Master Thesis

Trophoblast invasion of uterine glands in the second trimester placenta

submitted by

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for the Academic Degree of

Master of Science (MSc)

at the

Medical University of Graz

Division of Cell Biology, Histology and Embryology

Gottfried Schatz Research Center

under the supervision of

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2019

Acknowledgment

First, I would like to express my sincere gratitude to my supervisor Gerit. Thank you, for your support and guidance during the last months! I am grateful for your patience, motivation and subject-specific knowledge! The door to your office was always open whenever I had a question about my master thesis - I could not ask for a more caring supervisor!

Further, my appreciation goes to the Histo-Team. You welcomed me warmly and made feel part of the team from the first day on. Especially I would like to thank, Moni and Christine, for making me familiar with new lab-techniques. Also, I would like to thank Julia which helped me to solve statistical issues.

As well, I would like to thank Professor Huppertz, for the possibility to write this thesis at the Division of Cell Biology, Histology and Embryology, Gottfried Schatz Research Center.

My special thanks go to my family., to my loving parents, for their support and for giving me the opportunity to study. Your believing in me, motivated me the most. Further I want to thank my little brother, who cheered me always up and made me laugh even in hard times. You grew up to a great person.

Last, but not least, my special thanks go to a special person. Boro, I can not put it into words, how lucky I am that you were by my side on this journey. Thanks for your loving support and I am looking forward to the onward journey with you!

Abstract

The placenta is a very important organ and performs various functions during pregnancy. The decidua of the first trimester is well studied and described in the literature. It is known that endoglandular trophoblasts reach the uterine glands very early in pregnancy. This leads to the opening of the uterine glands, which enables glandular secretions to get into the intervillous space. In this way, the nutrition of the embryo is guaranteed in the first trimester. The second trimester is currently little described in the literature and various functions have not yet been examined.

The aim of this work was to study the presence of uterine glands and to analyze endoglandular and endovascular trophoblast invasion in placentas from the second trimester. Paraffine blocks of placentas of the second trimester (Gestational age=13-20 weeks) were obtained from the Biobank Graz. The placentas were formalin embedded serial sectioned and subsequent hematoxylin and eosin enabled to distinguish between invaded and not invaded decidua and to examine the presence of uterine glands. Only samples containing invaded decidua and uterine glands were subjected for further analysis. Serial sections were immunodouble stained with HLA-G (marker for trophoblasts) and CK7 (marker for glands) or HLA-G and CD31 (marker for vessels). Trophoblast invasion was quantitative evaluated with Vis Visiopharm Software and compared with previous data from first placentas. This work showed that uterine glands are present in the decidua of second trimester placentas. Uterine glands and vessels are invaded by extravillous trophoblasts (EVT). In the second trimester, uterine glands are invaded to a lesser extent by EVTs in relation to vascular invasion, compared to the first trimester. In future, more details have to be investigated to get insights into the second trimester and to understand different functions of the second trimester placenta.

Zusammenfassung

Die Plazenta ist ein sehr wichtiges Organ und erfüllt verschiedene Aufgaben während der Schwangerschaft. Die Dezidua des ersten Trimesters ist gut untersucht und in der Literatur beschrieben. Es ist bekannt, das endoglanduläre Trophoblasten die uterinen Drüsen sehr früh in der Schwangerschaft erreichen. Dies führt dazu, dass die uterinen Drüsen erodieren und anschließend eröffnet werden. Die Sekrete gelangen in den intervillösen Raum. So wird die Ernährung des Embryos im ersten Schwangerschaftsdrittel gewährleistet. Das zweite Trimester ist momentan noch wenig in der Literatur beschreiben und diverse Funktionen sind noch nicht erforscht.

Das Ziel dieser Arbeit ist es das Vorhandensein von uterinen Drüsen im zweiten Trimester zu untersuchen. Die endoglanduläre und endovaskuläre Trophoblasteninvasion während des zweiten Trimesters wurde ebenfalls analysiert. Paraffinblöcke von Plazenten des zweiten Trimesters (13. bis 20. Schwangerschaftswoche) wurden von der Biobank Graz bezogen und mit früheren Daten von Plazentas des ersten Trimesters verglichen. Von den Paraffinblöcken wurden Serienschnitte angefertigt. Anschließend wurde eine Hämatoxylin-Eosin-Färbung durchgeführt, und mit lichtmikroskopischen Methoden zwischen invadierter und nicht invadierter Dezidua unterschieden und auf das Vorhandensein von Drüsen untersucht. Serienschnitte von invadierter Dezidua wurden anschließend mit folgenden Antikörpern immunohistochemisch doppelgefärbt: HLA-G (Marker für extravillöse Trophoblasten), CK7 (Marker für Drüsen) und CD31 (Marker für Gefäße). Die Trophoblasteninvasion wurde mit Vis Visiopharm Software quantifiziert. Die Ergebnisse dieser Arbeit zeigen, dass in Plazenten des zweiten Trimesters uterine Drüsen und Gefäße vorkommen. Uterine Drüsen sowie Gefäße werden von extravillösen Trophoblasten (EVT) invadiert. Im Vergleich zum ersten Trimester, werden im zweiten Trimester weniger Drüsen in Relation zu Gefäßen von EVTs invadiert. In Zukunft müssen weitere Details untersucht werden, um einerseits Einblicke in das zweite Trimester zu bekommen und anderseits verschiedene Funktionen des zweiten Trimesters zu verstehen.

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1 Introduction

1.1 The human placenta

The placenta is an important organ during pregnancy. The placenta develops rapidly during the first weeks of gestation. It is required for the establishment of a physical and functional connection between the mother and the fetus, through which a normal growth and development of the fetus is ensured. The maternal blood is exposed to placental trophoblasts cells. This enables the contact to the developing embryo. The placenta fulfils different functions e.g. nutrition, protection and endocrine.

The membrane of the placenta is permeable to respiratory gases. This enables the diffusion of oxygen from maternal to fetal blood and of CO_2 from fetal to maternal blood. The main source of energy for the fetus is glucose that is supplied from the maternal circulation. Besides glucose, the placenta supplies the fetus with amino acids, lipids, vitamins and minerals. Another important function of the placenta is the protection of the fetus. In the maternal blood, it might come to a circulation of toxic substances, which are then transported through the placenta. As countermeasures, the placenta has several protective functions to reduce xenobiotic transfer to the fetus.

The placenta produces substances, which functions are not only confined in the placenta. They also play a role in the uterus. Endocrine, paracrine or/and autocrine factors e.g. progesterone, chorionic gonadotropin, are produced by the placenta.[1] [2] [3]

Defects during the formation of the placenta can contribute to fetal growth restriction and other pregnancy disorders, which can lead to miscarriage.[4]

1.2 Development of placenta

The placentation is an invasive and complex process in humans.[5] Implantation and the development of the placenta is a strictly controlled process, which includes interaction between maternal and fetal cells.[1]

This complex process is divided into four stages, which are briefly described in the below section.

Pre-implantation stage

The trophohectoderm is the precursor of all trophoblasts cells and differentiates 4-5 days post conception. The outer cell line of the blastocyst is made of trophohectoderm.[3] The blastocyst consists of two parts – an inner cell mass (embryoblast) and an outer layer of mononucleated trophoblasts.[6] The outer layer of trophoblasts provide nutrients and protects the embryo through the different development steps.[7] The trophoblast cells only occur in the placenta. The cells of the embryoblast are involved in the development of the embryo, the stroma and the chorion.[8]
All embryonic and fetal tissue e.g. umbilical cord and amnion, develop from inner cell mass.[9] The trophoblasts make a big part of the placenta and fetal membranes. 6-7 days (post-conception) the blastocyst attaches to uterine epithelium; this is the initiating step of placenta formation.[2]

Prelacunar stage

During the implantation, the blastocyst attaches to the uterine epithelium (day 6-7 p.c) and invade into the endometrium. The stem cells of the trophoectoderm develop the first trophoblast lineages: mononuclear cytotrophoblasts.[3] The mononuclated trophoblasts proliferate during implantation and fuse to generate a multinucleated syncytiotrophoblast. This enables blastocyst to penetrate the uterine epithelium.

Syncytiotrophoblast are no longer able to proliferate. In contrast to cytotrophoblast (single cells), which can proliferate continuous. At this stage of development, only the syncytiotrophoblast has direct contact with maternal cells and fluids.[6 8]

The process of differentiation causes changes in the surrounding maternal tissue. During the implantation, the endometrial stromal cells transform into decidual cells, now called decidua.

The decidua can be classified into three types: decidua basalis, decidua capsularis and decidua parietalis. The decidua basalis is defined as the site of the early placenta where trophoblast invasion begins. Decidua parietalis is in contact with the chorion. The layer which closes over the blastocyst is called decidua capsularis.[10 11]

Lacunar stage

The lacunar stage describes 8-13 days post conception. After day 8 post conception the first vacuoles develops in the syncytiotrophoblast. Form these vacuoles lacunas are arising and are filled with uterine secretions.[8 12]

The trabeculae separate the lacunas and are important for the development of the villous trees.[6] The development of lacunae facilitates the compartmentalization of the placenta.

The chorionic plate develops from the embryonic site of the placenta. The trabeculae are on the one hand important for the development of villous trees and on the other hand to connect the chorionic plate with the maternal site of placenta. The intervillous space arises from the lacunae.[8]

The implantation is completed at day 12 post conception. The embryo is embedded within the endometrium. Also, on 12 day post conception, cytotrophoblasts are starting to invade into the trabeculae. At about day 15 post conception, cytotrophoblasts reach the maternal side of placenta and come into contact with maternal tissue.[6]

Villous stage:

Villous development and morphogenesis begins around day 10 post conception.[3] On day 14 post conception, the cytotrophoblast starts to migrate into the trabeculae.[8] The finger-like formed structure proliferate into the intervillous space. The development of the villous trees begins. This structure, which is compromising trophoblasts, is called primary villi.[13] By reaching the maternal site, the migration stops and the extravillous trophoblasts (trophoblasts beyond the villous structure) start to invade the tissue of the placenta. After 2 days, the mesenchyme cells, of the embryonic mesoderm, invade into the trabeculae and primary villi. The secondary villi are formed. Fetal capillaries appear between days 18 and 20 post conception in the villous stroma and this marks the development of the tertiary villi.[14] [8] Around day 15 post-conception the cytotrophoblasts extend to form the trophoblastic shell. This is the outer layer of the placenta that surrounds the embryo. The trophoblastic shell leads to a further cell type: the extravillous trophoblasts (EVTs).[3]

1.3 Routes of extravillous trophoblast invasion

Trophoblast invasion serves several purposes. Different populations of EVTs are involved. The villous and extravillous trophoblast (EVT) originate from cytotrophoblast stem cells.[15] EVTs invade into the maternal tissue to the first third of the myometrium. The anchoring villi are placental villi connected to the basal plate. Their function is, to anchor and attach the placenta to the uterus. The anchoring of the placenta to the uterus takes place during the first trimester of the pregnancy. One site of the villous surface gets in contact with the maternal stroma. This leads to the following: cytotrophoblast proliferate and generate cell columns. The invasion into the interstitium of the uterus starts with the contact of cells with the uterine tissue (interstitial invasion). Endovascular trophoblasts, a subpopulation of EVT, invades from the uterine interstitium into maternal spiral arteries. The spiral arteries are remodeled and later in pregnancy opened towards intervillous space. Endovascular trophoblasts reach the lumen of the vessels and in this area, trophoblast plugs are formed. The task of the plug is to block the blood flow from the mother towards the placenta, to protect the early fetus from oxidative stress and enable a development in a low oxygen environment during early pregnancy. At the end of the first trimester, the trophoblast plugs disintegrate. The maternal blood reaches now the intervillous space (endoglandular invasion). Only from now on, the uteroplacental bloodflow is established for the hemotrophic nutrition of the embryo.[16 17 18]

Another subpopulation is the endoglandular trophoblast. The endoglandular invasion is the first phase of trophoblast invasion in humans. Single cells of endoglandular trophoblast reach the uterine glands during the early pregnancy. Endoglandular trophoblasts attach to uterine glands and replace the glandular epithelium. Thereby the lumen of the glands gets opened toward the intervillous space. The secretions are released into the intervillous space of the placenta. This secretion products are responsible for the nutrition of the embryo, called histotrophic nutrition. Endoglandular trophoblast express different proteins e.g. HLA-G, matrix metalloproteinase (MMP) 1 & 9 and integrin β1.[17 18 16 19]

A quantitative approach showed, that EVTs replace significantly more epithelial cells in glands compared to endothelial cells in blood vessels in the first trimester of pregnancy.[17] First connections between the uterine glands and the implanting blastocyst are visible as

early as implantation starts.[17 20] Endoglandular trophoblast invasion occurs mainly at the edges of the developing placenta,[20] due to the lateral growth of the placenta new glands are continuously connected with intervillous space.[21] At the edge of the placenta, EVTs may reach via the uterine glands directly the lumen of the uterus and migrate further on to the cervix, from where they can be serve as possible target for non-invasive prenatal diagnostics.[22] Uterine glands are invaded significantly more in the first trimester compared to the second trimester. This emphasizes, that endoglandular trophoblast invasion has more impact on early placental development, than hitherto assumed.

The described routes of extravillous trophoblast invasion are shown graphically in figure 1.

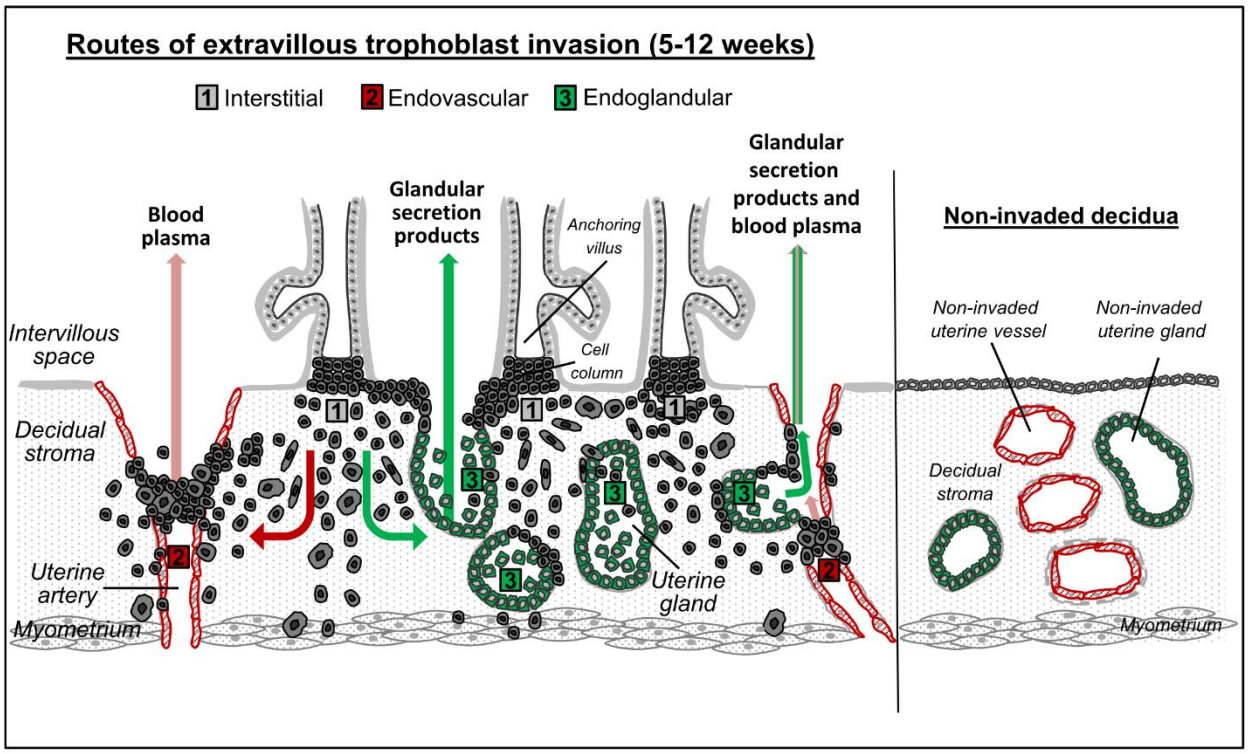

Figure1: Routes of extravillous trophoblast (EVT) invasion during the first trimester of pregnancy. (1) Interstitial trophoblast: EVT are the tips of cell columns of the anchoring villus. The EVTs invade into the decidual stroma and stop at the first third of the myometrium. Thereby the placenta is attached to the uterus. (2) Endovascular trophoblast: Arteries and veins are invaded by EVTs. The spiral arteries are remodeled, invaded and plugged during first trimester and opened towards intervillous space later in pregnancy. This enables the hemotrophic nutrition during the second and third trimester. (3) Endoglandular trophoblast replace the glandular epithelium and open the lumen of uterine glands towards intervillous space. The nutrition of the embryo during the first trimester via secretions of the uterine glands is enabled (histotrophic nutrition).[8 17]

1.4 The second trimester of pregnancy

The second trimester of pregnancy is rarely examined. Diverse functions are poorly understood. The uterine glands of the second trimester have not yet been described in the literature.

However, certain processes in the second trimester placenta are known. The placental villi run through dynamic morphological changes, which commence at first trimester and continue in the second trimester.[3] In the first trimester placenta pregnancy- specific leukocytes can be found. They fulfil crucial tasks e.g. uterine spiral artery remodeling and the development of the uteroplacental interface.[23] In addition to these cells, uterine NK cells and macrophages are present. Uterine NK cells can be found in the second trimester decidua too. A decrease of macrophages from the first trimester to second trimester placenta can be observed. The uterine NK cells and macrophages play an important role during the first 20 weeks. They induce structural changes which results into the remodeling of spiral artery. These maternal immune cells secrete chemokines and cytokines, support the actions of EVTs and contribute to tissue remodeling.[24]

1.5 Biological function of uterine glands during pregnancy

The adenogenesis describes the development of the uterine glands.[25] The process of development begins in the fetus and pursues postnatal. The adenogenesis is concluded during puberty.[26]

The uterus consists of two different layers - endometrium and myometrium.[27] The endometrium of humans can be divided in two layers: the upper stratum functionalis and lower stratum basalis. The first layer includes glands surrounded by loose stroma. The lower stratum basalis, which is the second layer, consist of bodies of glands and dense stroma.[28]

The key to a successful pregnancy is to provide nutrition to the fetus. The two pathways, histiotrophic and hemotrophic, transfer nutrients from the mother to the fetus. The hemotrophic nutrition describes the exchange of blood-borne materials between the maternal and fetal circulation.[29] Uterine glands directly synthesize and secrete substances into the lumen of the uterus, describing the histiotrophic nutrition. Their secretion products are essential for pregnancy and consist of different substances e.g. ions, sugars (glucose, fructose), amino acids, lipids and proteins. An important source of nutrition is glycogen. The secreted substances control uterine receptivity for blastocyst implantation – trophoblast attachment, growth and invasion. Besides that, they are also important for the stromal cell decidualization and conceptus growth.[30] [28.24]

The intervillous space is filled with secretions from the uterine glands during early pregnancy. Beside carbohydrate- and lipid-rich secretions for the nutrition also transcription factors are expressed by the glands. An example of a transcription factor is forkhead box a2 (Foxa2), which is only expressed by uterine glands. Foxa2 controls the differentiation and function of uterine glandular epithelium. A variety of growth factors are produced by glands which are involved in the regulation of development of the conceptus. Epidermal growth factor (EGF), leukemia inhibitory factor (LIF) and vascular endothelial growth factor (VEGF) and their receptors can be found in the first trimester placenta. LIF are important for the retention of trophoblast stem cells.[24] [31]

1.6 Infectious diseases during pregnancy - Chorioamnionitis

The infectious disease chorioamnionitis is an acute inflammatory reaction.[10] The placenta is structured into three major parts: the placental disc, the chorioamniotic membranes and the umbilical cord. Characterization of the inflammation of the placenta is the infiltration of neutrophils in the three above named structures.

Infectious organisms reach the chorioamnion and/or umbilical cord.[32] When the inflammatory process affects the chorion, amnion and placenta, it is termed as chorioamnitis (HC).[33] [34] Chorioamnionitis can be divided into two groups: clinical (CC) and histological (HC) chorioamnionitis. The CC is characterized by maternal fever and leukocytosis.

The rupture of fetal membranes and uterine contractions is induced by bacterial infection.[35] The organisms *E.coli*, *Bacteroides fragiliis* and *Strepotococci* are often associated with chorioamnionitis.[10] The bacterial infection is less common than HC.[31] It is often associated with fetal inflammatory response.

A major cause for perinatal mortality and long-term morbidity is preterm birth which can be cause by the above described infections.[34]

2 Aim

The invasion of extravillous trophoblasts in placenta, during the first trimester, is well examined. The endoglandular route of invasion is responsible for the establishment of histiotrophic nutrition during first trimester. The hemotrophic nutrition, enabled through endovascular route, begins with the beginning of the second trimester. However, the role of the uterine glands during the second trimester of pregnancy and if the glands are invaded by trophoblasts has not been investigated so far.

The task of this master thesis was, to determine the presence of uterine glands during the second trimester. Additionally, endoglandular and endovascular trophoblast invasion was analyzed. Experiments were performed with placentas from pregnancy pathologies, due to obvious ethical reasons there is no access to second trimester placentas from healthy pregnancies. Cases of chorioamnionitis were selected which is a common infectious disease during pregnancy, caused by virus, bacteria or parasites, which leads to preterm birth. Despite of the infection, the placenta develops putative normal and the infections do most likely not interfere the trophoblast development and routes of invasion.

Hematoxylin and Eosin staining, and immunohistochemistry was used to evaluate, on the one hand the presence of uterine glands and on the other hand to examine the trophoblast invasion in glands and vessels. The results of this work were compared with the previous data from first trimester placentas.

3 Methods and Materials

3.1 Tissue Collection

Placentas for this work were obtained from the Biobank Graz. In total, 146 formalin-fixed paraffin-embedded tissue of placentas at a gestational age of 11-28 weeks were provided. Only specimen with uterine glands, invaded decidua basalis and a gestational age of 13-20 weeks (n=20, Table 1) were included for this study. An additional criterion for this work was that only pathological placentas with chorioamnionitis were selected.
Data of trophoblast invasion from first trimester placentas (n=3) from healthy pregnancies were obtained from previously at the Department of Cell Biology, Histology and Embryology, Medical University of Graz.

Gestational age (weeks)	Amount (n)
13	3
16	3
17	2
18	9
19	1
20	4

Table 1: A list of placentas that were included for this study.

3.2 Preparation of Tissue

Sectioning:

The placentas, which are embedded in paraffin, were precooled (TUC 1, TUBE Cooler). The rotational microtome (HM 355S, MICROM, Zeiss, Germany) was used to make serial sections of six µm. In a warm water bath of 37°C, the sections were unfolded and afterwards placed on Superfrost Plus glass slides (Menzel-Gläser, Thermo Scientific, Germany). The sections were incubated for one hour at 42°C and overnight at 52°C (stretching table, TFP 40).

Deparaffination:

Paraffin embedded sections were deparaffinized by a rehydrating graded alcohol series. At first, the slides were slewed in Histolab-Clear (Histolab Products AB). Then they were rinsed in Tissue Clear and 100% ethanol (1:1 mixture). The slides were slewed in 100% ethanol, 96% ethanol, 70% ethanol and 50% ethanol and placed in distilled Aqua.

Antigen-Retrieval:

The heat induced epitope retrieval was carried out in KOS EM (Milestone Medical) in antigen retrieval buffer (Dako Target Retrieval Solution, pH 9) for 15 min at 93°C. Afterwards the slides cooled down in hot buffer for 20 min. In a new cuvette, filled with Aqua distilled, the slides were kept in the refrigerator (4°C) until next day.

3.3 Immunohistochemistry

Testing of antibodies:

The single immunohistochemistry was performed with the UltraVision LP Detection System HRP Polymer & AEC Chromogen (Thermo Scientific, USA). Antibodies were diluted in Dako AB Diluent with Background Reducing Components (Dako, Denmark). Table 2 shows details of all antibodies used and their respective dilutions. Counterstaining of sections was done with Mayer's haemalaun. Sections were mounted with Kaiser's glycerol gelatin (Merck, Austria). Further details regarding the procedure are provided in the supplemental protocols within the attachment.

Antibodies	Host	Company	Target	Stocks mg/ml	pH	Working dilution
HLA-G	Mouse	BD Pharminogen	EVT	0,5	9	100
CD31	rabbit	abcam	Vessels	0.316	9	100

Table 2: A list of primary antibodies used in immunohistochemistry and their working dilution.
Abbreviation: HLA-G: histocompatibility antigen, CD31: cluster of differentiation 31, EVT: extravillous trophoblast

Double immunohistochemistry:

Immunhistochemical double staining was performed with the Polink DS-MR-Hu A1 Kit (GBI labs, Golden Bridge International, USA). The staining of the slides was performed in a moisture chamber. Table 3 shows the antibodies which were used for the immunohistochemistry and respective optimized working conditions (working concentration, pH of Antigen retrieval buffer. Primary antibodies were diluted in antibody diluent (Dako AB Diluent with Background Reducing Components, Denmark).

Antibodies	Host	Company	Target	Stocks mg/ml	pH	Working dilution
HLA-G	mouse	BD Pharminogen	EVT	0,5	9	1:300
CK7	rabbit	Origene	Glands	1,0	9	1:2000
CD31	rabbit	abcam	Vessels	0,316	9	1:100
vWF	rabbit	Sigma-Aldrich	Vessels	7-13	9	1:2000 1:3000

Table 3: A list of primary antibodies used in immunohistochemistry and their working dilution.
Abbreviation: HLA-G: histocompatibility antigen, CK7: cytokeratin-7, CD31: cluster of differentiation 31, vWF: von Willebrand factor, EVT: extravillous trophoblast

From each placenta, two serial sections were double stained. Table 4 shows the used primary antibodies cocktail for immunohistochemistry.

Immunohistochemistry	Host	Company	Target
HLA-G	mouse (mc)	BD Pharminogen	EVT
CK7	rabbit (pc)	Origene	Glands
HLA-G	mouse (mc)	BD Pharminogen	EVT
CD31	rabbit (mc)	abcam	vessels

Table 4: Primary antibodies cocktail used for double staining of the serial sections.
Abbreviation: HLA-G: histocompatibility antigen, CK7: cytokeratin-7, CD31: cluster of differentiation 31, EVT: extravillous trophoblast, mc: monoclonal, pc: polyclonal

Further details regarding the procedure are provided in the supplemental protocols within the attachment.

Controls:

A negative control was proceeded through single staining with three serial sections. Instead of the primary antibody, IgG1 mouse and IgG rabbit (Dako Denmark) was used in respective concentration to the primary antibodies. Table 5 shows the used dilution.

Negative control	Host	Company	Dilution	Control to
IgG	Rabbit	Dako Denmark, X0936	1:4750 1:30000	CD31 CK7
IgG1	Mouse	Dako Denmark, X0931	1:20	HLA-G

Table 5: A list of the antibodies and working dilution, which were used for the negative controls. Abbreviation: IgG: Immunoglobulin G, IgG1: Immunoglobulin G1

3.4 Quantification of trophoblast invasion

Images were acquired using a microscope (DM6000B, Leica) equipped with a digital camera (DP72, Olympus). For the quantification, 10 images from the invaded decidua basalis were taken per slide. Overall, 600 pictures were taken. The same image section was selected and photographed within the serial section. Images were evaluated with the VIS software (VIS-Visiopharm Integrator System, Version 4.5.1.324). Every image was classified and counted in the following way: not invaded glands, not invaded vessels, glands attached with EVT, vessels attached with EVT, glands invaded by EVT glands, vessels invaded by EVT and luminal structures not assignable (Table 6). 150 images were excluded from the quantification, due the weak staining, blood clots, wrong region of interest.

Properties	Definition
Not invaded gland	EVTs not associated with gland
Not invaded vessel	EVTs not associated with vessel
Gland invaded	Epithelium replaced by EVTs
Vessel invaded	Endothelium replaced by EVTs
Glands attached	EVTs basal attached to glandular epithelium on the outer
Vessels attached	EVTs basal attached to vascular endothelium on the outer
Not assignable	Luminal structure, no clear annotation of glandular or vascular origin possible

Table 6: Classification of glands and vessels for the quantification.
Abbreviation: EVT: extravillous trophoblast

3.5 Statistical analysis

Data are reported as means ± standard error of the mean (SEM). Prism 8 (GraphPad, La Jolla, CA, USA) was used to perform non-parametric tests (Mann-Whitney-U, Kruskal-Wallis-Test). A p-value<0.05 was considered as significant. Data analysis was performed with Microsoft Excel. Figures were created with GraphPad Prism 8.

4 Results

4.1 Testing of antibodies

In a subset of two placentas (GA= 13 and 19 weeks), an immunohistochemical single staining was performed to determine the appropriate dilution factors for the antibodies for the subsequent immunohistochemical double staining. Serial sections were immuno-stained with antibodies CD31 (marker for vessels, Figure 1, A and B) and HLA-G (serves as a marker for EVT, Figure 1, C and D). Various dilution factors were tested; optimal staining was obtained for CD31 with a dilution factor of 1:100 and for HLA-G 1:300.

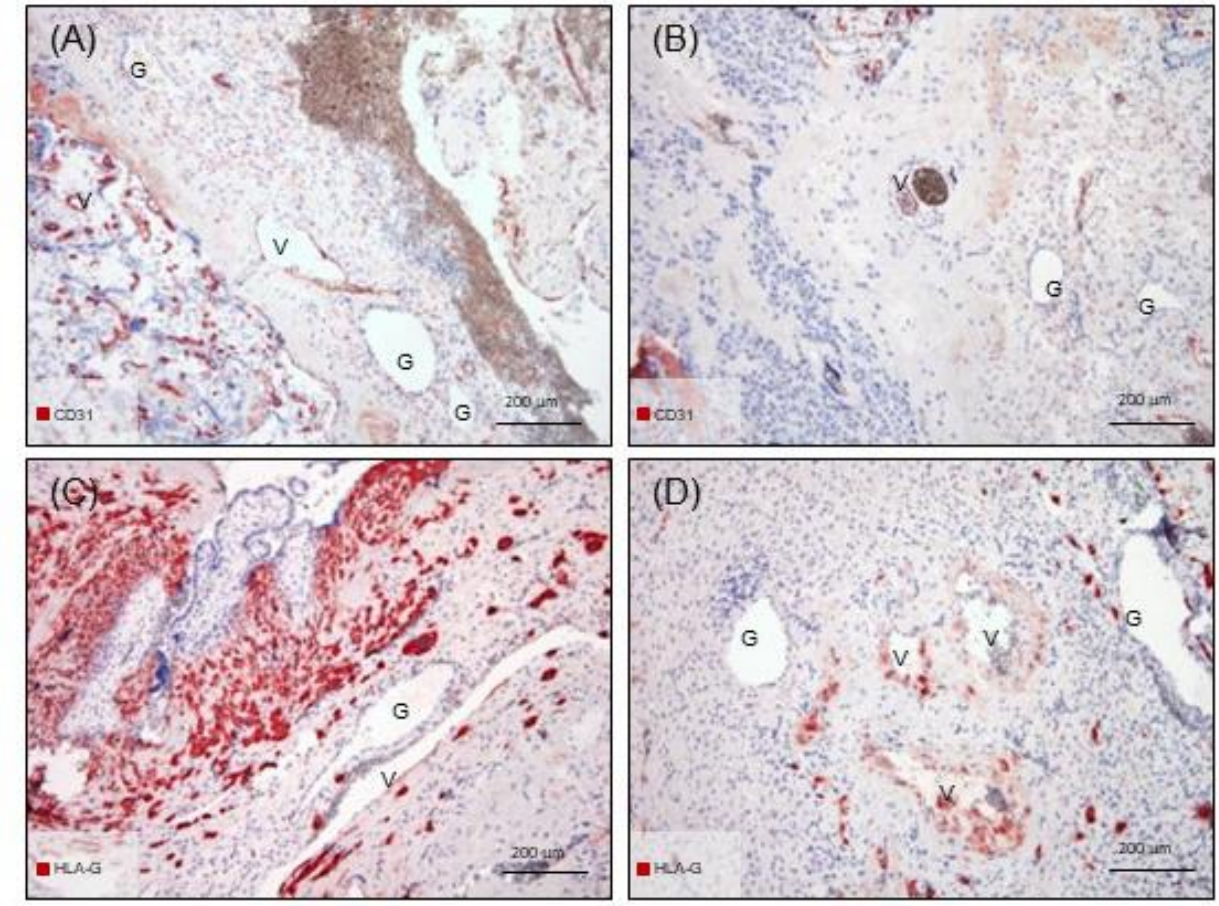

Figure 1: Determination of optimal antibody concentration for immunohistochemistry.
Immunohistochemical single staining of invaded decidua (GA=13 - 19 weeks) to determine the appropriate dilution factor for the immunohistochemical double staining for the antibodies with CD31 and HLA-G. (A), (B) shows a single staining with CD31 (1:100) as a marker for vessels, optimal staining was obtained at a dilution factor 1:100 In (C); (D) sections were immuno-single stained with HLA-G (1:100). The antibody HLA-G serves here as a marker for extravillous trophoblasts (red). Nuclear counter staining with Mayer's haemalaun.
Abbreviation: V=vessels, G=glands, HLA-G= major histocompatibility complex, class 1 G, CD31= cluster of differentiation 31, CK7= cytokeratin 7, GA= gestational age

4.1.1 Comparision CD31 and vWF

To find out which antibody is more suitable for marking the vascular endothelium, two antibodies were tested for the immunohistochemistry: Von Willebrand Factor (vWF) and CD31. The double staining with vWF was performed with two dilution factors. The figure 2 in B shows the double staining with 1:3000 as dilution factor. In D and F, 1:2000 was selected for dilution. A, C and E show serial sections that were immunohistochemically double stained with HLA-G (marker for EVTs) and CD31 (marker for endothelium).

vWF reacts with the vascular endothelium, but beside that the antibody also reacts unspecific within fibrinoid. Fibrinoid occurs frequently within the placenta, especially in regions of EVT invasion. The two differently chosen dilution factors did not show a major difference in the staining result. CD31 reacts, only with the endothelium of vessels and thus fibrinoid accumulations of the placenta were not stained with CD31.

Based on this results, further immunohistochemistry was performed with CD31 to mark vascular endothelium.

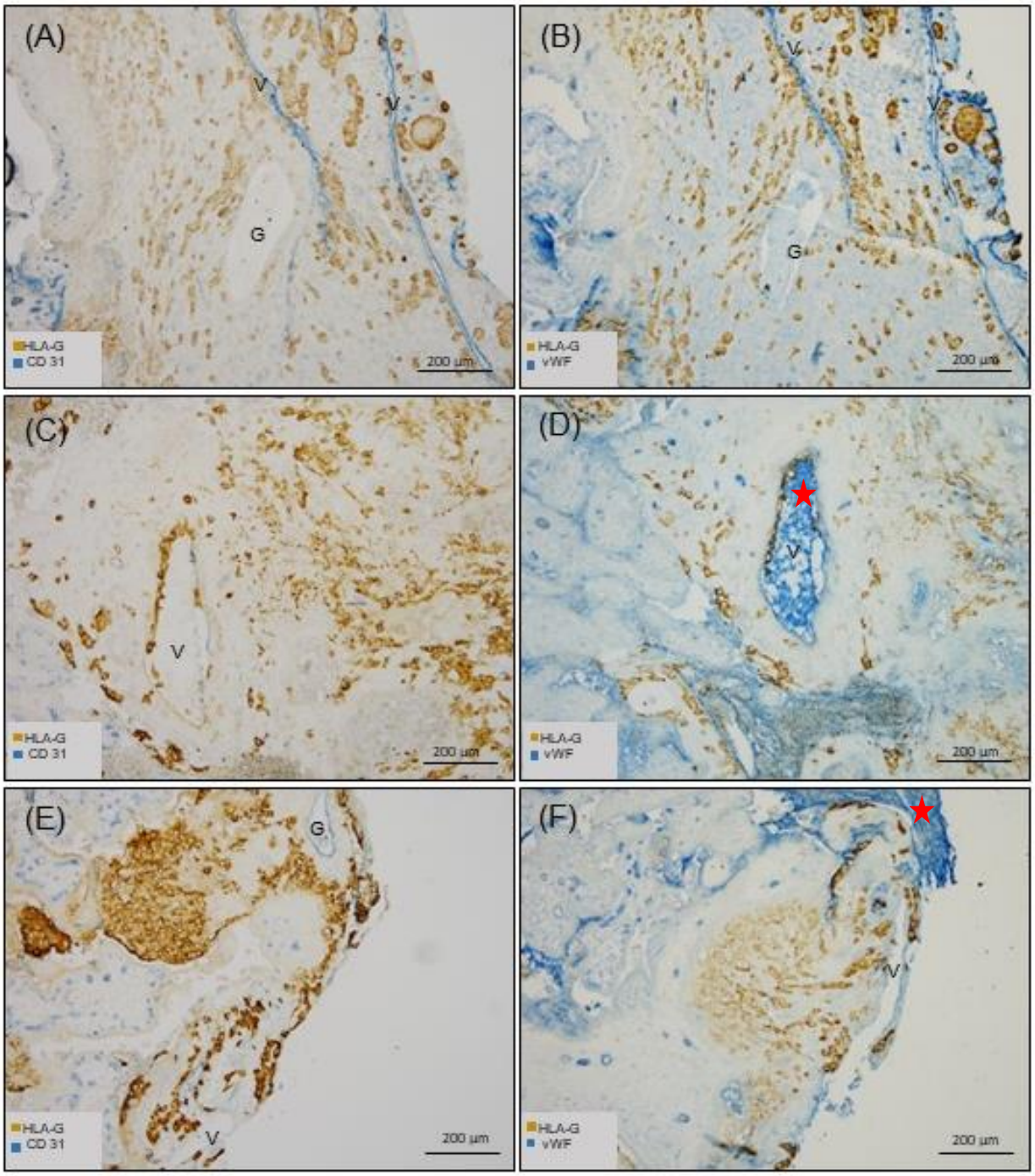

Figure 2: Testing of antibodies for the immunohistochemistry. Sections of second trimester placenta (GA=18 - 22 weeks) were double stained. In A, C and E a double staining was performed with HLA-G (marker for EVTs) and CD31 (serves as a marker for endothelium). As dilution factor for CD31, 1:100 was selected, based on the results of the single staining. In B, D and F immunohistochemistry was done with HLA-G (to mark EVTs) and vWF (to mark vessels). The double staining was performed with two different dilution factors for vWF: 1:3000 (B) and 1:2000 (B, D and F). The antibody vWF marks vessels and reacts unspecific with fibrinoid (asterisk) in the placenta. The two differently chosen dilution factors did not show a major difference. CD31 marks the endothelium of vessels, but does not react with fibrinoid. Based on these results, further immunohistochemistry was performed with CD31 to mark vessels.
Abbreviation: V=vessels, G=glands, HLA-G= major histocompatibility complex, class 1 G, CD31= cluster of differentiation 31, vWF= von-Willebrand-Faktor, GA= gestational age, red star= fibrinoid

4.2 Histological observations

Serial sections were stained with H&E (not shown in) to discriminate between specimens containing decidua and specimens without decidua. Sections without decidua and without uterine glands in the decidua were not used for further analysis. For the analysis, 146 placentas were obtained. Immunohistochemistry was performed to exclude invaded (Figure 3, B) form not invaded decidua (Figure 3, A). Overall, 20 placentas contained invaded decidua basalis and uterine glands, these were examined for quantification of trophoblast invasion.

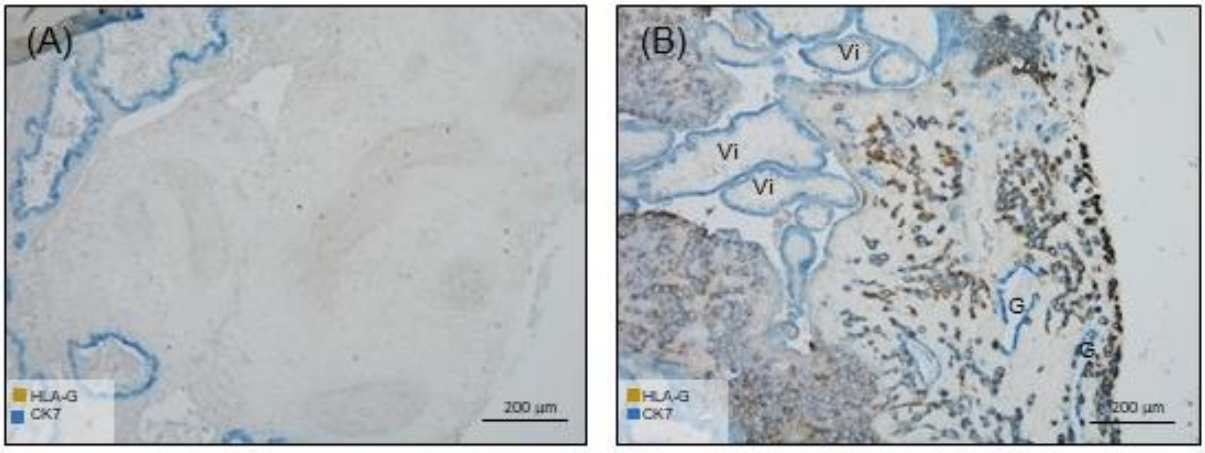

Figure 3: Invaded vs non invaded decidua. Serial sections of second trimester placenta (GA=18 weeks). Sections in (A) and (B) were immuno- double stained for HLA-G (serves as a marker for EVT, brown) and CK7 (serves as marker for glands, appears blue, but also reacts with all types of trophoblasts). In (A) a not invaded decidua is shown. In (B), the placenta is invaded by EVTs (appear brown) and uterine glands are present (blue).
Abbreviation: HLA-G= major histocompatibility complex, class 1 G, CK7= cytokeratin 7, GA= gestational age, G= glands, Vi= villi

4.3 Uterine glands are present in the second trimester of pregnancy

The presence of uterine glands within second trimester placentas was examined in H&E stained sections and additionally proved by immunohistochemistry (Figure 4). An antibody against major histocompatibility complex, class 1 G (HLA-G) was used as marker for EVT.

An antibody against Cytokeratin 7 (CK7) was used as a marker for glands - CK7 positive glandular epithelium appears blue in immunodoublestainings. Immunohistochemical double staining confirmed that EVTs are within the decidual stroma, attach to uterine glands from the basal side and invade uterine glands, thereby replacing the glandular epithelium.

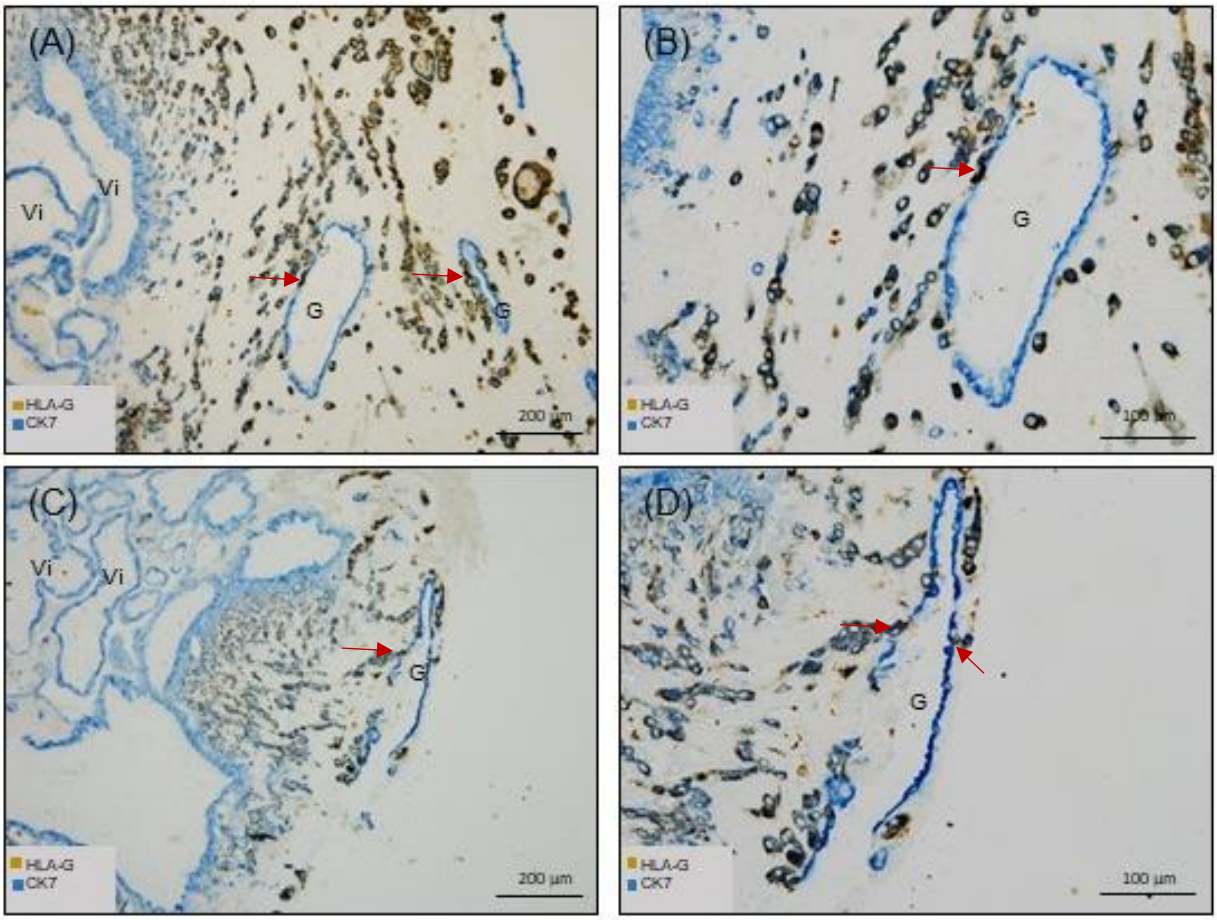

Figure 4: Uterine glands are invaded by EVTs placenta from in the second trimester of pregnancy (GA= 13 weeks). Serial sections of second trimester placenta were immuno-double stained with antibodies against HLA-G and CK7. HLAG- was used as a marker for EVTs; appears brown in (A)-(D). CK7 serves here as marker for glandular epithelium and appears blue. (A,C) Overview shows chorionic villi attaching to the maternal decidua. (B,D) higher magnification of the panel on the left side show EVTs (brown) attaching to the glandular epithelium and invading into the uterine glands (arrows).
Abbreviation: HLA-G= major histocompatibility complex, class 1 G, CK7= cytokeratin 7, GA= gestational age, G= glands, Vi= villi, EVT= extravillous trophoblast

4.4 Extravillous trophoblasts express HLA-G and CK7

Antibodies were used to discriminate between glands and vessels. The serial sections were double stained with HLA-G (serves as a marker for EVTs) and CD31 (to mark vascular endothelium). The antibody CK7 was used as a marker for glandular epithelium. Extravillous trophoblasts appear light brown in the immune double staining (Figure 5, A and C) and vessels blue. In (B) and (D) the EVTs appear dark brown compared to (A) and (C). The epithelium in (B) and (D) appears blue. EVTs express both – HLA-G and CK7. They appear dark brown in immunodouble staining.

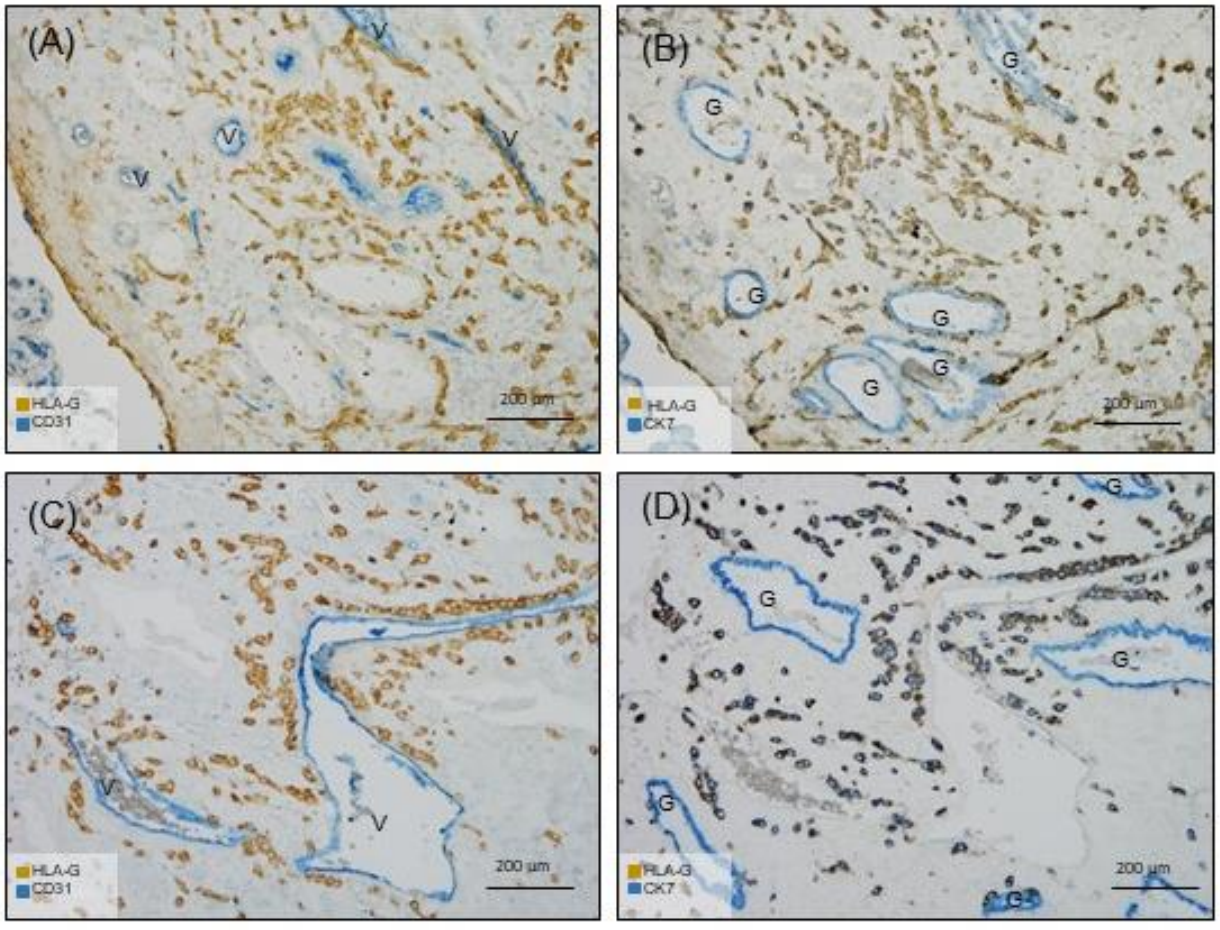

Figure 5: Trophoblasts express HLA-G and CK7. Immuno double staining was performed to distinguish between trophoblast invaded glands and vessels in the placenta (GA= 11 and 22 weeks). (A) and (C) show double staining with HLA-G (marker for EVT, brown) and CD31 (marker for vessels, blue). EVTs appear light brown, compared to (B). (B) and (D) show immuno double staining with HLA-G (marker for EVT, brown) and CK7 (marker for glands, blue). EVT express HLA-G and CK7, thus they appear dark brown in (B).
Abbreviation: V=vessels, G= glands HLA-G= major histocompatibility complex, class 1 G, CD31= cluster of differentiation 31, CK7= cytokeratin 7, GA= gestational age, EVT= extravillous trophoblast

4.5 Quantification of trophoblast invasion in serial sections

After the immunochemistry, images were taken of the serial sections for the quantification. Only images with invaded decidua included in quantification. For the quantification of trophoblast invasion from serial sections of placenta, seven categories were defined: gland invaded, vessel invaded by, not invaded gland, not invaded vessel, gland attached, and vessel attached (Table 6). Vessels and glands, where the endothelium or epithelium was replaced by EVTs, were classified as invaded. The category “attached” included vessels and glands, where ETVs were added to the endothelium or epithelium but not replaced. The third category “not invaded” include vessels and glands, which were not replaced or attached by EVTs. The category “not assignable” include luminal structures within the tissue that could not be clearly classified as glands or vessels. Examples for all categories are demonstrated in figure 6.

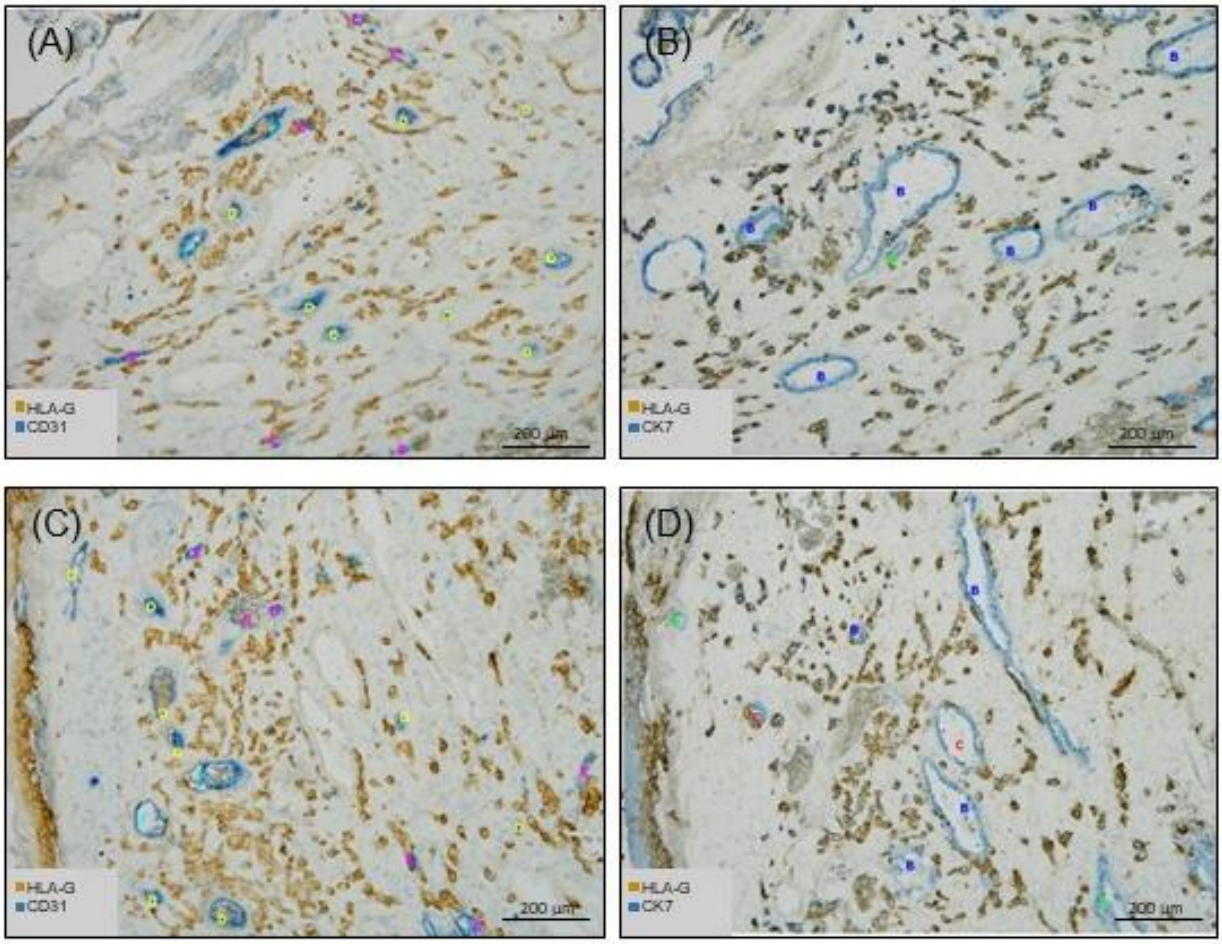

Figure 6: Quantification of trophoblast invasion in serial sections of second trimester placentas (GA=16 weeks). Sections in (A) and (C) were immuno-double stained for HLA-G (marker for trophoblasts) and CD31 (marker for vessels). Sections in (B) an (D) were immuno-double stained for HLA-G (marker for trophoblasts) and CK7 (serves here as marker for glands). For the quantification, seven categories were defined (gland invaded, vessel invaded, gland not invaded, vessel not invaded, gland attached, vessel attached not assignable). Within serial sections with the respective immune-double staining each structure was annotated respectively. Vessels and glands with extravillous trophoblasts within the lumen and/or epi/endothelium replaced by EVT were classified as invaded. Vessels and glands with attached EVTs were classified as attached. Uterine glands and vessels without EVT in the vicinity were classified as not invaded. Structures that could not be clearly classified as endothelium or epithelium were classified as not assignable. Abbreviation: HLA-G= major histocompatibility complex, class 1 G, CD31= cluster of differentiation 31, CK7= cytokeratin 7, GA= gestational age, A=glands not invaded, B=glands, attached, C= glands, invaded, D= vessels not invaded, E= vessels attached, F= vessels not invaded, G= not assignable

4.6 Negative control

In a subset of three placentas (GA=13-20 weeks) an immuno single staining with the respective Isotype negative control was performed to exclude an unspecific binding of antibodies. Negative control antibodies did not reveal any staining (Figure 7).

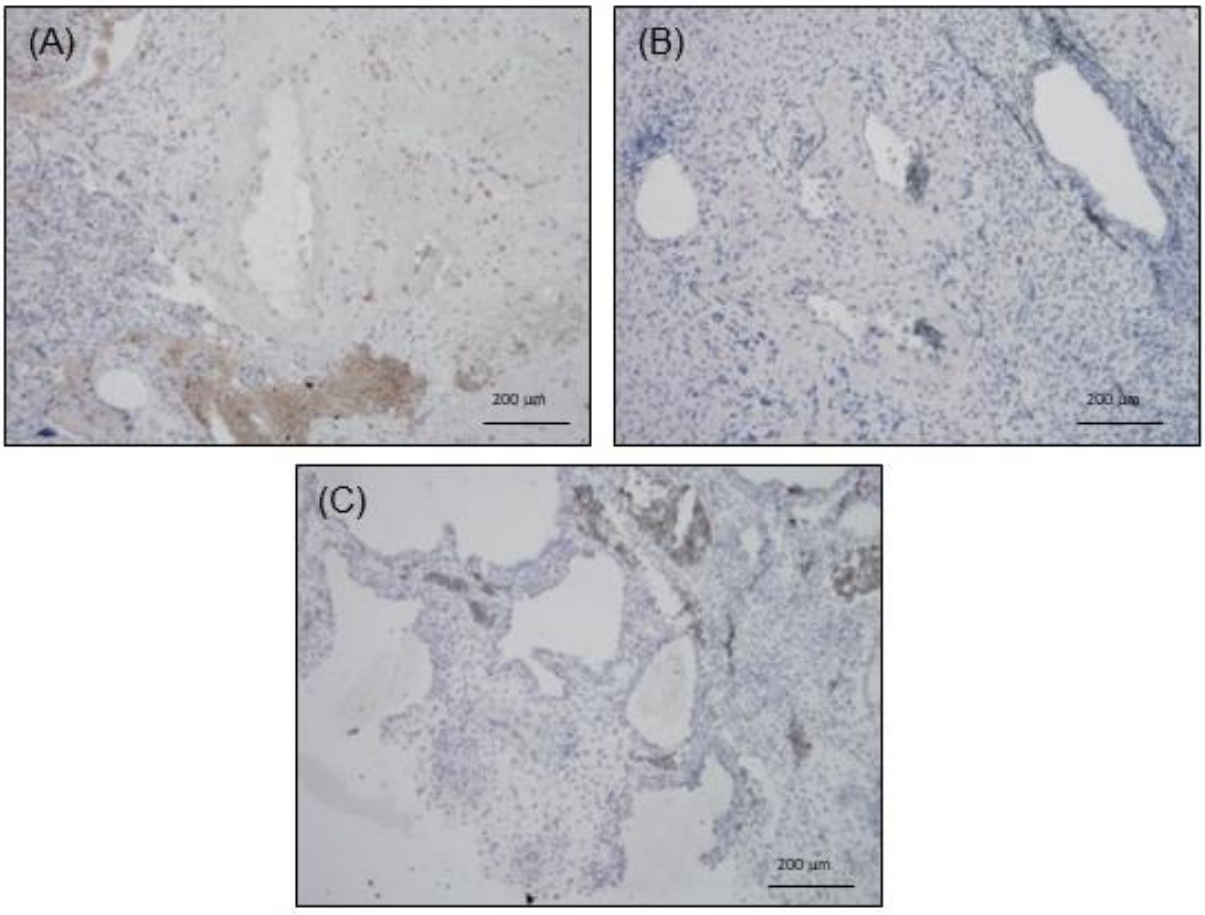

Figure 7: Negative controls. Sections of second trimester placentas (GA= 13-20 weeks) were used for the negative controls. Primary antibodies in an immunohistochemical single staining were replaced with respective IgG Isotype negative control (IgG (host rabbit) and IgG1 (host mouse)) in respective concentrations. In (A) the serial section was stained with IgG1 (dilution=1:20). The serial sections in (B) and (C) were single stained with IgG. In (B) the working dilution was 1:4750 and in (C) 1:30000. Negative control antibodies did not reveal any staining in non of the tested concentrations. Nuclear counterstain with hematoxylin.
Abbreviations: GA= gestational age, IgG=Immunoglobulin

4.7 Glands and vessels are present in the second trimester

Within the obtained images the second trimester placenta (GA= 13-20 weeks, n=20 placentas) structures like glands (27,14 ± 3,69) and vessels (30,96 ± 5,10) were present, shown graphically in figure 8. A small amount of luminal structures could not be clearly classified was also represented in the decidua (4,25 ± 0,63). Results were considered statistically significant when $p < 0.05$ (p= 0,8356).

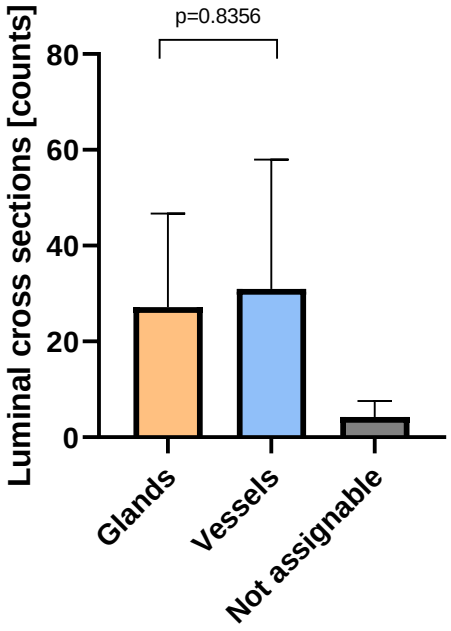

Figure 8: Structures that were found in the second trimester placenta (GA=13-20 weeks, n=20 placentas). Glands and vessels were present in the second trimester placenta (27,14 ± 3,69 and 30,96 ± 5,10 respectively). A small proportion of luminal structures could not be clearly classified (4,25 ± 0,63). Results were considered statistically significant when $p < 0.05$.

4.7.1 Glands and vessels were attached and invaded by EVTs

Quantification of trophoblast invasion within the second trimester placentas showed that both glandular and vascular structures within the decidua are attached and/or invaded by EVTs (Figure 9). Uterine glands (11,99 ± 1,74%) and vessels (8,56 ± 1,11%) are invaded by EVTs. Glands (13,98 ± 1,96%) and vessels (18,81 ± 1,80%), which were not attached or invaded by EVTs occurred too. EVTs attached to the epithelium (19,22 ± 1,77%) and endothelium (19,09 ± 1,71%) differed slightly. Structures that could not be clearly assigned as epithelium of endothelium were also present within decidua (8,36 ± 1,22%). There is no significant difference between invasion into glands and vessels.

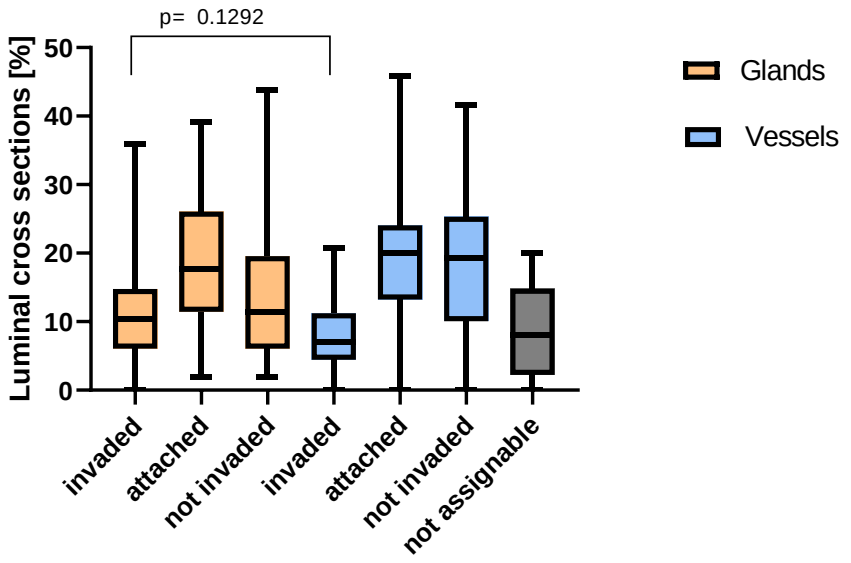

Figure 9: EVTs invade into uterine glands and vessels in second trimester placenta (GA=13-20 weeks, n=20 placentas). Seven categories of quantification (gland invaded, vessel invaded, gland attached, vessel attached, gland not invaded, vessel not invaded, not assignable). Analysis showed more replacement of epithelial cells in glands (11,99 ± 1,74%) compared with endothelial cells in vessels (8,56 ± 1,11%). Invasion of trophoblasts to glands or vessels was not significantly different. Results were considered statistically significant when $p < 0.05$.
Abbreviation: EVT=extravillous trophoblast, GA= gestational age

4.8 Trophoblasts invade into uterine glands in the second trimester placenta

A closer assessment of the uterine glands in placentas of the second trimester (n=20 placentas) showed that uterine glands were invaded and replaced by EVTS (27,47 ± 3,78%), attached by EVTs (42,99 ± 3,28%) or present but not invaded by EVTs (29,54 ± 3,25%) (Figure 10).

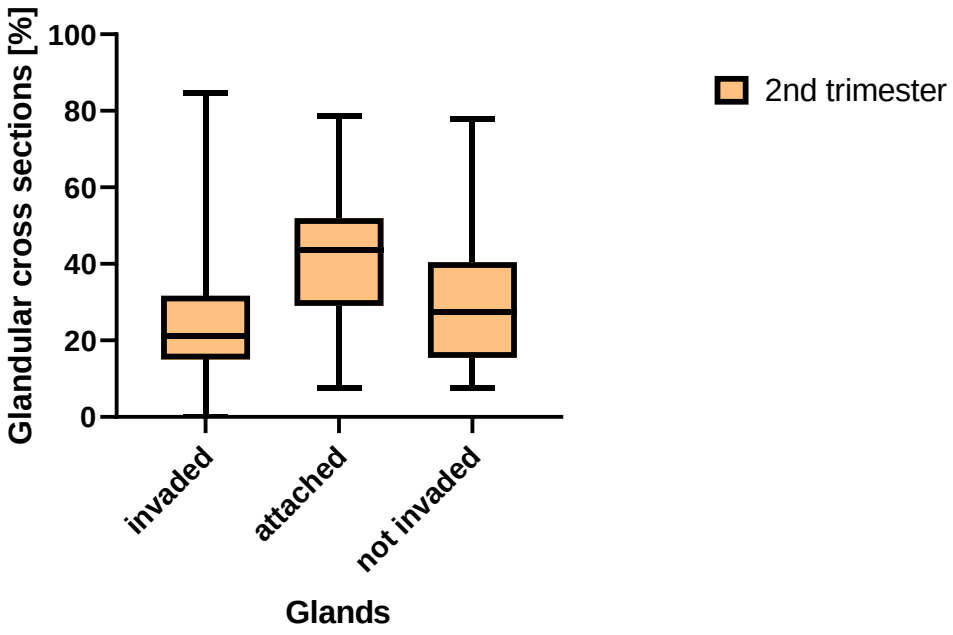

Figure 10: Quantification of trophoblast invasion into uterine glands in the second trimester (GA=13-20 weeks, n=20 placentas). Uterine glands were either invaded (24,47 ± 3,78%). A high percentage of uterine glands are attached by EVTs (42,99 ± 3,28%) or present but not invaded by EVTs (29,54 ± 3,25%).
Abbreviation: EVT=extravillous trophoblast, GA= gestational age

4.8.1 Trophoblasts invade into vessels in the second trimester of pregnancy

A closer assessment of the uterine vessels in placentas of the second trimester (n=20 placentas) showed that uterine vessels are invaded and replaced by EVTs (20,27 ± 3,04%), attached by EVTs (39,49 ± 2,64%) or present but not invaded by EVTs (40,24 ± 3,27%) (Figure 11).

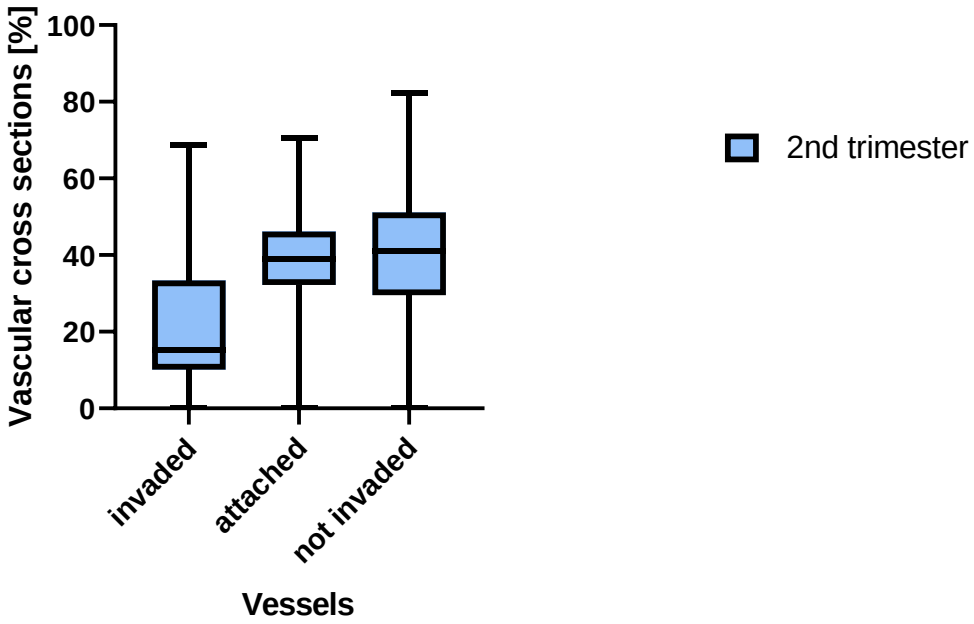

Figure 11: Quantification of trophoblast invasion into uterine vessels in the second trimester placenta (GA=13-20 weeks). Vessels are invaded and replaced by EVTs (20,27 ± 3,04%), attached by EVTs (39,49 ± 2,64%) or present but not invaded by EVTs (40,24 ± 3,27%).
Abbreviation: EVT=extravillous trophoblast, GA= gestational age

4.9 Frequency of uterine glands in second trimester placenta

Figure 12 shows graphically the amount of glandular cross sections from 13th weeks of gestation to the 20th weeks of gestation. Uterine glands are present in the decidua over the course of the second trimester. Although the number of glands within the decidua is slightly changing (from min. 15,50 to max 48,80) - between the weeks 13 – 20, there is no significant difference in the occurrence of glands over the course of the second trimester.

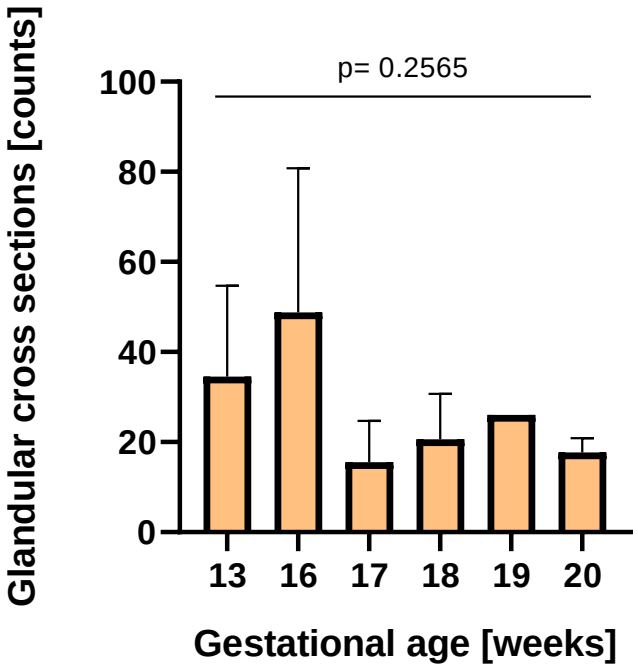

Figure 12: The frequency of uterine glands in second trimester placenta (GA=13-20 weeks, n=20 placentas) were observed. Uterine glands are present within the second trimester planta. The number of glands is changing between the weeks 13- 20. Results were considered statistically significant when $p < 0.05$.
Abbreviation: GA= gestational age

4.9.1 Trophoblast invasion at different stages of pregnancy

The trophoblast invasion into uterine glands and vessels was analyzed over the course of the second trimester and graphically presented in figure 13. In the early stage of the second trimester, 13th pregnancy week, the difference between invasion in glands (19,43 ± 4,00%) and vessels (17,46 ± 7,65%) is not major. Endothelium and epithelium are equally replaced. The trophoblast invasion into uterine glands decreased during progress of pregnancy. In the 17th week of pregnancy, there has been an enormous increase of invasion by EVTs into uterine glands. This rise decreased again. Results are not significant.

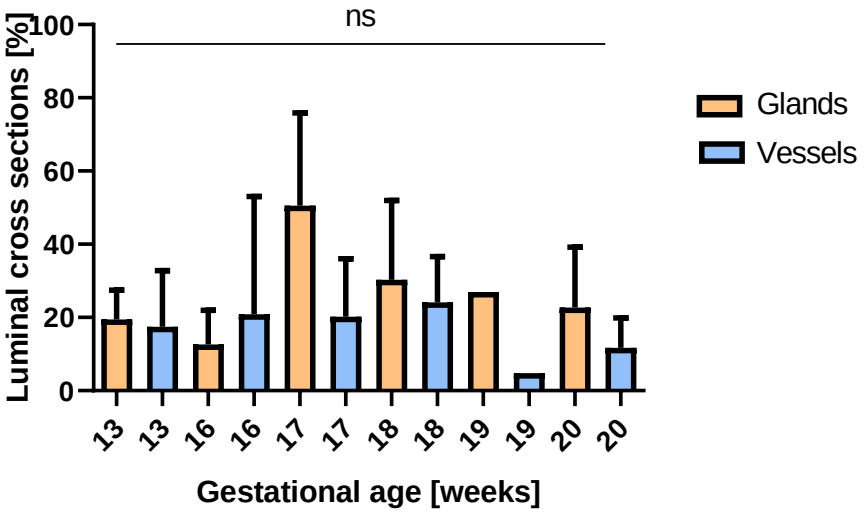

Figure 13: Trophoblast invasion into uterine glands and vessels over the course of the second trimester (GA=13-20weeks). At the beginning of the second trimester in pathological placenta, there is no big difference between glands (19,43 ± 4,00%) and vessels (17,46 ± 7,65%). Endothelium and epithelium are equally replaced. The trophoblast invasion increased at 17th week and decreased during progress of pregnancy. Results were considered statistically significant when $p < 0.05$.
Abbreviation: EVT=extravillous trophoblast, GA= gestational age

4.10 Comparison between mid-first and mid-second trimester placenta

Trophoblast invasion into uterine glands was compared between a subset of early first (GA= 5-7 weeks) and a subset of mid-second (GA= 18-20 weeks) trimester placentas, shown graphically in figure 14. In first trimester placentas, uterine glands invaded by EVTs (20,06 ± 2,96%) or attached by EVTs (11,94 ± 6,04 %) were present. A small number of uterine glands was not attached or invaded by EVTs (2,34 ± 0,84%). Compared to the second trimester, the invasion of EVTs into uterine glands increased (28,78 ± 4,82%). A rise is visible in the two defined categories: attached (45,21 ± 4,21%) and not invaded (26,00 ± 3,17%).

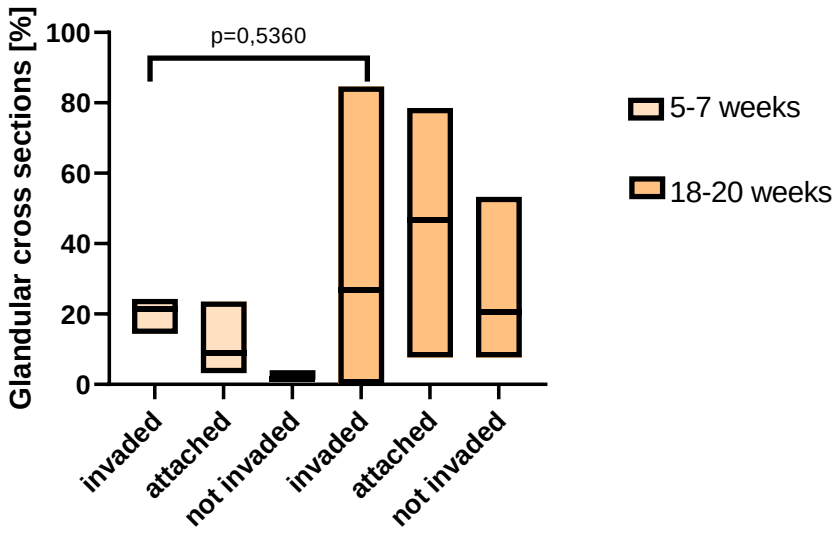

Figure 14: Quantification of trophoblast invasion: Comparison between early-first trimester (GA=5-7 weeks, n=3) and mid-second trimester (GA=18-20 weeks, n=20) placentas. In first trimester placentas, uterine glands were invaded by EVTs (20,06 ± 2,96%). EVTs attached to glands were present (11,94 ± 6,04%). Not invaded glands appeared within first trimester placenta (2,34 ± 0,84%). In second trimester placenta, the invasion into uterine glands increased (28,78 ± 4,82%). An increase is visible in the two categories: attached (45,21 ± 4,21%) and not invaded (26,00 ± 3,17%). Results were considered statistically significant when $p < 0.05$.
Abbreviation: EVT=extravillous trophoblast, GA= gestational age

4.10.1 Relative endoglandular trophoblast invasion in first and second trimester

The relative endoglandular invasion during (invaded vs non invaded glands) early-first (n=3) and mid-second (n=20) trimester pregnancy was calculated within a subset of placentas from the respective gestational age (Figure 15). To determine the significance, the p value was calculated with the raw data (p=0,0577). Results were considered statistically significant when $p < 0.05$.

Endoglandular trophoblast invasion during the mid-first trimester (gestational age of 5-7 weeks) is significantly higher (about 10-fold) compared to the mid-second trimester (gestational age 18-20 weeks).

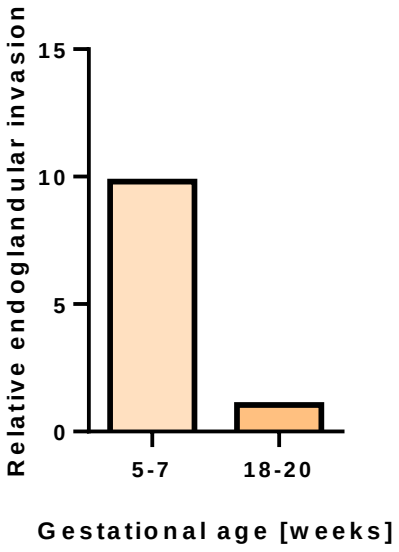

Figure 15: Comparison of the relative endoglandular invasion during mid-first trimester placenta (GA=5-7 weeks) and mid-second trimester placenta (GA=18-20 weeks). In the early-first placenta, the endoglandular invasion is significantly higher than in the mid-second trimester placenta.
Abbreviation: GA= gestational age

5 Discussion

The presence of uterine glands within the first trimester placenta is well examined and described in literature. The aim of this master thesis was to determine the presence of uterine glands within the decidua from second trimester placentas (GA= 13-20 weeks). This work show, that uterine glands are not only present in the first trimester placenta. With the help of immunohistochemistry, it was possible to show that uterine glands (Figure 4) are present in the second trimester placenta. During the first trimester of pregnancy, the uterine glands are invaded and replaced by EVTs.[19] This work showed, that endoglandular trophoblast invasion occurs as well in the second trimester of pregnancy but to a lesser extent than in the first trimester.

Until now, only placentas form the first trimester were examined with respect to endoglandular trophoblast invasion. Double-immunohistochemistry is a tool, which enables, on the one hand the discrimination between vessels and glands and on the other hand the possibility to examine the trophoblast invasion into uterine glands. The antibodies vWF and HLA-G are often used to stain serial sections of first trimester placentas.[34] vWF is a marker for endothelial cells, but reacts also unspecific with fibrinoid.[19] In the second trimester placenta a lot of fibrinoid is present. Serial sections that were double stained with vWF made it difficult to recognize vascular endothelium. For this reason, the immune-doublestainings were performed with CD31 instead of vWF. HLA-G was used as a marker for EVTs whereby the different HLA-G isoforms need to be considered.[36] CD31 served as a marker for vessels (veins and arteries).
Further, CD31 marks lymphatic vessels too.[37] The trophoblast invasion of glands and vessels within the first trimester was compared with the invasion of glands and vessels within the second trimester placenta. Therefore, it is possible that a false identification has occurred and may skew the comparison. Additional methods and stainings of serial sections with markers for the lymphatics (e.g. Lyve-1, Podoplanin) and markers for the smooth muscle layer of spiral arteries would be possible for discrimination between arteries, veins and lymphatics.[37]

In Moser et al. (2017) the invasion of EVTs into uterine glands and vessels during the first trimester was described.[20] The uterine glands are opened toward the intervillous space and the histiotrophic nutrition is established.[16] Only at the beginning of the second trimester the maternal blood flows towards the placenta.[20] So far this was the state of knowledge from previous research.

The work of this master thesis showed that the uterine glands are invaded, and the epithelium gets replaced by EVTs at the beginning of the second trimester (GA=13 weeks) and continues to the end of the second trimester (GA=20 weeks). The figure 15 represents the relative endoglandular invasion in earls-first trimester compared with the mid-second trimester. The invasion is about 10-fold higher compared to the mid-second trimester. During the first trimester, the nutrition is enabled by uterine glands (histiotrophic nutrition). At the end of the first trimester, the histiotrophic nutrition is replaced by the hemotrophic nutrition when the trophoblast plugs disintegrate, and the maternal blood reaches now the intervillous space.[16] [17] [18] [19] A reduction of endoglandular trophoblast invasion from first to second trimester would be expected during the switch from histiotrophic to hemotrophic nutrition and thus makes sense in terms of placental development.

Experiments were performed with placentas from pregnancy pathologies. For obvious ethical reasons there is no access to second trimester placentas from healthy placentas. All included second trimester placentas had the same pathology – chorioamnionitis. This a common infectious disease during pregnancy. This specific infection was selected, because the placenta develops putative normal and the infection probably does not interfere the trophoblast development and routes of trophoblast invasion.
In order to make a statement about the trophoblast invasion into uterine glands and vessels over the course of the second trimester, the amount of available second trimester pathologies were too low. For this research the amount of obtained second trimester placentas were different for the various weeks of pregnancy, shown in table 1. Placentas for the weeks 14 and 15 of pregnancy were not obtainable. For the week 19 only one case was available.

In conclusion, the presence of uterine glands and their invasion by extravillous trophoblasts in the second trimester placenta was proved. However, uterine glands are invaded significantly more in the first trimester compared to the second trimester. This emphasizes, that endoglandular trophoblast invasion has more impact on early placental development, than hitherto assumed. More research needs to be done on this field of work since the amount of obtainable placentas is limited due to ethical restrictions. Nevertheless, the quantity of placentas for the research needs to be much higher to give a significant statistical proof for the presence of glands and endoglandular trophoblast invasion during the second trimester. Therefore, the work of this master thesis can be seen as a fist steppingstone for further research in this field.

6 Abbreviations

CC	Clinical chorioamnionitis
CD31	Cluster of differentiation 31
CK7	Cytokeratin 7
EVT	Extravillous trophoblast
G	Glands
GA	Gestational age
H&E stain	Haematoxylin and eosin stain
HC	Histological chorioamnionitis
HLA-G	Major histocompatibility complex, class 1 G
lgG	Immunoglobulin G
lgG1	Immunoglobulin G1
mc	Monoclonal
NK cells	Natural killer cells
p.c.	post conception
pc	Polyclonal
V	Vessels
Vi	Villi
vWF	von Willebrand factor

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8 Protocols

Dissection:

Equipment	Company/Catalogue	Purpose
Cooling trough	TUC 1, Tube cooler, histo com	Cooling of paraffin block
Rotational microtome	HM 355 S, MICROM, Zeiss, Germany	Sectioning of paraffin block
Blades	Feather microtome blade, A35 Type	Trimming, cutting
Microscope slides	Menzel-Gläser, Superfrost Plus, Thermo Scientific	Adhesion of section
Filter paper		Filtering of excess water
Streching table	TFP 40, MEDITE, Germany	Drying of section
Needle		Separating and collection of sections

Steps	Time
Dissection • Precooling of the paraffin block in the cooling through • Paraffin block placed in Cool cuter and positioning • Trimming • Cutting of serial sections (11), 6 µm • Unfold serial sections in water bath • Placed on slides • Drain slides • Dry slides on heat plate (42°C) • Dry slides on heat plate (52°C)	20 min 1 h overnight

Deparaffination:

Equipment	Company/Catalogue	Purpose
Histolab-Clear		Deparaffination of sections
100% EtOH 96% EtOH 70% EtOH 50% EtOH		Dehydration of sections
Aqua distilled		

Steps	Time
Tissue Clear	4 x 5 min
Tissue Clear and 100% EtOH (1:1 mixture)	Slewing and drain to paper towel
100% EtOH	Slewing and drain to paper towel
96% EtOH	Slewing and drain to paper towel
70% EtOH	Slewing and drain to paper towel
50% EtOH	Slewing and drain to paper towel
Aqua distilled	Slewing and drain to paper towel

Antigen retrieval:

Equipment	Company/Catalogue	Purpose
Dako Target Retrieval Solution (10x), pH 9	Dako Denmark A/S, REF S2367	Heat induced epitope retrieval
KOS EM	Milestone Medical	Heat induced epitope retrieval

Steps	Time
Transfer slides into cuvette filled with buffer	
Choose antigenretrieval program, 93°C, 16 slides, 15 min, pH9	30 min
Cool down in buffer without lid	20 min
Transfer slides into cuvette filled with water	Overnight, in refrigirator

Immunohistochemistry:

Equipment	Company/Catalogue	Purpose
Moist chamber		Maintenance of humidity
Paper towel		Absorb liqiuds
Dako Pen	Dako, Denmark S2002	Tissue surrounding
Dako Dual Endogenous Enzyme Block	Dako, Denmark, S2003	Suppresses endogenous alkaline phosphatase and peroxidase
Polink DS-MR-Hu A1 Kit • HRP Polymer anti-Mouse • AP Polymer anti-Rabbitt • DAB Substrate • DAB Chromogen	GBI Labs, DS201A-6	Immunohistochemistry Staining
TBS (20x) + 0,05% Tween		Washing buffer
Vector Blue AP Substrate Kit	Vector Laboratories, USA, SK-5300	Chromogen
Kaiser´s glycerol gelatin	Merck, 109242	Aqueous mounting medium

Steps	Time
Prepare a moist chamber, with Aqua distilled	
Surround with Pap Pen the tissue	
Cover the tissue with Dako Dual Endogenous Enzyme Block	10 min
Wash with TBS-T	3 times
Incubate with primary antibody mix	30 min
Wash with TBS-T	3 times
Incubate with secondary antibody mix, 1:1 (for 1 slide: 1 drop of R1+1 drop R2)	30 min
Wash with TBS-T	3 times
Cover the tissue with Reagent 3A+3B (Two drops of Reagent 3B add to 1 ml Reagent 3A, adopt to the size of tissues, mix well, protect from light, DAB is carcinogenic)	5 min
Wash with Aqua distilled	1 time
Wash with TBS-T	3 times
Prepare Vector Blue Substrate 2,5 ml 100mM – 200 mM Tris-HCl, pH 8,2-8,5 Add 1 drop of Reagent 1+ 1 drop of Reagent 2+ 1 drop of Reagent 3 Mix well, protect from light, cover the tissue	10 min
Wash with Aqua distilled	1 time
Cover with Kaiser´s glycerol gelatin	