrophages) and then pass through the fetal microvasculature to reach fetal blood (Figure 2). On the maternal side, the decidua expresses cytotoxic, helper, and regulatory T cells, natural killer cells, macrophages, and neutrophils.[74]

Despite this multi-layered defence system, certain pathogens can cross the placenta and infect the fetus. These are referred to as TORCH pathogens, an acronym for *Toxoplasma gondii*, "Other" pathogens (including syphilis, Zika virus, and parvovirus B19), Rubella virus, CMV, and Herpes simplex virus (HSV).[75] TORCH pathogens have evolved different mechanisms to breach placental barriers, enter the fetal circulation, and in some cases cause severe congenital infection, developmental abnormalities, or pregnancy loss. These pathogens employ different strategies to enter the placenta. For example, CMV uses the cluster of differentiation 46 (CD46) receptor, a complement inhibitory receptor expressed on all nucleated cells, to enter trophoblasts and other cells.[76] The same mechanism is also employed by HHV-6A and HHV-6B to enter other cell types.[77]

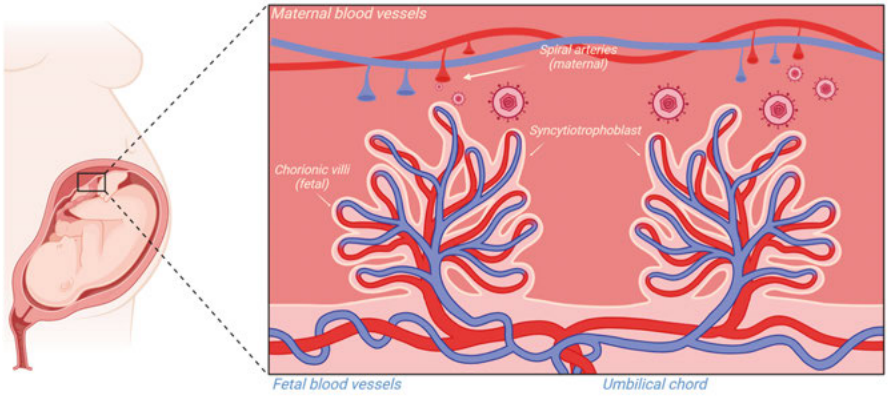

Figure 2. **Schematic representation of the maternal–fetal interface of the placenta and the barriers that pathogens must cross to reach the fetus**. Maternal blood flows into the intervillous space through spiral arteries and surrounds the chorionic villi that contain fetal blood vessels. The villi are covered by a continuous syncytiotrophoblast layer, which forms the primary barrier to pathogens. Beneath it lie the villous stroma and fetal microvasculature. To reach fetal blood, pathogens must cross the syncytiotrophoblast, the chorionic villi, and finally the fetal vessels.

Preeclampsia and microorganisms

In 1892, Gerdes (Centralblatt fur Gynäkologie, 1892, No. 20) hypothesized that preeclampsia was caused by a bacterium he found in women that had died of preeclampsia. He named this bacterium *Bacillus preeclampsia.* In the early 1980s, Lueck and colleagues observed worm-like structures in the blood of preeclamptic women that they named *Hydatoxi lualba,*[78] but these structures later turned out to be artefacts.[79] In the end, the hypothesis of microorganisms being involved in the pathogenesis of preeclampsia was abandoned.

More recently, the role of microorganisms in the development of preeclampsia has seen an increase in interest, not as the sole causative factor but as a contributing factor to its development. Multiple pathways that are activated during viral infections are similar to those that are activated during preeclampsia, most of them involving inflammation through the release of pro-inflammatory cytokines.[80]

Few viruses have been studied in relation to preeclampsia. The link between preeclampsia and viruses has recently been in the spotlight during the SARS-CoV-2 pandemic. The relationship between preeclampsia and COVID-19 is still not clear, although a number of studies have shown a probable association between the infection and preeclampsia, others have not. It has also been found that pregnant women with COVID-19 had higher levels of sFlt-1[81] and lower levels of PlGF,[82] similar to the biomarker profile seen in preeclampsia. It has also been reported that women who are Human immunodeficiency virus positive (HIV+) but have not yet started antiretroviral therapy have a lower risk of developing preeclampsia compared to the general population.[83] This could suggest that the virtual absence of the immune system could be protective against preeclampsia, suggesting a potential involvement of the immune system.[84] Other viruses that have been associated with an increased risk for preeclampsia are rubella, HSV-2,[85] adeno-associated virus-2,[86] and finally, HHV-6.[87]

Human herpesvirus-6

HHV-6 was first discovered in 1986 when it was isolated from the blood of six individuals with lymphoproliferative disorders.[88] Further research revealed that the virus infected isolated human B cells leading to their visible transformation into large cells.[88] By 1991, it became clear that there were differences between various strains of HHV-6 that warranted their classification into two groups, A and B.[89] In 2012, these variants were formally recognized as separate viral species: HHV-6A and HHV-6B.[90]

HHV-6A/B are double-stranded DNA viruses, part of the *Betaherpesvirinae* subfamily. Of the two viruses, HHV-6B is the most common. HHV-6B infection is usually acquired before 1 year of age and causes roseola, an infection characterized by high fever and a rash.[91] The clinical manifestations of HHV-6A are still not clear, but it is believed to cause asymptomatic infection.[92] Both HHV-6A and HHV-6B are so prevalent in the population that they are considered ubiquitous.

Structure and genome

HHV-6A and HHV-6B share the typical structure of other herpesviruses. An icosahedral capsid encloses the double-stranded viral DNA genome. An envelope that is derived from the host cell´s membrane surrounds the capsid and contains viral glycoproteins that are required for viral attachment and entry into host cells. Between the capsid and the envelope lies the tegument layer, which contains viral proteins involved in early stages of infection.[93,94]

The HHV-6 genome is composed of both unique and highly conserved regions. HHV-6A and HHV-6B share ~90% of their genomic sequence.[95] The genome, measuring 160-170 kb in length, is made up of unique regions (U regions) that contain core genes for viral replication and propagation, as well as tandem repeat regions (R regions) situated at the ends of the genome that play a role in the initiation of viral replication.[95,96]

Mechanisms of infection

Viral entry of HHV-6A and HHV-6B is initiated by interactions between the tetrameric envelope glycoprotein complex gH/gL/gQ1/gQ2 and specific host cell receptors. Although closely related, HHV-6A and HHV-6B differ slightly in the preferred receptor used for viral entry. HHV-6A uses primarily CD46 and HHV-6B uses primarily CD134, with CD46 serving as an alternative receptor in certain cell types, particularly via the C isoform.[77] Despite their ability to infect multiple cell types, both viruses have a strong tropism for $CD4^+$ cells, which serve as their primary target.[97]

In trophoblasts, CD134 is absent but CD46 is expressed, although the specific isoforms present have not been well characterized. Receptor use can also

differ by viral strains, with some HHV-6B strains having a higher affinity for CD46 than others.[77] Additionally, the heat shock protein 90 beta family member 1 (GP96) has been shown to facilitate viral entry and to enable infection even in cells lacking CD46,[98,99] a mechanism of particular relevance given that GP96 is expressed in trophoblasts.[100]

Integration and latency

One characteristic of all members of the *Herpesviridae* family that infect humans is that they are able to establish latency and persist indefinitely in a host.[101] The mechanisms behind HHV-6 integration are still largely unknown, what is known is that integration occurs in chromosomes´ telomeric or subtelomeric regions.[102] Reactivation of latent integrated virus can happen at any time, although it has been most clearly described during immunosuppressive states after a transplant.[103,104] HHV-6A/B is also more likely to reactivate during pregnancy compared to the general population,[105] yet the specific triggers and environmental factors that may cause reactivations are not well understood. While there is no definitive proof, it has been reported that telomeres with integrated HHV-6 are often short and unstable, and could theoretically contribute to the onset of cellular senescence.[106]

Endogenous HHV-6

If viral integration occurs into a germ cell that is passed down to a child, there is a 50% chance that this individual will carry HHV-6A/B in all cells of their body in what is referred to as endogenous HHV-6 (eHHV-6, previously known as chromosomally integrated HHV-6 or iciHHV-6). Endogenous HHV-6 is present in 1% of the population.[107,108] Since these individuals carry the HHV-6 genome in all cells of their body an abnormally high viral load is detected in their blood which can be mistaken for an active infection.[44] The consequences of eHHV-6 on the affected individuals are not clear, but it is more common in hospitalized people compared to healthy blood donors, suggesting a possible correlation with poor health.[109]

Response to HHV-6 infection

While there are not many recent studies on the response of the immune system to an HHV-6 infection, it has been shown that HHV-6 infection induces the production of pro-inflammatory cytokines IFN-α, TNF-α, IL-1β, IL-8 and IL-15.[110] A study examining the serum concentration levels of pro-inflammatory and anti-inflammatory cytokines in multiple sclerosis (MS) patients found significantly increased levels of pro-inflammatory cytokines TNF-α, IFN-γ, IL-1β and IL-6 and significantly lower levels of anti-inflammatory cytokines IL-12 and IL-10 in patients positive for HHV-6.[111]

HHV-6 also interacts with toll-like receptors (TLR), receptors that recognize pathogens. In particular HHV-6 interacts with TLR4, leading to reduced production of the anti-inflammatory cytokines IL-8 and IL-10.[112] In mouse models, HHV-6A infection has additionally been shown to activate TLR9, resulting in increased production of pro-inflammatory cytokines.[113]

HHV-6 has been implicated as a possible trigger for several immune diseases such as MS, Hashimoto´s thyroiditis, and autoimmune connective tissue diseases.[114]

HHV-6 and reproduction

The presence of HHV-6 has been linked to a number of reproductive conditions. HHV-6A has been linked to unexplained infertility and recurrent implantation failure, with studies showing that 43% of women with unexplained infertility and 37% of women with recurrent implantation failure had endometrial biopsies positive for HHV-6A, compared to none in the control groups.[115–117] In these studies, the authors suggest that HHV-6A infection could lead to an altered immune state that could potentially hinder embryo implantation and trophoblast invasion. Previous studies have shown that HHV-6A can infect the syncytiotrophoblast layer of the placenta,[118] whether primary cytotrophoblasts are permissive to infection is currently unknown.

The study that initiated my PhD project revealed that mothers carrying a fetus with eHHV-6 have a three-fold increase in the chance of developing preeclampsia.[87] This significant correlation underscores the potential impact of HHV-6 on maternal health outcomes during pregnancy.

Aims

The overarching aim of this project was to investigate whether HHV-6A/B reactivation may contribute to the development of preeclampsia and to explore potential mechanisms involved. Although eHHV-6 in the placenta has been associated with increased preeclampsia risk, it likely explains only a small fraction of cases. Since most individuals carry latent HHV-6A/B, these findings support the broader hypothesis that reactivation, of endogenous or exogenous virus, may influence disease pathways. Building on this rationale, this thesis examined both the general impact of viral infection during pregnancy and the specific effects of HHV-6A/B on primary human trophoblasts, with the following aims:

I. To investigate the association between antiviral medication use and the likelihood of developing preeclampsia.

II. To characterize HHV-6A/B induced changes in DNA methylation patterns in primary human trophoblast cells.

III. To analyse the effects of HHV-6A/B infection on bulk RNA and miRNA expression in primary human trophoblast cells.

IV. To investigate the effect of HHV-6A/B infection on the release of pro- and anti-angiogenic factors and ER stress pathways in primary human trophoblast cells.

Methods

Ethical consideration

All studies have been approved by the Swedish Ethical Review Authority. Paper I: registration number 2019-04925 with amendment number 2022-00922. Paper II, III and IV: registration number 2021-04324.

Women who donated their placentas received written and verbal information and provided written informed consent. Sensitive data were pseudonymized, with identifiers stored separately under restricted access. Data were hosted on secure Swedish academic infrastructures (Vesta, Uppsala UPPMAX/BIANCA).

Paper I

Study population and design

This was a population-based register study of all women giving birth to their first child in Sweden between 2007 and 2019 (n=618,814). Data were obtained from four different registers, the medical birth register (MBR), the national patient register (NPR), the national prescribed drug register (NPDR) and Statistics Sweden (SCB).

We obtained information on antiviral medication dispensation from NPDR and HSV diagnosis from MBR and NPR. The choice of including women that gave birth between 2007 and 2019 was motivated by the fact that the electronic reporting of data for MBR was introduced in 2007. We chose 2019 as our upper limit as we did not want to include any women that were diagnosed with COVID-19, as studying viral infections during a pandemic could introduce unwanted confounders. HSV-1 and HSV-2 diagnosis were included because it is the primary indication for antiviral medication prescription during pregnancy. Information on preeclampsia diagnosis was obtained from MBR and NPR. Information on prescription and dispensation of antiviral medication with Anatomical Therapeutic Chemical (ATC)-code J05AB was collected from NPDR. In this study, we lacked information about time of onset of preeclampsia, so the disease has been classified into different subtypes according to gestational age at delivery and fetal growth.

Statistical methods

Initially, three groups were compared using adjusted logistic regression since we noticed that many women who had a herpesvirus diagnosis were not prescribed antiviral medications. These groups were:

I. Women who filled a prescription for antiviral medications (Exposed to antivirals).
II. Women with a HSV diagnosis that were not prescribed antiviral medications (Unexposed to antivirals, HSV+).
III. Women without a HSV diagnosis that were not prescribed antivirals (Unexposed to antivirals, HSV-).

Since there was no difference in the odds of developing preeclampsia between unexposed groups (group II and III), these groups were pooled together for subsequent analyses.

Logistic regression with inverse probability of treatment weighting (IPTW) was employed to compare women exposed to antiviral medications with unexposed women and to examine associations across different preeclampsia subtypes. Likelihood of preeclampsia development was also investigated according to time point of first prescription filling using logistic regression with IPTW. Briefly, IPTW is a statistical method that is used to account for potential confounding in the baseline characteristics of the population. IPTW creates a weighted sample in which the distribution of measured covariates is balanced between exposed and unexposed groups, allowing estimation of the association between antiviral exposure and preeclampsia as if treatment assignment were random. Three separate imputations were made for missing BMI values, and the analysis was repeated three times for each imputed BMI dataset. Imputations were conducted using the mice[119] R package with predictive mean matching (pmm), using our confounders as predictors, and results were pooled together. Confounders were identified using a directed acyclic graph (DAG) considering clinical risk factors for preeclampsia. The following confounders were included in all adjusted models: maternal age, BMI, country of birth, in vitro fertilization use, smoking at first antenatal visit, pre-gestational diabetes, chronic hypertension, chronic kidney disease and systemic lupus erythematosus. All statistical analyses were performed using RStudio (RStudio 2023.09.1 + 494 “Desert Sunflower”).

Papers II, III and IV

Papers II, III, and IV are based on *in vitro* studies using primary trophoblast cells. A paired study design was used, in which cells from the same donor served as their own control. For each placenta, non-infected trophoblasts were directly compared with cells infected with HHV-6A or HHV-6B. All primary cells used in these experiments were derived from the same set of placentas.

The use of a paired design had several important advantages. First, it reduced biological variability, as primary trophoblast cells can differ a lot between individuals. By comparing infected and non-infected cells from the same placenta, genetic differences, differentiation state, and baseline cellular activity were controlled for. Second, this approach enabled the detection of smaller effects induced by the viruses. Finally, this design allowed us to more confidently attribute any changes to the HHV-6A/B infection rather than natural variations between placentas.

Trophoblast cells were collected from placentas that were donated by women undergoing planned Cesarean sections at Uppsala University Hospital. In total, sixteen placentas were collected, eight from pregnancies with a male fetus and eight from pregnancies with a female fetus, to account for sex-biased gene expression in the placenta.[120] Although all sixteen placentas were collected and used for preliminary infection experiments, only eight (four from pregnancies with a male and four from pregnancies with a female fetus) were included in the subsequent analyses. This reduction occurred because some primary cell preparations did not tolerate freezing and thawing, and others showed poor attachment to culture plates, issues that are common when working with primary trophoblast cells.[121]

Women undergoing Cesarean sections were selected for two main reasons. First, for practical purposes, as the trophoblast isolation protocol is time-consuming and requires substantial preparation. Second, placentas obtained after labor are known to exhibit altered gene expression profiles, which could introduce confounding factors.[122] The only exclusion criteria were preeclampsia or hypertensive disorders during the current or previous pregnancies. Placentas were collected and processed immediately after delivery. All participants provided written informed consent after receiving both a verbal explanation and a written summary of the study from a trained research nurse.

Placental cell isolation

The trophoblast isolation protocol was based on the method first described by Kliman et al. in 1986.[123] The whole placenta was collected and processed at our laboratory. The placenta was placed in a sterile tray with the decidua basalis facing up (chorionic plate with umbilical cord facing down).

Approximately five healthy looking cotyledons (avoiding calcifications, blood vessels, and blood clots) were dissected using scissors and the decidual

layer was removed. The tissue was then put in a beaker containing 0.9% saline solution at 37 °C. The tissue was then placed in gauze over a funnel and rinsed with approximately 600 ml of saline solution. Large blood vessels and other visible impurities, such as blood clots or minor calcifications were removed from the tissue that was then minced by cutting several times with scissors. The minced tissue was transferred to a conical flask to undergo three different digestions using Trypsin and DNase. Trypsin is used to release individual cells, whereas DNase prevents cell clumping by degrading extracellular genomic DNA. For this step, trypsin enzymatic activity was important to allow for optimal digestion so the manufacturer was contacted to know exact enzymatic activity of each trypsin batch. The supernatant of each digestion was collected, layered over heat-inactivated (HI) new born calf serum (NBCS, Gibco) and centrifuged to stop the enzymatic digestion. After centrifugation, the supernatant was discarded and the cells at the bottom were pooled together, resuspended in warm Dulbecco´s Modified Eagle Medium (DMEM, Gibco) and layered over a Percoll gradient that was prepared the same morning. Bands between 55 and 35% containing the trophoblast cells were collected. These cells were pelleted, counted and resuspended in complete trophoblast cell media (DMEM/Ham´s F-12 Nutrient mixture (F12, Gibco) with 10% HI fetal bovine serum (FBS, Gibco), 1% penicillin-streptomycin-glutamine (PSG) stock, and 0.1% gentamicin). For each placenta, cells were plated at a density of 3 million cells in a 6-well plate, and 200,000 per well in an 8-well chamber slide. The remaining cells were frozen using freezing media (90 % HI FBS, 10% Dimethyl sulfoxide (DMSO), Sigma-Aldrich).

Characterization

Trophoblast cell identity and purity were assessed through both morphological evaluation and molecular markers. Cells were cultured for five days to visually confirm syncytiotrophoblast formation, which typically appeared by day 3 (and in some cases as early as day 2). To further validate trophoblast functionality, 1 mL of culture medium was collected daily, and levels of hCG, a hormone specifically produced by trophoblast cells, were quantified using ELISA. An increase in hCG concentrations by day 3 was used as an additional indicator of successful syncytiotrophoblast formation.

Cell composition was assessed using immunocytochemistry for Cytokeratin-7 (clone OV-TL 13/30, Dako), a marker of trophoblast cells, and Vimentin (clone V9, Dako), a marker of stromal cells. Only cultures containing at least 85% Cytokeratin-7 positive trophoblast cells were included in the analyses. Images were analyzed in ImageJ,[124] where the proportion of Cytokeratin-7 positive cells was quantified within representative fields.

Herpesvirus-6A/B virus stock preparation

Aliquots of 1 ml of HHV-6A (strain U1102) infected cells and 1 ml of HHV-6B (strain Z29) infected cells were kindly donated to us by Prof. Louis Flamand of Laval University, Quebec City, Canada. HSB-2 and MOLT-3, both T lymphoblast cell lines, used to propagate the viruses were also donated.

First, both cell lines were cultured in Roswell Park Memorial Institute (RPMI, Gibco) medium (10% FBS, 0.1% gentamicin, Sigma-Aldrich) in order to obtain more uninfected cells that were needed for the viral production. HHV-6A was prepared in HSB-2 cells while HHV-6B was prepared in MOLT-3 cells, using the same media and the same protocol. The only difference between protocols was ideal cell density of the two different cell lines with HSB-2 at a density of 750,000 cells/ml and MOLT-3 at a density of 500,000 cells/ml.

One million infected cells were mixed with 10 million uninfected cells. Cells were left alone and fresh media was added either when the media turned too acidic (as indicated by cell media turning yellow) or to restore ideal cell density after cell counting. Cells were ready for collection when 80% of the cells showed signs of infection by being enlarged. Cells and cell culture media containing virus were collected, and the cells were disrupted to release viral particles through three cycles of rapid freezing in a dry ice bath followed by thawing at 37 °C. The lysates were centrifuged at 2,300 × *g* for 15 minutes to pellet cellular debris. The resulting supernatant containing the virus was filtered and subsequently ultracentrifuged at 38,420 × *g* for 2 hours and 40 minutes to pellet the virus. The viral pellet was resuspended in the minimal possible volume of RPMI medium and incubated on ice overnight. Aliquots of the purified virus were stored at −196 °C.

In this step, culturing HHV-6A proved particularly challenging (this is a known issue when culturing HHV-6A).[125] We hypothesize that the virus likely entered a latent state and did not replicate as efficiently as HHV-6B.

Viral titration

When working with viruses, it is essential to assess their infectious activity, or *titrate* the virus. This process can be challenging because several different titration methods exist. For this project, we chose to determine viral titers by infecting cells and quantifying the proportion that became infected.

First, we used uninfected HSB-2 and MOLT-3 cells and resuspended them in 50 µl of media with increasing amounts of virus for 5 hours, with agitation every 30 minutes to ensure viral mixing. Viral particles were then washed away, and cells resuspended in 1 ml of RPMI media in a 12-well plate and incubated for 42 hours. After 42 hours, 400 µl were collected, the supernatant was removed by centrifugation at 200 x g for 5 minutes and the cells were

resuspended in 12 µl of PBS. Ten µl of each resuspended cell pellet was transferred to a 10-well slide and left to dry. When dry, the slide was fixed using cold acetone for 10 minutes at -20 °C. The same process was repeated after 72 hours from plating.
The slides were then rehydrated and stained using fluorescent antibodies against viral proteins. The slides were stained against one early viral protein, p-41, and one late viral glycoprotein gp116/64/54.

After staining, the infected cells were counted using a fluorescence microscope and viral titers were determined using the formula:

$$\frac{\text{number of infected cells}}{\text{all cells}} = \frac{\text{x (number of infective particles)}}{\text{number of cells that were plated}} = number\ of\ infective\ particles$$

$$\frac{\text{Infected particles}}{\mu\text{l of virus used}} = \frac{\text{x (infective particles/ml)}}{\text{1 ml}} = number\ of\ infective\ particles/ml$$

Viral titration was also confirmed by counting the number of infected live cells by counting cells that were clearly enlarged and using the same formula as above.

Trophoblast infection

Trophoblasts seeded in a 12-well plate at a density of 413,000 per well (corresponding to 3 million in a 6-well plate) were infected with different multiplicities of infection (MOI). Three different MOIs (0.1, 1, and 5 MOI) were tested. After 24 hours, the trophoblasts were washed twice with warm sterile PBS to remove excess viral particles. Infection was confirmed using qPCR, with specific probes for HHV-6A and HHV-6B, and using immunofluorescence for one early and one late viral protein.

Infection validation

One of the most important steps of this project was to prove that our primary trophoblast cells were permissive to HHV-6A/B infection. First, reverse transcription quantitative polymerase chain reaction (RT-qPCR) with probes specific for HHV-6A or HHV-6B genes was done. Complementary DNA (cDNA) was generated from 19.2 ng of total RNA per sample using the SuperScript IV VILO Master Mix with ezDNase Enzyme (Invitrogen), according to the manufacturer's guidelines. To check for potential genomic DNA contamination and reagent carryover, reverse transcription–negative controls (no-RT) and no-template controls (NTC; nuclease-free water) were included in each run. Human RNase P was used as an internal control to verify successful cDNA synthesis and to ensure the absence of PCR inhibitors. Because RNase P is reliably present in all human samples, amplification of this gene

confirms that the reaction is functioning properly. Thus, failure to detect HHV-6A/B in samples with robust RNase P amplification can be interpreted as true absence of viral RNA rather than a false negative result.

RT-qPCR was carried out on a StepOnePlus Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Each 20 µl reaction contained TaqMan Fast Advanced Master Mix (Applied Biosystems), 300 nM of each primer, and 250 nM of the corresponding probe. Detection targeted the viral U67 gene, which enables differentiation between HHV-6A and HHV-6B. The oligonucleotide sequences used are presented in Table 1.

Table 1. Oligonucleotide sequences used for HHV-6 detection.

Target	**Type**	**Sequence (5′ → 3′)**
U67	Forward primer	TTCCGGTATATGACCTTCGTAAGC
U67	Reverse primer	GATGTCTCACCTCCAAATCTTTAGAAAT
HHV-6A	Probe	[FAM]-ACATTATATGTCGAACTTGACACTAC-CTTCCG-[IBFQ]
HHV-6B	Probe	[FAM]-CATTATATATCGAATCTGACGCTAC-CTTCCG-[IBFQ]

Thermal cycling was performed under the following conditions: initial denaturation at 95 °C for 20 seconds, followed by 40 cycles of 95 °C for 1 second and 60 °C for 20 seconds. Fluorescence was monitored in the FAM channel. All samples were analyzed in duplicate reactions. Quantification cycle (Cq) values were obtained using LinRegPCR,[126] which determines Cq based on the exponential phase of each individual amplification curve. LinRegPCR performs a sample-specific efficiency calculation by applying linear regression to the log-transformed fluorescence data within the exponential phase, allowing more accurate quantification and reducing bias introduced by assuming uniform PCR efficiency.

Immunofluorescence

Infection was further validated by immunofluorescence staining using fluorescently labelled primary antibodies targeting HHV-6 structural proteins. Primary trophoblast cells were fixed with cold acetone at day 3 post-infection, a time point chosen to allow sufficient viral replication for protein detection. Cells were incubated with anti-p41 (clone 9A5D12), which recognizes the viral early/late protein p41, and anti-gp116/64/54 (clone 6A5), which detects components of the HHV-6 envelope glycoprotein complex. These markers were selected because their expression indicates active viral gene expression and ongoing infection rather than residual input virus. After staining, nuclei were counterstained with DAPI, and images were acquired using a fluorescence microscope.

Paper II

DNA extraction, quality control and methylation profiling

Genomic DNA and total RNA isolation was done using the QiaCube with Qiagen´s AllPrep DNA/RNA/miRNA (Qiagen) according to manufacturer´s protocol (Figure 3). After five days of culture, the supernatant was removed and lysate buffer containing β-mercaptoethanol supplied in the kit was added to the cells. Cells were then scraped off the plate and flash-frozen on dry ice. DNA purity was evaluated by measuring the A260/A280 ratio on a NanoDrop spectrophotometer (Thermo Fisher Scientific), while DNA concentration was determined using a Qubit fluorometer (Invitrogen, Thermo Fisher Scientific) with the Qubit dsDNA BR Assay Kit (Invitrogen). DNA was too diluted for some samples so ethanol precipitation was performed to obtain more concentrated samples. In total we obtained 24 samples (eight controls, eight HHV-6A infected and eight HHV-6B infected). For sequencing, the receiving company (Life & Brain GmbH) required 20 µl of genomic DNA at 60 ng/µl (diluted in nuclease-free water). Acceptable purity thresholds values were A260/280 = 1.8–2.0 and A260/230 = 1.7–2.2. Samples were shipped on dry ice to Life & Brain GmbH, Bonn, Germany for methylation profiling with the Infinium Methylation EPIC v.2.0 BeadChip Array, the latest version of the platform, which profiles methylations at over 930,000 CpG sites. To minimize potential batch effects, samples from the same individual were placed on the same array chip whenever possible (eight samples per chip).

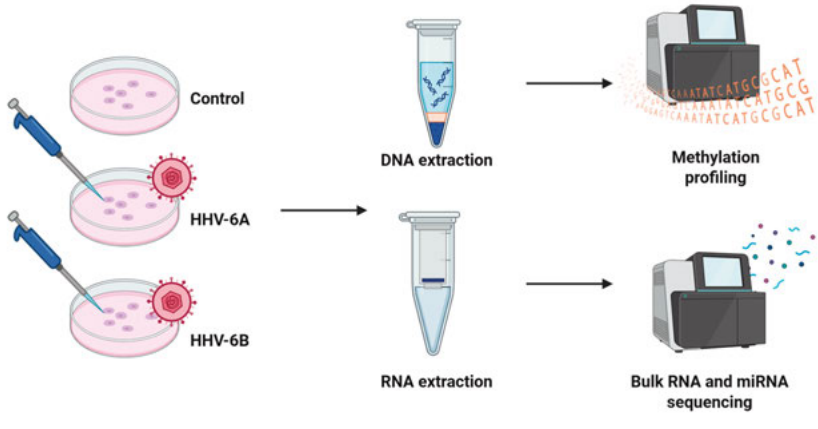

Figure 3. **Schematic overview of the experimental workflow used in Papers II and III.** Trophoblast cells were either uninfected (control) or infected with HHV-6A or HHV-6B. DNA and RNA were extracted from the same cultures and used for methylation profiling and bulk RNA/miRNA sequencing, respectively.

Cell viability

Cell viability after HHV-6 infection was tested using a lactate dehydrogenase (LDH) cytotoxicity assay kit. This kit measures the release of LDH, an enzyme that is commonly present in all cells that is released when cells are damaged or undergo apoptosis.[127] Culture medium was collected daily, and LDH levels were quantified to evaluate whether HHV-6A/B infected trophoblasts exhibited increased cell death compared with non-infected controls.

Data processing and statistics

Life & Brain provided the raw .idat files, which were securely processed on the Vesta server (the most secure server offered by Uppsala University) using the SeSAMe pipeline in R.[128] SeSAMe performs signal-level pre-processing, including background correction, dye-bias adjustment, and masking of unreliable probes based on out-of-band signal estimation, thereby improving data accuracy and reducing false positives. Since all samples originated from the same individuals, normalization was limited to technical normalization to preserve subtle within-individual methylation differences. To enhance the reliability of the dataset, probes with low detection confidence and probes known to cross-react were excluded prior to downstream analysis, ensuring that only high-quality methylation signals were retained.[129] Important to our analysis was to consider that the samples come from the same individual, therefore we implemented the duplicateCorrelation() function from the limma package[130] to account for this. Graphs for Figure 1 were made using the graph function of BioRender. All other graphs were made in R using the ggplot2 package.

Gene ontology

Gene ontology (GO) enrichment analysis was used to interpret the results from the differential methylation. GO enrichment analysis allows for biological interpretation of large gene lists by identifying overrepresented biological processes, molecular functions, or cellular components. This approach helps reveal shared functional themes among differentially methylated genes and provides insights into the underlying biological mechanisms.

For this analysis, significant differentially methylated probes were tested against a background consisting of all probes that remained after filtering out probes with low confidence (p-value > 0.05) and removing probes known to cross-hybridize. The top GO terms were extracted using the topGSA function of the missMethyl package,[131] which also calculates the false discovery rate (FDR) using the Benjamini-Hochberg method. This function accounts for variation in the number of CpG probes per gene, ensuring that genes with more or fewer overlapping probes do not bias the enrichment results.

Paper III

RNA extraction, quality control and sequencing.

RNA was extracted at the same time as DNA using the DNA/RNA/miRNA Universal Kit (Qiagen) according to manufacturer´s instructions. RNA integrity was assessed with a Bioanalyzer (Agilent Technologies) using the RNA 6000 Nano Kit; RNA concentration was determined with a Qubit fluorometer (Invitrogen) with the RNA HS kit (Invitrogen).

Samples were sent on dry ice to the National Genomic Infrastructure (SciLifeLab, Solna, Sweden) for sequencing. For placental transcriptome, sequencing libraries were prepared from twenty-four samples of total RNA using the SMARTer Pico Input RNA Library Preparation Kit (Takara Bio, Shiga, Japan). Prepared libraries were multiplexed and sequenced on half a lane on the Illumina NovaSeq X platform using the 25B-300 flow cell with a 2 x 150 bp paired-end configuration.

For sequencing of miRNA, libraries were prepared from the same twenty-four samples using the QIAseq miRNA Library Kit (Qiagen). Prepared libraries were multiplexed and sequenced on a single flow cell using the Illumina NextSeq 2000 platform with the P2-100 XLEAP (101-8-0-0) sequencing configuration.

Data analysis and statistics of bulk RNA

The data was pre-processed by the National Genomic Infrastructure (NGI) using their nf-core/rnaseq pipeline.[132] This pipeline included raw read quality control, adapter trimming and alignment. Both raw sequencing data and gene count matrices were provided for downstream analysis.

Transcript-level quantification files were imported into R using the tximport package, which allows for efficient summarization of transcript abundances to the gene level. Prior to downstream analyses, lowly expressed genes were filtered out to minimize noise and improve statistical power. Specifically, only genes with a minimum of ten read counts in at least eight samples were retained for further analysis. This filtering ensured that subsequent analyses focused on reliably detected transcripts across the dataset.

Differential gene expression analysis was carried out using the DESeq2 package.[133] A generalized linear model was fitted for each gene, incorporating both the infection condition and individual donor variability as explanatory factors (design formula: ~ condition + individual). This paired design accounts for inter-individual variation by comparing infected and control cells originating from the same donor. The control (non-infected) samples were used as the reference group. Statistical significance of differential expression was evaluated using Wald tests implemented in DESeq2. To control for FDR associated with multiple testing, p-values were adjusted using the Benjamini–Hochberg

procedure. Genes were considered significantly differentially expressed if they exhibited an adjusted p-value < 0.05 and an absolute $\log_2$ fold change greater than 1, corresponding to at least a twofold difference in expression between conditions.

To gain biological insight into the observed transcriptional changes, functional enrichment analyses were performed. Overrepresentation analysis (ORA) of GO terms was conducted using the clusterProfiler package. ORA identifies biological categories or pathways that contain a statistically significant number of differentially expressed genes relative to what would be expected by chance. ORA was done to investigate the GO terms common to both infection.

In addition, Gene Set Enrichment Analysis (GSEA) was performed using clusterProfiler in conjunction with GO annotations from the org.Hs.eg.db database. Unlike ORA, GSEA evaluates all genes ranked by a continuous metric, in this case, the Wald statistic derived from DESeq2, without requiring an arbitrary threshold for differential expression. This approach assesses whether predefined sets of genes (involved in specific biological processes, cellular components, or molecular functions) are enriched at the top or bottom of the ranked gene list, indicating coordinated up- or down-regulation of pathways. Only gene sets containing between 10 and 500 genes were included in the analysis to avoid overly broad or narrow categories. Statistical significance was defined at FDR < 0.05.

The resulting enrichment plots highlight the core enrichment genes, which are those contributing most strongly to the observed enrichment signal. Together, the differential expression and enrichment analyses provide complementary insights into both individual gene-level changes and broader biological processes affected by HHV-6A/B infection in primary trophoblast cells.

Data analysis and statistics miRNA

The data was pre-processed by NGI using their nf-core/smrnaseq pipeline.[132] The analysis for miRNAs was done in a similar way as for the bulk RNA. The same design for the generalized linear model was used (design formula: ~ condition + individual). Here, miRNAs with fewer than five reads in at least six samples were removed. Differential expression was again performed using DESeq2 and results were adjusted for multiple testing using the Benjamini–Hochberg method. An adjusted p-value threshold of 0.05 (FDR < 5%) and a $\log_2$ fold-change threshold of 1 were applied to define significantly differentially expressed miRNAs.

Paper IV

For this paper, we repeated the infection of trophoblast cells twice, once for protein collection and once for the analysis of pro- and anti-angiogenic factors released into cell media (Figure 4). This approach allowed precise quantification and normalization of protein levels based on total protein content.

For western blot analysis, proteins were collected using radioimmunoprecipitation assay buffer (RIPA) buffer supplemented with phosphatase inhibitors (Thermo Fisher Scientific) to prevent dephosphorylation by endogenous phosphatases. For this, proteins were measured from an aliquot of the collected lysate using the Pierce Rapid Gold BCA protein assay kit (Thermo Fisher Scientific).

For ELISA, total protein content was measured directly in the 24-well plate using a Lowry assay.[134]

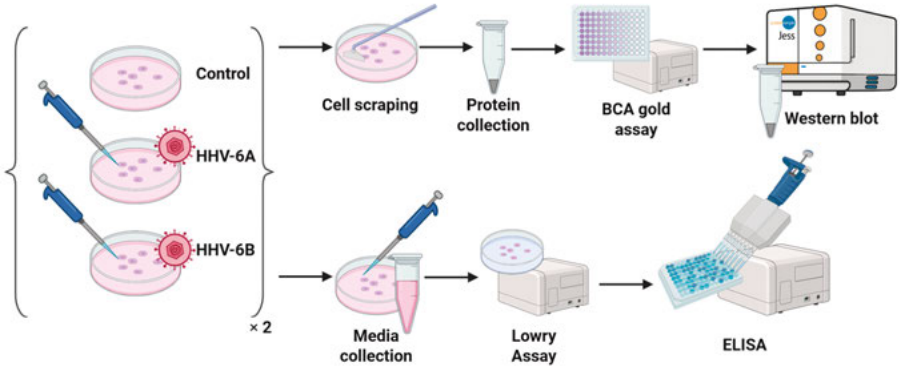

Figure 4. **Schematic overview of the experimental workflow used in Paper IV**. Primary trophoblast cells were infected in two separate experiments: once for protein collection and protein analysis, and another for assessing pro- and anti-angiogenic markers released into the cell culture media.

Protein analysis with simple western

To assess protein expression of ER stress pathways, the Jess Simple Western system (Bio-Techne, USA) was used. This is an automated capillary-based platform for Western blot. Protein lysates were prepared from trophoblast cells, and small aliquots (1.2 µg per sample) were loaded into capillaries for automated separation and immunodetection. Signal detection was performed using either horseradish peroxidase (HRP) or fluorescence-conjugated secondary antibodies. Total protein levels were quantified using the total protein module. Chemiluminescent and fluorescent signals were analyzed with the

Compass for Simple Western software (v6.1.0, Bio-Techne), and all target protein signals were normalized to total protein content to ensure accurate quantification.

Several antibodies were purchased and tested to investigate all main ER stress pathways. Antibodies highlighted in bold were those ultimately used in the final analysis (Table 2).

Table 2. Antibodies tested and used (bold) were:

Protein	Product number	Manufacturer
ATF4	mAb 11815	Cell signalling technology
ATF6	66563-1-Ig	Proteintech
CHOP	9C811554842	Invitrogen
eIF2α	mAb 2103	Cell signalling technology
GRP78 (BiP)	15855739	BD biosciences
IRE1	mAb 3294	Cell signalling technology
JNK	mAb 9252	Cell signalling technology
p-eIF2α	mAb 9721	Cell signalling technology
PERK	mAb 5683	Cell signalling technology
p-IRE1	Ab48187	Abcam
p-JNK	mAb 4668	Cell signalling technology
p-PERK	mAb 3179	Cell signalling technology
XBP-1s	E9V3E	Cell signalling technology

Release of anti-angiogenic factors

Release of pro- and anti-angiogenic factors by trophoblast cells in cell media was investigated with the following ELISA kits according to manufacturer's instructions. Multiple kits were tested, kits highlighted in bold were those used in the final analysis (Table 3).

Table 3. ELISA kits tested and used (bold) were:

Protein	Product number	Manufacturer
sFlt-1	BMS268-3	Invitrogen
PlGF	EHPGF	Invitrogen
Galectin-3	DY1154	R&D Systems
sENG	EHENG	Invitrogen
TGF-β1	BMS249-4	Invitrogen
Thrombospondin-1	EHTHBS1	Invitrogen
Vasohibin-1	EH480RB	Invitrogen
VEGF	BMS277-2	Invitrogen
Angiopoietin-2	KHC1641	Invitrogen

Statistical methods

For this study, a linear mixed-effects model was used to account for inter-individual biological variability in factor production that is independent of infection status. Infection category was included as a fixed effect, while individual donor was modeled as a random intercept to account for within-individual correlations. Including individual as a random effect allows each donor to have their own baseline level while estimating the overall effect of infection across all samples. The models were fitted using the lmer function from the lme4 package in R.[135] P-values < 0.05 were considered statistically significant. All analyses were conducted in RStudio version 2024.12.1+563 ("Kousa Dogwood").

Summary of results

Paper I

Use of antiviral medications during pregnancy and the likelihood of preeclampsia in a population-based register study.

In the first paper of this doctoral thesis, we showed with a large population-based register study that women who were prescribed antiviral medications at any point during pregnancy have a lower likelihood of developing preeclampsia. A total of 618,814 primiparous women who gave birth in Sweden between 2007 and 2019 were included. Among these, 18,004 women (2.9%) filled an antiviral medication prescription during pregnancy, and 27,135 (4.4%) developed preeclampsia.

Antiviral medication use during pregnancy was associated with a lower likelihood of preeclampsia compared with no antiviral use (3.8% vs. 4.4%, p-value < 0.001). After adjustment using IPTW, antiviral exposure remained associated with reduced likelihood of preeclampsia (aOR 0.88; 95% CI 0.81–0.96; p-value = 0.003). Reduced likelihood was observed across all preeclampsia subtypes examined. Sensitivity analyses showed that among women not exposed to antivirals, HSV diagnosis was not associated with an altered likelihood of developing preeclampsia.

The association between antiviral medication exposure and lower preeclampsia likelihood varied by timing of first prescription. Antiviral medication use initiated in the third trimester was associated with reduced likelihood of preeclampsia (aOR 0.77; 95% CI 0.67–0.90; p-value < 0.001), while antiviral use initiated before pregnancy or in the first or second trimester was not associated with a change in likelihood of preeclampsia.

Paper II

Herpesvirus-6A and 6B infection induces significant differential methylation in primary human trophoblast cells.

In paper I, we showed that the use of antiviral medication, regardless of indication, was associated with a reduced likelihood of developing preeclampsia.

Building on these findings, we sought to investigate whether viral infection or reactivation could induce differential DNA methylation in primary trophoblast cells, and whether such epigenetic alterations might contribute to the development of preeclampsia or other pregnancy disorders.

As a first step, we confirmed that trophoblast cells not only responded to viral exposure but were also permissive to viral infection. This was not a given, as trophoblasts are known to exhibit a natural resistance to infection.[136] Viral entry was verified using two approaches: qPCR targeting viral genes and immunofluorescence staining of viral proteins.

Although this was not the primary objective of our investigation, the observation that HHV-6A/B can infect primary cytotrophoblast cells (previous studies have shown that HHV-6 can infect syncytiotrophoblast)[118] represents a novel and important contribution to our understanding of viral–placental interactions.

After confirming successful infection, we proceeded with DNA methylation profiling and analysis. We identified 37 differentially methylated regions (DMRs), many of which correspond to genes previously implicated in preeclampsia or other pregnancy-related complications (*ROR1, PPARG, NOTCH3, LGMN, WNT1, WNT9B, CTHRC1, MME,* and *TJP1*). GO analysis further supported these findings, revealing enrichment of terms related to angiogenesis and placental development. In the case of HHV-6B infection, only a single DMR was detected, corresponding to the *PEG10* gene. Although, it is only a single DMR it is very interesting as it is a gene fundamental for placental development.

Together, these results provide the first evidence that HHV-6A/B can infect primary cytotrophoblast cells and can induce targeted epigenetic alterations, directly affecting genes linked to placental function and pregnancy disorders.

Paper III

Human herpesvirus-6A/B induces antiviral responses and suppresses placental development pathways in human trophoblasts.

In Paper II, we demonstrated that primary trophoblast cells are permissive to infection by both HHV-6A and HHV-6B, and that viral infection induces distinct patterns of differential DNA methylation. To further explore the functional consequences of these epigenetic changes, we investigated whether infection also alters gene and miRNA expression. Bulk RNA and miRNA were extracted from the same trophoblast cultures used for methylation profiling, allowing direct comparison between epigenetic and transcriptomic changes.

Transcriptomic analysis revealed that infection with HHV-6A and HHV-6B triggers significant alterations in gene expression, with 596 and 225 genes, respectively, showing differential expression compared to uninfected controls. GSEA indicated that both viruses strongly activate interferon-mediated antiviral pathways, reflecting a robust innate immune response to infection. Pathways related to wound healing, angiogenesis, and placental function were significantly downregulated in both infections, suggesting that viral infection may disrupt key processes essential for normal placental development.

The analysis of miRNA expression further supported these findings, identifying several miRNAs associated with inflammation regulation and pregnancy complications.

Together, these results suggest that HHV-6A and HHV-6B infection may impair placental development and function through combined epigenetic and transcriptional mechanisms. Overall, this study provides new insights into how HHV-6A/B infection could contribute to the development of preeclampsia, while also suggesting possible contributions to other pregnancy complications, including fetal growth restriction, preterm birth, and miscarriage.

Paper IV

Human herpesvirus 6A and 6B differentially disrupt placental function through angiogenic pathways and ER stress.

Following the investigation of HHV-6A/B–induced changes at the DNA and RNA levels, our final step was to assess how viral infection affects trophoblast function at the protein level, focusing on the release of angiogenic and anti-angiogenic factors and ER stress.

Using primary human trophoblast cells isolated from the same term placentas, we evaluated the effects of HHV-6A and HHV-6B infection on the release of pro- and anti-angiogenic factors and cellular stress markers. Notably, HHV-6B infection, but not HHV-6A, significantly reduced production of hCG four days post-infection. Both viral variants, however, increased secretion of sFlt-1 and Ang-2, indicating a shift toward an anti-angiogenic environment. HHV-6B further upregulated Vash-1 and Thbs-1 while downregulating PlGF, suggesting a stronger overall anti-angiogenic response compared to HHV-6A.

Examination of ER stress markers revealed that neither HHV-6A nor HHV-6B induced a global unfolded protein response, as evidenced by the absence of BiP (GRP78) upregulation. However, both infections reduced eIF2α levels, while HHV-6A uniquely increased phosphorylated eIF2α, indicating selective activation of the PERK-mediated ER stress pathway.

These findings show that HHV-6A and HHV-6B distinctly modulate trophoblast cell function. While HHV-6A appeared to more strongly affect ER stress signaling, HHV-6B had broader effects on angiogenic balance.

Discussion

Across four studies, we found that antiviral medication use during pregnancy was associated with a reduced likelihood of preeclampsia, and that HHV-6A/B infection induces epigenetic, transcriptional, and protein-level changes in primary human trophoblasts. These alterations are similar to biological processes known to be disrupted in preeclampsia, including angiogenesis, immune regulation, trophoblast differentiation, and cellular stress responses. Together, these findings support the possibility that HHV-6 reactivation may act as a co-factor in preeclampsia development.

In a nationwide cohort of more than 600,000 primiparous women, antiviral medication prescription during pregnancy was associated with a significantly reduced likelihood of developing preeclampsia. This pattern was consistent across all subtypes, with the strongest protective associations observed for early-onset disease and preeclampsia accompanied by fetal growth restriction, forms most closely linked to impairments in early placental development.

A significant reduction in likelihood of preeclampsia development was seen only among women who initiated antiviral treatment in the third trimester. The third trimester is characterized by a shift toward a more pro-inflammatory immune profile, often described as a return to the inflammatory state of early pregnancy.[137] This natural elevation in inflammatory mediators is thought to facilitate labor preparation but may also represent a period of vulnerability for women already experiencing placental stress. Viral reactivation may be more likely to occur during late pregnancy when the maternal immune system is altered, making the third trimester a critical window for intervention. Low-level viral activity during this period could also amplify pre-existing inflammatory stress, pushing inflammation to pathological levels, ultimately leading to the clinical manifestation of preeclampsia.

To investigate the biological mechanisms that might underlie these epidemiological observations, we used primary human trophoblasts to examine epigenetic, transcriptional, and protein changes. At the epigenetic level we found that HHV-6A and HHV-6B induced widespread DNA methylation alterations, with hypermethylation predominating. This pattern suggests transcriptional silencing of genes involved in trophoblast function and may reflect viral strategies to evade host defenses while manipulating cellular pathways for persistence. The most significant changes involved genes central to trophoblast invasion, vascular development, and placental barrier integrity. Several of the

genes found to be differentially methylated have already been studied in relation to preeclampsia or other pregnancy complications.

Gene ontology analysis of the methylation data highlighted processes such as tube morphogenesis, epithelial development, and regulation of transcription, supporting the concept that HHV-6A/B disrupt epigenetic regulation of genes required for proper placental formation. Although HHV-6B induced fewer methylation changes overall, the affected genes mapped to pathways critical for trophoblast differentiation and immune regulation.

Transcriptomic profiling showed that HHV-6A/B strongly induced interferon-stimulated genes and antiviral chemokines, reflecting robust innate immune activation. Sustained interferon signaling is inherently anti-proliferative and anti-angiogenic, and has been implicated in the pathophysiology of preeclampsia.[138,139]

Major metabolic pathways were also downregulated, including oxidative phosphorylation, purine biosynthesis, and translation, suggesting a shift from growth toward survival. HHV-6A/B also downregulated matrix metalloproteinases and suppressed pathways involved in implantation, uterine artery remodeling, and coagulation, all fundamental to early placental development.[140] This coordinated suppression of developmental programs in favor of antiviral defense mirrors the epigenetic changes observed in the previous study, where genes involved in invasion and vascular development showed altered methylation patterns. This suggests that an effective antiviral response might come at the cost of the normal developmental functions that trophoblasts must perform to establish a healthy placenta.

Strain-specific differences were also seen in response to infection. HHV-6A activated stronger innate antiviral responses but suppressed adaptive immunity, consistent with a strategy of immune evasion that might allow the virus to persist longer in infected cells. HHV-6B uniquely activated pyroptotic inflammatory pathways, suggesting inflammatory cell death that may impair placental integrity through a different mechanism.

MiRNA profiling added another layer of regulatory complexity, revealing downregulation of miR-223-3p in both infections. This particular miRNA suppresses the NLRP3 inflammasome, and its downregulation implicates potential activation of this inflammatory complex, a pathway that has been linked to several obstetric complications including preeclampsia and preterm labor.[53,141]

At the protein level, both viruses led to significant increases in sFlt-1 and Ang-2, two central drivers of endothelial dysfunction and impaired placental vascularization.[64] Infected trophoblasts showed elevated secretion in sFlt-1 and reduced secretion of PlGF, resulting in an increased sFlt-1/PlGF ratio, the key clinical biomarkers used for preeclampsia screening.[31,32] This finding is particularly significant because it demonstrates that viral infection can directly induce the anti-angiogenic state that can be used for preeclampsia screening.

HHV-6B elicited the most pronounced anti-angiogenic response at the protein level, with increased Vash-1 and Thbs-1 and reduced PlGF, resulting in a further increase of the sFlt-1/PlGF ratio. Analysis of ER stress pathways revealed selective activation of the PERK–eIF2α pathway, with no involvement of IRE1α-XBP1 or BiP. This indicates that HHV-6A/B induce a targeted integrated stress response rather than global ER stress. Elevated phosphorylation of eIF2α, particularly in HHV-6A infection, results in global suppression of protein synthesis, a feature observed in placentas from preeclamptic pregnancies and indicative of a shift toward a cellular survival state. This is also consistent with reports showing that HHV-6A elicits a limited eIF2α response.[142]

The difference between the cellular responses to HHV-6A and HHV-6B suggests that the two viruses may influence placental pathology through partially distinct mechanisms. While this could indicate true biological differences in pathogenic potential, with HHV-6A being inherently more capable of reprogramming trophoblast function, the apparently weaker response to HHV-6B may also reflect differences in infection kinetics or viral entry efficiency. Sampling at additional time points might have revealed a more complete picture of HHV-6A/B-driven changes and clarified whether the differences we observed represent true biological distinctions or simply phase shifts in a similar overall response.

Conclusion and Future Perspectives

The overarching aim of this work was to investigate whether HHV-6A/B could act as a contributing factor to the development or severity of preeclampsia. Our findings consistently supported this possibility.

This thesis offers new insight into the relationship between viral infection and placental dysfunction. First, we showed with a population-based register study that antiviral medications reduce the likelihood of developing preeclampsia. While observational, this association is consistent with our hypothesis in which viral infections and/or reactivations contribute to the development of preeclampsia, where antiviral treatment may mitigate this risk by suppressing viral activity.

Then, across the following three *in vitro* studies we showed that exposure and infection to HHV-6A/B influences trophoblast health at multiple biological levels, from DNA methylation, to bulk RNA and miRNA expression, and protein function. These effects primarily involved placental development, angiogenesis, immune regulation, and cellular stress responses, all processes known to be disrupted in preeclampsia.

Collectively, these findings support a biological framework in which HHV-6A/B activity may interfere with key processes required for healthy placentation, thereby contributing to the pathogenesis of preeclampsia.

Despite these important findings, several questions remain to be addressed:

- Timing of infection: Our *in vitro* model did not allow us to investigate temporal dynamics. The impact of HHV-6A/B infection may vary depending on the timing of infection or viral reactivation during pregnancy.
- Persistence and progression: We do not yet know how the observed molecular changes evolve over time or whether they resolve, persist, or intensify.
- *In vivo* validation: Further studies are needed to determine whether the molecular alterations observed *in vitro* also occur in placentas from women with confirmed HHV-6A/B infection or other viral infections.
- Consequences for the fetus and newborn: The potential impact of maternal HHV-6A/B reactivation on fetal development and neonatal outcomes remains unknown.

Although this work was primarily conducted *in vitro*, the findings carry potential clinical relevance. If confirmed *in vivo*, they open avenues for targeted antiviral strategies or screening for viral infection as part of pregnancy care, with the goal of preventing or mitigating placental dysfunction and preeclampsia. This is particularly promising given that safe and effective antiviral medications are already available and approved for use during pregnancy.

In the future, if HHV-6A/B infection is definitively proven to contribute to preeclampsia, it could lead to the development of new preventive or therapeutic approaches. Vaccine development, in particular, could become an important tool for reducing the incidence of preeclampsia in regions with limited access to advanced healthcare, potentially saving the lives of millions of women and infants and preventing the long-term consequences of the disease.

Popular science summaries

When the placenta meets a virus

Every year, hundreds of thousands of mothers and babies around the world die from a pregnancy complication called preeclampsia. It's a condition where the mother's blood pressure becomes dangerously high after the 20th week of pregnancy, often leading to problems with organs like the kidneys, liver, and brain. The only cure is to deliver the baby, sometimes far too early, which can cause serious risks for the newborn. Preeclampsia doesn't just affect pregnancy. Women who have had it are more likely to develop heart and kidney disease later in life. Despite decades of research, doctors and scientists cannot fully explain why preeclampsia develops. What they do know is that the condition begins in the placenta, the organ that nourishes and protects the growing baby. In preeclampsia, the placenta doesn't grow or function as it should, leading to inflammation, blood vessel damage, and stress on the mother's organs. But what sets this cascade in motion has remained a mystery.

My research explores the role of an unexpected agent: a common virus that almost everyone carries. Human herpesvirus 6 (HHV-6) is so widespread that most people catch it before the age of five, usually as a mild childhood fever. After the infection clears, the virus doesn't disappear, but hides inside our DNA, where it can remain for life. In some people, this viral DNA even becomes part of their genetic inheritance, passed from parent to child like any other gene.

To explore the role of HHV-6 during pregnancy, we combined large-scale population data with laboratory studies of placental cells. In a nationwide Swedish study, we found that women who used antiviral medications during pregnancy were less likely to develop preeclampsia, especially in the final trimester. While this doesn't prove that viruses cause the condition, it hints that viral activity may influence pregnancy health more than previously thought. In the lab, we examined how HHV-6 interacts with the cells that form the placenta. We discovered that infection could alter the way these cells regulate genes and disrupt signals needed for healthy blood vessel growth. These small molecular changes could lead to the kind of placental dysfunction seen in preeclampsia.

Our findings don't mean HHV-6 is the only cause of preeclampsia, but they suggest it could be a missing piece in a complex biological puzzle. Understanding how viral infections interact with the placenta may eventually help identify women at higher risk and even open the door to preventive treatments.

När moderkakan möter ett virus

Varje år dör hundratusentals mammor och barn runt om i världen till följd av en graviditetskomplikation som kallas preeklampsi (även kallat havandeskapsförgiftning). Det är ett tillstånd där den gravida kvinnans blodtryck blir farligt högt efter den 20:e graviditetsveckan, vilket ofta leder till problem med organ som njurar, lever och hjärna. Den enda boten är att förlösa barnet, ibland alldeles för tidigt, vilket i sin tur innebär stora risker för det nyfödda barnet.

Preeklampsi påverkar inte bara graviditeten. Kvinnor som haft tillståndet löper också större risk att senare i livet utveckla hjärt- och njursjukdomar. Trots decennier av forskning kan läkare och forskare ännu inte fullt ut förklara varför preeklampsi uppstår. Det man vet är att tillståndet börjar i moderkakan, organet som när och skyddar det växande barnet. Vid preeklampsi växer eller fungerar moderkakan inte som den ska, vilket leder till inflammation, skador på blodkärl och belastning på moderns organ. Men vad som sätter igång denna kedjereaktion har länge varit ett mysterium.

Min forskning undersöker en möjlig pusselbit: humant herpesvirus 6 (HHV-6), ett virus som nästan alla smittas av som små och som sedan ligger vilande i kroppen hela livet. Under graviditet är reaktivering av HHV-6 vanligare än hos andra, vilket väckt frågan om viruset kan påverka moderkakan. Genom att kombinera stora svenska registerdata med laboratoriestudier av moderkaksceller fann vi att kvinnor som använde antivirala läkemedel under graviditeten hade lägre risk att utveckla preeklampsi, särskilt i slutet av graviditeten. Det bevisar inte att virus orsakar sjukdomen, men antyder att viral aktivitet kan påverka hur graviditeten utvecklas. I våra cellstudier såg vi att HHV-6 påverkar moderkakans celler genom att förändra genreglering och störa signaler som behövs för normal kärlbildning. Dessa förändringar liknar de processer som ses vid preeklampsi, där moderkakan får svårare att försörja fostret och modern reagerar med inflammation och högt blodtryck

Våra resultat visar inte att HHV-6 är den enda orsaken till preeklampsi, men de tyder på att viruset kan vara en tidigare förbisedd del av sjukdomens komplexa biologiska bakgrund. En bättre förståelse av hur virus påverkar moderkakan kan i framtiden hjälpa oss att identifiera riskgraviditeter och utveckla nya förebyggande behandlingar.

Quando la placenta incontra un virus

Ogni anno, centinaia di migliaia di madri e neonati in tutto il mondo muoiono a causa di una complicanza della gravidanza chiamata preeclampsia (anche conosciuta come gestosi). Si tratta di una condizione in cui la pressione sanguigna della madre diventa pericolosamente alta dopo la ventesima settimana di gravidanza, provocando spesso danni a organi come reni, fegato e cervello. L'unica cura è il parto, talvolta molto prematuro, con tutti i rischi che questo comporta per il neonato. La preeclampsia non colpisce solo durante la gravidanza. Le donne che ne hanno sofferto hanno anche un rischio maggiore di sviluppare malattie cardiovascolari e renali nel corso della vita.

Nonostante decenni di ricerche, medici e scienziati non riescono ancora a spiegare completamente perché la preeclampsia si sviluppi. Quello che si sa è che la condizione ha origine nella placenta, l'organo che nutre e protegge il bambino in crescita. Nella preeclampsia, la placenta non cresce o non funziona come dovrebbe, causando infiammazione, danni ai vasi sanguigni e stress per gli organi della madre. Ma ciò che innesca questa catena di eventi resta un mistero.

La mia ricerca esplora un possibile tassello del puzzle: l'herpesvirus umano 6 (HHV-6), un virus molto comune che contraiamo da piccoli e che rimane latente per tutta la vita. Durante la gravidanza la sua riattivazione è più frequente, il che ha sollevato l'ipotesi che possa influenzare la placenta. Combinando grandi registri sanitari svedesi con studi su cellule placentari, abbiamo osservato che le donne che assumevano antivirali durante la gravidanza hanno un rischio minore di sviluppare la preeclampsia, soprattutto se consumati nell'ultimo trimestre. Questo non prova che il virus causi la malattia, ma suggerisce che l'attività virale possa incidere sulla salute della gravidanza.

In laboratorio abbiamo visto che HHV-6 altera la regolazione dei geni e i segnali necessari alla normale formazione dei vasi sanguigni della placenta, cambiamenti simili a quelli osservati nella preeclampsia.

I risultati non indicano che HHV-6 sia la causa unica della malattia, ma suggeriscono che possa rappresentare una parte finora trascurata. Comprendere meglio come i virus influenzano la placenta potrebbe in futuro aiutare a identificare le gravidanze a rischio e sviluppare nuove strategie preventive.

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Disclosures

In this thesis, I received limited assistance from AI-based language-editing tools, used solely to improve clarity and expression. I drafted all original text and completed all subsequent revisions, and I take full responsibility for the final content of this work. The initial version of the cover image was created by me using BioRender and subsequently modified with OpenAI´s GPT-5 model to achieve a water colour style appearance.

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