Dissertation

Maternal platelet activation and placental endocrine activity

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice and Ombuds Committee at the Medical University of Graz".

Graz, July 2022 Jacqueline Guettler

Disclosures

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- **Kupper N**, Pritz E, Siwetz M, Guettler J, Huppertz B. Placental Villous Explant Culture 2.0: Flow Culture Allows Studies Closer to the In Vivo Situation. *Int J Mol Sci*. 2021;22(14):7464. Published 2021 Jul 12. doi:10.3390/ijms22147464

- **Forstner D**, Guettler J, Gauster M. Changes in Maternal Platelet Physiology during Gestation and Their Interaction with Trophoblasts. *Int J Mol Sci*. 2021;22(19):10732. Published 2021 Oct 3. doi:10.3390/ijms221910732

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Foreword

This thesis is dedicated to the promising new direction of maternal platelet interaction with the extra embryonic trophoblasts in the early human placenta.

This project was based on previous findings (3–7) that platelets could enter the intervillous space of the placenta very early during gestation and that an interaction of these platelets with the trophoblasts causes a deregulation of different genes and proteins that are also found to be deregulated in pregnancy complications.

Within this project we identified an additional way for the platelets to enter the early intervillous space. Furthermore, the deregulation of the endocrine activity of the trophoblasts with special regard to steroid hormones was investigated, as these hormones play a key role in the development and maintenance of a healthy human pregnancy and are deregulated during pregnancy disorders.

We are contributing to a deeper insight into this promising topic and will continue to decipher the role of maternal platelets in the cross-talk with the human placenta during early pregnancy and in pregnancy pathologies.

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Cheers guys ♥

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Abbreviations

ABCP	ATP-binding cassette proteins
AEC	aminoethyl cabazole
AGR	antigen retrieval
Ang-1	Angiopoietin-1
Ang-2	Angiopoietin-2
APA	antiphospholipid antibodies
APS	Antiphospholipid Syndrome
BSA	bovine serum albumin
col-I	collagen type I
col-IV	collagen type IV
CT	cytotrophoblast
ECM	extracellular matrix
eEVT	endovascular extravillous trophoblast
Eng	endoglin
EV	extracellular vesicles
EVT	extravillous trophoblast
FBS	fetal bovine serum
FcR	Fc receptor
FFPE	formalin fixed paraffin embedded
FGR	fetal growth restriction
GA	gestational age
GPCR	G-protein coupled receptor
hCG	human chorionic gonadotrophin
HLA-G	histocompatibility complex G
ICM	inner cell mass
iEVT	interstitial extravillous trophoblasts
IF	immunofluorescence
IGF-I	insulin-like growth factor I
IgG	immunoglobulin G
IUGR	intra uterine growth restriction
IVS	intervillous space
MPV	mean platelet volume

MRP1	multidrug resistance protein 1
MRP	multidrug resistance-associated protein family
NO	nitric oxide
OCS	open canalicular system
PAF	platelet activating factor
PAI	plasminogen activator inhibitors
PAR-1	protease-activated receptor 1
PAR-4	protease-activated receptor 4
PC	platelet count
PE	preeclampsia
PF4	platelet factor 4
PFA	paraformaldehyde
PGI2	prostacyclin
PlGF	Placental growth factor
PO	propylene oxide
PRP	platelet rich plasma
qPCR	quantitative polymerase chain reaction
rHLA-G	recombinant HLA-G
RT	room temperature
s	seconds
SCT	syncytiotrophoblast
SD	standard deviation
sEng	soluble endoglin
sFlt1	soluble fms-like tyrosine kinase 1
SLE	Systemic Lupus Erythematosus
TBS	tris-buffered saline
TBS-T	tris-buffered saline with Tween
TE	trophectoderm
TEM	transmission electron microscopy
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TGF-β	transforming growth factor beta
TJP1	tight junction protein 1
TM	thrombomodulin

TP	thromboxane receptor
TXA_2	thromboxane A_2
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
ZO-1	zonula occludens-1 (also referred to as tight junction protein 1 (TJP1))

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Kurzzusammenfassung

In den frühen Stadien der humanen Schwangerschaft ist eine gesunde Entwicklung der Plazenta eines der wichtigsten Ereignisse, um eine erfolgreiche Schwangerschaft zu gewährleisten. Im ersten Trimester werden zirkulierende mütterliche Blutzellen durch extravillöse Trophoblastpfropfen in den mütterlichen Spiralarterien daran gehindert, in den intervillösen Raum der Plazenta einzudringen. Dadurch wird eine niedrige Sauerstoffkonzentration gewährleistet, die für eine gesunde Entwicklung der Zottenbäume wichtig ist.

Auf der Grundlage früherer Forschungsarbeiten und Pilotdaten stellten wir die Hypothese auf, dass Thrombozyten mit dem frühen villösen und extravillösen Trophoblasten über die klassischen bekannten entzündlichen Interaktionen hinaus interagieren, indem sie eine Vielzahl von Genen im menschlichen Trophoblasten verändern.

In dieser Arbeit haben wir einen neuen und alternativen Weg gefunden, wie mütterliche Blutplättchen in den intervillösen Raum der menschlichen Ersttrimesterplazenta gelangen können, bevor sich die extravillösen Trophoblastpfropfen auflösen. Diese kleinsten Blutzellen wurden durch Immunhistochemie und Transmissionselektronenmikroskopie in den interzellulären Lücken der distalen Bereiche der extravillösen Trophoblastzellsäulen nachgewiesen. Da die Thrombozyten in einem aktivierten Stadium erschienen, wurde spekuliert, dass die Aktivierung von einer Substanz herrührt, die in den Zwischenräumen der extravillösen Trophoblasten zu finden ist. Der extravillöse Trophoblastmarker HLA-G hat jedoch keinen Einfluss auf die Aggregationsfähigkeit der mütterlichen Thrombozyten und beeinflusst auch nicht die Adhäsion menschlicher Thrombozyten an Trophoblastzellen.

In einer archivierten „Embryo-in-Utero-Probe“ einer frühen menschlichen Schwangerschaft wurden die vorherigen Färbungen bestätigt und zeigten darüber hinaus eine Anhaftung von Thrombozyten an den villösen Trophoblasten im intervillösen Raum. Bei der Co-Kultur der differenzierten Trophoblast-Zelllinie Bewo mit isolierten menschlichen Thrombozyten konnte eine signifikante Deregulierung einiger Gene, die an der Steroidhormonsynthese beteiligt sind, festgestellt werden, und die Analyse der Kulturüberstände zeigte eine signifikante Herabregulierung der Freisetzung des Schwangerschaftshormons Progesteron. Diese Deregulierungen stimmen mit bekannten Veränderungen bei Schwangerschaftskomplikationen und -pathologien wie z.B. der Präeklampsie überein.

Zusammenfassend lässt sich sagen, dass diese Arbeit zu einem tieferen Verständnis der Interaktion zwischen extra embryonalen Trophoblastzellen und mütterlichen Thrombozyten im intervillösen Raum der Frühschwangerschaft beiträgt.

Abstract

During early stages of human pregnancy a healthy development of the placenta is one of the crucial events to ensure a successful gestation. In first trimester maternal blood cells are restrained from entering the intervillous space (IVS) of the placenta by extravillous trophoblast plugs in the maternal spiral arteries. This ensures a low oxygen concentration which is important for a healthy development of the villous trees.

Based on previous research and pilot data we hypothesized that platelets interact with the early villous and extravillous trophoblast beyond the classical known inflammatory interactions by altering a variety of genes in the human trophoblast.

In this thesis we found a new and alternative way for maternal platelets to enter the IVS of the human first trimester placenta before extravillous trophoblast plugs dissolve. These smallest blood cells were detected in intercellular gaps of distal parts of extravillous trophoblast cell columns by immunohistochemistry and transmission electron microscopy (TEM). As platelets appeared in an activated stage, it was speculated that the activation was derived from a substance found in the interstices of the extravillous trophoblasts (EVTs). However, the EVT marker HLA-G has no impact on the aggregation ability of the maternal platelets and also does not influence the adhesion of human platelets towards trophoblasts.

In an archival *in utero* specimen of an early human pregnancy the previous stainings were confirmed and furthermore showed adhering platelets to the villous trophoblast in the IVS. In co-culture of differentiated trophoblast cell line BeWo and isolated human platelets a significant deregulation of some genes that are involved in the steroid hormone synthesis could be identified and analysis of the cell culture supernatant showed a significant decrease of the release of the pregnancy hormone progesterone. These deregulations were in concordance with known alterations in pregnancy complications and pathologies like preeclampsia (PE).

In conclusion, this thesis will contribute to a deeper understanding of the interaction of extra embryonic trophoblasts and maternal platelets in the IVS of early pregnancy.

1. Introduction

1.1. The human placenta

During early human pregnancy a healthy development of the placenta is one of the critical events to ensure a proper and successful gestation. Next to other tightly regulated processes this organ is accountable for a lot of essential roles during gestation including transport of nutrients and gases, protection of the fetus and endocrinological changes to ensure the continuance of the pregnancy and to function as a substitute for yet immature embryonic and fetal organs (8,9).

Human placentation is called hemochorial placentation, which means that at term the maternal blood cells are in direct contact with the extra embryonic trophoblast cell layer of the placenta (3,10).

Placentation plays a major role in the health of mother and fetus. Defects in the development of the placenta can result in pregnancy disorders like PE, intra uterine growth restriction (IUGR), recurrent miscarriages and even still births. Furthermore it has a lifelong impact on the wellbeing of mother and child (11).

The diameter of a disc-like human term placenta measures 15-25 cm and the average thickness can be calculated with 3cm (12). The placental units are composed of two different plates: the fetal plate, also called chorion or chorionic plate, and the maternal plate, also called basal plate. These plates surround the IVS. In the first trimester placenta this space is filled with endometrial gland secretion and maternal blood plasma. From end of first trimester onwards the IVS is filled with maternal blood. The villi projecting into the IVS from the chorionic plate are complex tree-like structures. They are covered with two layers of extra embryonic trophoblasts, which are in direct contact with the maternal plasma or blood. The villi carry the extra embryonic blood vessels, which are branching from the umbilical vessels into the chorionic arteries. Maternal blood enters and leaves the IVS through the endometrial blood vessels. At term the exchange of gases and nutrients happens at the terminal regions of the chorionic villi where the maternal-fetal barrier is at its narrowest (10,13).

1.1.1. Implantation

A successful human implantation requires the timed arrival of a blastocyst into a susceptible endometrium. During the menstrual cycle the endometrium is remodeled and throughout a short period of time exhibits the receptiveness for the blastocyst, also known as ‘implantation window’ (14).

Before implantation and five to six days after fertilization the blastocyst persists of an outer layer of trophectoderm (TE) and the inner cell mass (ICM), which is asymmetrically attached

to the inside of the TE (15). Six to seven days after fertilization the blastocyst shows a polarity with the trophectoderm facing the endometrium. Once the blastocyst is in correct orientation, the zona pellucida surrounding the blastocyst is lost. The blastocyst attaches and penetrates the endometrial epithelium layer and invades the maternal stroma. Directly after attachment, the trophoblasts of the trophectoderm proliferate and differentiate into the inner cytotrophoblasts (CT) and the outer layer of the multinucleated syncytiotrophoblast (SCT). The early SCT invades further into the maternal endometrium, which during pregnancy transforms into a tissue called decidua, and the blastocyst immerses beneath the endometrial surface (11,13,14).

1.1.2. Development of the early placenta

After around eight days post conception lacunae start to form within the syncytial mass. These fluid-filled spaces magnify and fuse, leaving the left over syncytial partitions as a system of trabeculae (3,11). These progenitors of the IVS are already filled with a mixture of uterine glandular liquids, maternal blood plasma and blood.

At approximately 14 days post conception primary villi are formed. The proliferating CTs begin to migrate into the newly formed trabeculae. The newly formed primary villi consist of a center of mononucleated CTs surrounded by a layer of multinucleated SCT. Now, CTs can also migrate into the maternal decidua as so called extravillous trophoblasts (EVTs). Next, the CT core of the primary villi is being replaced by mesenchymal cells. These villi, that consist of the mesenchymal cells covered by two layers of trophoblasts (CT and SCT) are called secondary villi. At around day 18 to 20 post conception the vasculogenesis of the mesenchymal core starts, blood vessels are formed and the villi are termed tertiary villi now (Fig. 1) (10). Optimal villous vascularization is related to healthy fetal growth. This is made evident by some common pregnancy complications, that are associated with impaired vascularization of the villi as well as the placental decidua, that involve poor fetal growth (e.g. IUGR) (16–18). During first trimester of pregnancy a low oxygen concentration in the early IVS is physiological and of great importance for a healthy development of the villi, the placenta and a proper vasculogenesis. Responsible for the low oxygen during early gestation are the trophoblast plugs blocking the maternal spiral arteries and restraining maternal blood cells from entering the IVS, including oxygen bound erythrocytes (19).

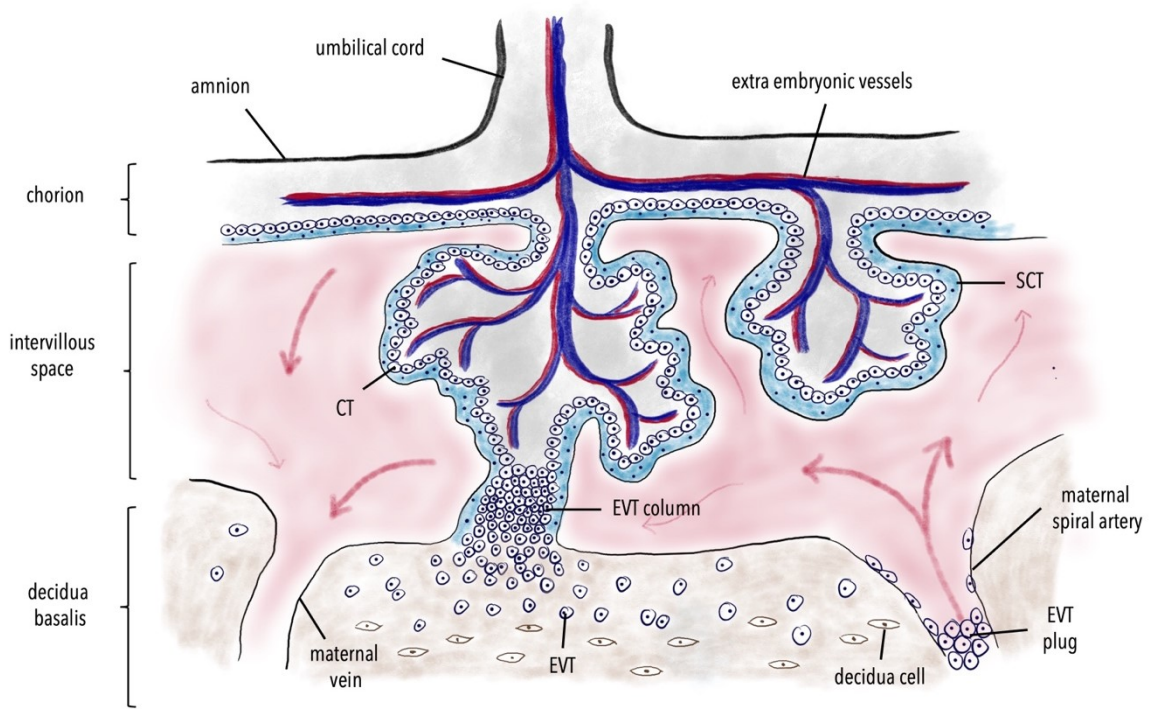


Figure 1: Schematic drawing of an early human placenta. *Extra embryonic vessels are projecting through the umbilical cord into villus trees in the IVS. The villi are covered with two layers of trophoblasts (CT and SCT). The IVS is filled with maternal plasma and secretion from uterine glandulae as the EVT plugs block spiral arteries and restrain maternal blood cells from entering the IVS during early pregnancy.*

1.1.3. Trophoblast differentiation

During pregnancy there are three major trophoblast subpopulations in the human placenta: the cytotrophoblast (CT), the multinucleated syncytiotrophoblast (SCT) and the extravillous trophoblast (EVT).

Following a successful implantation, the stem-cell like CT cells start to proliferate and differentiate. Their differentiation can undergo two different pathways: they either differentiate to fuse with the overlaying layer of the SCT or differentiate into an invasive phenotype and undergo partial epithelial-mesenchymal transition to migrate into the maternal decidua with an extravillous character (13,20).

1.1.3.1. Villous trophoblast

The villous CTs can proliferate and differentiate to become part of the outer multinuclear SCT layer by fusion. The SCT is the outer lining of the placental villi and at the beginning of pregnancy in direct contact with the maternal plasma and secretions from uterine glandulae. Later in pregnancy it provides the interface between the extra embryonic trophoblasts and the

maternal blood in the IVS. Here, the main maternal-fetal exchange of gases and nutrients is conducted. The SCT is a highly differentiated epithelial layer, which is covered on its apical side with microvilli to increase its surface area (21). This multinucleated layer is the primary site of placental transport, endocrine and protective functions. For example, the SCT secrets important pregnancy hormones, like human chorionic gonadotrophin (hCG) and placental lactogen, into the IVS and therefore the maternal circulation (11,13,20).

Furthermore, the SCT releases different extracellular vesicles (EVs) into the IVS. There are three main components of EVs originated from the SCT being detected in the maternal blood. At first syncytial knots are large multinucleated structures, that are results of apoptotic processes in the SCT and are constricted from the SCT to enter into the maternal circulation. Syncytial knots can later be found in lung tissue of the mother. Next, there are micro vesicles, that pinch off the plasma membrane, and exosomes, that are released by exocytosis of multivesicular bodies. All of these EVs carry specific molecules as an interaction between fetal tissue and maternal cells. For example, in pregnancy pathologies the quality and quantity of these placenta-derived EVs are altered resulting in an impact on the maternal system (22).

1.1.3.2. Extravillous trophoblast

CTs that are bound to differentiate into the extravillous pathway at the tips of the villi, proliferate rapidly to form EVT columns. The cells in the distal part of the column undergo an partial epithelial-mesenchymal change with the purpose of migrating and invading into the maternal tissue. These villi are termed anchoring villi as they anchor the villus part of the placenta to the maternal tissue. With the production of different enzymes (like type IV collagenase, matrix metalloproteinases, aminopeptidases, etc.) these cells are promoting the infiltration of the decidua by degradation of extracellular matrix (ECM) (13).

Based on their specific function, EVT can be further divided into subtypes: The interstitial extravillous trophoblasts (iEVT) are invading the maternal decidua up to the first third of the myometrium. This invasion has the purpose to attach the placenta to the uterus and interact with decidual cells. The terminal end-point of this extravillous pathway, when the iEVTs move even deeper into the maternal decidua, is the further differentiation into the multinucleated, rounded giant cell in the placental bed. The endovascular extravillous trophoblasts (eEVT) have the purpose of remodeling the maternal spiral vessels. Endoglandular EVT invade the uterine glands and open them up to the IVS enabling histotrophic nutrition early in pregnancy prior to the uteroplacental blood flow into the IVS (3,20,23). EVTs lose the ability to proliferate and no longer express cell cycle genes. However, they express other highly specific proteins, for

example the major histocompatibility complex G (HLA-G). HLA-G was first described in the 1980s and further on was known as predominantly expressed by EVTs (1). It is an important immune-inhibitory molecule and plays a major role in the interaction with peripheral maternal immune cells and is influencing the activity of the maternal natural killer cells, T-cells, B-cells and macrophages. It is therefore of grave importance for the immunological acceptance of the fetal and maternal tissues that are in direct contact with each other (24–26). In pregnancy complications, as for example PE, the level of HLA-G in maternal serum has been shown to be significantly decreased, suggesting complications in the immunological interplay of maternal and fetal tissues (27).

1.1.3.3. Remodeling of the maternal spiral arteries

The endovascular subtype of the EVTs play an important role in establishing a healthy utero-placental blood flow. This process of remodeling the vessels is critical for a successful gestation. The eEVTs breach the uterine vessel walls and remodel the vessels from tight spirals into wider conduits that guarantee a constant blood flow later in pregnancy. This process includes the depletion of smooth muscle cells in the vessel walls and the loss of the elastic lamina (28–31). In pregnancy pathologies like PE and IUGR the remodeling of the vessels is impaired (32,33).

During first trimester, eEVTs also plug the spiral arteries to prevent maternal blood cells to enter into the IVS (34–36). This process creates an environment of low oxygen concentration as it impedes oxygenated red blood cells to get in direct contact with the extra embryonic cells, which is beneficial for the development of the early placenta and the differentiation of trophoblasts (37). The eEVT plugs also assist in the remodeling process by promoting a supportive low shear environment for trophoblast retrograde movement against the low flow and their replacement of endothelial cells in the maternal vessels (38,39). Even though maternal blood cells are prevented from entering into the IVS, it is agreed upon that blood plasma can penetrate the plug. Recent studies with contrast-enhanced ultrasound found that the plugs seem to become loosely cohesive at around week 5-7 of pregnancy with capillary sized channels. However, this does not lead to a significant increase of flow into the placenta, which stays relatively constant until around week 12 of pregnancy and increases significantly by week 13 (40). Nevertheless, in some pregnancy pathologies maternal blood cells can be found in the placenta early in gestation suggesting problems with the spiral artery plugs and therefore the remodeling of the vessels (10,29).

1.1.4. Function of the placenta

The function of the human placenta changes throughout pregnancy to optimize maternal physiology and fetal outcome. The three main functions of the human placenta can be broken down into transport and metabolism, protection and endocrine function. The placenta provides gases and nutrients for the fetus, whereas also removing fetal waste products. Additionally, it can metabolize different substances, which can be released into maternal or fetal circulation. Furthermore, the placenta acts as a basic immunological barrier and takes part in the materno-fetal immunological acceptance. It releases hormones into both circulations that greatly affects the pregnancy, the metabolism and the fetal growth (13).

1.1.4.1.Transport

The transport function of the human placenta includes but is not limited to water, oxygen, carbohydrates, EVs, lipids, proteins, hormones, vitamins, different minerals and other nutrients for the fetus. (41–44)

Also, the early placental transport differs from the transport function at term in some aspects regarding the stage of establishment of the placenta and the demand of the developing fetus (45,46). Furthermore, the significantly decreased oxygen tension and the lower blood flow in the IVS in early gestation may also take part in altering the expression of some transporter proteins to fit the physiological necessities. In addition, during early pregnancy the nourishment of the placenta is histiotrophic due to the invasion of glands by endoglandular EVT (47).

After the first trimester of pregnancy, maternal blood cells are in direct contact with the villi of the placenta. Therefore, transfer of respiratory gases, nutrients and waste products occurs at the materno-fetal interface (13).

1.1.4.2.Protection

There are different protective features of the human placenta, which help to reduce placental transfer of xenobiotic substances from the maternal to the fetal circulation. The transfer of these potentially toxic substances could happen by simple diffusion or one of the large number of placental transport systems. Protective placental features include for example export pumps in the membrane of the SCT that is facing the maternal side, these include MRP1 (multidrug resistance protein 1), MRP (members of the multidrug resistance-associated protein) family, ABCP (placenta-specific ATP-binding cassette proteins), and many more (48). Furthermore, the human placenta contains a number of enzymes, for example of the cytochrome *P*450 family, that metabolize drugs and other potentially toxic substances (49).

During pregnancy the extra embryonic cells of the placenta are in direct contact to the maternal tissue but somehow the placenta is resisting the immunological rejection of the maternal immune system. There have been many debates about this phenomenon without a clear answer but some specific features (e.g. release of hormones into the maternal circulation and expression of specific proteins on the extra embryonic cells) are said to be playing a key role in this mechanism (50).

While most proteins are not able to cross the placental barrier, some maternal proteins are actively transported across as part of the transcytosis process. Very important for example are maternal antibodies of the IgG (immunoglobulin G) class to establish passive immunity for the newborn (50).

But even though the placenta has these protective features against some xenobiotic substances and the rejection of the maternal immune system, there are some substances that can cross the maternal-fetal barrier after all and by entering the fetal circulation these can have teratogenic effects on the fetus. These substances include for example alcohol, lithium, warfarin, and many more (13).

In general, the placenta forms a barrier against the transport of many bacteria and viruses from maternal to fetal circulation. (51) However, a number of bacteria and viruses can be transmitted across the barrier nether the less. A viral infection of the trophoblast has also been associated with pregnancy complications (52,53).

1.1.4.3. Endocrine functions

Endocrine, paracrine and autocrine factors are produced by the human placenta including steroid hormones like estrogens, progesterone, glucocorticoids and peptide hormones like hCG, placental lactogen and placental growth hormone. Furthermore, all kinds of other growth factors, cytokines and chemokines are created by this endocrine organ (13).

Most placental hormones are secreted by the SCT into the maternal circulation. These hormones are released in a highly regulated way and are of great importance for pregnancy establishment and maintenance, fetal and placental development, decidualization, angiogenesis and immunotolerance. As most of the hormones have a specific profile throughout pregnancy, some of them are also used as biomarkers in the maternal blood to predict pregnancy and prognose disorders (8).

One of the most important pregnancy hormones that is produced by the placenta and which concentration increases with ongoing pregnancy is progesterone. It is released into both maternal and fetal circulations and is essential for the establishment and persistence of

gestation. Progesterone has multiple immunomodulatory functions and inhibits uterine contraction and therefore preterm labor. It influences the maternal immune system to become more expectable of the pregnancy and also suppresses estrus and the release of luteinizing hormone (54–56).

At around 9 weeks of gestation, the human placenta becomes the main source of circulating estrogens, this includes estrone, estradiol and estriol. These hormones act as specialized growth factors for the maternal reproductive organs and are taking part in the main vasodilatory actions included in the uteroplacental vascular functions (57,58).

Glucocorticoids are present in the maternal and the fetal circulation and their concentration increases towards term. These hormones affect development of a wide range of fetal tissues. They replace divers functions previously carried out *in utero* by the placenta and activate many physiological systems that are vital at birth (59).

hCG is a glycoprotein that is mainly produced by the SCT and secreted into the maternal circulation. Its production increases in early pregnancy and peaks at about 8-10 weeks. hCG levels in maternal blood decline from about 12 weeks of gestation towards term (60). hCG plays an important role in the implantation process and the further maintenance of pregnancy. It is also a key player in the process of trophoblast differentiation and migration, vascularization and the adaption of the maternal immune system to pregnancy (8,21,61).

Human placental lactogen is synthesized by the SCT and released into both maternal and fetal circulations. In the fetus human placental lactogen takes part in the embryonic development and modulates the production of other hormones. It is also involved in angiogenesis (62,63).

Placental growth hormone is secreted into the maternal circulation and plays a role in maternal adjustment to gestation, control of maternal insulin-like growth factor I (IGF-I) levels, and placental development (64).

1.1.4.4 Pro- and anti-angiogenic factors

Anti- and pro-angiogenic factors need to exist in a proper balance to control blood vessel formation and sustain vascular function in the human placenta. The pro-angiogenic vascular endothelial growth factor (VEGF) is essential for endothelial cell differentiation (vasculogenesis) and for building new capillaries from already existing vessels (angiogenesis) (65). Placental growth factor (PlGF) promotes pathological angiogenesis under inflammatory or hypoxic conditions. It regulates endothelial cell migration and attracts macrophages, smooth muscle cells and pericytes (66,67). The pro-angiogenic, SCT-derived stress factor endoglin (Eng) binds the transforming growth factor beta (TGF-β) and prevents apoptosis in endothelial

cells under hypoxic conditions. The anti-angiogenic factor soluble Eng (sEng) is released from the SCT and antagonizes the activity of Eng. Its release might be stimulated by eighter hypoxia (68) or protease-induced shedding (69). Soluble Flt-1 (sFlt-1) is an anti-angiogenic factor and is shed from the cell surface. It binds VEGF, making it unavailable for direct angiogenesis (70). Circulating angiopoietins (Ang-1 and Ang-2) maintain important roles in regulating blood pressure and vascular homeostasis (71). Appropriate vascularization is crucial for the placental development. Important factors such as VEGF, PlGF, Ang-1, Ang-2, sFlt-1 and sEng regulate this blood vessel growth. A disturbed balance in these factors can have a severe impact on the placental vascular development. Furthermore, during pregnancy, these factors are released into the maternal circulation to help to adapt the maternal system to pregnancy. Increased levels of anti-angiogenic factors can contribute to the development of pregnancy pathologies such as PE and fetal growth restriction (FGR) (71). Studies found elevated levels of sFlt-1 in patients with PE. In addition, these patients exhibited a decrease in VEGF and PlGF (72,73). Another study reported increased serum levels of sENG in PE patients, which correlated with disease severity (74,75). FGR pregnancies show similar trends to PE with decreases in VEGF and PlGF levels. However, FGR placentas show a decrease in Ang-2 expression compared to an increase in PE placentas (71,76).

1.2. Human platelets

Platelets are anucleated and have a characteristic discoid shape. With a diameter of only 2-3 µm they are the smallest cell type in human blood. They have a life span of only 8-10 days before they are degraded in the spleen, liver or lung. Platelets are essential for processes such as hemostasis, wound healing, angiogenesis, inflammation, and innate immunity (2,77).

Pioneers William Osler and Julius Bizzozero first described circulating platelets in the blood with the ability to form aggregates when removed from their vascular environment in the 1870s and 1880s (78,79). Since then, with new technical and histological approaches the understanding of function and structure of human platelets has done a quantum leap (80). With electron microscopy detailed structures of human platelets could be revealed. Starting with the peripheral zone that consists of the platelet plasma membrane with a thick glycocalyx and a submembrane area. Furthermore, the platelet's surface shows numerous entrances into an extensive network of membrane tubules. This network is called the open canalicular system (OCS). The so-called sol-gel zone lays beneath the peripheral zone and resembles a liquid gel matrix. It consists of a circumferential coil of microtubules, microfilaments, and glycogen. There are also four types of secretory organelles, which are classified into α-granules, dense granules, lysosomes and multivesicular bodies (Fig. 2) (81). Platelet counts (PCs) in healthy humans range between 1.5–4.0 x 10^5/µl with a turnover rate of around 10^{11} platelets per day in a healthy adult (81).

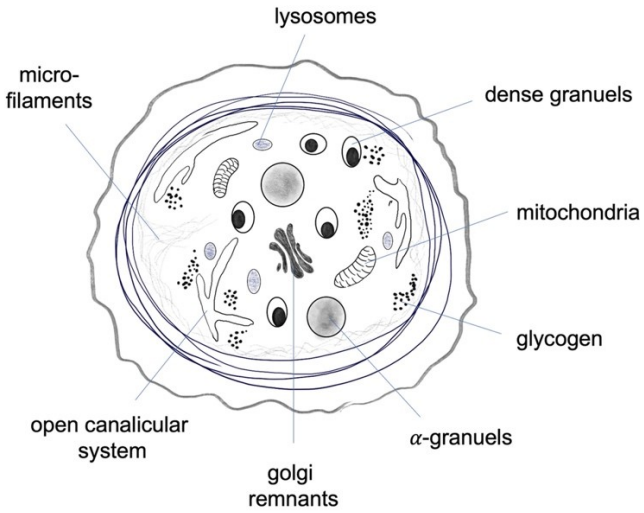


Figure 2: Schematic drawing of a resting human platelet. *Human platelets include secretory granules, like α-granules, dense granules and lysosomes. Through the open canalicular system the secreted cargo of these granules is released into the platelets environment. Moreover, platelets contain glycogen, mitochondria and the remnants of golgi.*

1.2.1. Platelet formation

In 1906 James Wright suggested megakaryocytes in the bone marrow to be the progenitor cells of human platelets (82). Megakaryocytes are the largest (50-100µm) and rarest (0.01%) of the nucleated cells in the bone marrow. Platelets are released from the cytoplasm of megakaryocytes (77).

For the process of platelet production megakaryocytes have to undergo maturation with the help of megakaryocyte-specific growth factors. During this process they become polyploid due to endomitosis. Parts of the enlarged cytoplasm is then packed into nucleus free protrusions that are called proplatelets. One megakaryocyte can extend up to 20 proplatelets. Platelets are formed exclusively at their tips, receiving all kinds of granules and organelles from the megakaryocyte. The process from megakaryocytes over proplatelets to pre-platelets to finally releasing discoid, anucleated platelets into the blood stream takes about 5 days in humans (77,83,84).

1.2.2. Platelet activation

Upon activation by vascular damage or biochemical stimuli platelets are quick to show a shape change by extending filopodia and rearranging their cytoskeleton from a discoid shape to an amoeboid shape. Furthermore, they are quick to interact with one another. The adhesion of activated platelets to the damaged area and therefore the exposed subendothelial matrix is the beginning of primary hemostasis. By an exocytosis process the granules fuse with the plasma membrane and the content of the platelet's granules is released. Some secretion products increase the stimulation and attract more platelets to the damaged area. For secondary hemostasis increased platelet activation leads to the activation of procoagulant processes including thrombin generation and development of stable platelet-fibrin plugs (2,85).

Upon vascular damage the subendothelial ECM is exposed to the circulating blood. Platelets immediately adhere to the site of damage in order to limit bleeding and support the healing. The ECM contains several adhesive macromolecules (e.g. collagen, von Willebrand factor (vWF), laminin, fibronectin and thrombospondin), which serve as ligands for different surface receptors of the circulating platelets. Among the subendothelial substrates, collagens type I and III are the strongest mediators of platelet adhesion (86). The platelet surface receptors that are involved in binding extracellular collagen are GPVI or α2β1 that can bind directly to the subendothelial collagen or GPIbα which binds vWF that in turn connects to the collagen. Stable binding of platelets to the ECM triggers further activation pathways. These involve tyrosine kinases and

signal transduction through G-protein coupled receptors (GPCRs). Ultimately, these pathways lead to increased cytosolic Ca^{2+} levels, cytoskeletal rearrangements and integrin activation (87). Signal transduction via tyrosine kinase activation is initiated by different platelet receptors and their agonists that mediate platelet activation. As two examples platelet surface receptor GPIb-V-IX binds VWF and GPVI-FcRγ binds directly to collagen. Amongst others these two receptor-ligand bindings initiate a tyrosine kinase cascade that results in the activation of PLCγ2 via Tyr-phosphorylation of the SH2 domain (88,89).

Signal transduction via GPCRs involve the following main effectors and corresponding platelet receptors next to others: Platelet's thromboxane receptor (TP) binds to thromboxane A_2 (TXA_2), platelet's protease-activated receptor 1 and 4 (PAR-1 and PAR-4) interact with thrombin and $P2Y_1$ and $P2Y_{12}$ bind ADP (Fig. 3). All of these receptors are bound to a G-protein (G_i, G_q or G_{13} in these cases), which further down the pathways influence the myosin light chain phosphorylation, the cytosolic Ca^{2+} concentration or negatively regulate the cAMP activity (90).

Inside-out activation of integrin αIIbβ3 by elevated Ca^{2+} and DAG levels generated upon platelet stimulation lead to the formation of an 'activation complex', which results in the rearrangement of the cytoskeleton (91,92).

Following platelet adhesion to the site of injury at the vessel wall and subsequent signaling to the platelet cytoplasm, a release of platelet granules that fuse with the outer plasma membrane takes place. The granules content of numerous bio-active molecules is released into the local environment. With para- and autocrine feedback the activation of nearby platelets is increased resulting in a secondary secretion, producing an amplification of the activation process of platelets. In platelets, three types of granules are defined: α-granules, dense granules and lysosomes, each with their individual distinctive content (93).

Following the processes of platelet adhesion and activation, platelets start to aggregate. This process is leading to the formation of a fibrinogen-rich thrombus at the damaged site of the vessel wall. Platelet aggregation is a complex process involving many ligands (e.g. fibrinogen and VWF), receptors (e.g. GPIbα and αIIbβ3), other blood cells and platelets in different activation states, resulting in a great heterogeneity of the thrombus (94,95).

1.2.3. Platelet-derived factors

1.2.3.1. Platelet granules

The intracellular secretory granules of human platelets can be differentiated into α-granules, dense granules (also called dense bodies) and lysosomes. Granules in general were first described at the end of the 19th century, but it was not until 1966 that dense bodies could be differentiated from α-granules, and it took another year to identify the difference between dense granules and lysosomes (80).

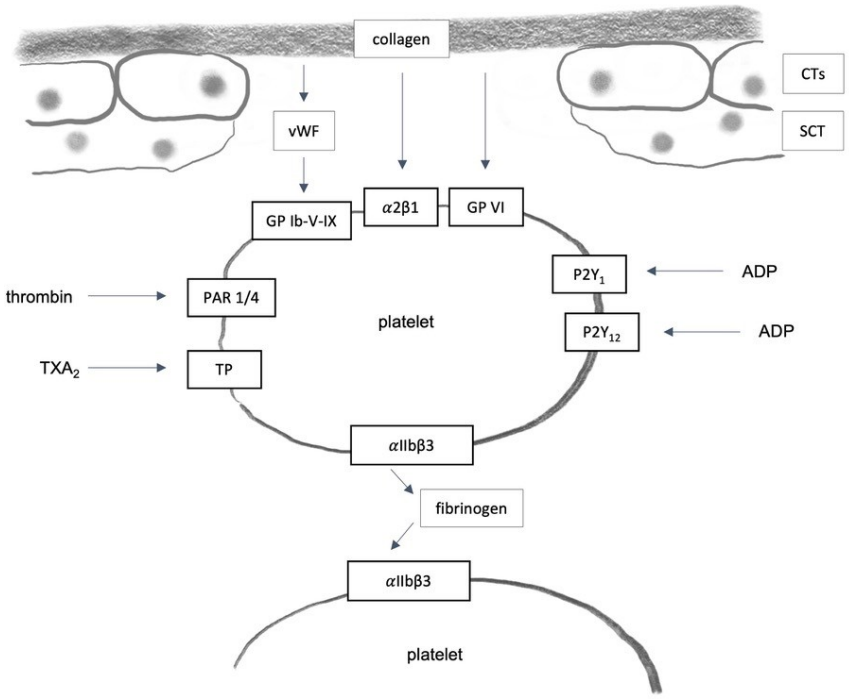


Figure 3: Receptors involved in the adhesion and activation of human platelets. *Receptors that are resembled here are involved in the direct or indirect adhesion of platelets towards collagen (GP Ib-V-IX, α2β1 and GP VI), in the activation by external agonists (TP, PAR 1/4, $P2Y_1$ and $P2Y_{12}$) or in the interaction and aggregation between human platelets (αIIbβ3).*

Upon platelet activation, the platelets undergo shape change and aggregation, furthermore the storage granules mentioned above migrate to the cell surface of the platelet. From these granules a multitude of different factors are released, containing different growth factors, coagulation proteins, adhesion and cell-activating molecules, a variety of cytokines, integrins, proinflammatory and angiogenic molecules. The composition of the released cargo is dependent on the involved agonist. A strong agonist like thrombin will cause the release of all granule

content whereas a weak agonist like ADP only causes the release of α-granules and dense bodies but not the lysosomes (96).

α-granules with the size of 200-500 nm originate from the trans-Golgi complex in the megakaryocyte and are the most numerous with about 80 α-granules per platelet. These granules are of great importance for platelet activation. They are the largest storage units in the human platelets and contain a divers cargo including platelet-specific proteins, glycoproteins, adhesion molecules, growth and coagulation factors (96).

There are about 7 dense bodies present in a human platelet. They are smaller than the α-granules and are key players in the processes of aggregation and vasoconstriction. Therefore, their cargo is mostly composed of molecules like ADP, ATD, Ca^{2+} and serotonin which is released by exocytosis when the dense body fuses with the plasma membrane (81,96).

Lysosomes are membrane bound and are stimulated upon thrombin activation. Their cargo contains hydrolases, cathepsins and lysosomal membrane proteins (81,96).

1.2.3.2. Platelet releasate

A vast variety of agonists can induce platelet activation. Depending on the strength of the agonist, the responses triggered are ranging from shape change to aggregation and also the amount of platelet secretion differs depending on the agonist. The released molecules enter the external milieu where they can act in an autocrine and/or paracrine manner. Over 350 proteins have been identified in thrombin activated platelet releasates. These proteins include growth factors, coagulation proteins, cytokines, adhesion and proinflammatory molecules (96). It has been shown that the secretion from platelet granules can be altered in diseases as well as in other exceptional circumstances like for example human pregnancy (81,97).

The cargo of α-granules composes membrane-associated and soluble proteins. They are associated with numerous processes including cell adhesion, coagulation and inflammation. Upon activation, membrane-bound granule proteins are presented on the surface of the platelet. On resting platelets there are already a variety of membrane-bound proteins present, for example, integrins, immunoglobulin family receptors and Fc receptors (FcR). Nevertheless, some membrane-associated proteins are exclusively expressed on the surface of activated platelets, for example P-selectin and are therefore known as markers for the activation state of platelets. Soluble granule proteins are released into the extracellular compartment upon activation. More than 300 soluble proteins released by α-granules have been identified with proteomic analyses (98,99).

Dense bodies contain high concentrations of ADP and ATP, uracil and guanine nucleotides, calcium, potassium and serotonin, which play a key role in aggregation and vasoconstriction. The pH within dense granules is slightly acid with a pH of approximately 5.4. It has been shown that the release of cargo from dense granule is faster than the release from the other two secretory granules (98,100).

Lysosome cargo of platelets consists of protein degrading enzymes (e.g. cathepsins, elastase, and collagenase), carbohydrate degrading enzymes (e.g. glucosidase and galactosidase) and acid phosphatase (phosphate ester cleaving enzyme). The lysosome's protective function is supported by proteins like CD63 that are found in the lysosomal membrane in a highly glycosylated state (101).

1.2.4 Wound repair

In physiological conditions, platelets circulate in the human blood vessels in close proximity to the endothelial walls. This healthy endothelial monolayer acts as a protective layer from unwanted platelet activation. With the release of inhibitory factors such as nitric oxide (NO) and Prostacyclin (PGI2) it ensures the inactive state of human platelets. With a disruption of the endothelial layer and the exposure of the underlying subendothelial matrix the platelet receptors interact with the now exposed collagen and vWF and thereupon activation signals are induced (102).

As described in chapter 1.2.2. (Platelet activation) the initial platelet plug is formed at the injured site. Subsequently, following the coagulation cascade a fibrin mesh is established that encapsulates the initial plug and strengthens the thrombus. This fibrin mesh is also serving as adhesion sites for coagulation factors (99,102).

Passing platelets can also become activated via GPCR signaling downstream from soluble agonists forming at the site of thrombus. Those soluble agonists released from activated platelets result in a number of positive feedback cascades leading to fast activation of large numbers of human platelets. Eventually, a set of many other secreted factors and involved cells results in restoration of the vessel wall and a recovery of its integrity (102).

1.3. Interaction of platelets and placenta in humans

1.3.1. Pro- and anticoagulants in the human placenta

Human pregnancy is known for its hypercoagulability and hypofibrinolysis. The increase in the expression of tissue factor (TF) and plasminogen activator inhibitors (PAI) in the placenta could contribute to the increased fibrin deposition by promoting coagulation and decreasing fibrinolysis (103,104).

During gestation the placenta allegedly is a source of TF. Normally TF is not present on cells that are in contact with blood, however, even though some studies showed contrary results, the presence of TF in the human placenta appears to be essential for the maintenance of hemostasis. In the placenta, TF probably functions as a coagulation activator and a signal for blood vessel damage (103).

In normal human placental tissue PAI-1 and PAI-2 have been identified and plasma levels of both inhibitors increase over the course of pregnancy. PAI-1 is synthesized mostly by endothelial cells and appears to be one of the primary regulators of the fibrinolytic system in vivo. Overexpression of this inhibitor may promote pathologic fibrin deposition when the clotting cascade is activated (105,106). Plasma and placental PAI-1 levels were significantly elevated in pregnant women with severe PE. Immunohistochemical and in situ hybridization analyses revealed a strong positive signal in the placental villous SCT from women with PE, but only a weak signal from those with healthy pregnancies (107). PAI-2 is mostly produced by macrophages and keratinocytes and has a possible role in placental cell invasion in the uterus.

The factors that are most involved in anticoagulant mechanisms in the human placenta are the Tissue Factor Pathway Inhibitor (TFPI), Thrombomodulin (TM) and Annexin V.

During pregnancy TFPI is one of the key inhibitors of the TF activation pathway. It is a negative modulator of the extrinsic pathway of coagulation. It is synthesized in endothelial cells, SCT and EVTs from 10 weeks of gestation to term at relatively constant concentrations (108). TFPI was most abundant in placental tissue compared to other examined organs. TFPI-2 is expressed by placental SCT and is found in maternal serum of normal pregnancies to prevent hypercoagulation. Serum level increased over the course of pregnancy and further increased in patients with trophoblastic disorders (109).

TM is a membrane receptor and localized in endothelium of placental vessels and on the apical side of the SCT (110,111). TM is speculated to play a role in the regulation of maternal hemostasis. Over the course of pregnancy, a 2.3-fold increase in protein and mRNA between first trimester and term placenta could be shown. Even further increased plasma levels have also been reported in pregnancies complicated with PE (112).

Annexin V is expressed on villous SCT, where it acts as an endogenous anticoagulant to regulate intervillous fluidity of the maternal blood (113). Reduction in annexin V expression on placental villi in vitro resulted in increased coagulation of plasma on cultured trophoblasts. These findings could be associated with recurrent fetal loss, which could be associated with pregnancy complications in context with antiphospholipid syndrome (APS) (114).

1.3.2. Hemodynamic changes during human pregnancy

The body of the pregnant mother undergoes a range of anatomical and physiological alterations to manage the demands of gestation. These changes include cardiovascular, respiratory, hematological, renal, gastrointestinal and endocrine adaptations (92).

In regard to the hemodynamic changes the plasma volume of the mother is increased by 30-50%, resulting in an elevation of blood volume of about 1-2 liters. Platelet concentration in healthy pregnancies generally decreases with ongoing gestation, eventually reaching a reduction of around 10% at term due to the expansion of plasma volume and the consumption in the utero-placental environment (115,116). Nevertheless, pregnancies are characterized as a prothrombotic state, accompanied with an increased risk of venous thrombosis, fibrinogen and procoagulant factors (e.g. vWF) (117). During pregnancy, there is also an increase in red and white blood cells. However, the increase of 18–25% of the red blood cells is disproportionate to the elevation of plasma volume of 30–50% (118–120).

1.3.3. Changes in steroid hormones during pregnancy

Adjustments in maternal physiology takes place to support human pregnancy. As one example the maternal immune responses are primed to prevent the inflammatory response and rejection of the fetal tissue cells. These adaptations of immunity are to some extent modulated by endocrine signals and the continuous rise of steroid hormones progesterone and estradiol over gestation (56).

To underline the importance of these hormone developments during pregnancy, a study by Lisova et al. investigated the fetoplacental hormone levels and their dynamic changes over pregnancy in women with recurrent fetal losses. Hormone levels of 50 healthy pregnant women

and 50 pregnant women with a history of miscarriage were determined at different stages of gestation. Women of the group with recurrent fetal losses had a significantly slower rate of elevation in hormone levels compared to the healthy control group. The estradiol levels during early pregnancy were 76.0% lower and during late pregnancy were 65.5% lower in women with miscarriage compared to the control group. Levels of placental lactogen and chorionic gonadotropin in early stage of gestation were also lower by 39.1% and 50.9% in women with miscarriage. At the end of pregnancy, these levels were still lower by 72.9% and 35.4%. Furthermore, progesterone levels in early and late gestation were lower by 67.4% and 68.4% compared to the control group. These data suggest a pronounced hormonal abnormality of the placenta in women with a history of miscarriages. This fetoplacental dysfunction, seems to develop early in pregnancy and continues to progress with ongoing gestation (121).

1.4. Pregnancy disorders linked to platelet and/or steroid hormone deregulation

With ongoing pregnancy PC in the maternal blood decreases compared to the non-pregnant state. At term a reduction of platelet concentration of approximately 10% is reached in uncomplicated cases. The platelet concentration returns to the normal, non-pregnant state a few weeks after birth. There are different reasons for the decreased PCs during gestation. While the most prominent cause is the dilution of maternal blood by plasma expansion, there are other causes like accelerated platelet consumption in the placental circulation (116,122,123). Pregnancies with a PC below the lower limit of 1.5 x 10^5/µl are referred to as gestational thrombocytopenia. However, also the cases of gestational thrombocytopenia return to the non-pregnant blood PC only a few weeks postpartum and do not have an increased risk for a poor pregnancy outcome (124,125).

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with several phenotypes and manifestations in different organs. Clinical symptoms vary from mild to severe multiorgan involvement (126). During gestation the maternal immune system is modified to tolerate the interaction of maternal and extra embryonic cells. These modifications are impacted by estrogens and progesterone whose blood concentrations increase with ongoing pregnancy. Steroid hormones have been found to be deregulated in pregnant patients with SLE compared with healthy mothers. In those patients whose pregnancy is complicated with SLE 17-β estradiol, progesterone and DHEAS concentrations have been found to be significantly reduced compared with the concentrations observed in healthy pregnancies. The women with complicated pregnancies do not display a peak of estrogen, progesterone, testosterone and DHEAS serum levels in the 3rd trimester, which can be witnessed in healthy women. This might be due to placental damage in these patients. Vascular changes and coagulation abnormalities (such as infarction, edema and villous thrombosis) have been observed in patients complicated with SLE (127,128).

During pregnancy the Antiphospholipid Syndrome (APS) is associated with recurrent fetal loss. Pregnancies that are complicated with APS and still proceed to the third trimester are often linked with vascular complications. In many cases these pregnancies show thrombotic processes and inflammatory damages of the uteroplacental vessels, including fibrinoid necrosis and arthrosis (10,129).

Increased expression of TF by endothelial cells or monocytes of nonpregnant patients with APS could play a role in thrombus formation. Therefore, the potential roles of TF, TM, and annexin

V have been determined in placentas from 12 women with APS and matched healthy controls by immunohistochemistry. No differences were found in the stainings (129). Placental villous cultures were exposed to antiphospholipid antibodies (APAs) for 24 hours. This resulted in reduced levels of annexin V on the SCT, reducing the protection against coagulation processes it usually provides. By binding to annexin, APAs can therefore reduce its protective mechanism, increasing coagulation processes and potential thrombotic complications during pregnancy (114).

Preeclampsia (PE) is a pregnancy-specific syndrome associated with hypertension, proteinuria and several plasma changes in coagulation parameters that affects 3-8% of pregnancies and that can ultimately result in maternal and fetal morbidity and mortality. (103,130) Regarding the topic of platelets, the general PC has been decreased in pregnancies complicated with PE when comparing them to healthy pregnant controls (130–132). Furthermore, some platelet activation markers like mean platelet volume (MPV) (132–135), platelet surface marker and plasma levels of P-Selectin (135–137) were significantly elevated in PE cases compared with control groups. The exact reason for the development of a PE has not been elucidated, although some pre-existing conditions and the involvement of the placenta have been considered. In placentas obtained from PE patients excess fibrin deposition could be observed, which could affect the exchange at the materno-fetal interface. The placenta produces a large quantity of steroid hormones to maintain pregnancy. Those steroid hormone concentrations, in particular progesterone and estrogen, in the serum of women with PE were significantly downregulated (103,138).

It also has been proposed that progesterone may reduce the risk of PE. HLA-G is reduced in the placenta and serum of pregnancies complicated with PE and this may contribute to impaired placentation and subsequent PE. As progesterone enhances the expression of HLA-G in placental trophoblasts, progesterone may promote immunological tolerance of the extra embryonic cells to the maternal circulation and therefore reduce the risk of PE (139).

Another severe pregnancy complication is the Hemolysis Elevated Liver Enzyme and Low Platelet (HELLP) syndrome. This HELLP syndrome has an incidence of 0.1–0.8% of all the pregnancies and it occurs in 80% of all cases with PE. It is associated with elevated liver enzymes, low PC and hemolytic anemia (140,141).

2. Aims

Based on previous research and pilot data we hypothesized that platelets interact with the early villous and extravillous trophoblast beyond the classical known inflammatory interactions by altering a variety of genes in the human trophoblast.

The following aims were addressed in this study:

I. Identification of platelets in between extravillous trophoblasts in early human first trimester placenta

Previous studies have shown platelets in between EVTs by immunohistochemical staining. This alternative route of the maternal platelets to enter the IVS of the first trimester placenta would be another variant next to small channels in the extravillous trophoblast plugs of the maternal spiral arteries. We wanted to further characterize the way of platelets through intercellular interstices of the distal part of EVT columns to confirm and strengthen this finding and the presence of maternal platelets in the IVS of the human first trimester placenta. Therefore, immunohistochemistry with the markers HLA-G, CD42b, CXCL4, CD34, ZO-1, occludin and β-hCG and TEM was used on human first trimester placental tissue.

II. The effect of HLA-G on the aggregation of human platelets and their adhesion towards trophoblasts

HLA-G has an immunomodulatory role during pregnancy. Due to the fact that EVTs express the specific marker HLA-G and slightly shape changed platelets were found in between EVTs, we wanted to test whether recombinant HLA-G would cause aggregatory effects in human platelet rich plasma (PRP). Furthermore, the trophoblast cell line JAR was transfected to stably overexpress HLA-G, which was confirmed with immunocytochemistry and Western Blot. Here, the adhesion of human platelets to the HLA-G overexpressing cells was analyzed in a flow assay. Slides were stained with immunofluorescence (IF) and data were analyzed with specific software.

III. Influence of human platelets on the expression of genes that could be involved in the steroid hormone synthesis of villous trophoblasts

It is validated that maternal platelets enter the IVS early during pregnancy and get in direct contact with the extra embryonic villous trophoblasts early in gestation. In previous studies we have shown that platelets decrease the trophoblast's expression of the gene that encodes for the pregnancy hormone hCG. As two of the most important hormones for the maintenance of a healthy pregnancy are the steroid hormones progesterone and estrogen, we wanted to investigate the differences in genes involved in the steroid hormone synthesis upon co-incubation of syncytium and isolated platelets. Genes of interest were investigated via qPCR, PrimePCR and RNA-Sequencing. Furthermore, immunohistochemistry confirmed the presence of the proteins that resembled the genes of interest in the first trimester human tissue. At last, the corresponding supernatant was analyzed with progesterone-ELISA and further supernatants of co-incubation experiments with platelets of pregnant women were analyzed for progesterone with Immulite assay.

In conclusion, this study was conducted to obtain a deeper insight into the topic of maternal platelet-placenta cross-talk and interaction during early human pregnancy.

3. Methods

The chapter "Methods" was based on and extended from Guettler, J., et al., "Maternal platelets pass interstices of trophoblast columns and are not activated by HLA-G in early human pregnancy." (2021) (1).

3.1. Human Placenta Tissue

3.1.1. Human placenta tissue collection

Placental first trimester tissue was collected from women undergoing elective termination of pregnancy (gynecologist's practice in Graz - Dr.med.univ. Glasner - Femina Med). Gestational age (GA) of placentas collected was between week 5 and week 12. Healthy patients signed written informed consents. Ethical approval was obtained from the Medical University of Graz Ethics Committee (31-019 ex 18/19; 26-132 ex 13/14).

Within 1-4h after the medical intervention, the tissue samples were processed at the Division of Cell Biology, Histology and Embryology at the Medical University of Graz. Placental tissue samples were dissected under the stereoscopic microscope (Leica MZ6 in combination with Leica CLS 150) and were immediately fixed in 4% paraformaldehyde (PFA). Afterwards fixed first trimester placenta tissue was embedded in paraffin (Sakura Tissue-Tek VIP and Medite, Tissue Embedding System TES Valida).

3.2. Cell Culture

3.2.1. BeWo cell line

For *in vitro* studies of the SCT the choriocarcinoma cell line BeWo (European Collection of Cell Cultures (ECACC, Salisbury, UK)) was used. BeWo cells were cultured in DMEM/F12 medium (1:1, Gibco, life technologies; Paisley, UK) supplemented with 10% fetal bovine serum (FBS, HyCloneTM; Gibco; heat inactivated for 1 h at 56°C), 1% L-Glutamine (Gibco) and Penicillin/Streptomycin (Gibco) in a humidified incubator with conditions of 5% CO_2 at 37°C. For passaging, cells were detached with accutase (Biowest®) and used for *in vitro* experiments between passage 10 and 30. Cell count was obtained by CASY® (SCHÄRFE SYSTEM) measurements. The syncytialization of the trophoblast cell line was induced by 20 µM forskolin (Tocris, Bio-techne, Abingdon, UK) treatment for 48 h.

3.2.2. JAR cell line

For *in vitro* studies of EVTs, the cell line JAR (originally purchased from ATCC) was used. HLA-G overexpressing JAR-G cells were generated by transfection of the full-length HLA-G1 cDNA as previously described (142). Briefly described, to amplify HLA-G1 cDNA the high-fidelity DNA polymerase Pwo (Eurogentec, Seraing, Belgium) was used. Thus, Xbal and Kpnl restriction sites were introduced. Subsequently, the amplified HLA-G1 cDNA was cloned into PGEM-T (Promega, Charbonnieres, France) and sequenced using a Sequenase DNA sequencing kit (Amersham, Les Ulis, France). Afterwards a fragment was produced using the digestion by Xbal/Kpnl, which was in return cloned into a pcDNA3 plasmid (Invitrogen,San Diego, CA). The pcDNA-G1 plasmid included a neomycin resistance. Now the calcium phosphate method was used to transfect the plasmid into the trophoblast cell line JAR. Cells that showed a stable transfection were extracted by use of the newly obtained neomycin resistance with treatment of a selection medium containing 500 pg/mL of G418 (Life Technologies Inc, Cergy-Pontoise, France). Culture medium for the cell lines was RPMl 1640 medium (Gibco, life technologies; Paisley, UK.), supplemented with 10% FBS (HyCloneTM; Gibco; heat inactivated for 1 h at 56°C) and 1% Penicillin/Streptomycin (Gibco). Mycoplasma tests were conducted for both cell line every 6 weeks with negative test results.

3.2.3. Isolation of human platelets

Citrated whole blood was collected from healthy donors at the Division of Pharmacology (Otto Loewi Research Center, Medical University of Graz) or from pregnant donors at the Department of Obstetrics and Gynecology (Medical University of Graz). All donors signed written informed consents. Collected blood samples were subjected to centrifugation of 100 x g for 15 min at RT. Afterwards supernatant, also called PRP, was gently mixed with wash buffer (supplemental table 1) at ambient temperature, supplemented with prostaglandin E1 (50 ng/mL; CaymanChemical, Michigan, USA) before usage. After the next centrifugation step at 3000 rpm for 15 min at room temperature (RT) the platelet pellet was resuspended in wash buffer and centrifuged again at the same conditions. Finally, the platelet pellet was resuspended in the equivalent amount to the former PRP volume of eighter RPMI 1640 ((Sigma Aldrich) supplemented with 1 % penicillin/streptomycin (Gibco)) or DMEM/F12 medium (1:1, Gibco, life technologies; Paisley, UK) supplemented with 1% L-Glutamine (Gibco) and Penicillin/Streptomycin (Gibco). PC was determined with a Sysmex KX-21NTM.

3.2.4. Aggregometry

Aggregometry of PRP was conducted with a Chrono-log Model 700. 1 mL of PRP was prewarmed to 37°C. The electrodes were inserted and with a stable electrical current in the electrodes, the baseline was established. For activation different agonists (2-MeS-ADP (10 µM; Tocris), recombinant HLA-G1 (600 ng/mL; abcam), collagen IV (20 µg/ mL; Sigma-Aldrich), collagen I (2 µg/mL; Chrono-Log) were added to the PRP. The increasing electrical resistance (ohm) in the electrodes was measured for 6 min, protocolling the aggregation curve. Aggregation experiment was repeated five times for every agonist with different platelet donors.

For analyzing the of trophoblast-derived HLA-G effect on platelet aggregation, 500 µL of double-concentrated isolated platelet suspension was prewarmed to 37°C, as with addition of the agonist the platelet suspension is diluted to a normal PC. The electrodes were inserted and the baseline established again. As an agonist either 500 µL prewarmed supernatant of JAR cells with addition of 20 µM 2-MeS-ADP (Tocris) or 500 µL prewarmed supernatant of JAR-G cells was added to the concentrated platelet suspension. Aggregation was again measured by documenting the rising electrical resistance (ohm) in the electrodes for 6 min. These aggregation experiments were repeated three times with different platelet donors and supernatant from different cell passages of JAR and JAR-G cells.

3.2.5. Co-incubation of platelets and JAR/JAR-G cells under flow (IBIDI system)

Citrated whole blood was drawn with written informed consent from healthy donors at the Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics at the University of Leipzig (179/20-ek, 507/19-ek). Platelet isolation was conducted as described before (3.2.3. Isolation of human platelets).

For the platelet adhesion experiment under flow, the ibidi Pump System (Ibidi, Germany) was used. This included the red perfusion set (red tubing set; length 15 cm, ID 1.6 mm, 10 mL reservoirs), fluidic units and the ibidi pumps. The ibidi Pump System was prepared, preincubated and calibrated overnight at 37 °C, including the filling of each fluidic unit by 13 mL of RPMI 1640 medium (Sigma Aldrich), supplemented with 1% penicillin/streptomycin, air bubble-free. Next day, JAR and JAR-G cells were seeded with a density of 3.5×10^6 cells/mL in a channel slide (ibiTreat, 0.6 µm height; Ibidi, Germany) for 3 h at 37 °C, previous to the flow and co-incubation with platelets. Afterwards the ibidi channel slides were connected to the tubing system and flow parameters were set. After a constant flow on the cells was established, freshly isolated human platelets with a concentration of $6.85 \times 10^6 – 1.27 \times 10^7$ per

ml were added to each flow system. The two cell lines JAR and JAR-G were co-incubated with platelets in presence or absence of ADP (10µM). The conditions for the flow experiment were 1.7 dyn/cm^2 for 2 h at 37 °C and 5% CO_2. After this co-incubation, JAR and JAR-G cells were washed with (-/-) DPBS (no calcium, no magnesium; Thermo Fischer Scientific) under flow conditions until incubation medium and non-adherend platelets were completely removed. For further staining experiments, cells in the slide were fixed with 4% PFA for 30 min at RT und afterwards washed with (-/-) DPBS for three times. Ibidi slides of the flow experiment were stored at 4 °C in (-/-) DPBS until further use. The experiment was repeated three times with different platelet donors and different cell passages of JAR and JAR-G cells.

3.2.6. Co-incubation of platelets and BeWo cells

For co-incubation with isolated and washed human platelets, BeWo cells with a density of 2 x 10^5 cells per well were seeded in a 12-well culture dish. After 24 h, culture medium was exchanged with medium containing eighter 20 µM forskolin for induction of syncytialization or 0.1% DMSO as a vehicle control. After further 48 h of pre-differentiation the medium was exchanged for eighter freshly isolated human platelets at a density of 1.44-2.70 x 10^8 platelets/ml or normal BeWo culture medium. Cells were co-incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37°C.

3.3. Gene expression analysis

3.3.1. RNA isolation and cDNA synthesis

After incubation, cells were washed with PBS and cells were lysed with RNA-Lysis-Puffer T (peqGOLD VWR, Cat.Nr.: 12-TRK-88). RNA was isolated with PeqGOLD Total RNA Kit (C-Line) (peqGOLD VWR, Cat.Nr.: 12-6634-02) from cells according to the manufacturer's instructions. Cell sample purification and concentration was determined with NanoDrop® ND-1000 (peqlab Biotechnologie GmbH).

Afterwards, Reverse Transcription was performed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems by Thermo Fischer Scientific, Cat.Nr.:4368814). Reverse Transcription Puffer (10x), dNTP (100 mM), Reverse Transcription Random Primer (10x), nuclease free water and MultiScribe Reverse Transcriptase (50 U/µl) were added to the defined concentration of 1 µg RNA. Thermo Cycler was programmed to 10 min at 25°C, 120 min at 37°C, 5 min at 85°C and subsequent cooling at 4°C. Thereafter, originated cDNA was diluted to a concentration of 1 ng/µl and stored at -80°C.

3.3.2. qPCR

Quantitative Polymerase Chain Reactions (qPCRs) were performed in 96-well and 384-well plates (BioRad) with cDNA concentration of 0.25 ng/µl. A 2:1 master mix of Blue S'Green qPCR (Biozym, Cat.Nr.: #331416) and cDNA were prepared and added to the pre-pipetted primer (800 nM) (table 1) to each well. After performing the qPCR in the C1000^{TM} Thermal Cycler (BIO –RAD, CFX96^{TM} Real-Time System) data was analyzed with Bio-Rad CFX Manager 3.1. Cq values and expression values were quantified by referring to the used housekeeping gene GAPDH and YWHAZ.

Table 1: List of primers

Primer	forward	reverse
CGB3_5_8	TGA GCC ACT CCT GCG CCC	CAG CCC CTG GAA CAT CTC CA
CYP19A1	CCC AAG TTT GCT GCC GAA TC	TGC GAG TCT GGA TCT CTG GA
GAPDH	ACC CAC TCC TCC ACC TTT GA	CTG TTG CTG TAG CCA AAT TCG
STS	CCC TCA GAA CAG CAC ATC CAT	AAC TCC CGA AAC AGA AGC ACA
TBP	TGA CCC AGC ATC ACT GTT TC	CCA GCA CAC TCT TCT CAG CA
YWHAZ	GGT GGC CAA TAT GGG GAT GT	TCC CTT TTA TTC CCC GCC AG

Furthermore, three replicates of costum-designed PrimePCR plates (BioRad) were performed. A panel of user designed primers (genes involved in the steroid hormone synthesis) was provided on the plates by the company and the further procedure was conducted as described above.

3.3.3. RNA-Sequencing

Samples of co-incubation of BeWo cells with platelets were send to ZMF (Center for Medical Research, Medical University of Graz) and subjected to RNA Sequencing. In short, BeWo cells were seeded in 12-well culture dishes at a density of 1 x 10^5 cells/ml. After 24 h, cells were treated with 20 µM forskolin to differentiate into a syncytium. After another 48 h of BeWo-differentiation isolated human platelets at a density between 1.44 x 10^8 platelets/ml and 2.70 x 10^8 platelets/ml were added to the BeWo syncytium. In addition, a control without platelets was cultured. The experiments were incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37°C and repeated three times with different healthy platelet donors and BeWo cell passages.

RNA was isolated and transcribed as previously described (3.3.1. RNA isolation and cDNA synthesis).

Additionally, Qiagen RNeasy Mini Kit (Qiagen; Hilden, Germany; 74004) for DNAse treatment and purification was used. Quantification was performed on the NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific; Waltham, MA, USA). The integrity of each RNA sample was determined using an Agilent 2100 Bioanalyzer (Agilent; Foster City, CA, USA) using the RNA 6000 Nano LabChip (Agilent; Foster City, CA, USA; Cat.No. 5065-4476). Only RNA samples with a RIN factor above 9 were used for the preparation of the transcriptomic library.

To eliminate the rRNA NEBnext rRNA Depletion Kit V2 (New England Biolabs; Ipswich, MA, USA; Cat.No. E7405) was used according to manufactures instruction. Input of total RNA was 200 ng. Library preparation was conducted with NEBnext Ultra II Directional RNA Library Prep Kit for Illumina Kit (New England Biolabs; Ipswich, MA, USA; Cat.No. E7760). In detail: fragmentation time was set to 15min; the adapter dilution chosen was 1:25; for PCR library enrichment we used 8 cycles. For proper identification of the samples after next generation sequencing, we labeled the samples with NEBNext Multiplex Oligos, Set 1 (New England Biolabs; Ipswich, MA, USA; Cat.No. E7335S). Afterwards, libraries were quantified on Quantus Fluorometer (Promega; Madison, WI, USA) with QuantiFluor ONE dsDNA System (Promega; Madison, WI, USA; Cat.No. E4870). For quality check of the fragment sizes of the library all samples were analyzed with Agilent 2100 Bioanalyzer (Agilent; Foster City, CA, USA) using the DNA High Sensitivity LabChip (Agilent; Foster City, CA, USA; Cat.No. 5067-4626). A mean peak size of 379 bp was observed, which is in the range of the manual description. The Non Template Control (Aqua Dest.) of the RNA transcriptomic library showed marginal outcome. A pool of all samples was sequenced by VBCF (Vienna Biocenter Core Facilities GmbH, Dr.-Bohr-Gasse 3, 1030 Vienna, Austria) on an Illumina HiSeqV4 flow cell (Illumina; San Diego, CA, USA) in a SR50 read mode. After demultiplexing, FASTQ files were used for data analysis. We reached between 36.3 x 10^6 and 41.8 x 10^6 total raw reads.

Calculated and summarized results of the RNA-Sequencing were further analyzed with DAVID software.

3.4. Protein analysis

3.4.1. Protein Isolation

After incubation, cells were washed with PBS and subsequently homogenized in RIPA buffer containing 1x protease inhibitor cocktail (Roche Diagnostics; Mannheim, Germany) and 1x phosSTOP (Roche Diagnostics) and frozen at -80 °C. Thereafter, thawed and homogenized cell lysates were centrifuged at 8000 rpm and 4 °C for 10 min. After centrifugation, the clear supernatant was transferred to a fresh eppendorf tube and total protein concentration of the clear protein lysate was measured by the Lowry Protein Assay (143), a method that is based on the reaction of cooper ions and their peptide bonds under alkaline conditions. Subsequent, the colorimetric reduction of the Folin-Ciocalteu reagent can be measured. As standard protein sample Bovine serum albumin (BSA) was used (0 to 8mg/ml diluted with RIPA buffer). Recipes for solution A, B and C can be found in supplemental table 2. In brief, these solutions were mixed (100:1:1) and incubated with the protein sample for 10 minutes at RT. Afterwards Folin–Ciocalteus Phenol Reagent (Merck) (1:1 with Aqua dest) was added and after 30 min of incubation the total protein content was measured by anthos 2010 Microplate Reader (Biochrom®) at a wavelength of 620 nm. Protein content was calculated by referring to the standard protein type curve.

3.4.2. Western Blot

Total protein (20 µg) was mixed with 1x LDS (invitrogen NuPAGE®, Cat.Nr.: NP0007) and 1x Reducing agent DTT-1M (invitrogen NuPAGE®, Cat.Nr.: NP0004) and was preheated to 95 °C for 5 minutes before loading samples on a 10% Bis-Tris Gel (NuPAGETM 10% Bis-Tris Gel 1.0 mm X 10 well, Invitrogen, Cat.Nr.: NP0301BOX). Afterwards SDS gel electrophoresis (PowerPacTM HC, Bio-Rad) with 1x MES SDS Running Buffer (novex® NuPAGE®, 20x, Cat.Nr.: NP0002) at 140 volt was performed. The standard protein ladder contained a mixture of MagicMark XP Western Protein Standard (Invitrogen, Cat.Nr: LC5602) and See Blue Pre-Stained Protein Standard (10 to 180 kDa, PageRulerTM, Cat.Nr.: 26616).

Blotting on a 0.45 µm nitrocellulose membrane (Hybond, Amersham Biosciences, GE Healthcare Life Sciences, Little Chalfont, UK) was conducted in 1x Transfer-Buffer (novex® NuPAGE®, Cat.Nr.:NP0006-1) and 20% (v/v) Methanol (Carl Roth®, Cat.Nr.:4627.5) for 1.5 h at 160 volt and 250 mA (PowerPacTM HC, Bio-Rad). After a subsequent loading control with Ponceau Red staining (Ponceau S Solution (Sigma® Life Science, LOT: SLBR3445V)), the membrane was blocked with 5% milk (Carl Roth ®, Cat.Nr.: T145.2) in Tris-buffered saline with Tween (TBS-T) for 1 h. Afterwards the membrane was incubated overnight with various

primary antibodies (table 2) at 4 °C. The next day, after 3 washing steps with TBS-T, HRP conjugated goat anti-rabbit (BioRad) and goat anti-mouse IgG (BioRad) were used as secondary antibody for 2 h at RT. After a subsequent washing step, chemiluminescence was development with WesternBrightTM Quantum and WesternBrightTM Peroxide (WesternBright Chemiluminescence Substrate Quantum (Biozym, Art.Nr.:541015)) according to the manufacturer's instructions to detect immunolabeled proteins with the iBright CL 1000 Imaging System (Thermo Fischer Scientific). Quantitative analysis was performed with Image Studio Lite Version 5.2.

3.4.3. ELISA

Progesterone levels were measured in supernatants of co-incubated BeWo cells with isolated human platelets in duplicates using the progesterone ELISA kit (Enzo Life Sciences, Switzerland), according to the manufacturer's manual.

3.4.4. Measurement of secreted proteins in cell culture supernatants

Progesterone was analyzed via Immulite at the diagnostic laboratory of the division of endocrinology and diabetology (Medical University of Graz).

3.5. Histological methods

3.5.1. Preparation of sections

The formalin fixed and paraffin embedded (FFPE) tissue of first trimester placenta was sectioned (5 µm; Mikrotom Microm HM 355 S (Thermo Fisher ScientificTM)). Subsequently, these sections (glass slide; Superfrost® plus Thermo scientific, Cat.Nr.: J1800AMNZ) were deparaffinized (protocol: supplemental table 4) and antigen retrieval (AGR) was performed for 15 minutes at 93 °C either in citrate buffer (pH 6) or Tris EDTA Puffer (pH 9; Novocastra™, Leica) in the laboratory microwave KOS (Milestone Medical, Sorisole, Italy).

3.5.2. Immunohistochemistry

Immunohistochemistry stainings were performed using the UltraVision LP-Detection System (ThermoScientific) according to manufacturer's instructions (protocol: supplemental table 5). In brief, with Hydrogen Peroxidase endogenous enzymes were blocked for 10 min, afterwards slides were washed (3x tris-buffered saline with tween, TBS-T) and then incubated for 5 min with Ultra-V Block (Thermo Scientific). These were followed by a 45 min incubation step of various primary antibodies (table 2), that were diluted in Antibody Diluent (Dako). Slides were

washed and only for mouse antibodies an additional incubation step of Enhancer (Thermo Scientific) was performed for 15 min with subsequent washing steps with TBS-T. Afterwards slides were incubated with Large Volume HRP Polymer (Thermo Scientific) for 15 min, followed again by washing steps with TBS-T and another 10 min incubation step with substrate aminoethyl cabazole (AEC Chromogen Single Solution). After a washing step in aqua dest., a counter staining with hemalaun according to Mayer's Hemalaun staining (Thermo scientific) was performed. Slides were covered with Kaiser's Glycerin Gelatin (Merck) and microscoped with Olympus BX3-CBH (Olympus, Tokyo, Japan).

3.5.3. Immunofluorescence double staining

For immunofluorescence (IF) staining, ibidi slides were incubated with a blocking solution of PBS, that was supplemented with 3% BSA and 0.05% Tween for 30 min at RT. The primary antibody CD42b was diluted in blocking solution (table 2), added to the slide and incubated overnight at 4°C in a humidified chamber. Thereafter, ibidi slides were washed three times with PBS and the secondary fluorescence-labelled antibody (donkey anti-rabbit IgG 488, Invitrogen; diluted 1:300 in blocking solution) with an addition of 1x Phalloidin Solution was added to the ibidi slides and incubated 2 h at RT. Subsequently three washing steps with PBS were performed and antifade mounting medium including DAPI (Vectashield) was added (protocol: supplemental table 6). Images were received with BZ-X810 All-in One Fluorescence Microscope (Keyence; Japan). Adhesion experiment images were analyzed and evaluated with HALO software (v3.1.1076.342). Data were given as % platelet positive trophoblasts, referring to the number of JAR and JAR-G cells that showed positive staining for adhesion of a platelet in regard to the total number of trophoblasts.

Table 2: List of antibodies

antibody	**company** (clone/*order* numbers)	**host species**	**AGR for IHC**	**Dilution IHC**	**Dilution IF**	**Western Blot**
HLA-G	BP Pharmingen (4H84)	mouse	pH6	1:2000		1:1000
CD42b	Biotechne (*12860-1-AP*)	rabbit	pH9	1:1000	1:500	
CXCL4	LSBio (RTO)	mouse	pH9	1:2000		
CD34	Dako (QBEnd-10)	mouse	pH9	1:500		
Occludin	CellSignaling (E6B4R)	rabbit	pH9	1:750		
ZO-1	CellSignaling (D6L1E)	rabbit	pH9	1:50		
hCG	ThermoFischer (*RB-059-A*)	rabbit	pH6	1:5000		
STS	Sigma (*HPA002904*)	rabbit	pH6	1:100		
CYP19A1	CellSignaling (D5Q2Y)	rabbit	pH6	1:100		
Neg. Control Mouse	Dako (*X0943*)	mouse	pH6	1:2000		
Neg. Control Rabbit	Dako (*X0936*)	rabbit	pH9	1:5000		
Fluorescence-labelled 2nd anti rabbit (IgG 488)	Invitrogen (*A-21206*)	donkey			1:300	
β-Actin	Abcam (AC-15)	mouse				1:500000
HRP conjugated anti-mouse	BioRad (*170-6516*)	goat				1:5000
HRP conjugated anti-rabbit	BioRad (*170-6516*)	goat				1:5000

3.6. Transmission electron microscopy (TEM)

First trimester placental tissue (GA 7+0) was dissected into small pieces (< 1 mm^2) and fixed in cacodylate buffer (supplemental table 3) including 2% paraformaldehyde and 2.5% glutaraldehyde for 2 h at RT. Tissue samples were stored in sole 0.1 M cacodylate buffer at 4 °C over night. Subsequently, samples were post fixed with 2% osmium tetroxide (Electron Microscopy Sciences, Hartfield, USA) in 0.1 M cacodylate buffer for 2.5 h. After washing samples in 0.1 M cacodylate buffer, the dehydration process was conducted with a graded ethanol series (50–96%) for 30 min each, ending with two times 100% ethanol for 15 min. Thereafter, a propylene oxide (PO) step as an intermedium followed for 1 h. Afterwards, resin infiltration was initiated with 1:1 TAAB embedding resin (TAAB Laboratories Equipment Ltd., UK) in PO for 3 h, followed by 2:1 TAAB embedding resin in PO over night at 4 °C. Ultimately two changes of sole TAAB embedding resin at 45 °C for 1 h. Placental tissue samples were embedded in sole TAAB resin in silicone forms and left in an incubator at 60 °C for polymerization for 3 days. Thereafter, samples were sectioned with an ultramicrotome (Leica, Vienna, Austria). For an overview, semi-thin sections of 500 nm were produced and stained with 1% toluidine blue solution (Sigma-Aldrich, USA) to determine the area of interest. Sample blocks were trimmed and ultra-thin sections of 70 nm were acquired and collected with 200 mesh copper grids. The sections of the grids were stained with lead citrate and platinum blue prior to EM imaging (Ultra-stainer, Leica) and examined with a Zeiss 900 TEM (Carl Zeiss Microscopy GmbH, Jena, Germany) operated at 80 kV.

3.7. Statistical analysis

Produced data were analyzed using GraphPad Prism Version 8.4.3. Results are presented as means ± standard deviation (SD). Performed normality test was the Shapiro-Wilk test. For normally distributed data, differences in between groups were analyzed with the two-tailed t-test and for multiple comparison between more than two groups one-way ANOVA was followed by a Tukey Multiple Comparison test. In case of not normally distributed data Wilcoxon signed rank test was applied. P-values of less than 0.05 were considered statistically significant.

4. Results

4.1 Alternative route of how platelets may enter the first trimester IVS

To identify whether maternal platelets could enter the IVS during first trimester of human pregnancy we subjected FFPE samples of first trimester human placental tissue to a series of immunohistochemical stainings. Another recent study by our group revealed the way of platelets through narrow channels in the EVT plug that is obstructing the maternal spiral arteries during early pregnancy (7). Here however, we discovered another possible way for the platelets to enter the IVS of the early human placenta. For that, we showed the positive staining for EVT marker HLA-G (Fig. 4A) in trophoblast columns of anchoring villi. Furthermore, maternal platelets were identified in intercellular gaps of these EVT by staining for platelet marker CD42b (Fig. 4B), which was further confirmed by immunohistochemical staining of serial sections for CXCL4 (also called platelet factor 4 (PF4)). This staining was localized in similar areas where the CD42b signals could be found (Fig. 4C). As a positive control FFPE platelets were stained for platelet markers CD42b and CXCL4, while serial sections of the same sample served as a negative control for all other antibodies used in this experimental series (Suppl. Fig. 1). Endothelium was stained with CD34 antibody. This endothelial marker identified small extra embryonic blood vessels in the mesenchyme of the placental villi, but also revealed that the narrow intercellular gaps in between the EVTs had no endothelial lining (Fig. 4D). In order to investigate whether plasma or platelet could leak into distal parts of EVT column regions, the distribution of tight junction proteins was analyzed with antibodies against tight junction components occludin and ZO-1 (zonula occludens-1, also known as tight junction protein 1 (TJP1)). Occludin staining showed specific staining of the microvilli at the apical side of the SCT, as well as a strong staining surrounding the villous CTs (Fig. 4E). ZO-1 marker was mostly visible in the extra embryonic endothelium inside the mesenchymal stroma of the villi, whereas it could also be faintly detected surrounding the villous CT and also the trophoblasts at the proximal end of the trophoblast cell columns (Fig. 4F). Both tight junction proteins hardly showed any staining after the second EVT layer of the cell column. The CTs that are located in this area started to undergo partial mesenchymal to endothelial transition and differentiate into EVTs. In distal parts of EVT columns both markers showed no staining, indicating an absence of tight junctions, which enables plasma flow in between the EVTs and might also suggest for small blood components like platelets to pass into these interstices (1).

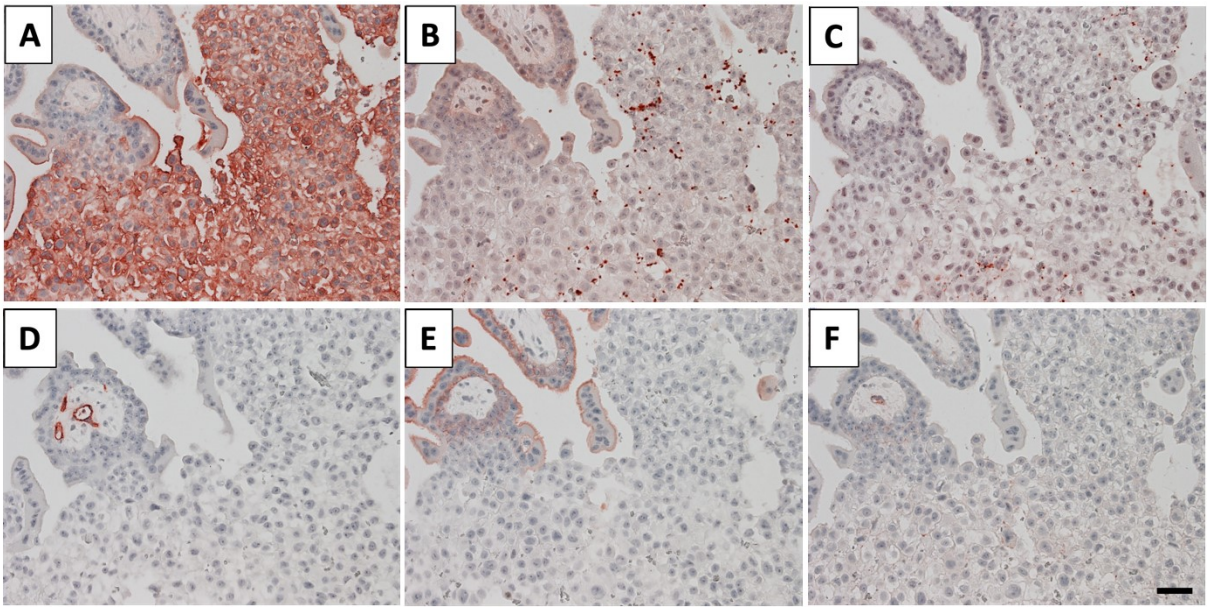


Figure 4: Human first trimester placental tissue showed positive staining for platelet markers in interstices in between EVTs. *IHC for EVT marker HLA-G showed the extravillous character of trophoblast population in anchoring villi of human first trimester placenta (shown placenta: GA 8 + 4) (A). Furthermore, serial sections were stained for two platelet markers: CD42b (B) and CXCL4 (C). These stainings showed positive platelet staining between the EVTs. Extra embryonic endothelium was detected by CD34 (D) in the stroma of villi and moreover showed that platelets in between the EVTs left their vascular environment and were therefore not surrounded by an endothelial layer. Also, with occludin (E) and ZO-1 (F) tight junction proteins were detected surrounding villous trophoblasts and the proximal trophoblasts of the EVT column of the anchoring villus. Scale bar represents 50 µm. Reproduced from Guettler et al., 2021 with permission of publisher Elsevier covered under the CC BY license (1).*

In the distal part of EVT columns occasionally also bigger almost vessel-like channels could be identified. These channels contained maternal erythrocytes, but lacked positive staining for CD34 and therefore an endothelial lining. At the rims of these channels in between EVTs positive staining of platelet marker CD42b could be shown (Fig. 5). This accumulation of platelets suggested that maternal plasma including platelets was leaking into the intercellular gaps in between the EVTs, as the channels were missing a surrounding endothelium and also lacked tight junction proteins, as shown in Fig. 4 (1).

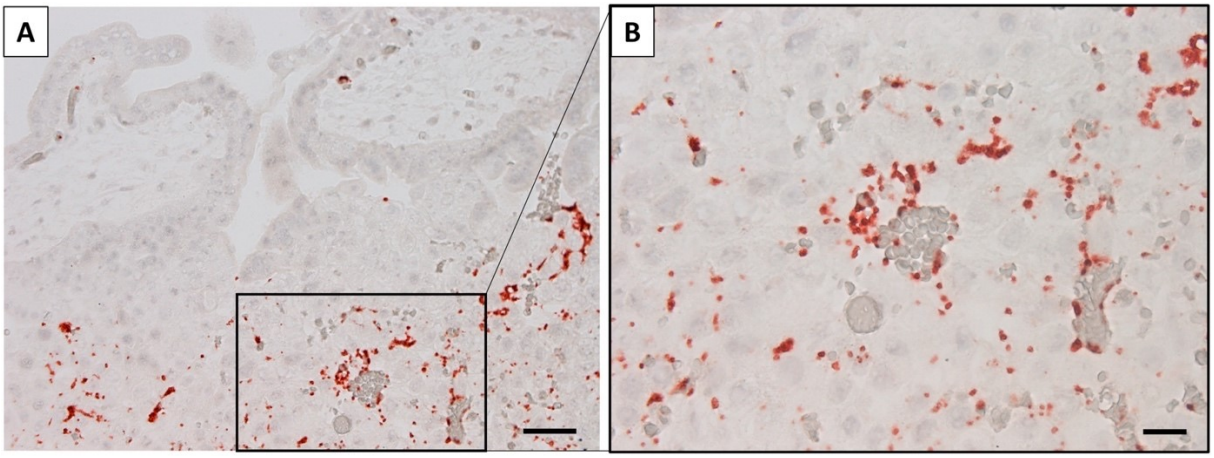


Figure 5: Positive platelet staining with CD42b of human first trimester placenta (GA 11+1) identified platelets leaking from vessel-like channels containing maternal red blood cells in distal parts of trophoblast columns (A). *A higher magnification of the region with channel like structures containing maternal erythrocytes shows platelets leaking into intercellular EVT gaps (B). Scale bar represents 50 µm in (A) and 20 µm in (B). Reproduced from Guettler et al., 2021 with permission of publisher Elsevier covered under the CC BY license (1).*

Placental tissue was transported quickly to the Division of Cell biology, Histology and Embryology after termination of pregnancy and processed as soon as possible to ensure a good quality of the tissue. In case of FFPE samples the tissue was directly transferred into the formalin fixans (4% PFA), where all residual blood components were washed away. Therefore, any adherence of platelet to villous trophoblasts or appearance in between EVT, which was revealed by staining for CD42b, has probably already appeared *in vivo* and persisted throughout the fixation and embedding processes. Therefore, adhering maternal platelets on the placental villous surface and in between EVTs are not the result of blood clotting during the termination process of the pregnancy. This conjecture could be confirmed by IHC of an unique archival specimen of a human first trimester placenta *in utero*, that was obtained by hysterectomy. IHC revealed the villous SCT by staining for β-hCG (Fig. 6A) and EVT marker HLA-G identified the invading trophoblasts (Fig. 6B) at the interface between the extra embryonic part of the placenta and the decidua. Lastly platelet marker CD42b confirmed the accumulation of platelets on the surface of the villous SCT in the IVS and furthermore in the anchoring parts of the EVT columns and some areas that are possibly the entrance of the maternal vessels into the IVS (Fig. 6C-F) (2).

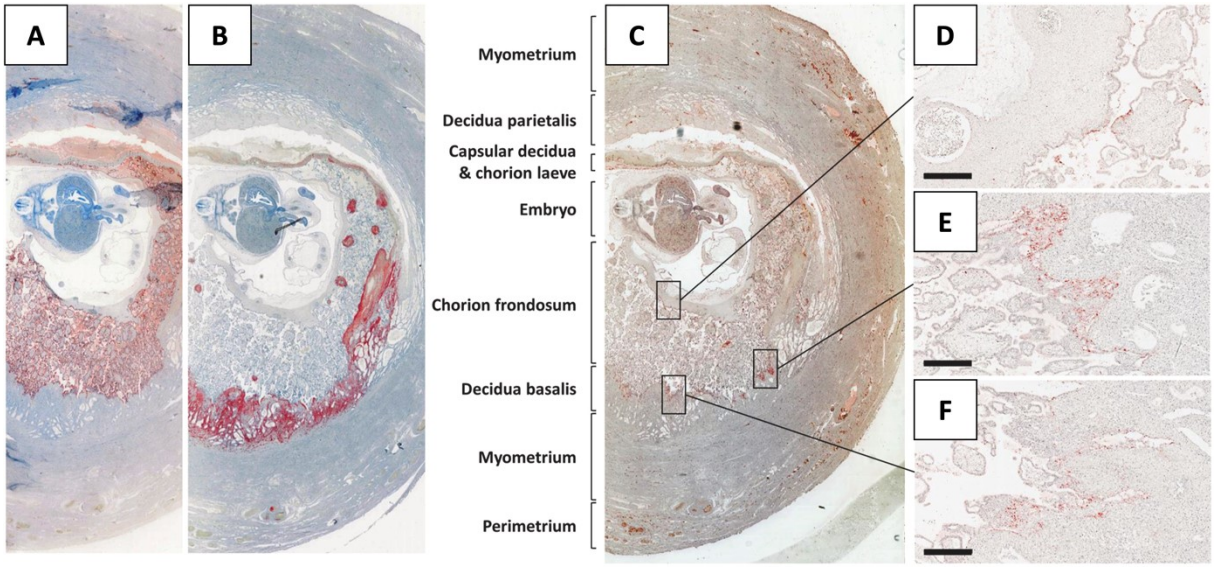


Figure 6: Localization of villous SCT, EVTs and platelets in human first-trimester placenta in utero specimen. *This archival specimen of a first-trimester human placenta was obtained after a hysterectomy. Sections of this sample were stained for the SCT marker β-hCG (A), EVT marker HLA-G (B) and platelet marker CD42b (C). Maternal platelet staining could be confirmed on the surface of the villous SCT (D), as well as in areas where possibly maternal vessels enter the IVS (E) and furthermore in distal parts of anchoring trophoblast cell columns (F). Scale bars in D-F represent 400 µm. Reproduced from Guettler et al., 2021 with permission of publisher Elsevier covered under the CC BY license (2).*

To identify human platelets in between EVTs even further and to confirm the immunohistochemical stainings of CD42b and CXCL4 antibodies, we wanted to detect the platelets in this area of interest with TEM. However, first we wanted to analyze human platelet solitarily with this method. Therefore, platelets were isolated from human whole blood and embedded for TEM. Here, the detailed structure of a human platelet was revealed. Resting platelets (Fig. 7A) could be differentiated from an activated platelet (Fig. 7B) as the activated platelets undergo a shape change from the discoid form into an amoeboid form and they develop filopodia. Inside the platelets the OCS could be identified with its extensive network of membrane tubuli. Next to that, ultrastructure clearly revealed platelet specific structures like glycogen, α-granules and dense bodies inside of the human platelets (2).

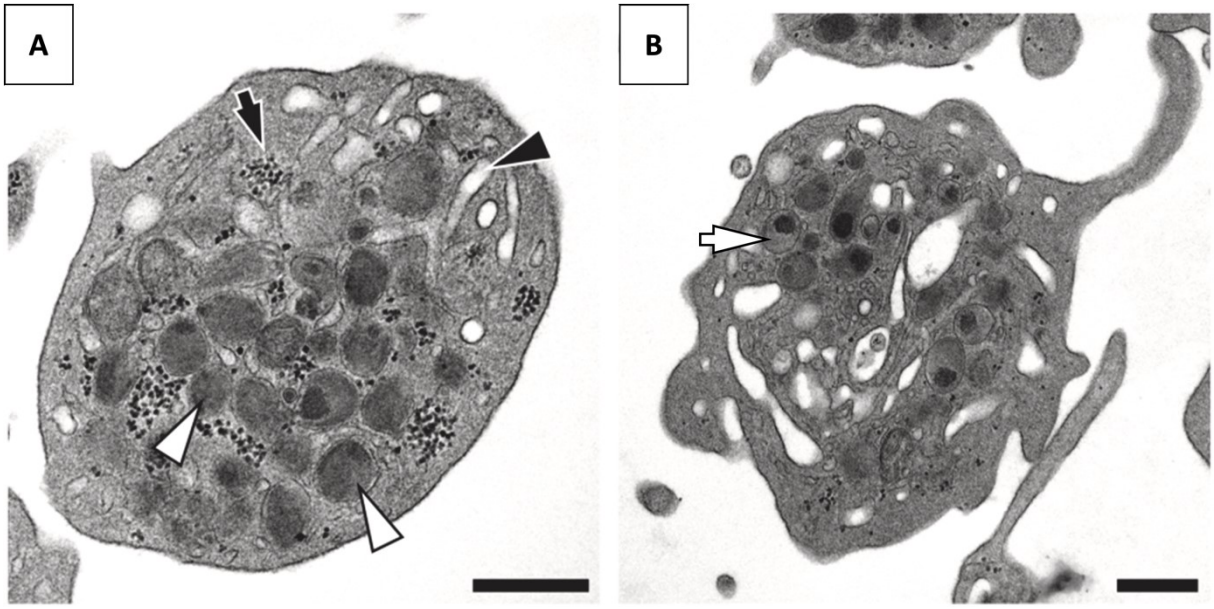


Figure 7: TEM of isolated human platelets in their resting and activated form. *Ultrastructural analysis revealed the shape change between the resting platelet (A) and the activated platelet (B). Furthermore, glycogen could be recognized (black arrow). Also, α-granules (white arrowhead) and dense bodies (white arrow) could be identified, which release their content upon activation via the OCS (black arrowhead). Scale bars represent 0.5 µm. Reproduced and adapted from Guettler et al., 2021 with permission of publisher Elsevier covered under the CC BY license (2).*

TEM was performed on anchoring villi of first trimester placenta that were identified under a stereoscopic microscope and further embedded for TEM. With a semithin section of 500 nm an overview (Fig. 8A) was given over the tissue area to confirm the right area of interest. In the proximal regions of the EVT columns an arrangement of trophoblasts with close proximity to each other and narrow gaps could be observed (Fig. 8B). In the distal region of the cell column however, the EVTs were only loosely connected by desmosomes (Fig. 8C). Here, the trophoblasts had many surface projections, that were extending into intercellular gaps of up to 4 µm. Within these gaps maternal platelets were found (Fig. 8D). They could be characterized by their OCS, accumulations of glycogen and different organelles (e.g. dense bodies and α-granules). For example, dense granules could be identified as they have an electron-opaque spherical body within itself, which however is separated from the surrounding membrane by a seemingly empty space. The platelets had already undergone a shape change into an amoeboid form, what suggested an activation of platelets including a discharge of granule cargo into the OCS and from there into the direct platelet environment. Furthermore, microvesicles and multivesicular cargo could be found in the intercellular gaps of the distal areas of EVT columns (1). Multivesicular cargos had been described previously as structures containing tightly packaged high numbers of endomembrane vesicles enclosed by a plasma membrane (144).

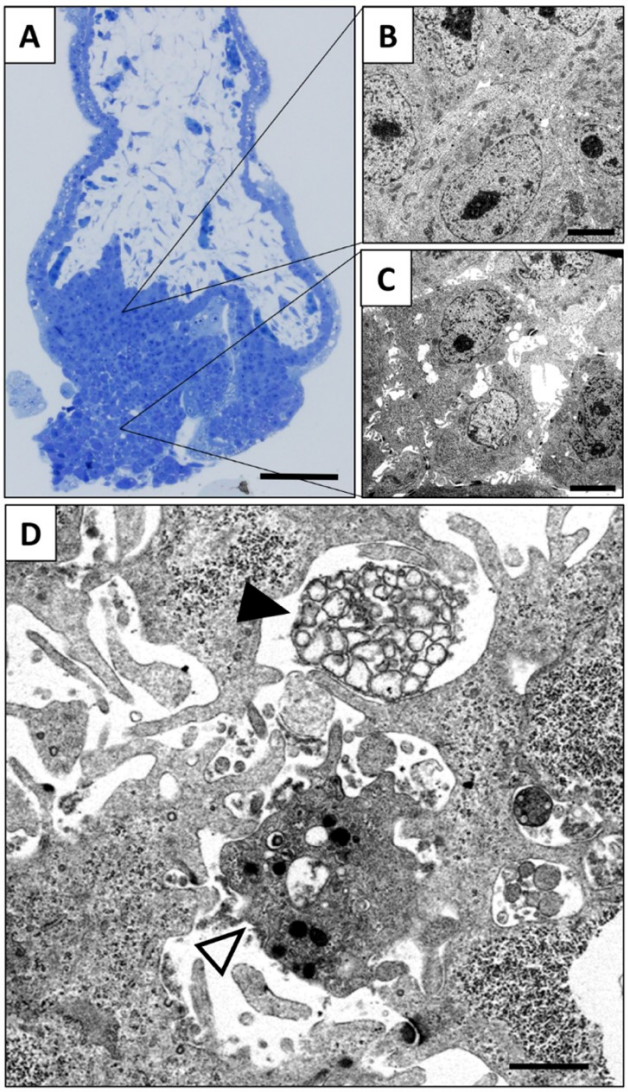


Figure 8: TEM of an anchoring villus could confirm platelets in intercellular gaps of EVT columns. *TEM of an anchoring villus including an EVT column of a human first trimester placenta (GA 7 + 0) was performed. The 500 nm semithin section, that was stained with toluidin-blue, provided an overview over the area of interest (A). TEM of the ultrathin sections revealed the trophoblasts in close proximity with narrow intercellular gaps in the area of the cell column close to the mesenchymal core of the villus (B). In the distal part of the EVT column the trophoblasts were only loosely cohesive, connected only by desmosomes (C). The intercellular gaps of distal area of the EVT column had widths of up to 4 µm. In such intercellular gaps a maternal platelet (open arrowhead) could be identified by the characteristic OCS, accumulations of glycogen and platelet specific granules like dense bodies and α-granules. In these intercellular gaps of the EVT columns also multivesicular cargos (black arrowhead) could be identified. Scale bar in (A) represents 100 µm, scale bars in (B) and (C) represent 4 µm and in (D) 1 µm. Reproduced from Guettler et al., 2021 with permission of publisher Elsevier covered under the CC BY license (1).*

4.2 The effect of HLA-G on platelet aggregation and adhesion

As the immunohistochemical findings of platelets in between EVTs could be confirmed by TEM and the found platelets showed all signs of being in the activated state, the involvement of the extra embryonic trophoblast cell on platelet activation/aggregation was investigated. EVTs show a strong expression of their specific marker HLA-G. The hypothesis that we tested was based on the question whether or not maternal platelets get activated by interacting with EVTs and therefore HLA-G while they pass through the narrow intercellular gaps in distal areas of EVT columns.

At first, different agonists including recombinant HLA-G (rHLA-G) were analyzed on what effect they would have on the aggregation behavior of human PRP of healthy donors. On one hand, well known activation agonists like 2-MeS-ADP and collagen type I (col-I) were tested and as expected considerably induced the aggregation of PRP. On the other hand, addition of rHLA-G did not induced aggregation of PRP and furthermore also did not inhibited aggregation that was induced by ADP (Fig. 9). Oefner et al. has previously shown that collagen type IV (col-IV) can be found between HLA-G positive EVTs in the distal area of the EVT columns (145). Therefore, we also tested col-IV on its aggregation response in human PRP. However, PRP did not show an aggregation response to the administration of col-IV.

In order to investigate the response of human platelets to shed soluble HLA-G, the trophoblast cell line JAR was used. This cell line lacks HLA-G expression (146), therefore the calcium phosphate method was used to introduce genetic material to overexpress HLA-G in this cell line (JAR-G). With IHC the absence of HLA-G expression in the control JAR cells could be confirmed (Fig. 10A), whereas staining for the HLA-G marker in the JAR-G cells showed considerable staining for the EVT marker (Fig. 10B). Furthermore, Western Blot analysis of protein lysates and supernatants of JAR and JAR-G cells confirmed the overexpression of cell-bound HLA-G in JAR-G lysates and release of soluble HLA-G in supernatants of JAR-G cells (Fig. 10C). As a confirmation of the specific binding of the used HLA-G antibody, JAR-G cell lysates were compared to the recombinant HLA-G. The recombinant protein had a slightly higher molecular weight as a result of its His-Tag (Fig. 10D). In the supernatants of different passages of JAR-G cells double bands could be detected, which implied that next to the released and cleaved fraction of the protein also a full-length molecule was detected, that could have been a result of dead cells and/or released vesicles of the cell membrane as previously suggested by Blaschitz et al. (147). Supernatant of JAR-G cells containing endogenous HLA-G showed no effect on the aggregation response of isolated human platelets, while they responded to the 2-MeS-ADP agonist (Fig. 10E). To summarize, the collected data indicated that neither soluble

nor recombinant HLA-G has an effect on the aggregation of maternal platelets at the materno-fetal interface (1).

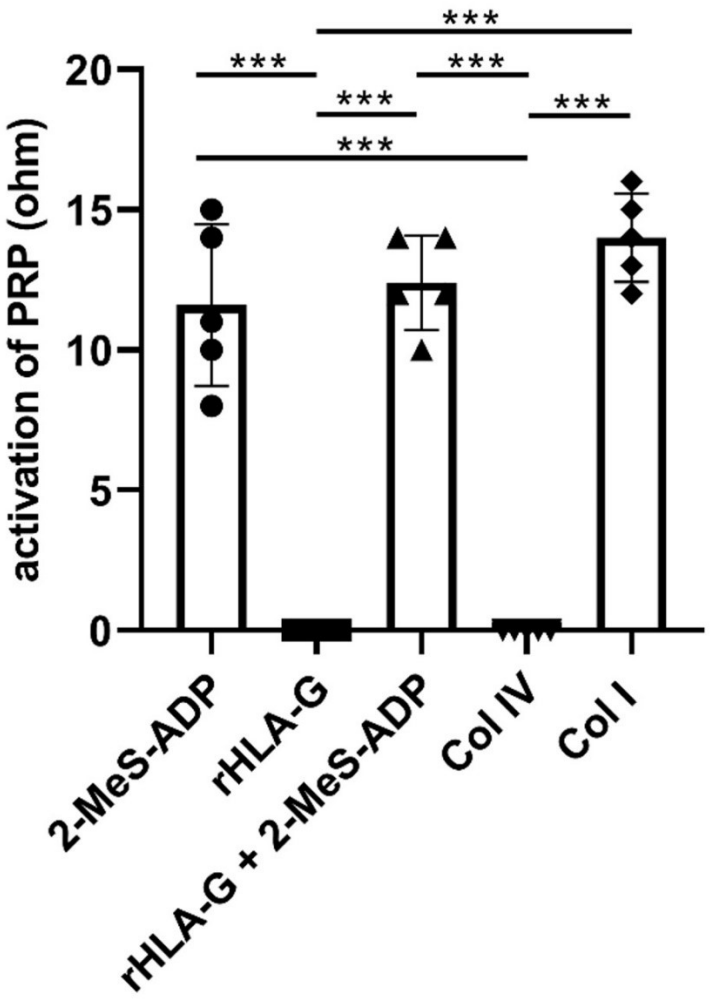


Figure 9: rHLA-G has no effect on the aggregation of human PRP. *Aggregation of human PRP (n = 5) was tested by aggregometry experiments. The known agonists 2-MeS-ADP (10 µM) and col-I (2 µg/mL) induced constant high aggregation responses. However, rHLA-G (600 ng/mL) as well as col-IV (20 µg/mL) did not show any responses and in addition, rHLA-G did also not impair ADP-induced aggregation. Collected data are presented as means ± SD from five independent experiments using five different PRP donors (n=5). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Reproduced from Guettler et al., 2021 with permission of publisher Elsevier covered under the CC BY license (1).*

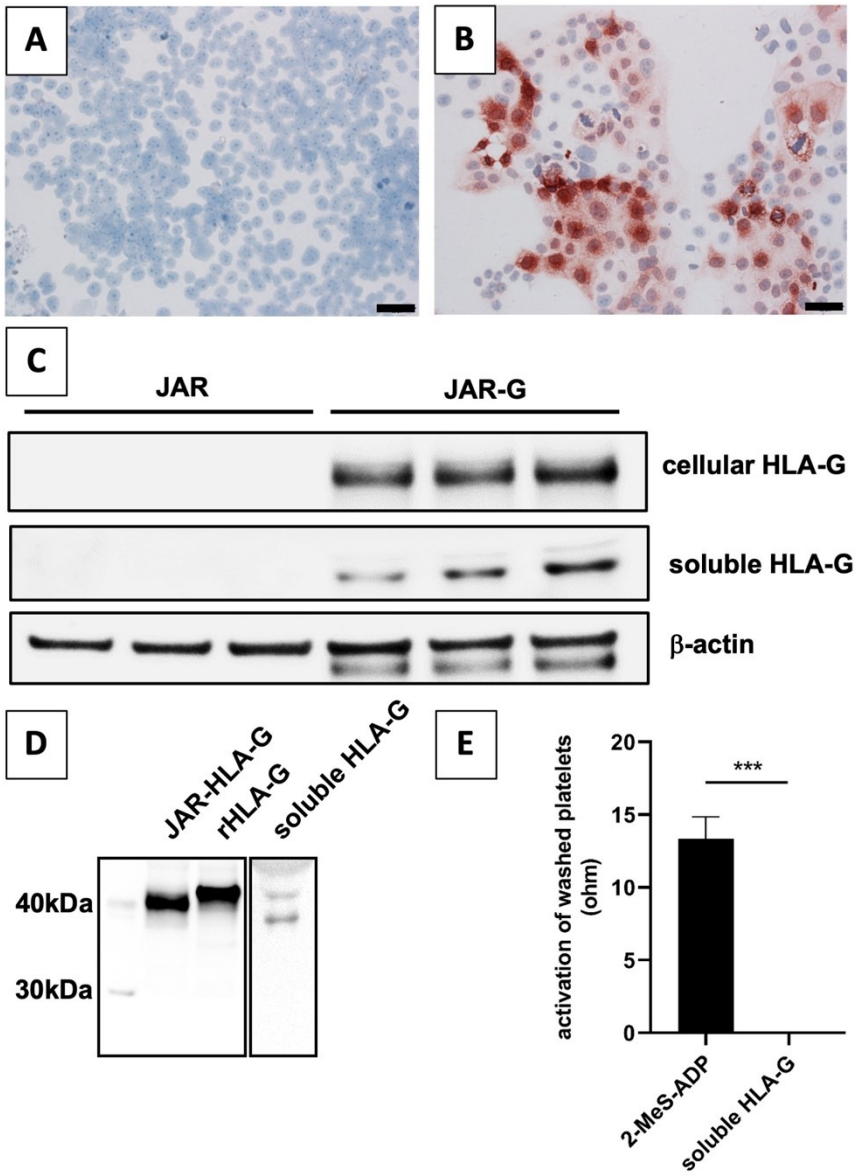


Figure 10: Overexpression of HLA-G in JAR-G cells was confirmed and soluble HLA-G did not induce a platelet aggregation response. *Staining with HLA-G antibody showed the absence of HLA-G in the trophoblast cell line JAR (A), and confirmed the presence of HLA-G in the transfected JAR-G cells (B). Furthermore, the successful transfection and overexpression of HLA-G was validated by Western Blots of cell lysates of JAR and JAR-G cells and the associated supernatants (n=3) (C). To verify the specificity of the used HLA-G antibody JAR-G cell lysates were compared with soluble HLA-G from supernatants and rHLA-G (D). Isolated human platelets (n = 3) of healthy patients showed an aggregation response to 2-MeS-ADP (10 µM), while in comparison soluble HLA-G from the supernatants of JAR-G cells had no effect (n=3) (E). Collected data presented as means ± SD from independent experiments using isolated human platelets from three different individuals. Scale bars represent 50 µm. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Reproduced from Guettler et al., 2021 with permission of publisher Elsevier covered under the CC BY license (1).*

Next, cell-bound HLA-G was tested on its ability to mediate the adhesion of isolated human platelets under flow conditions. Here, JAR-G and control cells were subjected to constant flow in the ibidi flow system. They were further incubated with isolated human platelets in either the presence or the absence of ADP. Afterwards IF was performed (Fig. 11A-D) and software-based analysis with HALO software (Fig. 11E) indicated no significant differences in the adherence of platelets to JAR-G and control cells, even with and without ADP stimulation. Nevertheless, the presence of the agonist ADP showed a slight decrease of platelet adhesion, in both cell lines. This could be explained due to an increased platelet clearance and therefore slightly less adhesion counts in presence of ADP in this experimental setup. In summary, these results referring to aggregation and adhesion of platelets to trophoblasts suggested that trophoblastic HLA-G, membrane-bound or soluble, has no indication on affecting maternal platelet activation at the maternal-fetal barrier (1).

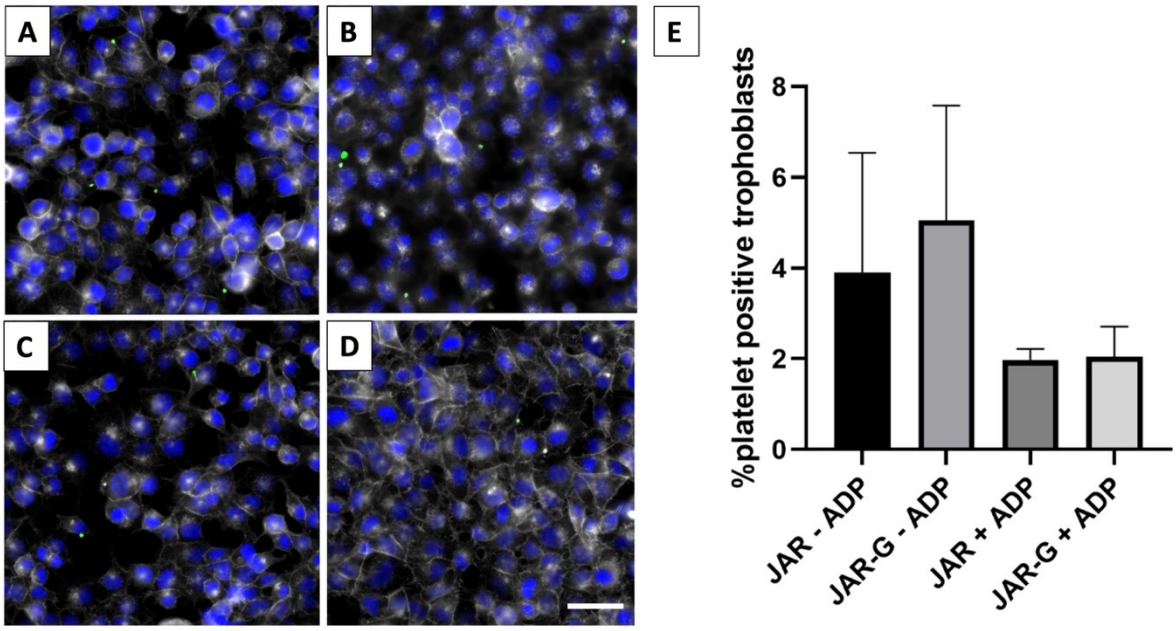


Figure 11: HLA-G overexpression did not have an effect on platelet adhesion to trophoblast cell lines. *In the ibidi flow system JAR control cells (A + C) and HLA-G overexpressing JAR-G cells (B + D) were subjected to isolated platelets in presence (C + D) and absence (A + B) of 10 µM ADP. Slides were stained with platelet marker CD42b (green), actin marker phalloidin (grey) and nucleus staining DAPI (blue). Analysis with HALO-software of stained slides showed that platelet adherence to HLA-G-overexpressing cells and controls had no significant differences (E). Collected data in (E) are presented as means ± SD from three independent experiments using platelets from three different donors (n=3). Scale bar represents 50 µm. Reproduced from Guettler et al., 2021 with permission of publisher Elsevier covered under the CC BY license (1).*

4.3 Effects of isolated human platelets on villous trophoblasts

For the last aim we tested what influence human maternal platelets would have on the villous SCT. For this reason, pre-differentiated BeWo cells, which represented the villous SCT, were co-incubated with isolated and washed human platelets. Afterwards supernatant and RNA lysates were collected and analyzed with qPCR, PrimePCR, RNA-Sequencing and ELISA.

4.3.1. RNA expression

At first, samples of three individual experiments of control BeWo cells and BeWo cells treated with human platelets were analyzed with qPCR for *CGB* expression (Fig. 12). The gene *CGB* encodes the β-hCG protein. This was done as a quality control to ensure that the results from a previous study by Forstner et al. could be reproduced (7). As expected the *CGB* expression of the trophoblasts was significantly impaired by co-incubation with human platelets.

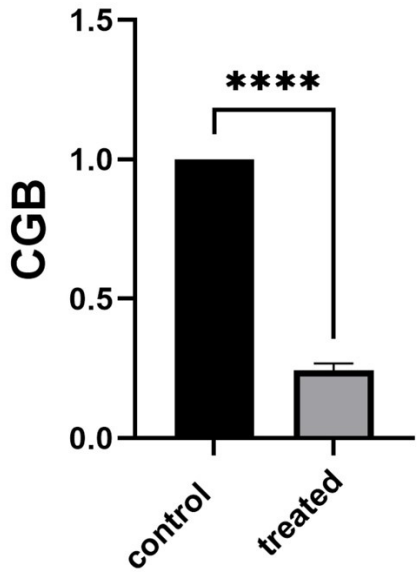


Figure 12: Co-incubation of pre-differentiated BeWo cells with isolated human platelets significantly impaired CGB expression. *Collected data presented as mean ± SD from three independent experiments using isolated human platelets from three different individuals (n=3). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.*

Furthermore, samples of the platelet co-incubation were subjected to an individual designed PrimePCR (BioRad). Here, different genes that are involved in the steroid hormone synthesis of the human placenta were analyzed for deregulation upon co-incubation.

The fold changes of some of the investigated genes showed promising results, although the high SD had to be taken into account in some cases. Nevertheless, the deregulation of *CYP1A1*, *SULT2B1*, *CYP19A1* and *STS* (Fig. 13) were promising and therefore further analysis were conducted to get a deeper insight into genes involved in the steroid hormone synthesis.

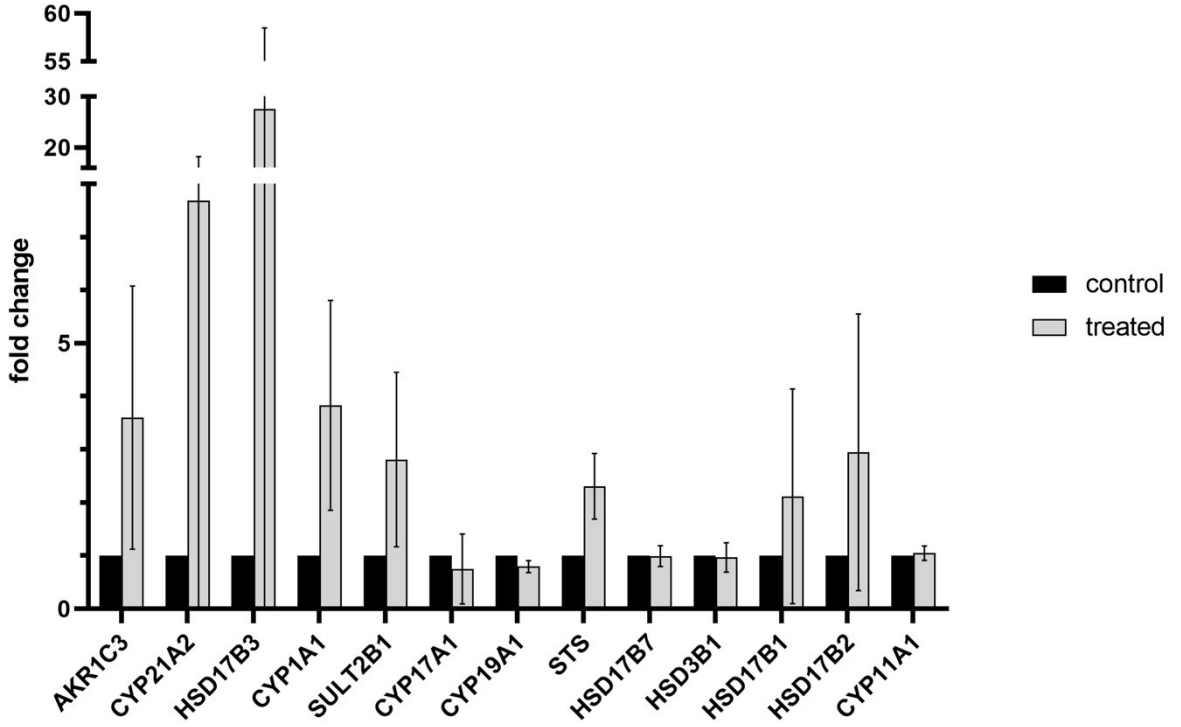


Figure 13: PrimePCR of some genes that could be involved in the steroid hormone synthesis of the human placenta. *Pre-differentiated BeWo cells were treated with and without isolated human platelets for 24 h. Results showed deregulation in the expression of genes that are involved in steroid hormone synthesis. Collected data presented as means ± SD from three independent experiments using isolated human platelets from three different individuals (n=3).*

The samples generated from the co-incubation experiment were subjected to a RNA-Sequencing analysis at the ZMF (Core facility, Medical University of Graz).

The total count of genes that were analyzed was 58051. From these analyzed genes 1462 were significantly deregulated by a fold change of at least 2 (Fig. 14). Thereof, 385 genes showed a significant downregulation and 1077 genes were significantly upregulated. With the DAVID functional annotation analysis different pathways that were influenced by the interaction of platelets with differentiated BeWo cells were identified (e.g. inflammatory response, immune response and neutrophil chemotaxis).

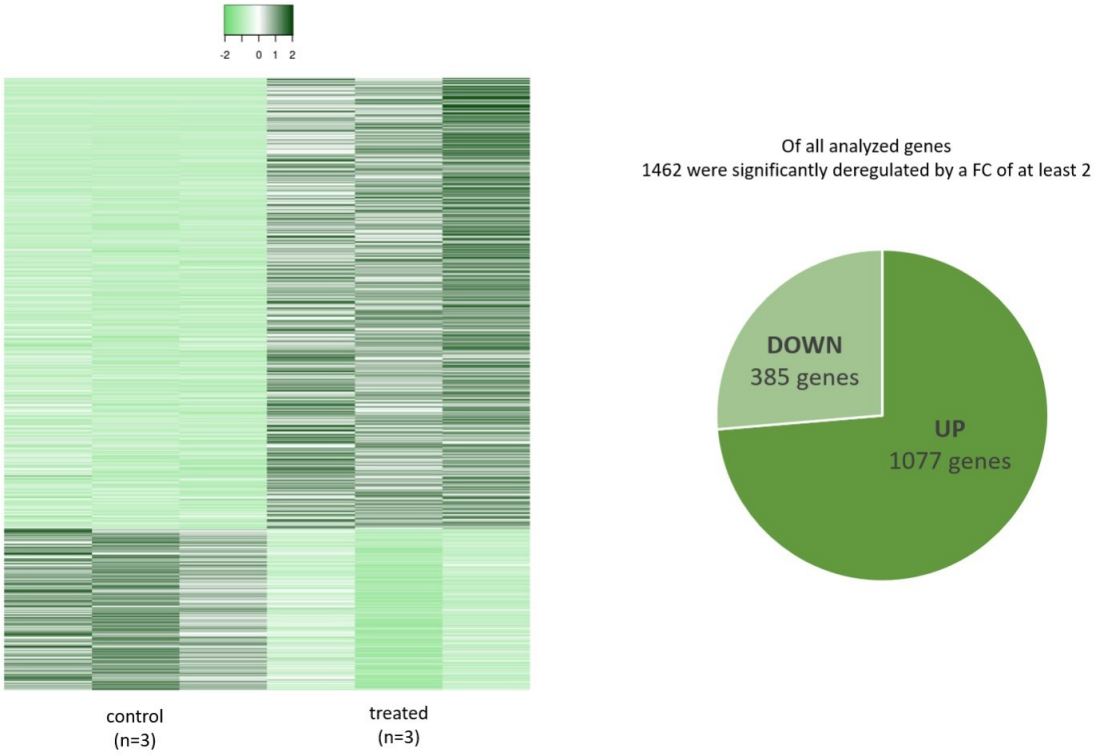


Figure 14: RNA-Sequencing of trophoblast-platelet co-incubation showed significant deregulation of 1462 genes by a fold change of at least 2. *Trophoblast cell line BeWo was pre-differentiated (20 µM forskolin for 48 h) and co-cultured with (treated) and without (control) isolated and washed human platelets from healthy donors for 24 h (n=3). RNA samples were analyzed via RNA-Sequencing. 1462 were found to be significantly deregulated by a fold change (FC) of at least 2. From these significantly deregulated genes 1077 genes were upregulated and 385 genes were downregulated.*

As the analysis resulted in the deregulation of many genes, specific genes that were involved in cell survival, proliferation, apoptosis or cell death were checked for deregulation. The genes *LIMS1*, *TP53* and *Blc-2* were analyzed to examine whether the co-culture influenced the trophoblasts' viability (Fig. 15). *LIMS1* encodes for an adapter protein that is involved in cell proliferation, differentiation and general cell survival. *TP53* encodes for a suppressor protein which is involved in cell apoptosis, senescence, DNA repair and changes in the metabolism. *Bcl-2* encodes for a protein that suppresses apoptosis and attenuates inflammation. All three genes were not significantly deregulated. These results embraced the hypothesis that the treatment with isolated human platelets did not impair the viability of the trophoblasts.

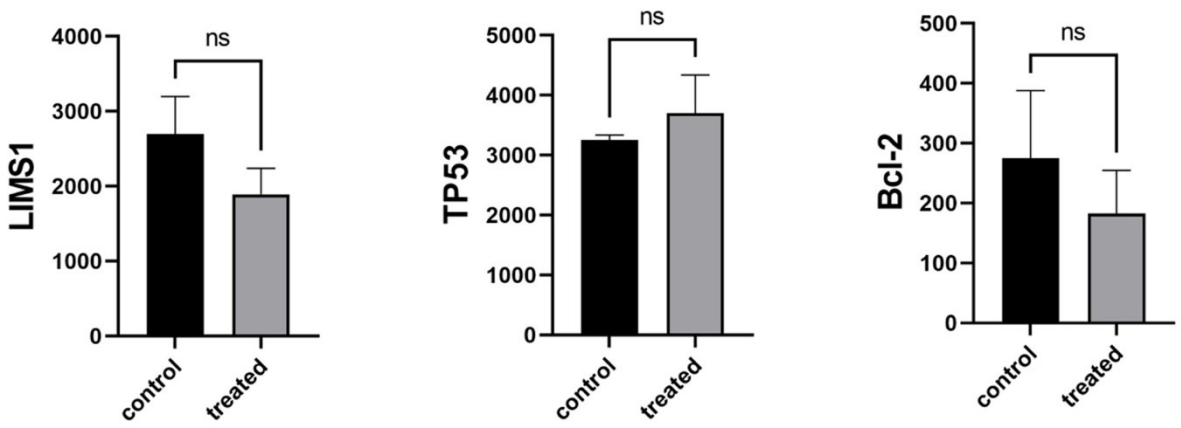


Figure 15: Genes involved in cell survival and apoptosis indicated that platelet treatment had no influence on trophoblast cell viability. *LIMS1, TP53 and Bcl-2 as genes of interest for cell survival and apoptosis did not result in a significant deregulation. Collected data presented as means ± SD from three independent experiments using isolated human platelets from three different individuals (n=3). ns = not significant, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001*

The same genes that have already been investigated with PrimePCR, as they are involved in the steroid hormone synthesis, were analyzed again with the RNA-Sequencing data. Five of the former target genes had to be dismissed due to the minimal representation in the sequencing data. From the seven remaining genes two genes were identified to be most promising due to their high representation in RNA-Sequencing data, their presence in RNA- and protein data in the placenta and their significant deregulation upon platelet co-incubation on BeWo cells from our data. In RNA-Sequencing data *STS* (Fig. 16A) revealed a significant upregulation with of fold change of over 2 and *CYP19A1* (Fig. 16B) showed a significant downregulation. These results could also be confirmed with qPCR (Fig. 16C+D)

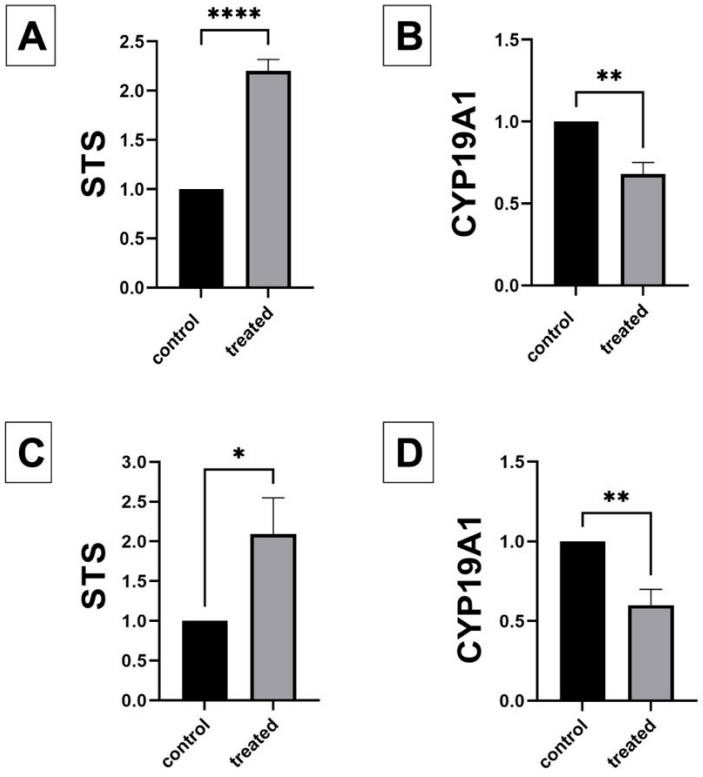


Figure 16: RNA-Sequencing data revealed deregulation of STS and CYP19A, which could be confirmed with qPCR. *Seven of the former analyzed genes could be identified with promising results in RNA-Sequencing data. Of these seven genes STS (A) and CYP19A1 (B) are the most auspicious as they provided good presence in RNA and protein content in the human placenta and showed significant deregulation upon co-incubation of pre-differentiated trophoblast cell line BeWo with human platelets (treated) for 24 h. RNA-Sequencing data could be confirmed with qPCR (C+D). Collected data presented as means ± SD from three independent experiments using isolated human platelets from three different individuals (n=3). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.*

For future analysis and experiments the regulation of possible housekeeping genes was analyzed (Fig. 17). From six possible housekeeping genes *YWHAZ* indicated almost no difference between control and treated cells. However, *CYC1* and *B2M* revealed a significant deregulation upon platelet treatment and should therefore be avoided for the application as a housekeeping gene in this setting. *GAPDH*, *TBP* and *HPRT1* displayed a slight deregulation although without significance.

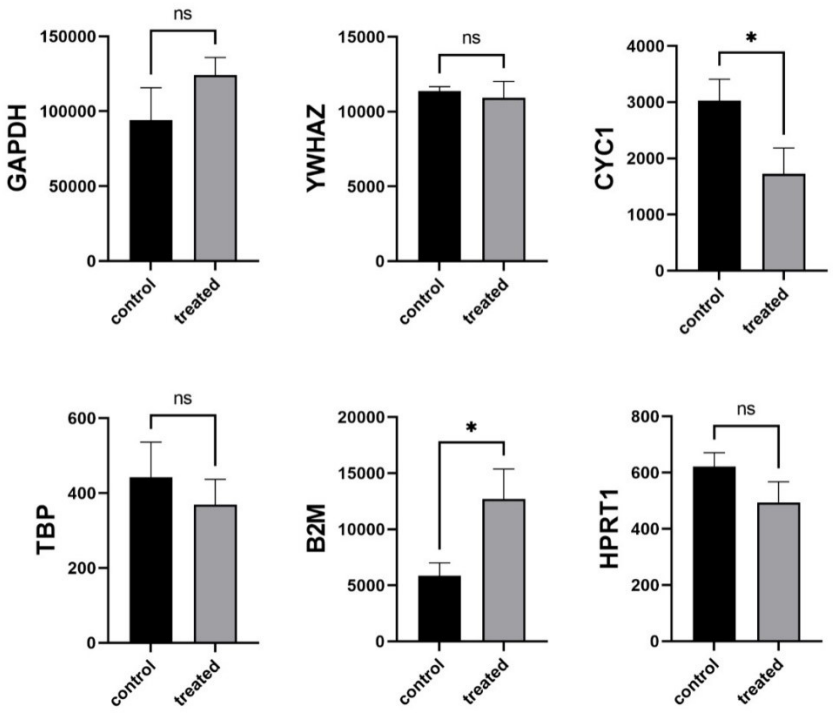


Figure 17: Regulation of housekeeping genes in BeWo cell line upon platelet treatment. *YWHAZ indicated to be the least regulated gene of the tested housekeeping genes. GAPDH, TBP and HPRT1 also revealed only a slight deregulation without significance. However, CYC1 and B2M are significantly deregulated and therefore not suitable to function as housekeeping genes in this setting. Collected data presented as means ± SD from three independent experiments using isolated human platelets from three different individuals (n=3). ns = not significant, *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001*

4.3.2. Protein expression

The two identified target genes *STS* and *CYP19A1* encode for proteins STS and Aromatase. The protein expression in the human first trimester placenta (GA 9+5) was localized with IHC stainings for STS (Fig. 18A) and Aromatase (Fig. 18B). Both stainings could be localized in the SCT of the human first trimester placental tissue, while mesenchymal structures, CTs and endothelium revealed no staining. Therefore, the corresponding proteins to the identified target genes are located in the human SCT and are interesting targets for future experiments, when analyzing the effect of platelets on the villous SCT and the deregulation of steroid hormones.

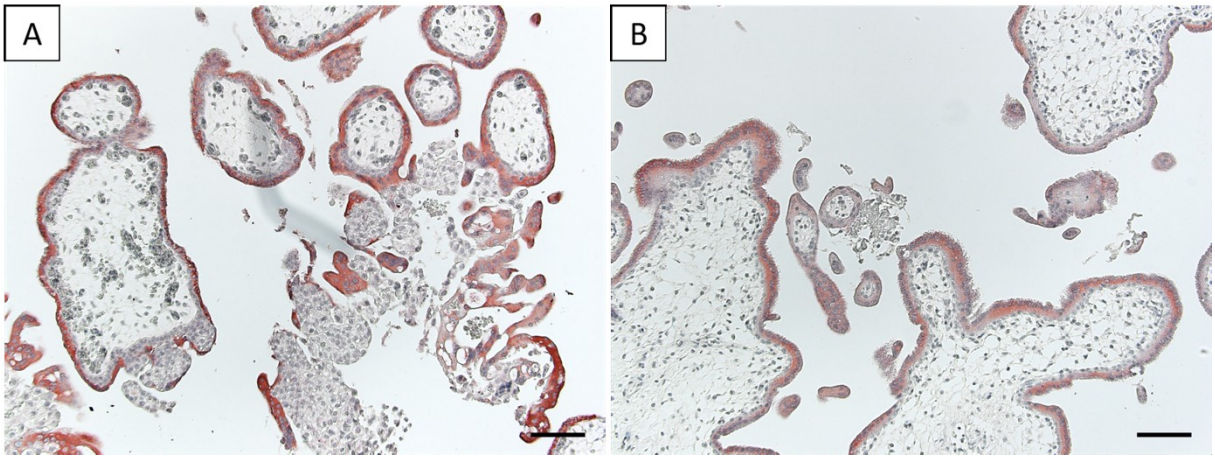


Figure 18: IHC staining of STS and Aromatase on human first trimester placenta. *The stainings for antibodies STS (A) and Aromatase (B), which the identified gene targets encode for, were localized in the SCT of human first trimester placenta (GA 9+5). Scale bars represent 100 µm.*

4.3.3. Released hormones

Furthermore, the supernatant of the co-incubation of isolated human platelets on differentiated BeWo cells was analyzed with progesterone ELISA (Enzo) to investigate the effect the co-incubation would have on the progesterone release of the simulated SCT. Analysis of the supernatant showed a significant decrease (Fig. 19) of progesterone that was released by the trophoblasts.

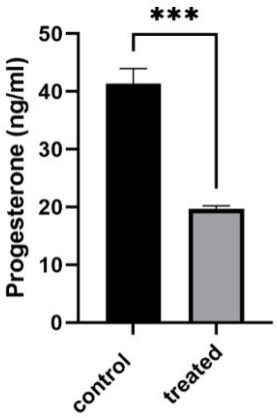


Figure 19: Co-incubation of BeWo cells with isolated human platelets revealed a significant downregulation of released progesterone. *With absolute data the amount of progesterone in the supernatant of the treated cells was significantly down regulated. Collected data presented as means ± SD from three independent experiments using isolated human platelets from three different individuals (n=3). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001.*

In addition, the experiment of co-incubation of pre-differentiated BeWo cells with isolated human platelets was performed again with isolated platelets derived from healthy pregnant women. The analysis of the supernatant was conducted in the diagnostic laboratory of the division of endocrinology and diabetology (Medical University of Graz) with an Immulite assay. Also, co-incubation with platelets originating from the blood of pregnant mothers resulted in the significant decrease of progesterone released into the supernatant of the cell culture (Fig. 20).

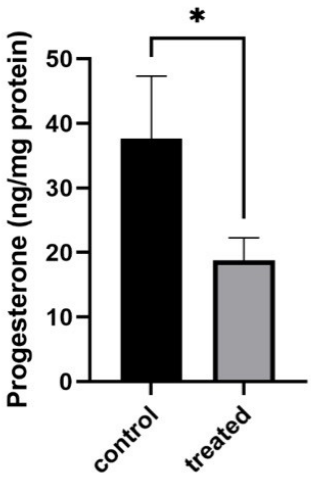


Figure 20: Supernatant of differentiated BeWo cells showed a significant decrease of released progesterone upon co-incubation with platelets derived from pregnant women. *With normalized data the amount of progesterone in the supernatant of the treated cells was significantly down regulated. Collected data presented as means ± SD from three independent experiments using isolated human platelets from three different individuals (n=3). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001*

5. Discussion

The human placenta is a hemochorial organ and thus maternal blood cells are in direct contact with the extra embryonic cells. However, in the first weeks of pregnancy, maternal spiral arteries are plugged with EVTs to restrain maternal blood from entering into the IVS and to keep a low oxygen concentration for the developing villi (21).

In this thesis we found an alternative way for maternal platelets to enter the IVS as the first maternal blood cells during first trimester of human pregnancy next to the way through small channel like structures in the EVT plug. Platelets can find their way through interstices in between EVT, enter into the IVS as the first maternal blood cells and, upon activation, can deregulate the gene expression of the villous trophoblasts. They influence the release of steroid hormones into the maternal circulation and could therefore impact the course of pregnancy (Fig. 21).

5.1 Alternative route of platelets entering the IVS in early human pregnancy

With IHC it was shown that during first trimester of pregnancy there were platelets in intercellular gaps of EVTs. These small interstices at the distal part of EVT cell columns probably arose due to absent tight junction proteins and lacked an endothelial lining (Fig. 4). The found absence of tight junction proteins ZO-1 and occludin confirmed earlier stainings of Marzioni et al. (148). and is furthermore in concordance with the finding of Davies et al. who showed in mRNA expression of partial epithelial to mesenchymal transition in trophoblasts that these tight junction proteins could be found in SCT and CTs but not in EVTs (149). Also, some bigger gaps in between EVTs filled with erythrocytes (Fig. 5) were visible and also lacked the endothelial lining. From here, maternal plasma and platelets might be able to further penetrate between the EVTs into smaller interstices. Also, an archival specimen of a human first trimester placenta *in utero* confirmed the finding of platelets in between EVTs early in pregnancy and showed platelets already adhering to the SCT in the IVS (Fig. 6). These findings proved that the platelets in the interstices and adhering to the villous trophoblast are there due to *in vivo* situations and not due to the elective surgical termination of pregnancy. Furthermore, the platelet staining of FFPE tissue samples could be validated even further, as an anchoring villous with an adjacent EVT column showed platelets in between EVTs in TEM (Fig. 8). The platelet was identified due to its characteristic open canalicular system and its common platelet

structures, like α-granules, dense bodies and glycogen. This was the first time that maternal platelets could be shown travelling in distal parts of the EVT cell columns with TEM.

Next to this possible way of platelets travelling in between EVTs, earlier studies already revealed that the EVT plugs are getting loosely cohesive from 6-7 weeks of gestation onwards. These small channels also have a diameter big enough for these smallest maternal blood cells to pass the EVT plug and enter the IVS of the early placenta before the rest of the maternal blood cell components (40,150,151).

At the end of first trimester the EVT plug is dissolving, which has been shown by earlier studies with contrast-enhanced ultrasonography, which could detect a significant increase in the flow through the maternal vessel from 11-13 weeks of gestation onwards (40,152). From this time point onwards the maternal whole blood is entering into the IVS and the oxygen concentration is rising to around 8% in the IVS. In earlier stages of pregnancy the reduced oxygen concentration of 2.5% was important for a healthy development of the early extra embryonic villi, which otherwise would have been compromised (19,153). However, Allerkamp et al. showed that the EVT plugs do not dissolve completely but are still partly present in the maternal vessels. During that time however, the vessels enlarge and grow to such an extent that the remainders of the plugs do not obstruct the blood flow into the IVS anymore (154).

Nevertheless, the EVT plugs are of great importance for human pregnancy. Without the maternal blood cells being restrained from entering into the IVS early in pregnancy the higher oxygen concentration would seriously affect the development and differentiation of the early trophoblasts and therefore the extra embryonic villi. Furthermore, the obstruction of the flow enables the EVT additionally to remodel the maternal spiral arteries to highly dilated vessels. The remodeling of the maternal spiral arteries with the depletion of smooth muscle cells and the loss of the elastic lamina in the vessel walls ensure a constant blood flow later in pregnancy (155,156). In many pregnancy disorders like PE or IUGR an incomplete remodeling of the maternal vessels is claimed to play a role in the development of the disease. Therefore, it is of great importance for the maternal spiral arteries to be blocked early in pregnancy (155,156).

As shown in this thesis and other studies, platelets seem to play an important role in the interaction with the extra embryonic trophoblasts as well as presumably in reparation and wound healing processes that are important for the development of the early placenta (2). Therefore they may enter the IVS eighter through the channel like structure in the EVT plug in the maternal spiral arteries from early in first trimester onwards or as an alternative through interstices in between the distal part of the EVT cell columns (1,7).

With TEM the platelet in between EVTs in the distal parts of the EVT column showed signs of activation by built extensions. The question of how and why the platelet got activated in the interstices and whether the EVTs are playing a role in this process is further analyzed in this thesis.

A study by Sato et al. showed that activated platelets released a compound of chemoattractants. They performed an invasion assay where they showed an increased EVT invasion under the influence of platelet derived soluble factors (4). Therefore, it was proposed, that platelets play an important role in the remodeling of maternal spiral arteries. Platelets and ECM can be found within the EVT plugs that restrain the maternal blood cells from entering into the first trimester IVS. The platelets release a variety of soluble factors and create a chemokine gradient around the maternal vessel that is remodeled. Attracted by this gradient EVT are migrating towards the vessels and are encouraged to take part in the remodeling process (156,157). Next to platelet-derived soluble factors uterine natural killer cells and macrophages are taking an active part in the remodeling of the maternal spiral arteries. (158) In the early stages vascular changes occur without the presence of EVTs, suggesting the involvement of another cell type. Uterine natural killer cells are the main maternal immune cell component of the decidua and accumulate around maternal spiral arteries even before EVTs invasion. During this early time of remodeling, vascular smooth muscle cells show disruption and disorganization combined with signs of apoptosis and ECM degradation. Decidual natural killer cells and macrophages infiltrate vascular smooth muscle cell layers and are positive for proteolytic enzymes like matrix metalloproteinase -7 and -9. Spiral artery remodeling occurs in trophoblast-dependent and -independent stages and there is evidence that decidual natural killer cells and macrophages are involved in the trophoblast-independent stages. (31,158,159) Various other factors have been proposed to promote or inhibit the invasion of EVT including cytokines, chemokines, growth factors, cell adhesion molecules, ECM-degrading enzymes and oxygen concentration. Nevertheless, the overall pictures of the mechanism of EVT invasion and spiral artery remodeling is not completely understood. Potential factors that direct EVTs towards maternal spiral arteries are high oxygen concentration in the spiral arteries, maternal platelet-derived factors and vascular smooth muscle cells. (160)

5.2 HLA-G does not affect aggregation or adhesion characteristics of human platelets

As EVTs express the marker HLA-G, which is said to play an important immunomodulatory role during pregnancy (161,162), we wanted to investigate whether this marker could influence the activation status of maternal platelets. With aggregometry the ability of known platelet agonists ADP and col-I to activate human platelets in PRP samples was confirmed. In comparison, recombinant HLA-G did not trigger an activation or adherence of the platelets in PRP and also did not impair the further activation with ADP. Here, HLA-G has no influence on the aggregation of platelets (Fig. 9). Also, col-IV was tested, as it was found in between EVTs in the decidua of the human placenta (145). However, col-IV did not affect the aggregation of human platelets in PRP.

As recombinant HLA-G did not affect the aggregation nor impair further activation with known agonists, we nevertheless wanted to test, if HLA-G would have an effect on platelets adhesion behavior. Therefore, we were working with the JAR cell line as a control cell line and as an additional cell line JAR cells were transfected to stably overexpress HLA-G (JAR-G). Overexpression was confirmed with IHC of EVT marker HLA-G on JAR and JAR-G cells. In addition, Western Blot analysis of protein lysates and supernatants of both cell lines for HLA-G were conducted and the clear overexpression could be acknowledged in the JAR-G protein lysates and supernatant, while JAR cells showed no expression at all. With shed HLA-G in the supernatant of JAR-G cells another aggregometry was performed on isolated human platelets. Again, no aggregation could be observed. In comparison human platelets did show aggregation when analyzed with ADP as an agonist (Fig. 10).

As the overexpression of HLA-G in JAR-G cells was confirmed the ability of HLA-G to increase adhesion of platelets towards trophoblasts was investigated. Therefore, a flow assay with the ibidi pump system was conducted. Platelets were introduced into flow over JAR and JAR-G cells each in presence and absence of ADP. Afterwards ibidi slides were stained for cells and platelets and analyzed with the HALO software for the percentage of trophoblasts that were also positive for the platelet marker. In summary, no difference could be found in the adhesion of platelets towards trophoblasts either with or without HLA-G nor with or without activation of platelets by ADP (Fig. 11). One important aspect that needs to be mentioned is that the calculation of the flow rate in the early human placenta is challenging and complex. However, this ongoing discussion is an important factor when investigating the adhesion of platelets towards trophoblasts. The used flow rate of 1.7 dyn/cm^2 in this experiment was an

informed decision based on earlier trophoblast flow studies (163) (especially by James et al. (164)), but could of course have an fundamental impact on the adhesion capabilities of platelets towards trophoblasts.

In conclusion, HLA-G does not influence the activation and therefore the aggregation of platelets or the adhesion of platelets towards trophoblasts.

There are some known agonists of platelets including ADP, TXA_2 and thrombin and also multiple structures to which platelets bind due to special receptors, e.g. collagen, vWF and fibrinogen (87). With these experiments however it was confirmed that HLA-G is not one of these structures. Another study also showed that platelets can get activated by and trigger pathways of the complement system. Clinical data suggest that enhanced platelet-derived activation of the complement system correlates with an increased risk for arterial thrombosis in patients with SLE and APS with APA. In vivo, activation of the complement system is linked with APA-induced pregnancy complications like fetal loss and growth retardation. Under physiological conditions, activation of the complement system may result in the clearance of used platelets from circulation to regulate pro-thrombotic effects. Under pathological conditions, dysregulated activation of the complement system on or by platelets may result in thrombotic events and thrombocytopenia (165).

However, at this point we can not rule out that shear stress might have played a role in the process of activated platelet in between the EVTs as presumably there is increased pressure in the uterine arteries with which the plasma and platelets are encouraged to enter the interstices in between the EVTs. Another interesting aspect has been first mentioned by Lowenhaupt et al. in the 70s. They first described that platelet mobility might be an active process (166). A more recent study of Gaertner et al. in a mouse model monitored platelet migration *in vivo*. The platelets moved actively and independently of the blood flow, of clot formation or migrating leukocytes. Moreover, to investigate the mechanistic basis of platelet migration an *in vitro* experiment was conducted and the platelet's leading edge showed actin polymerization and at the trailing edge myosin-mediated contraction could be observed (167).

Furthermore, there can be other substances in the intercellular gaps that could be involved in the activation process of platelets. Another substance that was found as an ECM protein in between EVTs recently was col-IV. That however was also tested by us with aggregometry and showed no activation capability of PRP in contrast to e.g. col-I. However, it is well known that ECM proteins play an important role in the invasion process of trophoblasts. Invading EVTs express collagen receptors and also secrete col-IV *in vivo* as well as *in vitro.* As neutralizing antibodies to col-IV block EVT invasion *in vitro,* EVTs seem to have an impact on the invasive

character of other trophoblasts at the implantation site by their secreted extracellular proteins (145,168). The influence of decidual factors on trophoblast differentiation towards the extravillous pathway, migration and invasion has been intensively investigated. Next to EVTs, fibroblasts, macrophages and natural killer cells express cytokines, chemokines and soluble factors, that could be involved in the control of EVT invasion and migration. Besides their role in trophoblast motility, chemokines and cytokines could also play a role in immune cell recruitment and activation of macrophages, natural killer cells, fibroblasts and other immune cells of the fetal-maternal interface (149,169). Next to the ECM there are other physical factors that play a role in the regulation of invasion of EVTs, like oxygen and tissue stiffness (170). Another speculative factor that could influence the partial epithelial to mesenchymal transition of the villous CTs to become invasive EVTs could be the released cargo of platelets in the interstices of EVTs. This released cargo contains all sorts of chemokines, cytokines and growth factors (1).

Slight maternal platelet activation in between EVTs and adherence in the IVS, as shown in this project by IHC and TEM, could be involved in wound healing or repair mechanisms in a healthy placenta. However, excessive activation of platelets together with the release of platelet-derived factors may cause a sterile inflammation in the placenta. This is considered to be an inflammatory process without the presence of any infectious pathogens. Activated maternal platelets are suggested to be highly involved in this process. This so-called thrombo-inflammation in the trophoblast may further contribute to renal and endothelial dysfunction causing pregnancy disorders like PE (171,172).

5.3 Platelet-derived factors deregulate genes that are involved in the steroid hormone synthesis and impair the release of progesterone into the maternal circulation

Forstner et al. have shown that platelets decrease the expression of the gene that encodes for the pregnancy hormone hCG in the human SCT (7). Two of the most important hormones for the maintenance of a healthy pregnancy are the steroid hormones progesterone and estrogen. Therefore, in this part of the project we focused on the differences that platelet co-incubation could have in genes involved in the steroid hormone synthesis in the extra embryonic syncytium and whether this could influence certain pathways that impact a healthy pregnancy.

With co-incubation of differentiated BeWo cells and isolated human platelets the deregulation of genes involved in the synthesis of steroid hormones were investigated with PrimePCR (Fig. 13) and RNA-Sequencing (Fig. 14) and later confirmed with qPCR (Fig. 16). The most promising target genes were identified to be *STS,* which was upregulated upon platelet co-incubation, and *CYP19A1*, which was downregulated upon platelet co-incubation.

STS is localized in the endoplasmatic reticulum and belongs to the sulfatase family. It hydrolyses proteins that function as metabolic precursors of estrogens, androgens and cholesterol and therefore also for progesterone. CYP19A1, also called aromatase or estrogen synthase, is a member of the cytochrome P450 superfamily and is responsible for a key step in the synthesis of estrogens. Both genes are important players in the synthesis of steroid hormones especially during human pregnancy where these hormones are crucial for the development and maintenance of a healthy gestation.

Next, the presence of STS and Aromatase (encoded by CYP19A1) proteins were confirmed in first trimester placental tissue by IHC (Fig. 18). Both proteins were located in the extra embryonic SCT of early human placentas, confirming that platelets and platelet-derived factors are in direct contact with the SCT where the genes and proteins of interest are expressed.

Earlier studies also localized STS expression in the SCT (173,174) and STS mRNA and protein expression was found to be significantly increased in placentas from women whose pregnancies are complicated with early onset PE (175). In PE, factors like sFlt1 disrupt the maternal endothelial lining causing PE like symptoms such as dysregulation of placental perfusion (72). Silencing experiments of STS with primary trophoblasts cells resulted in a significant downregulation of sFlt1 secretion and a significant decrease of sFlt1 transcription. Therefore, it was speculated that increased STS expression could contribute to the development of PE via increased sFlt1 expression (175). In a study by Perez-Sepulveda et al. CYP19A1 expression

and function by trophoblasts are significantly downregulated in pregnancies complicated with PE compared to healthy controls (176). Thus, both identified genes of interest that are significantly deregulated in trophoblasts upon platelet co-incubation, are also deregulated in pregnancy complications like PE and could therefore be a promising new future direction in deciphering the role of maternal platelets in the human placenta, pregnancy and pregnancy pathologies.

Furthermore, the viability of the trophoblast cell line BeWo under platelet treatment was examined and according to *LIMS1*, *TP53* and *Bcl-2* genes, that are involved in different apoptosis and cell survival processes, the viability of BeWo cells was not significantly influenced by the treatment (Fig. 15).

These results should be confirmed on protein level. For that a variety of assays are available that measure different metabolic markers to estimate the number of viable cells in a cell culture experiment. Each viability test has its advantages and disadvantages. The ATP detection assay for example is the most sensitive, the fastest and has less interferences in comparison to other assays. However, as another possibility the MTT assay offers a less expensive alternative that may also achieve a sufficient performance depending on the experimental setting (177).

For further experimental analysis possible housekeeping genes were identified that were not regulated by platelet treatment. *CYC1* and *B2M* are not suitable for these experiments as they showed a significant deregulation, whereas *YWHAZ* was not regulated by the treatment. As an additional housekeeping gene *GAPDH*, *TBP* of *HPRT1* are possible (Fig. 17).

Also, supernatants of the co-incubation experiment were analyzed with progesterone ELISA, revealing a highly significant downregulation of released progesterone into the supernatants of treated cells (Fig. 19). These findings could be confirmed in another co-incubation of differentiated BeWo cells with platelets isolated from whole blood of pregnant women. The supernatants were analyzed in the routine lab with Immulite analysis and also revealed a significant downregulation (Fig. 20). These findings indicate that upon contact of maternal platelets or presumably platelet-derived factors and extra embryonic trophoblasts the release of progesterone into the maternal circulation is significantly decreased. Progesterone is one of the most important pregnancy hormones, that is involved in establishment and persistence of gestation and has multiple immunomodulatory functions. Therefore, a decline of progesterone concentration could influence the acceptance of the extra embryonic cells by the maternal system and influence the pregnancy tremendously.

A study by Palomares et al. showed that fetal derived exosomes contained the platelet activating factor (PAF). This factor activates a pathway including the progesterone receptor A that in the

end results in a reduction of progesterone and leads to the initiation of labor. The study could also show that in the placentas from mothers, that had recurrent preterm labor and births, reduced levels of progesterone receptor A could be found. This indicated that progesterone receptor A could play a role in some cases of preterm births (178).
Further it can be discussed, that the co-incubation of human platelets and trophoblasts resembles a pathological situation as it induces an activated state of the platelets, due to instability in their isolated form, and the direct contact to trophoblasts for 24 h. In human first trimester placenta this situation should only occur due to injury of the SCT and exposure of the ECM that results in platelet activation or high shear stress due to increased turbulences or similar situations. The exposure of trophoblasts to direct platelet contact and high concentrations of platelet-derived factors usually only occurs during wound healing or repair and construction mechanisms in a healthy placenta. Thus, the experimental approach could be seen as simulation of the platelets highly activated state in case of inflammation, injury or pregnancy complication and not as a normal process of healthy trophoblast and platelet interaction.

In general, the most essential role of platelets as part of the coagulation system is well known. However, the critical role of platelets in immune responses is much less investigated. The platelets' small size and high number in human blood circulation, combined with the OCS and a multitude of pathogen receptors, allow them to deal with smaller immunological threats and to mark larger pathogens and viruses for further immune-cell recruitment. Once stimulated, platelets release a variety of cytokines, chemokines and growth factors that contribute to cell growth and wound repair, and potentially to immune suppression (179).
Increased turbulences in the IVS e.g. due to insufficient remodeling of the maternal spiral arteries and the therefore resulting damage of the SCT can contribute to the activation of maternal platelets within the IVS. This activation state is involved in different wound healing processes and has an important influence in the formation of the villous trees. For instance, maternal platelets and the associated coagulation system contribute to the generation of fibrin-type fibrinoid, which can be detected in areas where the IVS is not lined by the SCT due to turbulences and the hence resulting damage of the SCT, especially in the term placenta. Due to this damage the basal membrane underneath the SCT is exposed to the maternal blood. Components of the basal membrane like collagen, laminin and fibronectin are functioning as procoagulant substances influencing the activation state of maternal platelets. Therefore, so-called perivillous fibrin-type fibrinoid is found at the surface of placental villi, where it replaces the villous SCT (10,180–182). Two different theories of how fibrin-type fibrinoid is generated

in the human placenta are currently accepted. One includes the initial damage of the SCT, the exposition of the ECM of the basal membrane, which results in the activation of maternal platelets and the coagulation cascade. Here, fibrin-type fibrinoid functions as a replacement for the damaged SCT. The other theory proposes that turbulence of maternal blood in the IVS induces aggregation and activation of maternal platelets and the degeneration of SCT is a succeeding consequence. However, both theories are thought to be valid, and it is now well-accepted that fibrin accumulation at the placental villi are normal findings and increase towards term (3,180). Particularly on the surface of stem villi fibrin contributes to the mechanical stability of the human placenta (10,103). Nevertheless, increased fibrin accumulation can result in placenta insufficiency, pregnancy complications and late fetal loss (103). As this thesis focused on first trimester placenta tissues, it was not possible to determine whether one of the pregnancies would have developed a pregnancy complication like PE later in gestation. However, there are studies that investigated the increased fibrin-type fibrinoid deposition in the IVS of term placentas from pregnancies complicated with FGR and PE (183).

Furthermore, hyperactivated maternal platelets at the maternal-fetal interface can also result in so called thrombo-inflammation in the IVS, causing a sterile inflammation in the placenta and an inflammatory response of the mother. Therefore, the degree of activation determines whether maternal platelets are beneficial or harmful for human pregnancy. Increased activation of maternal platelets can have a direct influence on the development of a placenta-associated pregnancy pathology, e.g. PE (3).

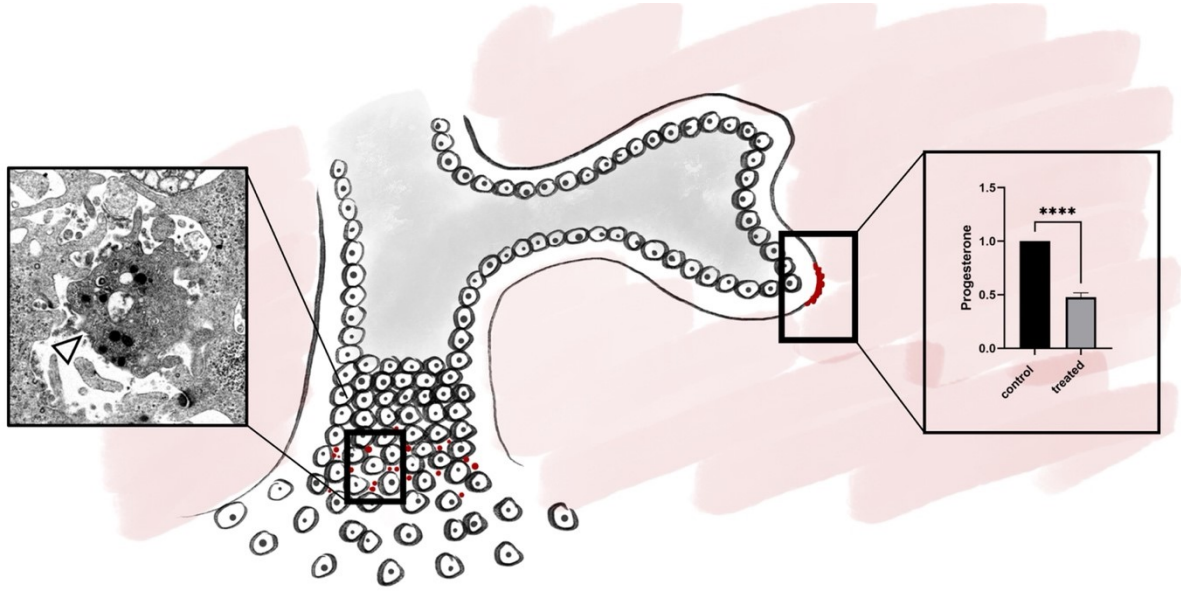


Figure 21: Thesis summary of maternal platelet activation and placental endocrine activity. *In this thesis maternal platelets could be identified in intercellular gaps in between EVTs as an alternative route into the IVS during early pregnancy. Also, platelets could be located adhering to the villous SCT in the IVS. Further analysis of cell culture experiments showed a significant downregulation of progesterone released by the trophoblasts.*

For future experiments, we would like to investigate whether we can confirm the results of deregulated genes involved in the steroid hormone synthesis and downregulated progesterone in the supernatant in experiments with human first trimester and term placental tissue and platelets and platelet derived factors from pregnant women.

Also, the question whether the platelets from women with a pregnancy pathology like severe PE would induce a difference in the results when compared to the results obtained from platelets from non-pregnant women or with healthy pregnancies would be interesting for further investigation.

5.4 Conclusion

In conclusion, this study found a new and alternative way for maternal platelets to enter the IVS of the human first trimester placenta. These smallest blood cells were detected in interstices of distal parts of EVT columns by immunohistochemistry and TEM. However, the EVT marker HLA-G has no impact of the aggregation ability of the maternal platelets and also does not influence the adhesion of human platelets towards trophoblasts.

In an archival *in utero* specimen of an early human pregnancy the former stainings of the maternal platelets in intercellular gaps of EVTs were confirmed and furthermore showed adhering platelets to the villous trophoblast in the IVS. These findings indicate that platelets do play a role in the maternal-fetal cross talk in the IVS from a very early stage of human pregnancy. In co-culture of differentiated trophoblast cell line BeWo and isolated human platelets a significant deregulation of some genes that are involved in the steroid hormone synthesis could be found and analysis of the cell culture's supernatant showed a significant downregulation of the release of pregnancy hormone progesterone. Therefore, maternal platelets could have a significant effect on the development and maintenance of human pregnancy when influencing the production and release of important pregnancy hormones. Further studies are necessary to get a deeper inside into this promising and new future direction in deciphering the role of maternal platelets in human placenta, pregnancy and pregnancy pathologies.

6. References

1. Guettler J, Forstner D, Cvirn G, Maninger S, Brugger BA, Nonn O, et al. Maternal platelets pass interstices of trophoblast columns and are not activated by HLA-G in early human pregnancy. J Reprod Immunol. 2021 Apr;144:103280.

2. Guettler J, Forstner D, Gauster M. Maternal platelets at the first trimester maternal-placental interface - Small players with great impact on placenta development. Placenta. 2021 Dec 9;S0143-4004(21)00658-5.

3. Moser G, Guettler J, Forstner D, Gauster M. Maternal Platelets—Friend or Foe of the Human Placenta? Int J Mol Sci. 2019 Nov 11;20(22):E5639.

4. Sato Y, Fujiwara H, Zeng BX, Higuchi T, Yoshioka S, Fujii S. Platelet-derived soluble factors induce human extravillous trophoblast migration and differentiation: platelets are a possible regulator of trophoblast infiltration into maternal spiral arteries. Blood. 2005 Jul 15;106(2):428–35.

5. Blaschitz A, Siwetz M, Schlenke P, Gauster M. Adhering maternal platelets can contribute to the cytokine and chemokine cocktail released by human first trimester villous placenta. Placenta. 2015 Nov;36(11):1333–6.

6. Moser G, Huppertz B. Implantation and extravillous trophoblast invasion: From rare archival specimens to modern biobanking. Placenta. 2017 Aug;56:19–26.

7. Forstner D, Maninger S, Nonn O, Guettler J, Moser G, Leitinger G, et al. Platelet-derived factors impair placental chorionic gonadotropin beta-subunit synthesis. J Mol Med Berl Ger. 2020 Feb;98(2):193–207.

8. Costa MA. The endocrine function of human placenta: an overview. Reprod Biomed Online. 2016 Jan;32(1):14–43.

9. Sacks G, Sargent I, Redman C. An innate view of human pregnancy. Immunol Today. 1999 Mar;20(3):114–8.

10. Benirschke K, Kaufmann P. Pathology of the Human Placenta. Springer Science & Business Media; 2013. 887 p.

11. Turco MY, Moffett A. Development of the human placenta. Development. 2019 Nov 15;146(22):dev163428.

12. Sadler TW, Langman J. Medizinische Embryologie, German Language. Thieme; 2008. 530 p.

13. Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. Thromb Res. 2004 Jan;114(5–6):397–407.

14. Diedrich K, Fauser BCJM, Devroey P, Griesinger G. The role of the endometrium and embryo in human implantation. Hum Reprod Update. 2007 Jul 1;13(4):365–77.

15. Rossant J, Tam PPL. Early human embryonic development: Blastocyst formation to gastrulation. Dev Cell. 2022 Jan;57(2):152–65.

16. te Velde EA, Exalto N, Hesseling P, van der Linden HC. First trimester development of human chorionic villous vascularization studied with CD34 immunohistochemistry. Hum Reprod Oxf Engl. 1997 Jul;12(7):1577–81.

17. Egbor M, Ansari T, Morris N, Green CJ, Sibbons PD. Pre-eclampsia and fetal growth restriction: how morphometrically different is the placenta? Placenta. 2006 Jul;27(6–7):727–34.

18. Mayhew TM, Ohadike C, Baker PN, Crocker IP, Mitchell C, Ong SS. Stereological investigation of placental morphology in pregnancies complicated by pre-eclampsia with and without intrauterine growth restriction. Placenta. 2003 Mar;24(2–3):219–26.

19. Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN, Burton GJ. Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. Am J Pathol. 2000 Dec;157(6):2111–22.

20. Xiao Z, Yan L, Liang X, Wang H. Progress in deciphering trophoblast cell differentiation during human placentation. Curr Opin Cell Biol. 2020 Dec;67:86–91.

21. Huppertz B, Schleußner E, editors. Die Plazenta: Grundlagen und klinische Bedeutung. 1. Auflage. Berlin, Heidelberg: Springer; 2018. 382 p.

22. Kupper N, Huppertz B. The endogenous exposome of the pregnant mother: Placental extracellular vesicles and their effect on the maternal system. Mol Aspects Med. 2021 Feb 18;100955.

23. Craven CM, Zhao L, Ward K. Lateral placental growth occurs by trophoblast cell invasion of decidual veins. Placenta. 2000 Apr;21(2–3):160–9.

24. Bainbridge DRJ, Ellis SA, Sargent IL. HLA-G suppresses proliferation of CD4+ T-lymphocytes. J Reprod Immunol. 2000 Aug;48(1):17–26.

25. Li C, Houser BL, Nicotra ML, Strominger JL. HLA-G homodimer-induced cytokine secretion through HLA-G receptors on human decidual macrophages and natural killer cells. Proc Natl Acad Sci. 2009 Apr 7;106(14):5767–72.

26. Naji A, Menier C, Morandi F, Agaugué S, Maki G, Ferretti E, et al. Binding of HLA-G to ITIM-Bearing Ig-like Transcript 2 Receptor Suppresses B Cell Responses. J Immunol. 2014 Feb 15;192(4):1536–46.

27. Yie S mian, Li L hong, Li Y mei, Librach C. HLA-G protein concentrations in maternal serum and placental tissue are decreased in preeclampsia. Am J Obstet Gynecol. 2004 Aug;191(2):525–9.

28. Burton GJ, Woods AW, Jauniaux E, Kingdom JCP. Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy. Placenta. 2009 Jun;30(6):473–82.

29. Burton GJ, Jauniaux E. The cytotrophoblastic shell and complications of pregnancy. Placenta. 2017 Dec;60:134–9.

30. Harris LK. Review: Trophoblast-vascular cell interactions in early pregnancy: how to remodel a vessel. Placenta. 2010 Mar;31 Suppl:S93-98.

31. Smith SD, Choudhury RH, Matos P, Horn JA, Lye SJ, Dunk CE, et al. Changes in vascular extracellular matrix composition during decidual spiral arteriole remodeling in early human pregnancy. Histol Histopathol. 2016 May;31(5):557–71.

32. Brosens I, Dixon HG, Robertson WB. Fetal growth retardation and the arteries of the placental bed. Br J Obstet Gynaecol. 1977 Sep;84(9):656–63.

33. Meekins JW, Luckas MJ, Pijnenborg R, McFadyen IR. Histological study of decidual spiral arteries and the presence of maternal erythrocytes in the intervillous space during the first trimester of normal human pregnancy. Placenta. 1997 Aug;18(5–6):459–64.

34. Hustin J, Schaaps JP. Echographic [corrected] and anatomic studies of the maternotrophoblastic border during the first trimester of pregnancy. Am J Obstet Gynecol. 1987 Jul;157(1):162–8.

35. James JL, Saghian R, Perwick R, Clark AR. Trophoblast plugs: impact on utero-placental haemodynamics and spiral artery remodelling. Hum Reprod Oxf Engl. 2018 Aug 1;33(8):1430–41.

36. Jauniaux E, Hempstock J, Greenwold N, Burton GJ. Trophoblastic oxidative stress in relation to temporal and regional differences in maternal placental blood flow in normal and abnormal early pregnancies. Am J Pathol. 2003 Jan;162(1):115–25.

37. James JL, Stone PR, Chamley LW. The regulation of trophoblast differentiation by oxygen in the first trimester of pregnancy. Hum Reprod Update. 2006 Apr;12(2):137–44.

38. James JL, Cartwright JE, Whitley GS, Greenhill DR, Hoppe A. The regulation of trophoblast migration across endothelial cells by low shear stress: consequences for vascular remodelling in pregnancy. Cardiovasc Res. 2012 Jan 1;93(1):152–61.

39. Xie B, Wu H, Li J, Lv X, Zhou Y, Yu Q, et al. Mechanical forces on trophoblast motility and its potential role in spiral artery remodeling during pregnancy. Placenta. 2022 Jun;123:46–53.

40. Roberts VHJ, Morgan TK, Bednarek P, Morita M, Burton GJ, Lo JO, et al. Early first trimester uteroplacental flow and the progressive disintegration of spiral artery plugs: new insights from contrast-enhanced ultrasound and tissue histopathology. Hum Reprod. 2017 Dec 1;32(12):2382–93.

41. Malek A, Sager R, Schneider H. Transport of proteins across the human placenta. Am J Reprod Immunol N Y N 1989. 1998 Nov;40(5):347–51.

42. James SR, Franklyn JA, Kilby MD. Placental transport of thyroid hormone. Best Pract Res Clin Endocrinol Metab. 2007 Jun;21(2):253–64.

43. Czernek L, Düchler M. Exosomes as Messengers Between Mother and Fetus in Pregnancy. Int J Mol Sci. 2020 Jun 15;21(12):E4264.

44. Griffiths SK, Campbell JP. Placental structure, function and drug transfer. Contin Educ Anaesth Crit Care Pain. 2015 Apr;15(2):84–9.

45. Glazier JD, Jansson T. Placental Transport in Early Pregnancy—A Workshop Report. Placenta. 2004 Apr;25:S57–9.

46. Walker N, Filis P, Soffientini U, Bellingham M, O'Shaughnessy PJ, Fowler PA. Placental transporter localization and expression in the Human: the importance of species, sex, and gestational age differences†. Biol Reprod. 2017 Apr 1;96(4):733–42.

47. Burton GJ, Watson AL, Hempstock J, Skepper JN, Jauniaux E. Uterine Glands Provide Histiotrophic Nutrition for the Human Fetus during the First Trimester of Pregnancy. J Clin Endocrinol Metab. 2002 Jun 1;87(6):2954–9.

48. Marin JJG, Macias RIR, Serrano MA. The Hepatobiliary-like Excretory Function of the Placenta. A Review. Placenta. 2003 May;24(5):431–8.

49. Pasanen M. The expression and regulation of drug metabolism in human placenta. Adv Drug Deliv Rev. 1999 Jun;38(1):81–97.

50. Moffett A, Loke YW. The Immunological Paradox of Pregnancy: A Reappraisal. Placenta. 2004 Jan;25(1):1–8.

51. Robbins JR, Bakardjiev AI. Pathogens and the placental fortress. Curr Opin Microbiol. 2012 Feb;15(1):36–43.

52. Arechavaleta-Velasco F, Koi H, Strauss JF, Parry S. Viral infection of the trophoblast: time to take a serious look at its role in abnormal implantation and placentation? J Reprod Immunol. 2002 May;55(1–2):113–21.

53. Soilleux EJ, Coleman N. Transplacental transmission of HIV: a potential role for HIV binding lectins. Int J Biochem Cell Biol. 2003 Mar;35(3):283–7.

54. Di Renzo GC, Giardina I, Clerici G, Brillo E, Gerli S. Progesterone in normal and pathological pregnancy. Horm Mol Biol Clin Investig [Internet]. 2016 Jan 1 [cited 2022 May 21];27(1). Available from: https://www.degruyter.com/document/doi/10.1515/hmbci-2016-0038/html

55. Druckmann R, Druckmann MA. Progesterone and the immunology of pregnancy. J Steroid Biochem Mol Biol. 2005 Dec;97(5):389–96.

56. Solano ME, Arck PC. Steroids, Pregnancy and Fetal Development. Front Immunol. 2020 Jan 22;10:3017.

57. Mandalà M. Influence of Estrogens on Uterine Vascular Adaptation in Normal and Preeclamptic Pregnancies. Int J Mol Sci. 2020 Apr 8;21(7):2592.

58. Shu C, Han S, Xu P, Wang Y, Cheng T, Hu C. Estrogen and Preeclampsia: Potential of Estrogens as Therapeutic Agents in Preeclampsia. Drug Des Devel Ther. 2021 Jun;Volume 15:2543–50.

59. Fowden AL, Forhead AJ. Glucocorticoids as regulatory signals during intrauterine development: Glucocorticoids and intrauterine development. Exp Physiol. 2015 Dec 1;100(12):1477–87.

60. Malassine A, Cronier L. Hormones and Human Trophoblast Differentiation. Endocrine. 2002;19(1):3–12.

61. Nwabuobi C, Arlier S, Schatz F, Guzeloglu-Kayisli O, Lockwood CJ, Kayisli UA. hCG: Biological Functions and Clinical Applications. Int J Mol Sci. 2017 Sep 22;18(10):E2037.

62. Corbacho A, Martinez De La Escalera G, Clapp C. Roles of prolactin and related members of the prolactin/growth hormone/placental lactogen family in angiogenesis. J Endocrinol. 2002 May 1;173(2):219–38.

63. Handwerger S, Freemark M. The Roles of Placental Growth Hormone and Placental Lactogen in the Regulation of Human Fetal Growth and Development. J Pediatr Endocrinol Metab [Internet]. 2000 Jan [cited 2022 May 21];13(4). Available from: https://www.degruyter.com/document/doi/10.1515/JPEM.2000.13.4.343/html

64. Lacroix MC, Guibourdenche J, Frendo JL, Muller F, Evain-Brion D. Human Placental Growth Hormone—A Review. Placenta. 2002 Apr;23:S87–94.

65. Zachary I. Signaling mechanisms mediating vascular protective actions of vascular endothelial growth factor. Am J Physiol Cell Physiol. 2001 Jun;280(6):C1375-1386.

66. Ahmad S, Ahmed A. Elevated placental soluble vascular endothelial growth factor receptor-1 inhibits angiogenesis in preeclampsia. Circ Res. 2004 Oct 29;95(9):884–91.

67. De Falco S. The discovery of placenta growth factor and its biological activity. Exp Mol Med. 2012 Jan 31;44(1):1–9.

68. Barsoum IB, Renaud SJ, Graham CH. Glyceryl trinitrate inhibits hypoxia-induced release of soluble fms-like tyrosine kinase-1 and endoglin from placental tissues. Am J Pathol. 2011 Jun;178(6):2888–96.

69. Brownfoot FC, Hannan N, Onda K, Tong S, Kaitu'u-Lino T. Soluble endoglin production is upregulated by oxysterols but not quenched by pravastatin in primary placental and endothelial cells. Placenta. 2014 Sep;35(9):724–31.

70. Flint EJ, Cerdeira AS, Redman CW, Vatish M. The role of angiogenic factors in the management of preeclampsia. Acta Obstet Gynecol Scand. 2019 Jun;98(6):700–7.

71. Umapathy A, Chamley LW, James JL. Reconciling the distinct roles of angiogenic/anti-angiogenic factors in the placenta and maternal circulation of normal and pathological pregnancies. Angiogenesis. 2020 May;23(2):105–17.

72. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest. 2003 Mar;111(5):649–58.

73. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, et al. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med. 2004 Feb 12;350(7):672–83.

74. Venkatesha S, Toporsian M, Lam C, Hanai J ichi, Mammoto T, Kim YM, et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med. 2006 Jun;12(6):642–9.

75. Levine RJ, Lam C, Qian C, Yu KF, Maynard SE, Sachs BP, et al. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N Engl J Med. 2006 Sep 7;355(10):992–1005.

76. Nadar S, Karalis I, Al Yemeni E, Blann A, Lip G. Plasma markers of angiogenesis in pregnancy induced hypertension. Thromb Haemost. 2005;94(11):1071–6.

77. Machlus KR, Italiano JE. The incredible journey: From megakaryocyte development to platelet formation. J Cell Biol. 2013 Jun 10;201(6):785–96.

78. Bizzozero J. Ueber einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung: Untersuchungen. Arch Für Pathol Anat Physiol Für Klin Med. 1882 Nov;90(2):261–332.

79. Osler. VI. An account of certain organisms occurring in the liquor sanguinis. Proc R Soc Lond. 1874 Dec 31;22(148–155):391–8.

80. Tomaiuolo M, Litvinov RI, Weisel JW, Stalker TJ. Use of electron microscopy to study platelets and thrombi. Platelets. 2020 Jul 3;31(5):580–8.

81. Michelson AD, Cattaneo M, Frelinger AL, Newman PJ, editors. Platelets. Fourth edition. London: Elsevier/Academic Press; 2019. 1230 p.

82. Wright JH. The Origin and Nature of the Blood Plates. Boston Med Surg J. 1906 Jun 7;154(23):643–5.

83. Italiano JE, Lecine P, Shivdasani RA, Hartwig JH. Blood Platelets Are Assembled Principally at the Ends of Proplatelet Processes Produced by Differentiated Megakaryocytes. J Cell Biol. 1999 Dec 13;147(6):1299–312.

84. Richardson JL, Shivdasani RA, Boers C, Hartwig JH, Italiano JE. Mechanisms of organelle transport and capture along proplatelets during platelet production. Blood. 2005 Dec 15;106(13):4066–75.

85. Jurk K, Kehrel BE. Platelets: Physiology and Biochemistry. Semin Thromb Hemost. 2005 Aug;31(04):381–92.

86. Farndale RW, Siljander PR, Onley DJ, Sundaresan P, Knight CG, Barnes MJ. Collagen-platelet interactions: recognition and signalling. Biochem Soc Symp. 2003;(70):81–94.

87. Broos K, Feys HB, De Meyer SF, Vanhoorelbeke K, Deckmyn H. Platelets at work in primary hemostasis. Blood Rev. 2011 Jul;25(4):155–67.

88. Du X. Signaling and regulation of the platelet glycoprotein Ib-IX-V complex. Curr Opin Hematol. 2007 May;14(3):262–9.

89. Watson SP, Herbert JMJ, Pollitt AY. GPVI and CLEC-2 in hemostasis and vascular integrity. J Thromb Haemost JTH. 2010 Jul;8(7):1456–67.

90. Brass LF, Manning DR, Cichowski K, Abrams CS. Signaling through G proteins in platelets: to the integrins and beyond. Thromb Haemost. 1997 Jul;78(1):581–9.

91. Banno A, Ginsberg MH. Integrin activation. Biochem Soc Trans. 2008 Apr;36(Pt 2):229–34.

92. Han J, Lim CJ, Watanabe N, Soriani A, Ratnikov B, Calderwood DA, et al. Reconstructing and deconstructing agonist-induced activation of integrin alphaIIbbeta3. Curr Biol CB. 2006 Sep 19;16(18):1796–806.

93. Reed GL. Platelet secretory mechanisms. Semin Thromb Hemost. 2004 Aug;30(4):441–50.

94. Jackson SP. The growing complexity of platelet aggregation. Blood. 2007 Jun 15;109(12):5087–95.

95. Munnix ICA, Cosemans JMEM, Auger JM, Heemskerk JWM. Platelet response heterogeneity in thrombus formation. Thromb Haemost. 2009 Dec;102(6):1149–56.

96. Coppinger JA, Maguire PB. Insights into the platelet releasate. Curr Pharm Des. 2007;13(26):2640–6.

97. Szklanna PB, Parsons ME, Wynne K, O'Connor H, Egan K, Allen S, et al. The Platelet Releasate is Altered in Human Pregnancy. Proteomics Clin Appl. 2019 May;13(3):e1800162.

98. Gremmel T, Frelinger A, Michelson A. Platelet Physiology. Semin Thromb Hemost. 2016 Feb 29;42(03):191–204.

99. Maynard DM, Heijnen HFG, Horne MK, White JG, Gahl WA. Proteomic analysis of platelet alpha-granules using mass spectrometry. J Thromb Haemost JTH. 2007 Sep;5(9):1945–55.

100. Jonnalagadda D, Izu LT, Whiteheart SW. Platelet secretion is kinetically heterogeneous in an agonist-responsive manner. Blood. 2012 Dec 20;120(26):5209–16.

101. Flaumenhaft R. Platelet Secretion. In: Platelets [Internet]. Elsevier; 2013 [cited 2022 May 24]. p. 343–66. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780123878373000183

102. Golebiewska EM, Poole AW. Platelet secretion: From haemostasis to wound healing and beyond. Blood Rev. 2015 May;29(3):153–62.

103. Lanir N, Aharon A, Brenner B. Procoagulant and Anticoagulant Mechanisms in Human Placenta. Semin Thromb Hemost. 2003;29(2):175–84.

104. Roland CS, Hu J, Ren CE, Chen H, Li J, Varvoutis MS, et al. Morphological changes of placental syncytium and their implications for the pathogenesis of preeclampsia. Cell Mol Life Sci CMLS. 2016 Jan;73(2):365–76.

105. Astedt B, Hägerstrand I, Lecander I. Cellular localisation in placenta of placental type plasminogen activator inhibitor. Thromb Haemost. 1986 Aug 20;56(1):63–5.

106. Kruithof EK, Tran-Thang C, Gudinchet A, Hauert J, Nicoloso G, Genton C, et al. Fibrinolysis in pregnancy: a study of plasminogen activator inhibitors. Blood. 1987 Feb;69(2):460–6.

107. Estellés A, Gilabert J, Keeton M, Eguchi Y, Aznar J, Grancha S, et al. Altered expression of plasminogen activator inhibitor type 1 in placentas from pregnant women with preeclampsia and/or intrauterine fetal growth retardation. Blood. 1994 Jul 1;84(1):143–50.

108. Edstrom CS, Calhoun DA, Christensen RD. Expression of tissue factor pathway inhibitor in human fetal and placental tissues. Early Hum Dev. 2000 Aug;59(2):77–84.

109. Sprecher CA, Kisiel W, Mathewes S, Foster DC. Molecular cloning, expression, and partial characterization of a second human tissue-factor-pathway inhibitor. Proc Natl Acad Sci U S A. 1994 Apr 12;91(8):3353–7.

110. Maruyama I, Bell CE, Majerus PW. Thrombomodulin is found on endothelium of arteries, veins, capillaries, and lymphatics, and on syncytiotrophoblast of human placenta. J Cell Biol. 1985 Aug;101(2):363–71.

111. Salem HH, Maruyama I, Ishii H, Majerus PW. Isolation and characterization of thrombomodulin from human placenta. J Biol Chem. 1984 Oct 10;259(19):12246–51.

112. Fazel A, Vincenot A, Malassiné A, Soncin F, Gaussem P, Alsat E, et al. Increase in expression and activity of thrombomodulin in term human syncytiotrophoblast microvilli. Placenta. 1998 May;19(4):261–8.

113. Krikun G, Lockwood CJ, Wu XX, Zhou XD, Guller S, Calandri C, et al. The expression of the placental anticoagulant protein, annexin V, by villous trophoblasts: immunolocalization and in vitro regulation. Placenta. 1994 Sep;15(6):601–12.

114. Rand JH, Wu XX, Guller S, Scher J, Andree HA, Lockwood CJ. Antiphospholipid immunoglobulin G antibodies reduce annexin-V levels on syncytiotrophoblast apical membranes and in culture media of placental villi. Am J Obstet Gynecol. 1997 Oct;177(4):918–23.

115. Bernstein I. Plasma volume expansion in early pregnancy*1. Obstet Gynecol. 2001 May;97(5):669–72.

116. Reese JA, Peck JD, McIntosh JJ, Vesely SK, George JN. Platelet counts in women with normal pregnancies: A systematic review. Am J Hematol. 2017 Nov;92(11):1224–32.

117. Tan EK, Tan EL. Alterations in physiology and anatomy during pregnancy. Best Pract Res Clin Obstet Gynaecol. 2013 Dec;27(6):791–802.

118. Choi JW, Pai SH. Change in erythropoiesis with gestational age during pregnancy. Ann Hematol. 2001 Feb 7;80(1):26–31.

119. Hytten F. Blood Volume Changes in Normal Pregnancy. Clin Haematol. 1985 Oct;14(3):601–12.

120. Peck TM, Arias F. Hematologic changes associated with pregnancy. Clin Obstet Gynecol. 1979 Dec;22(4):785–98.

121. Lisova KM, Kalinovska IV, Pryimak SH, Tokar PY, Varlas VN. Changes in the level of fetoplacental complex hormones in pregnant women with miscarriage. J Med Life. 2021 Aug;14(4):487–91.

122. Reese JA, Peck JD, Yu Z, Scordino TA, Deschamps DR, McIntosh JJ, et al. Platelet sequestration and consumption in the placental intervillous space contribute to lower platelet counts during pregnancy. Am J Hematol. 2019 Jan;94(1):E8–11.

123. Ushida T, Kotani T, Moriyama Y, Imai K, Nakano-Kobayashi T, Kinoshita F, et al. Platelet counts during normal pregnancies and pregnancies complicated with hypertensive disorders. Pregnancy Hypertens. 2021 Jun;24:73–8.

124. McCrae KR. Thrombocytopenia in pregnancy. Hematol Am Soc Hematol Educ Program. 2010;2010:397–402.

125. Shehata N, Burrows R, Kelton JG. Gestational thrombocytopenia. Clin Obstet Gynecol. 1999 Jun;42(2):327–34.

126. Justiz Vaillant AA, Goyal A, Varacallo M. Systemic Lupus Erythematosus. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2022 [cited 2022 Jun 8]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK535405/

127. Doria A, Cutolo M, Ghirardello A, Zampieri S, Vescovi F, Sulli A, et al. Steroid hormones and disease activity during pregnancy in systemic lupus erythematosus. Arthritis Rheum. 2002 Apr 15;47(2):202–9.

128. Zen M, Ghirardello A, Iaccarino L, Tonon M, Campana C, Arienti S, et al. Hormones, immune response, and pregnancy in healthy women and SLE patients. Swiss Med Wkly. 2010 Apr 3;140(13–14):187–201.

129. Lakasing L, Campa JS, Poston R, Khamashta MA, Poston L. Normal expression of tissue factor, thrombomodulin, and annexin V in placentas from women with antiphospholipid syndrome. Am J Obstet Gynecol. 1999 Jul;181(1):180–9.

130. AlSheeha MA, Alaboudi RS, Alghasham MA, Iqbal J, Adam I. Platelet count and platelet indices in women with preeclampsia. Vasc Health Risk Manag. 2016;12:477–80.

131. Thalor N, Singh K, Pujani M, Chauhan V, Agarwal C, Ahuja R. A correlation between platelet indices and preeclampsia. Hematol Transfus Cell Ther. 2019 Jun;41(2):129–33.

132. Temur M, Taşgöz FN, Çift T, Serpim G, Üstünyurt E. Role of platelet indices in prediction of preeclampsia. Ginekol Pol. 2021;92(11):792–6.

133. Wang ZM, Zhu QY, Zhang JF, Wu JL, Yang R, Wang DM. Changes of platelet parameters in early severe preeclampsia. Clin Exp Obstet Gynecol. 2017;44(2):259–63.

134. Dundar O, Yoruk P, Tutuncu L, Erikci AA, Muhcu M, Ergur AR, et al. Longitudinal study of platelet size changes in gestation and predictive power of elevated MPV in development of pre-eclampsia. Prenat Diagn. 2008 Nov;28(11):1052–6.

135. Jakobsen C, Larsen JB, Fuglsang J, Hvas AM. Platelet function in preeclampsia - a systematic review and meta-analysis. Platelets. 2019;30(5):549–62.

136. Harlow FH, Brown MA, Brighton TA, Smith SL, Trickett AE, Kwan YL, et al. Platelet activation in the hypertensive disorders of pregnancy. Am J Obstet Gynecol. 2002 Sep;187(3):688–95.

137. Macey MG, Bevan S, Alam S, Verghese L, Agrawal S, Beski S, et al. Platelet activation and endogenous thrombin potential in pre-eclampsia. Thromb Res. 2010 Mar;125(3):e76-81.

138. Shin YY, Jeong JS, Park MN, Lee JE, An SM, Cho WS, et al. Regulation of steroid hormones in the placenta and serum of women with preeclampsia. Mol Med Rep. 2018 Feb;17(2):2681–8.

139. Bezerra Maia E Holanda Moura S, Marques Lopes L, Murthi P, da Silva Costa F. Prevention of preeclampsia. J Pregnancy. 2012;2012:435090.

140. Várkonyi T, Nagy B, Füle T, Tarca AL, Karászi K, Schönléber J, et al. Microarray profiling reveals that placental transcriptomes of early-onset HELLP syndrome and preeclampsia are similar. Placenta. 2011 Feb;32 Suppl:S21-29.

141. Abildgaard U, Heimdal K. Pathogenesis of the syndrome of hemolysis, elevated liver enzymes, and low platelet count (HELLP): a review. Eur J Obstet Gynecol Reprod Biol. 2013 Feb;166(2):117–23.

142. Blaschitz A, Lenfant F, Mallet V, Hartmann M, Bensussan A, Geraghty DE, et al. Endothelial cells in chorionic fetal vessels of first trimester placenta express HLA-G. Eur J Immunol. 1997 Dec;27(12):3380–8.

143. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951 Nov;193(1):265–75.

144. Fertig ET, Gherghiceanu M, Popescu LM. Extracellular vesicles release by cardiac telocytes: electron microscopy and electron tomography. J Cell Mol Med. 2014 Oct;18(10):1938–43.

145. Oefner CM, Sharkey A, Gardner L, Critchley H, Oyen M, Moffett A. Collagen type IV at the fetal–maternal interface. Placenta. 2015 Jan;36(1):59–68.

146. Hammer A, Hutter H, Blaschitz A, Mahnert W, Hartmann M, Uchanska-Ziegler B, et al. Amnion Epithelial Cells, in Contrast to Trophoblast Cells, Express All Classical HLA Class I Molecules Together With HLA-G. Am J Reprod Immunol. 1997 Feb;37(2):161–71.

147. Blaschitz A. The soluble pool of HLA-G produced by human trophoblasts does not include detectable levels of the intron 4-containing HLA-G5 and HLA-G6 isoforms. Mol Hum Reprod. 2005 Dec 5;11(10):699–710.

148. Marzioni D. Expression of ZO-1 and occludin in normal human placenta and in hydatidiform moles. Mol Hum Reprod. 2001 Mar 1;7(3):279–85.

149. E. Davies J, Pollheimer J, Yong HEJ, Kokkinos MI, Kalionis B, Knöfler M, et al. Epithelial-mesenchymal transition during extravillous trophoblast differentiation. Cell Adhes Migr. 2016 May 3;10(3):310–21.

150. Moser G, Sundl M, Lichtensteiner M, Weiss G, Huppertz B. A revised picture of extravillous trophoblast invasion. J Reprod Health Med. 2016 Dec;2:S9–14.

151. Weiss G, Sundl M, Glasner A, Huppertz B, Moser G. The trophoblast plug during early pregnancy: a deeper insight. Histochem Cell Biol. 2016 Dec;146(6):749–56.

152. Roberts VHJ, Lo JO, Salati JA, Lewandowski KS, Lindner JR, Morgan TK, et al. Quantitative assessment of placental perfusion by contrast-enhanced ultrasound in macaques and human subjects. Am J Obstet Gynecol. 2016 Mar;214(3):369.e1-8.

153. Burton GJ, Cindrova-Davies T, Yung HW, Jauniaux E. HYPOXIA AND REPRODUCTIVE HEALTH: Oxygen and development of the human placenta. Reprod Camb Engl. 2021 Jan;161(1):F53–65.

154. Allerkamp HH, Clark AR, Lee TC, Morgan TK, Burton GJ, James JL. Something old, something new: digital quantification of uterine vascular remodelling and trophoblast plugging in historical collections provides new insight into adaptation of the utero-placental circulation. Hum Reprod Oxf Engl. 2021 Feb 18;36(3):571–86.

155. Pijnenborg R, Vercruysse L, Hanssens M. The uterine spiral arteries in human pregnancy: facts and controversies. Placenta. 2006 Oct;27(9–10):939–58.

156. Sato Y. Endovascular trophoblast and spiral artery remodeling. Mol Cell Endocrinol. 2020 Mar 1;503:110699.

157. Fujiwara H. Do circulating blood cells contribute to maternal tissue remodeling and embryo-maternal cross-talk around the implantation period? Mol Hum Reprod. 2009 Jun 1;15(6):335–43.

158. Meakin C, Barrett ES, Aleksunes LM. Extravillous trophoblast migration and invasion: Impact of environmental chemicals and pharmaceuticals. Reprod Toxicol. 2022 Jan;107:60–8.

159. Wallace AE, Fraser R, Cartwright JE. Extravillous trophoblast and decidual natural killer cells: a remodelling partnership. Hum Reprod Update. 2012 Jul;18(4):458–71.

160. Sato Y, Fujiwara H, Konishi I. Mechanism of maternal vascular remodeling during human pregnancy. Reprod Med Biol. 2012 Jan;11(1):27–36.

161. Ferreira LMR, Meissner TB, Tilburgs T, Strominger JL. HLA-G: At the Interface of Maternal-Fetal Tolerance. Trends Immunol. 2017 Apr;38(4):272–86.

162. Xu X, Zhou Y, Wei H. Roles of HLA-G in the Maternal-Fetal Immune Microenvironment. Front Immunol. 2020;11:592010.

163. Brugger BA, Guettler J, Gauster M. Go with the Flow-Trophoblasts in Flow Culture. Int J Mol Sci. 2020 Jun 30;21(13):E4666.

164. James JL, Whitley GS, Cartwright JE. Shear stress and spiral artery remodelling: the effects of low shear stress on trophoblast-induced endothelial cell apoptosis. Cardiovasc Res. 2011 Apr 1;90(1):130–9.

165. Peerschke EI, Yin W, Ghebrehiwet B. Complement activation on platelets: Implications for vascular inflammation and thrombosis. Mol Immunol. 2010 Aug;47(13):2170–5.

166. Lowenhaupt RW, Glueck HI, Miller MA, Kline DL. Factors which influence blood platelet migration. J Lab Clin Med. 1977 Jul;90(1):37–45.

167. Gaertner F, Ahmad Z, Rosenberger G, Fan S, Nicolai L, Busch B, et al. Migrating Platelets Are Mechano-scavengers that Collect and Bundle Bacteria. Cell. 2017 Nov;171(6):1368-1382.e23.

168. Damsky CH, Librach C, Lim KH, Fitzgerald ML, McMaster MT, Janatpour M, et al. Integrin switching regulates normal trophoblast invasion. Dev Camb Engl. 1994 Dec;120(12):3657–66.

169. Pollheimer J, Vondra S, Baltayeva J, Beristain AG, Knöfler M. Regulation of Placental Extravillous Trophoblasts by the Maternal Uterine Environment. Front Immunol. 2018;9:2597.

170. Abbas Y, Turco MY, Burton GJ, Moffett A. Investigation of human trophoblast invasion in vitro. Hum Reprod Update. 2020 Jun 18;26(4):501–13.

171. Kohli S, Ranjan S, Hoffmann J, Kashif M, Daniel EA, Al-Dabet MM, et al. Maternal extracellular vesicles and platelets promote preeclampsia via inflammasome activation in trophoblasts. Blood. 2016 Oct 27;128(17):2153–64.

172. Kohli S, Isermann B. Placental hemostasis and sterile inflammation: New insights into gestational vascular disease. Thromb Res. 2017 Mar;151 Suppl 1:S30–3.

173. Chatuphonprasert W, Jarukamjorn K, Ellinger I. Physiology and Pathophysiology of Steroid Biosynthesis, Transport and Metabolism in the Human Placenta. Front Pharmacol. 2018;9:1027.

174. French AP, Warren JC. Steroid-3-beta-sulfatase in fetal and placental tissues. Steroids. 1965 Dec;6(6):865–9.

175. Gratton AM, Ye L, Brownfoot FC, Hannan NJ, Whitehead C, Cannon P, et al. Steroid sulfatase is increased in the placentas and whole blood of women with early-onset preeclampsia. Placenta. 2016 Dec;48:72–9.

176. Perez-Sepulveda A, Monteiro LJ, Dobierzewska A, España-Perrot PP, Venegas-Araneda P, Guzmán-Rojas AM, et al. Placental Aromatase Is Deficient in Placental Ischemia and Preeclampsia. PloS One. 2015;10(10):e0139682.

177. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. Cell Viability Assays. In: Markossian S, Grossman A, Brimacombe K, Arkin M, Auld D, Austin C, et al., editors. Assay Guidance Manual [Internet]. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2004 [cited 2022 Jul 13]. Available from: http://www.ncbi.nlm.nih.gov/books/NBK144065/

178. Palomares KT, Parobchak N, Ithier MC, Aleksunes LM, Castaño PM, So M, et al. Fetal Exosomal Platelet-activating Factor Triggers Functional Progesterone Withdrawal in Human Placenta. Reprod Sci Thousand Oaks Calif. 2021 Jan;28(1):252–62.

179. Menter DG. Where is Waldo? or find the platelet. Cancer Metastasis Rev. 2021 Sep;40(3):649–55.

180. Kaufmann P, Huppertz B, Frank HG. The fibrinoids of the human placenta: origin, composition and functional relevance. Ann Anat Anat Anz Off Organ Anat Ges. 1996 Dec;178(6):485–501.

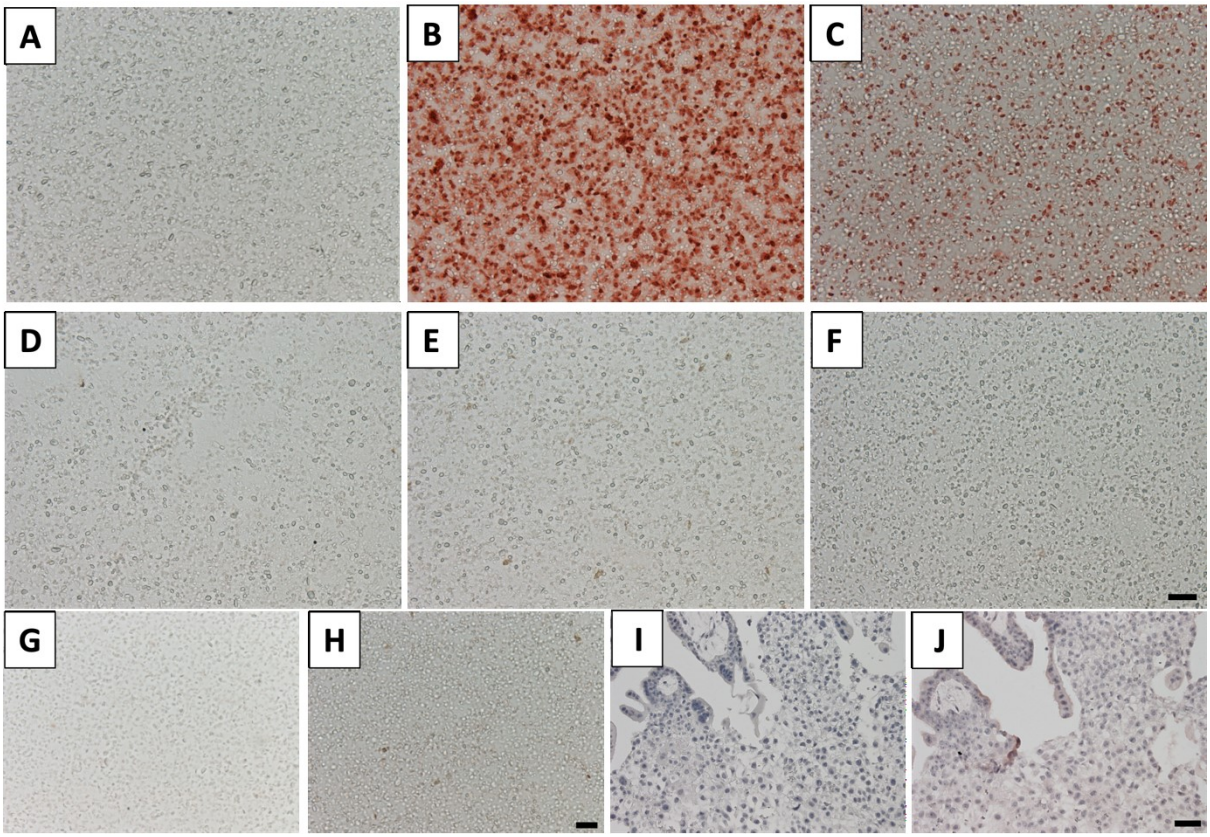
181. Pierleoni C, Castellucci M, Kaufmann P, Lund LR, Schnack Nielsen B. Urokinase receptor is up-regulated in endothelial cells and macrophages associated with fibrinoid deposits in the human placenta. Placenta. 2003 Jul;24(6):677–85.

182. Nelson DM, Crouch EC, Curran EM, Farmer DR. Trophoblast interaction with fibrin matrix. Epithelialization of perivillous fibrin deposits as a mechanism for villous repair in the human placenta. Am J Pathol. 1990 Apr;136(4):855–65.

183. Burton GJ, Jauniaux E. Pathophysiology of placental-derived fetal growth restriction. Am J Obstet Gynecol. 2018 Feb;218(2):S745–61.

7. Appendix

7.1. Results



Supplemental figure 1: *IHC of FFPE human platelets for EVT marker HLA-G revealed no staining (A).* Platelet marker CD42b (B) and CXCL4 (C) gave a positive signal. CD34 (D), occludin (E) and ZO-1 (F) showed no staining. The negative controls for mouse antibodies (G and I) and rabbit antibodies (H and J) confirmed the specificity with no signal on FFPE platelet block and on human first trimester placenta (GA 8+4). Scale bars of (F) and (H) represent 20 µm and scale bar in (J) represents 50 µm.

7.2. Buffers and Solutions

Supplemental table 1: Recipe of wash buffer for platelet isolation

NaCl	140 mM
$NaHCO_3$	10 mM
KCl	2.5 mM
Na_2HPO_4* $2H_20$	0.9 mM
$MgCl_2$	2.1 mM
$C_6H_5Na_3O_7$	22 mM
D(+)-Glucose monohydrate	0.055 mM
Bovine Serum Albumin (BSA)	0.35%

Supplemental table 2: Solutions for Lowry Protein Assay

Solution A	2% Na_2CO_3 in 0.1M NaOH
Solution B	1% $CuSO_4$ x $5H_2O$ in Aqua dest
Solution C	2% $KNOC_4H_4O_8$ x $4H_2O$ in Aqua dest

Supplemental table 3: Cacodylatbuffer

Cacodylatbuffer	0.1 M dimethyl arsenic acid sodium buffer; pH 7.4

7.3. Protocols

Supplemental table 4: Protocol for deparaffinization of tissue sections

Histolab-Clear (Histolab) 1a	5 min
Histolab-Clear (Histolab) 1b	5 min
Histolab-Clear (Histolab) 2a	5 min
Histolab-Clear (Histolab) 2b	5 min
Histolab-Clear/100% EtOH	slew round
100% EtOH	slew round
96% EtOH	slew round
70% EtOH	slew round
50% EtOH	slew round
Aqua dest.	3x

Supplemental table 5: Protocol for immunohistochemistry

TBS-T	
Hydrogen Peroxidase Block	10 min
TBS-T	3x
Ultravision Protein Block	5 min
Tab off	
diluted primary antibody	45 min
TBS-T	3x
For mouse antibody: enhancer	15 min
TBS-T	3x
HRP-Polymer	10 min
TBS-T	3x
AEC substrate	10 min
Aqua dest.	3x
Hemalaun	10 min
Aqua dest.	3x
NH_3 water	Slew around
Aqua dest.	3x
Remove excessive water	
Mount with Kaiser's glycerol gelatine	

Supplemental table 6: Protocol for immunofluorescence double staining of ibidi slides

Blocking solution	30 min
Primary antibody	overnight at 4 °C
PBS	3x
Secondary fluorescence-labelled antibody with 1x Phalloidin solution	2 h at RT
PBS	3x
Mount with antifade mounting medium including DAPI (Vectashield)	