UiT Norges arktiske universitet

Faculty of Health Sciences

Isolation of trophoblast cells from first trimester placentas

Optimizing a protocol for future investigation of placenta and platelet alloimmunization

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Preface

For as long as I can remember, I have taken a special interest in women's health and mother-child related issues. Ever since I started my medical studies, my interest in this field has increased. Going through the subjects of embryology, gynaecology and obstetrics, I knew without a doubt that this was the field of subject in which I wanted to write my master thesis. Furthermore, I wanted to challenge myself and go beyond my own comfort zone, and a project including laboratory work sounded like a good fit.

This project is part of the larger project "Platelets and placenta – the new hotspot in fetal-maternal crosstalk", which is led by Heidi Tiller of the Women's Health and Perinatology Research Group at UiT – The Arctic University of Tromsø. The goal of this project was to optimize the first steps of an in-house protocol for isolating trophoblast cells from first trimester placentas, so that these cells could be used for further research.

There are several people who deserve thanks in relation to this project. I would like to thank the nurses at the Maternity Outpatient Clinic at UNN Tromsø for their contribution by helping to recruit women to the project and for the good communication we have had during the project period. Thank you to Mona Nystad, for contributing with proof reading and academic input and feedback.

Furthermore, I would like to thank my supervisor Nora Hersoug Nedberg and co-supervisor Heidi Tiller. Thank you, Heidi, for introducing me to the project, encouraging me to go beyond my comfort zone, and for your academic inputs and feedback. Thank you, Nora, for all the hours spent walking me through and helping with the laboratory work, the feedback, for the good brainstorms and conversations we have had, and for giving me invaluable pep-talks along the way.

Finally, I would like to thank my family, friends and most especially my partner, Kristoffer, for the support, for enduring my endless babbling about the project and for keeping me motivated during the process of work and writing.

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Summary

Background: Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a pregnancy complication caused by an HPA-incompatibility between mother and fetus. In Caucasians, mainly maternal anti-HPA-1a antibodies cause FNAIT. It has been hypothesized that maternal anti-HPA-1a antibodies might affect not only the fetal platelets, but also the placenta, in alloimmunized pregnancies. However, the link between maternal platelet antibodies and placenta is far from fully understood. Investigating the properties of live trophoblast/EVT cells in the context of anti-HPA-1a antibodies would be an important part in unravelling these mechanisms. Therefore, the aim was to optimize the first steps of a protocol for isolation of trophoblast cells from first trimester placentas. If successful, the protocol could be used in future research on how anti-HPA-1a antibodies are related to placenta.

Method: Existing protocols were used as basis to test two different strategies for trophoblast isolation, which mainly differed in the enzymatic processing of the tissue. Strategy 1 utilized trypsin-EDTA and collagenase I, whereas strategy 2 utilized trypsin-EDTA and DNase I.

Results: Of eight conducted experiments, four were considered partly successful, in that cells had adhered to the fibronectin coated plate, in turn indicating that the cells had differentiated overnight.

Conclusion: Strategy 2 seemed to be superior to strategy 1. However, the protocol still requires further testing and adjusting before it is fully optimized. Flow cytometry is warranted to confirm differentiation of trophoblasts to EVT cells.

Abbreviations

EVT cells	extravillous trophoblast cells
FBS	fetal bovine serum
FNAIT	fetal and neonatal alloimmune thrombocytopenia
hCG	human chorionic gonadotropin
HLA	human leukocyte antigen
HPA	human platelet antigen
ICH	intracranial haemorrhage
ICM	inner cell mass
IUGR	intrauterine growth restriction
IVIg	intravenous immunoglobulins
MHC	major histocompatibility complex
PBS	phosphate buffered saline
PVO	Norwegian: Personvernombud (English: Data Protection Official)
P/S	Penicillin/Streptomycin
RhD	rhesus D
RPMI	Roswell Park Memorial Institute
RT	room temperature
SGA	small for gestational age
TE	trophectoderm

1 Introduction

In the following sections, topics related to the thesis will be presented. First, a general presentation of the condition fetal and neonatal alloimmune thrombocytopenia (FNAIT). Next, placental development in the first trimester will be presented, highlighting why normal placental function is so vital and why even more extensive knowledge of cell properties and cell interactions in early placentation is warranted. Finally, how FNAIT and placenta may be linked, and how human platelet antigen antibodies may affect the placenta as well as the fetus in a pregnancy.

1.1 Fetal and neonatal alloimmune thrombocytopenia

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is a pregnancy complication caused by an incompatibility in human platelet antigens (HPA) between mother and fetus. If the fetal platelets possess a paternally inherited platelet antigen which the mother does not have, it can lead to alloimmunization of the mother (1). This causes the mother to produce IgG alloantibodies targeting the incompatible fetal platelet antigens. These antibodies may cross the placenta and bind the fetal platelets, in turn leading to platelet destruction, causing thrombocytopenia and increased risk of bleeding in the fetus and/or newborn. Of the possible complications caused by FNAIT, intracranial haemorrhage (ICH) is the most feared.

1.1.1 Epidemiology

Although FNAIT is rare, it is the leading cause of severe term neonatal thrombocytopenia (2). FNAIT has an incidence of approximately 1 in 1000-1100 live births (2-7), but it has been proven to be significantly underdiagnosed (8, 9). FNAIT-related ICH occurs in approximately 1 per 10 000 neonates, most often before birth (2).

1.1.2 Pathophysiology and HPA-1-alloimmunization

The most common cause of FNAIT in Caucasian populations is HPA-1a-incompatibility; a pregnancy in which the mother has the less common HPA-1bb-allotype and the fetus has a

paternally inherited more common HPA-1a-allotype (**Figure 1**). HPA-1a incompatibility causes up to 85% of FNAIT cases in Caucasians (1, 3, 5). There are several other possible HPA-incompatibilities that can cause FNAIT; in African-Americans, incompatibilities of HPA-2 and HPA-5 are more frequent (10), and in Japanese populations, HPA-4 and HPA-5 incompatibilities are the most common (11). To further complicate matters, combinations of different HPA-incompatibilities can also be present in the same pregnancy.

Human platelet antigen 1 (HPA-1) is expressed on the β3 integrin as part of the αIIbβ3 integrin complex, also known as the fibrinogen receptor, and is widely expressed on platelets (3). HPAs are expressed on fetal platelets as early as gestation week 16-18 (12). The β3 integrin also makes part of the αVβ3 integrin complex (the vitronectin receptor), which is expressed on other cell types, such as syncytiotrophoblast cells, invasive trophoblasts, and vascular endothelial cells (3, 5). The αVβ3 integrin complex is important for angiogenesis (3).

The HPA-1-system is defined as a leucine/proline polymorphism at residue 33 in the β3 integrin (13), meaning that only one amino acid differs between genotype HPA-1a and HPA-1b. HPA-1a is the most frequent allelic genotype in Caucasian populations, while only 2% of women have HPA-1bb. Only 10% of HPA-1bb women develop anti-HPA-1a antibodies during an HPA-1a incompatible pregnancy (8). Furthermore, there is a strong correlation between HPA-1a-alloimmunization and the presence of HLA-DRB3*0101 (9, 13-15), which is a haplotype expressed on human MHC class II.

Fetomaternal HPA-1 incompatibility is the main foundation for maternal alloimmunization to take place. In addition, there must be a break of tolerance, mainly due to the maternal immune system having a direct “encounter” with fetal cells that express the incompatible HPA-1a epitope. This could happen during a smaller fetomaternal haemorrhage, a common occurrence, as fetal blood platelets could enter the maternal circulation and thereby alert the maternal immune system. It is also believed that the HPA-1a antigen from shed trophoblast microparticles can cause maternal HPA-1a alloimmunization if they were to enter the

maternal blood circulation (16). The exact mechanism that leads to the alloimmunization in FNAIT is not fully known, and there is still further research needed on the matter.

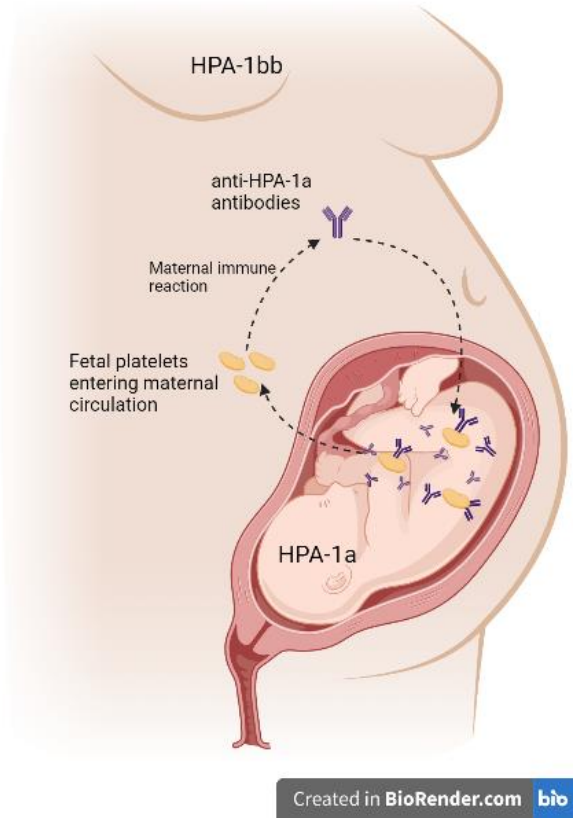

Figure 1: Pathophysiology of an HPA-1-incompatible pregnancy in which the mother is HPA-1bb and the fetus is HPA-1a. If fetal cells (platelets or trophoblasts expressing HPA-1a on the cell surface) enters the maternal blood circulation, the mother's immune system will recognize the fetal cells as foreign, triggering an immune response producing antibodies (anti-HPA-1a). These antibodies may cross the placenta and target fetal platelets expressing HPA-1a for destruction, leading to thrombocytopenia, and thus FNAIT. Illustration created with BioRender.com.

A previous large prospective study in Norway found that 75% of HPA-1a immunizations happened during delivery and only 25% during the first pregnancy (17). Williamson *et al* had similar findings in an English observational study (14). The Norwegian study also found a strong correlation between levels of anti-HPA-1a antibodies in maternal blood and severity of thrombocytopenia in the neonate. Although some studies have disputed such a correlation (9, 18), a systematic literature review concluded that an association is present between maternal anti-HPA-1a antibody levels and neonatal platelet count (19). Studies done in mice

have also found a similar correlation between levels of maternal anti-HPA-1a antibodies and severity of thrombocytopenia (20).

1.1.3 Clinical presentation and diagnosis

Generally, the lower the platelet count, the higher the risk of bleeding. Thrombocytopenia is defined as a platelet count < 150×10^9/L. Thrombocytopenia can also be divided into different grades of severity. Mild thrombocytopenia is defined as a platelet count between 100-149 × 10^9/L, moderate thrombocytopenia as a platelet count between 50-99 × 10^9/L, and severe thrombocytopenia is defined as a platelet count < 50×10^9/L (21, 22). In the case of FNAIT however, thrombocytopenia is probably not the only trigger for bleeding; the current focus revolves a direct antibody-mediated endothelial damage. Clinical manifestations of FNAIT vary from none to mild skin bleedings (petechiae) to severe ICH (5). There have also been reports of internal bleeding and organ haemorrhage (23). FNAIT is the most frequent cause of neonatal severe ICH in otherwise healthy term newborns (24), and it is the most known and feared bleeding complication in neonates as it can cause severe lifelong neurological damage and deficits, or in worst case, death. FNAIT-neonates with ICH often have worse outcomes than neonates with ICH from other causes (24).

The Norwegian guidelines for FNAIT state that a complete examination and confirmation of diagnosis includes blood samples from mother, father and child – to confirm HPA-incompatibility, measurement of maternal anti-HPA-antibodies and platelet count in the neonate (21). Some neonates display no symptoms of FNAIT and in these cases the diagnosis may go by unnoticed and unregistered. Undiagnosed, the neonate is left with increased risk of ICH or other bleeding complications until spontaneous increase in platelets occurs. In addition to the clear risk for the neonate, it also leaves the mother unknowing of increased risk of fetal/neonatal bleeding complications in a subsequent pregnancy (2).

1.1.4 Screening

As of today, there is no screening programme for FNAIT, but it has for a long time been, and still is, under discussion whether it should be implemented or not (4-6, 9, 15, 25-29). Many who advocate for screening programmes, argue that it should be done antenatally. Antenatal screening would, unlike neonatal screening, enable early detection of FNAIT among primigravidas as well as multiparas. This would further enable early intervention, a closer follow-up of the pregnancy and plan for facilitation of delivery with necessary resources available. A majority of FNAIT-related ICH develop in utero (2, 30), which makes for another strong argument that screening should be done antenatally if it is to have any preventative effect of the current pregnancy. A study by Tiller *et al* showed that only 14% of the expected cases of FNAIT in Norway are diagnosed without a screening programme (8), meaning that 86% of cases go by undetected and untreated.

Despite the expenses of large-scale testing and costly treatments (e.g., intravenous immunoglobulins (IVIg)), screening for FNAIT may prove to be cost-effective. The high burden of disease for the affected child and their family, in addition to high costs for health care and society in case of FNAIT-related ICH with brain damage, most likely outweigh the expenses of large-scale testing and treatment (2, 27).

An argument that complicates the screening debate is that FNAIT, per now, does not fully meet the World Health Organization's "checklist" for screening programmes (25). To fulfil the criteria, the incidence and consequences of severe cases still needs further investigation on a populational level. Especially, good data on a low-risk population is needed to better establish incidence, consequence, and severity of FNAIT. A review article on screening studies by Kamphuis *et al* found that all the reviewed studies offered antenatal or intrapartum interventions, meaning that the outcomes of the studies were biased by the interventions (2). The same article pointed out that studies without interventions during pregnancy are needed to determine unbiased outcomes of FNAIT-pregnancies (2).

1.1.5 Risk stratification and management

Currently, there is no preventative treatment that protects against immunization, but an antibody-mediated prophylaxis is in the stage of clinical trials (31). FNAIT was previously not believed to benefit from a prophylactic treatment due to a general perception that HPA-1a-immunization happens mainly during the first pregnancy. However, this has been disproved, as results from larger prospective studies indicate that most immunizations seem to happen during delivery (14, 31). A prophylaxis with anti-HPA-1a could possibly work similarly to anti-RhD. Studies done in mice suggest that anti-β3 antibodies may prevent maternal immunization and thrombocytopenia and bleeding complications in the fetus/neonate (32). In similarity to RhD, HPA-1-typing could be done early in the pregnancy in order to identify those eligible for the prophylaxis (31).

Women who are identified to be of high risk for having a child with FNAIT (i.e., women with a prior history of a FNAIT pregnancy or HPA-1bb-typed women) should be assessed more closely to determine degree of risk of severe FNAIT and decide the most appropriate management for each woman/pregnancy (21). One factor that could be used for further risk assessment could be a thorough obstetric history, i.e., if the woman has a history of previous intrauterine fetal death, previously given birth to FNAIT-neonate and severity of the bleeding symptoms in the neonate, etc. Further, the mother could be HLA-DRB3*0101-typed – if she is HLA-DRB3*0101-positive, she is at greater risk for alloimmunization than if she is HLA-DRB3*0101-negative (14, 33). The pregnant woman could also be monitored for levels of anti-HPA-1a antibodies throughout the pregnancy. According to the Norwegian guidelines, if maternal anti-HPA-1a antibody levels exceed 3 IU/ml, the woman should be assessed at a centre for fetal medicine and conduct control ultrasound to assess for ICH and fetal growth, as well as taking blood samples every fourth week to monitor antibody levels (21). One could also conduct HPA-1-typing of the father to determine whether he is HPA-compatible to his partner or not. Another option for risk assessment is non-invasive prenatal testing (NIPT), which detects cell-free fetal DNA in maternal blood and could be used to HPA-1-type the fetus in suspected high-risk pregnancies (21). This is not yet common practice in Norway, but it is

currently being used clinically in The Netherlands, and it is possible to send samples from Norway if indicated.

There are treatments available for identified FNAIT-pregnancies or assumed high-risk pregnancies to potentially lessen the risk of severe bleedings in the fetus/neonate. Currently, preventative measures are mainly taken in subsequent pregnancies of those with known previous FNAIT-pregnancies (2). Antenatal treatment can be divided into invasive interventions and non-invasive interventions. Examples of invasive interventions are fetal blood sampling and intrauterine platelet transfusion. Fetal blood samplings can be used to detect antenatal thrombocytopenia, but this comes with inherent risks of bleeding (e.g., umbilical haemorrhage), possible boosting of antibody levels, emergency (preterm) caesarean section and fetal death (34, 35), and is therefore disrecommended by most.

Treatment protocols vary between nations, sometimes even between hospitals, and there is no international consensus about the antenatal management of high-risk pregnancies. The Norwegian guidelines and management model differ from most other Western countries, as the use of maternal IVIg treatment is almost solely restricted for those who have previously given birth to a FNAIT-neonate with ICH (36). Meanwhile, many other Western countries practice off-label administration with weekly high-dose IVIg to all pregnant women with a previous history of a FNAIT-pregnancy (37). IVIg is said to reduce the incidence of fetal ICH in high-risk pregnancies (12, 38). However, a newly published retrospective study from Norway comparing the frequency of FNAIT-related ICH in IVIg-treated and non-treated pregnancies, found that omitting antenatal IVIg treatment did not increase ICH-risk in low-risk pregnancies (37), thereby adding defence to the Norwegian guidelines. Caesarean section is often part of management protocol of FNAIT, though it needs further investigation to see if it has any impact on risk of ICH (30, 36).

1.2 Placenta

The placenta origins mostly from fetal tissue, and it is a vital part of pregnancy. It is responsible for hormone production (e.g. human chorion gonadotropin (hCG)) and controls nutrient, gas (oxygen) and waste (carbon dioxide) exchange between the mother and the fetus (39). Maternal IgG antibodies may cross the placenta, providing the fetus with passive immunity against various infectious diseases (39).

1.2.1 Placental development in the first trimester

Placental development starts early after fertilization and develops alongside the embryo (**Figure 2**). Approximately 3 days after fertilization, the embryo is in its morula-stage, consisting of one inner and one outer layer of cells. The inner cell mass (ICM) make for embryonic tissues and the outer cell layer forms the trophectoderm (TE), which later gives rise to the trophoblast. Trophoblast cells are vital for implantation and placentation – processes in which trophoblast cells form placental tissue and infiltrate the uterine mucosa (endometrium), so that the blastocyst (pre-stage embryo) can attach itself to the uterine wall within the end of the first developmental week. Proper implantation depends on mutual contribution from trophoblastic and endometrial cells (39, 40).

The site where the TE adjoins the ICM is called the polar TE (41). Cells at the polar TE develop, forming a structure in which proliferative, mononucleated cytotrophoblast cells make up the inner part, lined with a non-proliferative, multinucleated cell mass of primitive syncytium. The primitive syncytium is highly invasive, and it starts invading the uterine tissue as soon as the blastocyst attaches to the endometrial epithelium. The endometrium decidualizes, i.e., it becomes more oedematous and vascularized, facilitating for optimal conditions of implantation. By the end of the second gestational week, the embryo is enclosed by primitive syncytium and is fully implanted in the endometrium (41, 42).

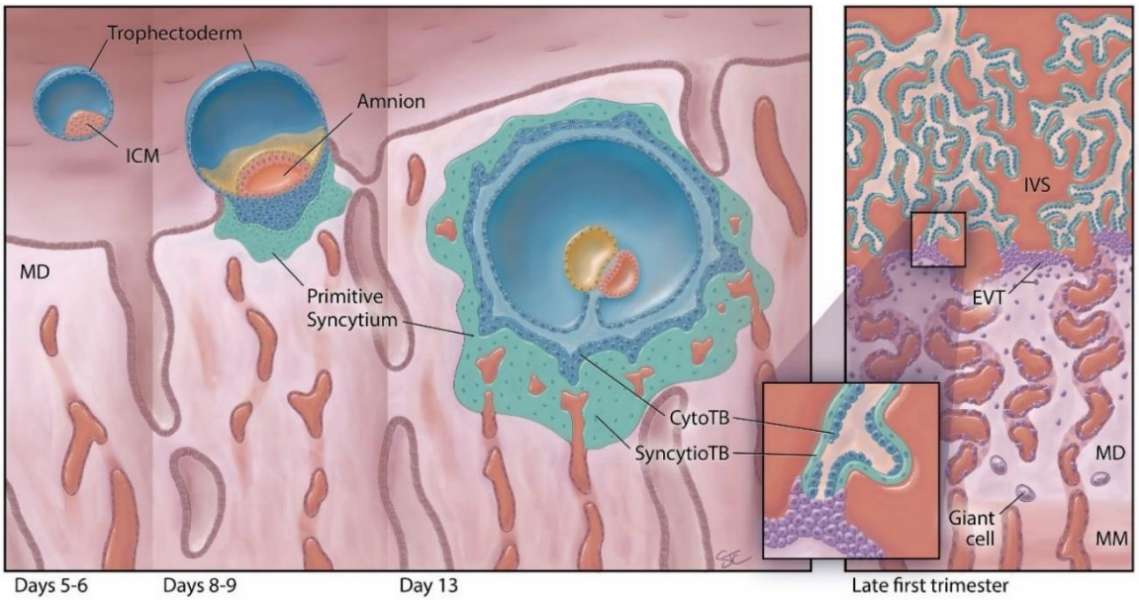

Figure 2: Placental development of the first trimester. ICM = inner cell mass, MD = maternal decidua, CytoTB = cytotrophoblast cells, SyncytioTB = syncytiotrophoblast cells, IVS = intervillous space, EVT = extravillous trophoblast cells, MM = maternal uterine myometrium. Illustration made by medical illustrator Stacy Cheavens at MIZZOU – University of Missouri, reused with permission from authors and publisher (42).

From the cytotrophoblasts, primary villi arise, protruding out into the syncytium and creating invaginations of the cytotrophoblast layer (42). Small lacunae start to form in the syncytium, gradually fusing and enlarging, eventually forming the intervillous space. The primary villi develop into secondary villi when extraembryonic mesenchymal cells grow into the core of the villi. As the extraembryonic mesenchymal cells differentiate and create villous capillaries, the villi are turned into tertiary villi (also called definitive placental villi) (39-42). The villous capillaries connect to the chorionic plate and the connecting stalk (which later becomes the umbilical cord), thereby establishing the fetoplacental circulation. As the villi develop from primitive to secondary to definitive, some of the cytotrophoblast cells proliferate and infiltrate their way through the tips of the villi and the surrounding syncytium. These cytotrophoblast cells connect with cytotrophoblastic extensions from other villi, forming an outer cytotrophoblast shell, which eventually encloses the syncytium entirely, meaning that cytotrophoblast cells constitute the barrier between the placenta and the uterine tissue (41, 42). Syncytiotrophoblast cells make up the outer lining of the definitive placental villi and are thus in direct contact with maternal blood flowing into the intervillous space (41). It is over

this very layer of cells that nutrients, gaseous products, hormones, proteins and waste products are transported (41, 43). Villi that stretch from the chorionic plate to the uterine tissue are called anchoring villi, and they constitute a mechanical anchor of the placenta, hence why they are named anchoring villi (41, 42). By the beginning of the second trimester, the placenta is characterized by villous structures and further development mainly consists of growth and further expansion of the villous tree (**Figure 3**).

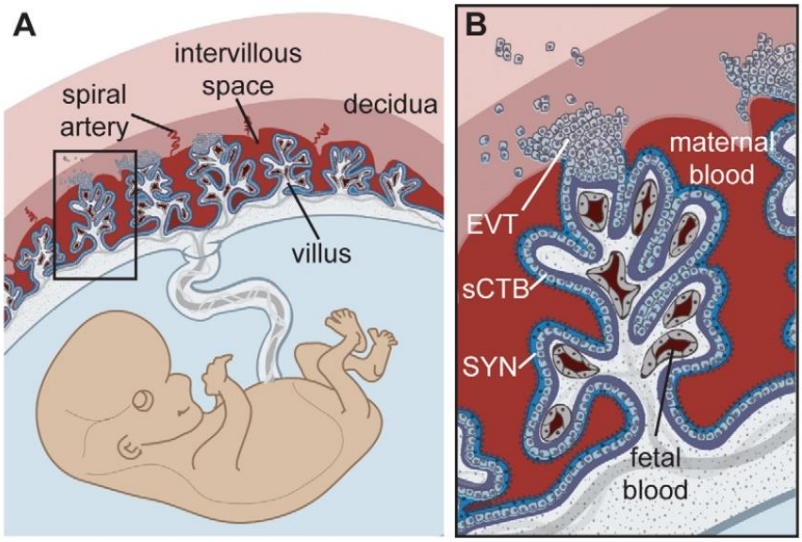

Figure 3: Placental structures when fully and normally developed. EVT = extravillous trophoblast cells, sCTB = subsyncytial cytotrophoblasts cells, SYN = syncytium. Illustration reused from Zeldovich *et al* (44).

1.2.2 Extravillous trophoblast cells

From the cytotrophoblasts of the anchoring villi, extravillous trophoblast cells (EVT) arise (41, 45, 46). These cells are pivotal in anchoring the placenta to the decidua of the uterine wall. EVT cells break free of the anchoring villi, invade the uterine spiral arteries and remodel them by replacing their arterial structure with amorphous fibrinoid material and endovascular trophoblastic cells. This creates a low-resistance, high-capacity perfusion system from the spiral arteries to the intervillous space of the villous tree (40, 47), thus establishing the placental blood supply. Proper differentiation of EVT cells is a crucial part of the "dialogue" between the decidua and placental tissue (40).

Lunghi *et al* phrased it well, "*The physiology of pregnancy depends upon the orderly progress of structural and functional changes of villous and extravillous trophoblast, whereas a derangement of such processes can lead to different types of complications* (…)" (40). It is well established that inadequate development/differentiation of EVT cells or inadequate EVT-invasion of the spiral arteries could lead to complications of the pregnancy, affecting the mother and/or fetus (40, 45, 47). It could affect the fetomaternal exchange of nutrients and gaseous products, in turn affecting fetal growth (45, 47). Complications of placental malfunction could be e.g., preeclampsia, intrauterine growth restriction (IUGR), intrauterine fetal death, stillbirth or recurrent miscarriages (5, 41).

1.3 FNAIT and placenta

Findings in previous studies makes for the plausibility that reduced placental function could be a potential complication of FNAIT (3). As the β3 integrin (in which HPA-1a is expressed), is also expressed on vascular endothelial cells, syncytiotrophoblasts and invasive trophoblasts (aka. EVT cells) (3, 5), it is plausible that anti-HPA-1a antibodies can bind to, and thus affect, placental cells (i.e., EVT and syncytiotrophoblasts). High levels of anti-HPA-1a antibodies have been found to be associated with reduced birthweight and risk for small-for-gestational-age (SGA) in male neonates (3). However, it is not fully understood, nor investigated, why boys seem to be more susceptible to endure complications of FNAIT than girls.

Anti-HPA-1a antibodies have also been found to affect adhesion, migration and invasive capacity of EVT's from a commercial cell-line (HTR8/SVneo cells) (48). A study by Nedberg *et al* found an association between maternal HPA-1a-alloimmunization and chronic histiocytic intervillositis in placental tissue (4). Nedberg *et al* also found that those with placental lesions of chronic histiocytic intervillositis and maternal alloimmunization had worse clinical outcomes (4). A smaller French study by Dubruc *et al* reported similar findings (49).

A study in murine models by Li *et al* suggested that maternal infection status could spark an immune response leading to maternal alloimmunization, although this has not been reported nor properly investigated in humans (50). Li *et al* also observed cases of severe "non-classical" FNAIT in their murine models, i.e., miscarriage but no bleeding in the pups (50). Women with children affected by FNAIT often have a previous history of miscarriages, suggesting a link between FNAIT and miscarriage (5, 50). However, it is not fully understood nor investigated how the two are linked in humans, but it has been hypothesised that the placenta might be part of the explanation. Findings in studies from murine models of FNAIT show that maternal anti-integrin β3 might stimulate or increase the risk of miscarriage (20, 51). This makes for the suggestion that similar mechanisms may take place in human pregnancies and that the maternal anti-HPA-1a alloantibodies might not only target fetal platelets, but also placental receptors and thus the placenta itself.

Findings such as those listed, makes for the possibility that HPA-1a alloimmunization might affect the placental function, and thereby may be affecting the fetal development and pregnancy as a whole. Although we have knowledge of development and pathophysiology of both FNAIT and the placenta, current understanding of how the two may be connected is still poor.

2 Aim

The aim of this project was to optimize the first steps of a protocol for isolating trophoblast cells from first trimester placentas. This project is part of the bigger project "*Platelets and placenta – the new hotspot in fetal-maternal crosstalk*". When such a protocol is established, the protocol will be used in future functional studies of how anti-HPA-1a antibodies may affect early placentation and further pregnancy development.

Several different protocols for isolating trophoblast cells already exist, but it was desirable to develop an in-house protocol optimized for use in the research laboratory. A functional

protocol for isolating trophoblast cells would enable the conduction of experiments on live cells, thus eliminating the need to solely rely on commercial cell lines. Even though commercial cell lines have prolonged life spans, are at low-cost and have lower risk of contamination, the advantage of primary cell cultures is that they more closely resemble the original tissue in their physiological properties and expression surface molecules (i.e., proteins and receptors) (52).

3 Materials and methods

3.1 Obtaining consent

Placental tissue was obtained from women undergoing legal termination of pregnancy before the end of gestational week 12 at the University Hospital of North Norway in Tromsø (UNN). Nurses at the Maternity Outpatient Clinic asked women coming in for abortion consults if they would like to participate after taking the medical history and completing the general examination on day 0 of the abortion. All women who contributed gave written informed consent before undergoing the abortion.

3.2 Ethical approval

As the project is part of the larger project "Platelets and placenta – the new hotspot in fetal-maternal crosstalk", ethical approval of the project and collecting of tissue had already been granted by the local ethics committee (REK NORD, 2015/2192 *Plater og placenta – samspill for livet*). Approval for the consent forms and storage of the consent forms was granted by the Data Protection Official (PVO 2022/2826 *Metodeutviklingsprosjekt for å studere morkakeceller*, nr. 02961). The consent forms signed by patients were stored as per protocol and shredded at the end of the project period (June 1st, 2022). No identifying data were attached to the tissue samples used. See Appendix 1-3 for copies of the approvals and forms.

3.3 Data access

No data access or data collection was needed for the project as no patient data were associated with the tissue samples. All information needed for developing a protocol was collected from previously publicised research and academic literature.

3.4 Study population – Inclusion and exclusion criteria

Inclusion criteria for the project were pregnant women who underwent medically induced abortions before gestation week 12 at UNN and agreed to donate their aborted placentas to research purposes. Exclusion criteria applied to those who had surgical abortions, those who did not agree to donate their abortion material to research purposes, those who had their abortion elsewhere than at the hospital and women who underwent abortion after gestation week 12. Women with miscarriages were not considered for inclusion.

Factors such as age, race and ethnicity were not taken into consideration in this project, as they were considered to have little or no relevance when developing a protocol of how to isolate trophoblast cells. Exact gestation age and parity was not taken into consideration in this project for logistical reasons. Women who did not understand or speak Norwegian, Swedish, Danish, or English, who needed a translator, were not asked to participate for logistical reasons as well.

3.5 Choice of strategy

Various existing protocols for isolation of trophoblast cells were used as a basis for adapting different strategies (**Table 1**). Among the protocols used were the Cambridge protocol by Male *et al* (53), the method used by James *et al* (54) and basic protocol 1 published by Breman & Patel (55). Furthermore, an unpublished, preliminary in-house protocol developed by Nora H. Nedberg and medical student Meike Hofer from 2019, was also used as basis (Appendix 4).

Table 1: A comparison of the protocols used as basis for the strategies which were tested for isolation of trophoblast cells.

Protocol	Preparation of tissue	Enzymes and time exposure	Inactivation of enzymatic reaction	Reference
1	Washed and dissected in RPMI 1640. Bottle with sterile magnetic stirrer bar used to wash off blood residue. Weight/amount of tissue not specified.	75 ml trypsin-EDTA (of their own blend), prewarmed to 37˚C, was added to the tissue suspension, 25 ml at a time, to digest for 8-9 min.	Ham's F12	Male *et al* (53)
2	Approx. 8 mg wet weight of cells were cultured in Matrigel-coated flasks with cell medium for 10 days.	20 ml of 0.25% trypsin-EDTA (prewarmed to 37˚C) and 0.2 ml DNase I was added to the tissue suspension to digest for 10 min × 5.	1 ml of FBS and 1000 U of trypsin inhibitor	James *et al* (54)
3	Approx. 20-25 mg tissue.	2 ml of 0.25% trypsin-EDTA (temperature not specified) was added to the tissue suspension and digested for 15 minutes. The tissue was transferred to a new dish, 2 ml of Collagenase was added and digested for 30 min. After each enzymatic step, the cell suspension was evaluated in a microscope and if the cell dissociation was not satisfactory, the suspension was incubated for another 15 and 30 min respectively.	MEM CellGro was used to inactivate the trypsin. Complete cell medium (AmcelGrow or Amniomax) was used to inactivate the collagenase.	Breman & Patel (55)
4	Rinsed in PBS, dissected in Ham's F12.	0.05% trypsin-EDTA (prewarmed to 37˚C) digested for 10 min × 3. DNase I digested for 10 min × 1.	FBS	Nedberg & Hofer (Appendix 4)

	Weight/amount of tissue not specified.			

Based on these four protocols for isolation of trophoblast cells, two different strategies were chosen, differing mainly in the type of enzymatic treatment of the tissue (**Table 2**). Strategy 1 involved the use of trypsin-EDTA and collagenase I. Strategy 2 involved the use of trypsin-EDTA and DNase I. An explanation as to why these specific enzymes were used, as well as their properties, is given in section 3.5.4 Enzymatic processing of the tissue.

Table 2: Comparison of strategy 1 and 2 for isolation of trophoblast cells.

Strategy	Experiment	Estimated* amount of tissue per experiment	Rinse and dissection of tissue	Time of enzymatic exposure	Inactivation of enzymatic reaction
1	1-4	40-50 mg* (ca. 1-2 ml)	Rinsed in PBS, dissected in RPMI.	Trypsin-EDTA: 55-60 min Collagenase I: 40-60 min	Ham's F12
2	5-8	Not estimated, but the maximum amount possible was harvested from the samples.	Rinsed in PBS, dissected in Ham's F12.	Trypsin-EDTA: 10 min × 3 DNase I: 10 min × 1	FBS

* The tissue was not weighed, the amount of tissue was estimated based on a photograph from a book by the Association of Genetic Technologists (56).

Each experiment was numbered chronologically, i.e., the first tissue sample received was experiment 1, and so on. Strategy 1 was tested out on experiment 1-4, and strategy 2 was tested out on experiment 5-8. Detailed descriptions of the two strategies can be found in Appendix 5 and 6 respectively. Figure 4 illustrates a simplified comparison of the two strategies. Strategy 2 is almost identical to the preliminary protocol by NH Nedberg and M Hofer, with only minor adjustments.

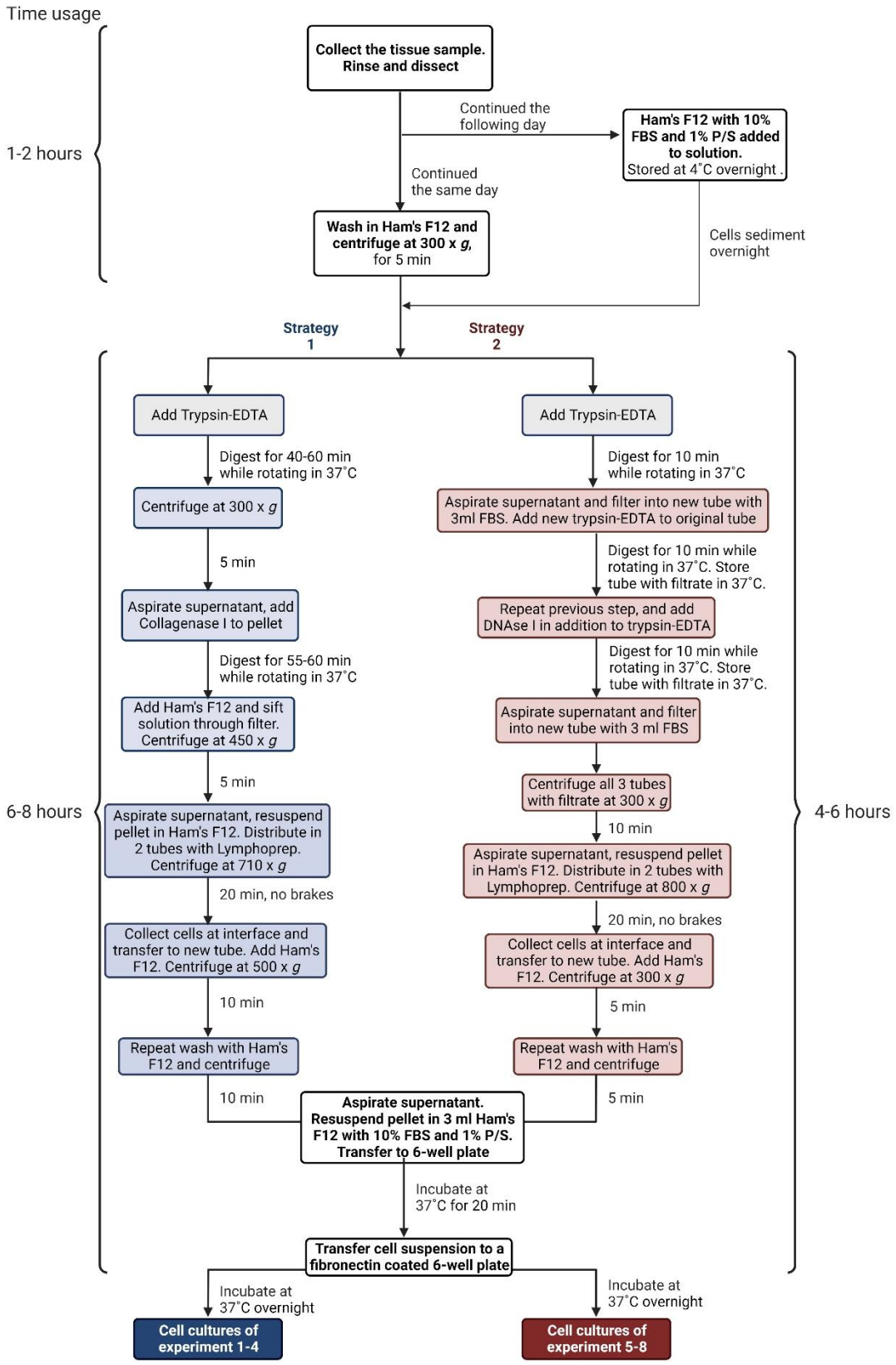

Figure 4: A simplified schematic comparison of strategy 1 vs. strategy 2. Illustration created with BioRender.com.

3.5.1 Tissue sampling

Nurses from the Maternity Outpatient Clinic called the laboratory when the abortions were complete and tissue material was ready. Tissue samples were collected and transported from the Outpatient Clinic to the laboratory in 150 mm petri dishes inside a Styrofoam box lined with aluminium foil and filled with ice.

3.5.2 Equipment and reagents

Equipment used in the experiments are listed in the strategies, found in Appendix 5 and 6 respectively. Supplier, item numbers/models are also included. The reagents used for the experiments are listed and described in **Table 3**.

Table 3: Reagents used in strategy 1 and 2 for isolation of trophoblast cells.

Reagent	Description and purpose	Supplier/batch nr.
Dulbecco's Phosphate Buffered Saline (PBS)	An isotonic buffer solution, non-toxic to villous trophoblast cells. Used to rinse the tissue of blood, clots, and other possible contaminants.	Sigma Lot No. RNBK8735
RPMI 1640 (Roswell Park Memorial Institute medium)	Growth medium used to nurture cell cultures. Contains glucose, salts, amino acids, and vitamins.	Sigma Lot No. RNBK7760
Ham's F12 medium (without calcium and magnesium)	Growth medium designed to mimic extracellular fluid. Contains amino acids, vitamins, glucose, and salts – which all contribute to nurture cell in a cell culture. A medium especially well fit for culturing of trophoblast cells.	Sigma Product nr. 1651C Batch nr. LCC0499
Fetal bovine serum (FBS)	Supplement which provides hormones, vitamins, lipids, transport proteins, attachment factors, spreading factors and growth factors. Often used in in vitro models.	Gibco Lot nr. 2412072
Penicillin/Streptomycin (P/S)	Antibiotic, used to prevent bacterial growth in the cell culture.	VWR Order nr. 15140-148
Trypsin-EDTA (1X) liquid, 0.05%, with phenol red. 500 ml	Because of its digestive properties, it is widely used for tissue and cell dissociation.	Thermo Fischer Scientific Order nr. 25300-054

Collagenase I (350 U/ml) <u>*Used in experiment 1-3*</u>	Enzyme which catalyses cleavage of peptide bonds in collagen.	Sigma Order nr. C2799 Originally 7500 U/ml, but batched out with RPMI to 350 U/ml
Collagenase I (125 U/ml) <u>*Used in experiment 4*</u>	Enzyme which catalyses cleavage of peptide bonds in collagen. This collagenase had a concentration of 1 mg/ml, which equals to 125 U/ml, meaning that it is gentler on the cells than the collagenase with 350 U/ml.	Sigma Order nr. C9263
DNase I (1 mg/ml)	Enzyme used to reduce clumping of the cell suspension.	Stemcell Technologies Cat. 07900, 100-0762 Lot. 19E102783
Lymphoprep	Density gradient medium. Used to isolate cells – they are distributed in different layers when centrifuging, depending on cell density.	Stemcell Technologies Cat. #07801/07811 Lot #00321
Fibronectin (type I)	Glycoprotein expressed in the plasma and on cell surfaces. Important for e.g., cell differentiation, growth, and migration.	Sigma Cat No. F4759

3.5.3 Rinsing and dissecting the tissue

The tissue samples were brought to the laboratory and taken into a sterile biosafety cabinet. Then, the tissue was carefully rinsed with PBS before pieces of decidua and blood clots were removed with sterile forceps. After the rinse, the tissue was transferred into a 60 mm petri dish with either RPMI 1640 medium or Ham's F12 medium and brought under a microscope (Leica MZ6) for dissection by scalpel and forceps. In the microscope, the villous tissue could be seen as "pink, feathery and fluffy" structures (53). Membranes (such as the chorionic), placental blood vessels and the umbilical cord were removed, leaving only villous tissue. The villous tissue was then carefully cut into small pieces by scalpel. Villi were aspirated by pipette and transferred into 50 ml tubes. Figures 5 and 6 show examples of tissue samples during the steps of rinsing and dissection.

If the experiment was continued the same day, the cells were washed with 20 ml Ham's F12 and centrifuged for 5 minutes at 300 × *g*, so that the cells would sediment. If the experiment was done over the course of two days, 10-20 ml Ham's containing 10% FBS and 1% P/S was added and left in the fridge at 4˚C overnight. The steps for collection, rinse and dissection of the placenta were the same for strategy 1 and 2.

3.5.4 Enzymatic processing of the tissue

The enzymatic processing of the tissue differs between strategy 1 and strategy 2; the enzymes used, the quantity and time exposure of the enzymes. In strategy 1, trypsin-EDTA and collagenase I was used, while in strategy 2, trypsin-EDTA and DNase I was used. Trypsin-EDTA has proteolytic properties, enabling it to cleave proteins and weaken cell adhesion in cell suspensions. However, the treatment with trypsin gives the tissue a slimy consistence, making it hard to filter it through cell strainers. DNase I is an endonuclease that contributes to tissue dissociation by breaking down leaked DNA from damaged cells in the cell suspension. Collagenase I cleaves the peptide bonds of collagen in the extracellular matrix, thereby dissociating the tissue into a single cell suspension.

In strategy 1, pre-warmed 0.05% trypsin-EDTA was added to the pellet of tissue and digested for approximately 40-60 minutes while rotating in a 37˚C heating cabinet. The tubes were regularly checked. When the tissue had become slimy, the tubes were centrifuged at 300 × *g* for 5 minutes. The supernatant was aspirated and discarded, and collagenase I (350 U/ml) was added to digest for approximately 55-60 minutes while rotating in an incubator at 37˚C. The tubes were checked regularly. When the tissue had dissociated, Ham's F12 was added to the solution to stop the enzymatic reaction before filtering the suspension through a 100 µm cell strainer. The tubes were then centrifuged at 450 × *g* for 5 minutes to sediment the cells.

In strategy 2, pre-warmed 0.05% trypsin-EDTA was added to the pellet of tissue and digested for 10 minutes, while rotating in a 37˚C heating cabinet. When the cells had sedimented, the

supernatant was aspirated and filtered through a 100 µm cell strainer into a new tube containing 3 ml of FBS (to stop the enzymatic reaction). The filtrate was kept in the heating cabinet while the whole process was repeated twice (total enzyme exposure time of 30 minutes). On the last round, DNase I was added together with trypsin-EDTA, i.e., the DNase digested for 10 minutes total. The supernatant and remaining tissue was filtered through a 100 µm cell strainer, resulting in a total of three tubes of filtrate. The tubes were then centrifuged at 300 × *g* for 10 minutes to sediment the cells.

All enzymatic digestion processing was done while the tubes were placed in a rotator inside a 37˚C heating cabinet. Every centrifugation was conducted using the same centrifuge (Eppendorf® Centrifuge 5810/5810R) with a swing-bucket rotor, meaning that the tubes were in horizontal position when centrifuged. The speed and time of centrifugation was adjusted as needed, but the temperature was set at 18˚C for every centrifugation. Brakes were always set at level 9, except for one step, which is clearly stated in text.

3.5.5 Cell separation using lymphoprep

After centrifugation, the supernatant was aspirated, and the pellets were resuspended in Ham's F12. The cell suspension was then distributed between two tubes containing lymphoprep. Lymphoprep is a density gradient medium, used to isolate cells. By centrifugation the different cell types distribute throughout the gradient, depending on their different densities. This allows for cells of the same type to gather in the same layer (as their density will be the same). The tubes with lymphoprep and cell suspension were centrifuged for 20 minutes, no brake, at respectively 710 × *g* and 800 × *g* in strategies 1 and 2. Trophoblast cells are expected to gather in the interface between the lymphoprep and cell suspension, and therefore the interface was collected by a pipette to gather these cells. The collected solution was transferred to new tubes and the cells were washed twice by adding Ham's F12 before the tubes were centrifuged at 500 × *g* in strategy 1 and at 300 × *g* in strategy 2.

3.5.6 Incubation of cell cultures

After centrifugation, the supernatant was aspirated from the tubes and discarded. The pellet was resuspended in 3 ml of Ham's F12 containing 10% FBS and 1% P/S. The cell suspension was then transferred to a 6-well plate, the contents of each tube being transferred into their own well. The plate was then incubated in an incubator with 5% CO_2 (Forma Scientific, 3110 Water Jacketed CO_2 incubator) for 20 min, so that placental macrophages (Hofbauer cells) would adhere to the plate. The placental macrophages were plated down because they bind antibodies non-specifically, which could be a problem in flow cytometry at a later stage (53).

After the 20 minutes of incubation, the cell suspension was aspirated and plated onto fibronectin coated wells. The fibronectin helps the cells adhere to the plate and grow. For each well prepared, a 20 µl aliquot of fibronectin was thawed and diluted in 1 ml Ham's F12. The solution was plated onto the 35 mm well in the 6-well plate and left to incubate for 45 minutes at room temperature (RT). Remaining fibronectin solution was discarded right before use.

The plate was then incubated at 5% CO_2 at 37˚C. Development and differentiation of the cell cultures were monitored on day 1 and 3 after plating. This was done by using a microscope with camera function (Nikon Eclipse Ts2), which allowed for documenting the development of the cell cultures both by inspection and by photography. If there were many cells floating around when checking in on the cell cultures, the medium was aspirated and replaced with 3 ml Ham's containing 10% FBS and 1% P/S. The cell cultures were inspected to see if the cells had adhered to the plate by flattening their shape and stretching out overnight (55, 57). If the majority of the cells (approximately 80-90 %) had adhered to the plate the next day, the plan was to perform cell counting and proceed with flow cytometry in order to identify EVT-cells.

4 Results

In total, eight placentas were included in the project for cell isolation and testing. These placentas were obtained during the period of April 19th to May 20th of 2022. Figure 5 and 6 illustrate how these placentas appear after being rinsed and dissected.

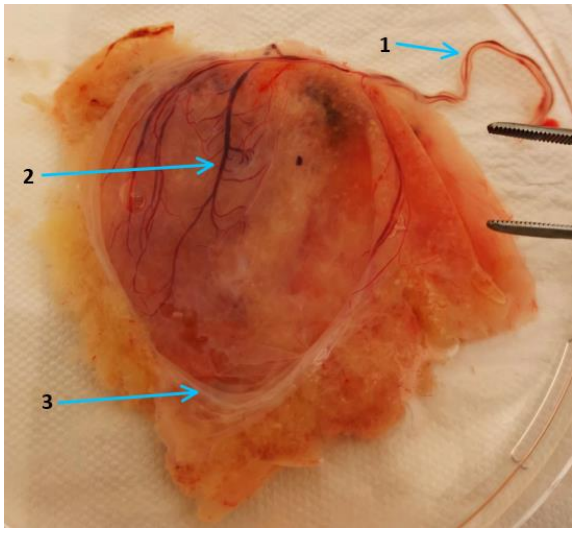

Figure 5: The fetal side of the placenta. 1) The umbilical cord peripherally placed towards the top of the picture, 2) branches of placental blood vessels and 3) the chorionic membrane which lies folded. Picture: private.

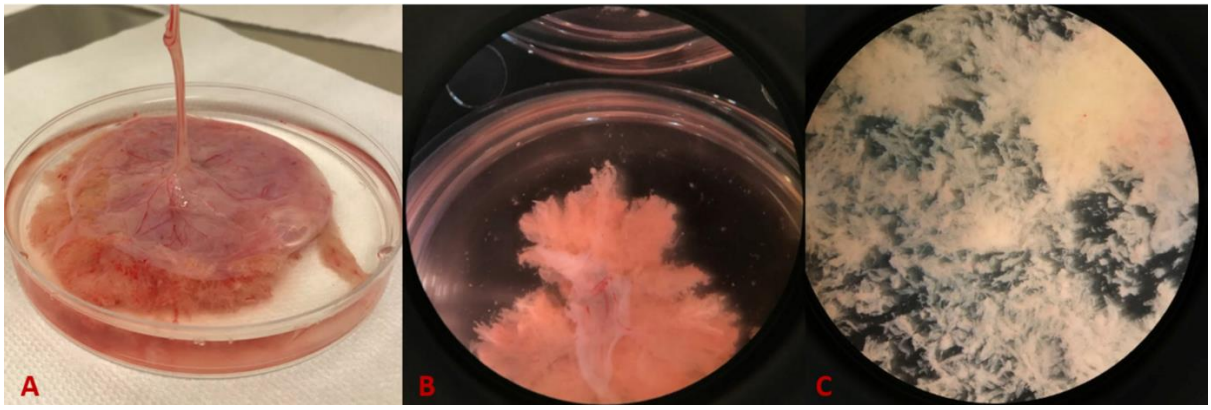

Figure 6: Macroscopic images of first trimester placentas. Image A) shows a placental tissue sample, which has been rinsed, removing blood and clots. It shows the fetal side of the placenta with a centrally placed umbilical cord. Image B) shows a piece of villous tissue in the microscope just before dissection. The light pink membrane seen centrally in the tissue piece is part of the chorion membrane. Image C) shows dissected tissue in the microscope, ready to be transferred into a new tube for further processing. Pictures: private.

Experiment 1-4 were conducted using strategy 1, varying mostly in the amount of tissue used and time of enzymatic exposure. Experiment 5-8 were conducted using strategy 2. A summarizing comparison of all the experiments and their respective results can be found in Table 4.

Table 4: Results from the eight experiments that were conducted.

Experiment	Time	Preparation	Enzymatic process and exposure time	Results on day 1 and 3 of culturing
Nr. 1	1 day	Washed in PBS. Dissected in RPMI.	0.05% trypsin-EDTA: 60 min Collagenase I (350 U/ml): 60 min Enzymatic process inactivated by Ham's F12.	A few cells had adhered to the plate overnight, but most cells floated in the cell medium (**Figure 7**).
Nr. 2	2 days	Washed in PBS. Dissected in RPMI.	Not completed.	Not completed due to unforeseen absence of laboratory supervision.
Nr. 3	1 day	Washed in PBS. Dissected in RPMI.	0.05% trypsin-EDTA: 55 min Collagenase I (350 U/ml): 60 min Enzymatic process inactivated by Ham's F12.	No cells were seen in the microscope.
Nr. 4	2 days	Washed in PBS. Dissected in RPMI.	0.05% trypsin-EDTA: 60 min Collagenase I (125 U/ml): 40 min Enzymatic process inactivated by Ham's F12.	Rich amounts of cells were seen floating in the suspension, but none had adhered to the plate.
Nr. 5	1 day	Washed in PBS. Dissected in RPMI.	0.05% trypsin-EDTA: 10 min × 3 DNase I: 10 min × 1	Rich amounts of cells, many of which had adhered to the plate and stretched out, were seen both on day 1 and 3 (**Figure 8**).

			Enzymatic process inactivated by FBS.	
Nr. 6	2 days	Washed in PBS. Dissected in Ham's F12.	0.05% trypsin-EDTA: 10 min × 3 DNase I: 10 min × 1 Enzymatic process inactivated by FBS.	Some cells were seen in the microscope, but none had adhered to the plate.
Nr. 7	1 day	Washed in PBS. Dissected in Ham's F12.	0.05% trypsin-EDTA: 10 min × 3 DNase I: 10 min × 1 Enzymatic process inactivated by FBS.	Rich amounts of cells, many of which had adhered to the plate and stretched out, microscopically detected on day 1 and 3 (**Figure 9**).
Nr. 8	1 day	Washed in PBS. Dissected in Ham's F12.	0.05% trypsin-EDTA: 10 min × 3 DNase I: 10 min × 1 Enzymatic process inactivated by FBS.	Rich amounts of cells, many of which had adhered to the plate and stretched out, microscopically detected on day 1 and 3.

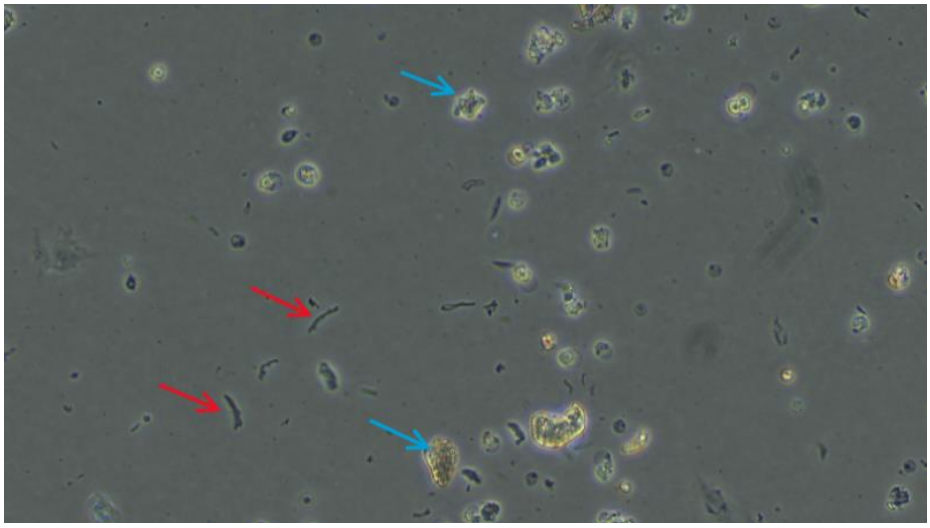

Figure 7: Both floating and adhered cells were seen in the cell culture of experiment 1, on day 3 of culturing. The red arrows point to cells that had adhered to the plate, flattened in shape, and stretched out. The blue arrows point to clumps of dead cells. Picture: private.

Of eight conducted experiments, four (experiments 1, 5, 7 and 8) were considered partly successful, in that cells that had adhered to the plate by flattening their shape and stretching

out, in turn indicating that cells had differentiated overnight. These four experiments were all conducted from start to finish on the same day as the tissue samples were received.

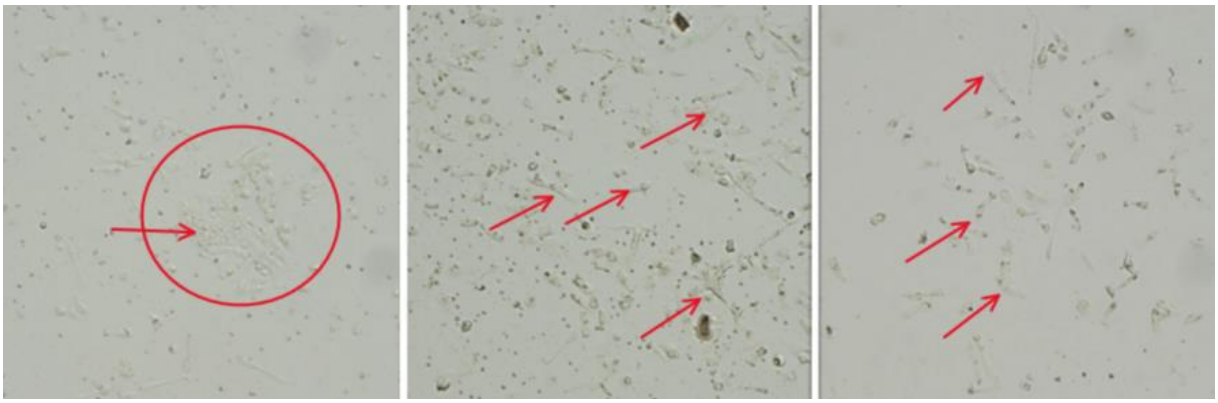

Figure 8: Development of the cell culture of experiment 5. From left to right; day 1, day 3 before changing medium and after changing medium. All pictures show red arrows pointing to cells that had adhered to the plate by flattening their shape and stretching out. Pictures: private.

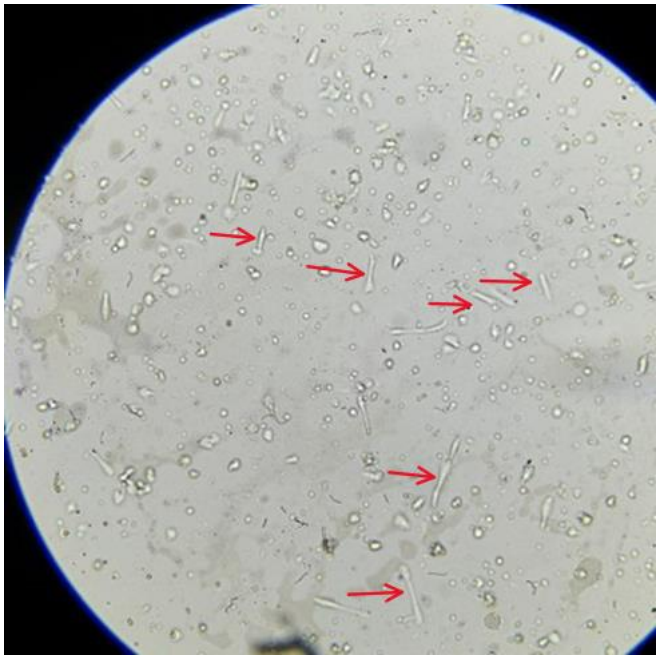

Figure 9: The red arrows point at cells that had adhered to the plate by flattening their shape and stretching out overnight in the cell culture of experiment 7. Picture taken on day 1 of culturing. Picture: private.

5 Discussion

Isolation of trophoblast cells is important when studying the cellular properties and mechanisms of early placental development. Regarding placenta research, cell culture studies are especially important. Animal models are of limited use because each mammal species has their own unique placental development. The goal of this project was to optimize the first steps of a protocol for isolating trophoblast cells from first trimester placentas, with the long-term ambition being to implement isolation of trophoblast cells into the research group's ongoing placenta research. First, findings are shortly discussed focusing on comparing the two strategies tested. Next, both strengths and limitations of the project are discussed before commenting on some future perspectives.

Cells examined immediately after isolation of trophoblasts are a mixture of villous trophoblast cells and extravillous trophoblast cells. When cultured overnight on fibronectin, 70-90 % of the trophoblasts are expected to show an extravillous phenotype (53). To estimate percentage of adhered EVT cells, one would have to aspirate cell medium from the culture, add trypsin-EDTA to detach the cells from the plate. Then, a small amount of cell suspension could be collected for cell counting and continue with flow cytometry. Only then could an estimated percentage of adhered EVT cells be given. As neither cell counting nor flow cytometry was conducted in this project, the amount of adhered cells in the cell cultures were solely based on observation by eye/microscope. Of the eight conducted experiments, four of them had partly successful outcomes albeit viability and cell specificity were not confirmed. In the cell cultures of experiment 1, 5, 7 and 8, it was observed on day 1 and 3 that cells had adhered to the fibronectin coated plate by flattening their shape and stretching out, suggestive of being EVT cells. To confirm that they indeed were EVT cells, as well as to assess purity and cell viability, flow cytometry could have been done. "Life expectancy" of cells plated onto fibronectin is approximately one week (53). However, when studying trophoblast cells, these must be used within the first couple of days after cultivation. This is because contaminating cells, such as fibroblasts cells, proliferate in vitro, whereas trophoblast cells do not (53). If cultivated for too long, the cultures would be overgrowing with contaminating cells. In this

project, cell cultures were checked up on, on day 1 and 3 of incubation. All cell cultures were discarded after day 3.

Strategy 1 resulted in observation of adhered cells in one out of four cell cultures. The cells had flattened their shape and stretched out. Strategy 2 resulted in observation of adhered and stretched out cells in three out of four tries. There are several plausible explanations to this. Due to the continuous learning curve of the student during the project period, the hands-on quality of the many and time-consuming steps may have improved from using strategy 1 to strategy 2. Several minor errors were made in all four of the experiments conducted with strategy 1, supporting this hypothesis. On the other hand, failure of strategy 1 could also be explained by other factors, such as the quantity, concentration and/or exposure time of the reagents. Strategy 1 had a considerably longer enzyme exposure time than strategy 2. The trypsin-EDTA had the same concentration (0.05%) in both strategies, but the total exposure time was 40-60 minutes in strategy 1, compared to 30 minutes in strategy 2. Furthermore, collagenase, used in strategy 1 with the exposure time of 55-60 minutes, can be quite hard on the cells and therefore requires careful monitoring of the cell suspension. In order to implement a protocol for isolation of trophoblast cells in the laboratory, it would be easier to follow strategy 2. Strategy 2 has established times of exposure of the enzymes, whereas strategy 1 relies on judgement by the researcher whether or not the cell suspension has the correct/wanted consistency. The quantity of enzymes is also established in strategy 2, in contrary to strategy 1, where the quantity of enzyme is reliant on amount of tissue processed (e.g., 5ml/mg trypsin-EDTA). However, larger amounts of tissue would take longer time to filter than smaller amounts of tissue.

An estimated amount of 40-50 mg wet weight of tissue was used in strategy 1, whereas as much tissue as possible (no estimated weight) was applied when testing strategy 2, meaning that the experiments of strategy 2 had considerably more tissue than those of strategy 1. The protocol by Male *et al* does not mention amount or weight of tissue (53), but the starting point of their protocol is a whole first trimester placenta. Both James *et al* and Breman & Patel only

state approximate amounts or weight of tissue (54, 55), and their starting points are with small biopsies taken from first trimester placentas, thus much less tissue than Male *et al*. Based on this fact, a decision was made early in the process of developing the strategies that amount/weight of tissue would not be emphasized. A greater focus was directed towards the amount of and time exposure of the enzymes. Another factor that could have had an impact on results of the cell cultures could be if the experiments were conducted all in one sitting or over the course of two days. All experiments that showed adhered cells and observational signs of differentiation (stretched out cells) were conducted in one sitting.

5.1 Strengths and limitations of the project

Even though the clinic collaborated excellently to assist in recruiting patients and placenta samples for the project, the main obstacle was the logistics of obtaining the tissue samples. During the planning of the project, placenta samples could be collected two days a week. When the project started, however, the clinic had changed their routines and samples were only available once a week, and only on Fridays. For future work on first trimester placentas, a change in the clinical routines would be necessary to have time to also do cell counting, standard trypan blue exclusion assay and flow cytometry.

Another problem that occurred was a delay in the PVO-approval. A delayed response time from PVO caused a delay in the start-up of the hands-on lab part of the project. The initial goal was to collect tissue material from 10-20 placentas. There is an obvious weakness in the limited number of experiments that were conducted, as only 7 out of 8 experiments were completed. Limited availability of placenta samples was however replaced with reading articles and literature on FNAIT, HPA-1a alloimmunization and EVT cells. The student also participated in FNAIT placenta-related research on term placenta (preparing histological sections and adding markers with immunofluorescence to term placentas from FNAIT pregnancies).

Risk of human error is always possible when doing manual laboratory work. The starting point of the student in this project was no previous laboratory training, and thus the lack of such experience likely increased the risk of human error. Inexperience also makes for a slower workflow. The slower workflow might cause longer reagent exposure of the tissue, due to slower progress of work, e.g., aspiration of supernatant or filtering solution through cell strainers. However, it can be considered a strength that all experiments were conducted within a short period of time (April 19th to May 20th of 2022), giving the work process of the experiments a good continuity, parallel with the student gaining experience at laboratory work. Furthermore, for all collected tissue samples, processing was started within 1 hour of delivery, ensuring the viability of the tissue/minimizing the degree of tissue decay. In addition, all tissue samples were rinsed and dissected under a microscope (Leica MZ6). This can be considered a strength, as the tissue samples were finely rinsed and contaminating tissue/cells (e.g., maternal blood, chorionic membrane etc.) were largely removed. The execution of rinsing and dissection improved for each conducted experiment, as the student gained more experience and improved the technique. The dissected tissue samples could therefore be assumed to have minimized amounts of contamination.

Another strengthening aspect is that the strategies tested were mainly based on published protocols (53-55), which have been successfully used by their authors. This should provide a good foundation for the strategies which were applied in the experiments of this project. The protocols used as basis were chosen as they were publications of renowned researchers or research facilities/groups, with aims similar to this project and they were properly described in text. Furthermore, they utilized several of the same reagents, although varying somewhat in concentration, quantity, and time exposure. In the strategies tested, there were some differences in centrifugation forces and times. In retrospect, the same centrifugation forces and times could have been applied in both strategies.

5.2 Future perspectives

What can be done differently in future experiments? What is warranted for future experiments, based on the experiences from this project? A longer time period for collecting tissue samples and conducting experiments would definitively improve the chances of successfully optimizing a functional protocol for isolation of trophoblast cells from first trimester placenta, as it would allow for more experiments to be conducted. Collaboration with the clinic in facilitating for placenta research is also essential. In this case, it would mean a change in the clinical routines regarding which days of the week these placenta samples can be obtained. Experience on the researcher's part would also be beneficial, both to decrease risk of human error and to ensure more efficient workflow. Furthermore, an estimate of the rinsed tissue according to the same figure used in strategy 1 (56), should be incorporated into strategy 2 in order to establish a ratio of the amount of tissue and the amount of enzyme added. Standard trypan blue exclusion assay should be used to ensure viability of the cells in the cell culture. Cell counting should be implemented in order to determine how many cells are isolated after the processing and cultivation. This can be done by aspirating the cell medium from the culture, adding trypsin-EDTA to detach the cells from the plate. Then, a small amount of cell suspension can be collected for cell counting. Finally, flow cytometry is warranted to verify viable EVT cells in the cell cultures. EVT cells can be labelled with anti-HLA-G antibodies as EVT cells are HLA-G positive (46).

6 Conclusion

Strategy 2 seemed to be superior to strategy 1. In three out of four cell cultures from strategy 2 (compared to one out of four cultures from strategy 1), cells cultured on fibronectin overnight, had adhered to the plate and showed microscopically observational signs of differentiation by flattening their shape and stretching out. However, the protocol still requires further testing and adjusting before it is fully optimized. Flow cytometry is also warranted to confirm differentiation of trophoblasts cell to EVT cells.

7 References

1. Hoffbrand AV, Moss PAH. Hoffbrand's essential haematology. 7th ed. Chichester: Wiley; 2016.
2. Kamphuis MM, Paridaans NP, Porcelijn L, Lopriore E, Oepkes D. Incidence and Consequences of Neonatal Alloimmune Thrombocytopenia: A Systematic Review. Pediatrics. 2014;133(4):715-21.
3. Tiller H, Killie MK, Husebekk A, Skogen B, Ni H, Kjeldsen-Kragh J, et al. Platelet antibodies and fetal growth: maternal antibodies against fetal platelet antigen 1a are strongly associated with reduced birthweight in boys. Acta Obstet Gynecol Scand. 2012;91(1):79-86.
4. Nedberg NH, Turowski G, Guz K, Przytuła E, Uhrynowska M, Roald B, et al. Platelet alloimmunization is associated with low grade chronic histiocytic intervillositis - A new link to a rare placental lesion? Placenta. 2021;112:89-96.
5. Tiller H, Husebekk A, Ahlen MT, Stuge TB, Skogen B. Current perspectives on fetal and neonatal alloimmune thrombocytopenia - increasing clinical concerns and new treatment opportunities. Int J Womens Health. 2017;9:223-34.
6. de Vos TW, Winkelhorst D, de Haas M, Lopriore E, Oepkes D. Epidemiology and management of fetal and neonatal alloimmune thrombocytopenia. Transfus Apher Sci. 2020;59(1):102704.
7. Regan F, Lees C, Jones B, Nicolaides K, Wimalasundera R, Mijovic A, et al. Prenatal Management of Pregnancies at Risk of Fetal Neonatal Alloimmune Thrombocytopenia (FNAIT). BJOG. 2019;126(10):e173-e85.
8. Tiller H, Killie M, Skogen B, Øian P, Husebekk A. Neonatal alloimmune thrombocytopenia in Norway: poor detection rate with nonscreening versus a general screening programme. BJOG 2009;116(4):594-8.
9. Turner ML, Bessos H, Fagge T, Harkness M, Rentoul F, Seymour J, et al. Prospective epidemiologic study of the outcome and cost-effectiveness of antenatal screening to detect neonatal alloimmune thrombocytopenia due to anti-HPA-1a. Transfusion. 2005;45(12):1945-56.
10. Kim HO, Jin Y, Kickler TS, Blakemore K, Kwon OH, Bray PF. Gene frequencies of the five major human platelet antigens in African American, white, and Korean populations. Transfusion. 1995;35(10):863-7.
11. Ohto H, Miura S, Ariga H, Ishii T, Fujimori K, Morita S. The natural history of maternal immunization against foetal platelet alloantigens. Transfus Med. 2004;14(6):399-408.
12. Kamphuis MM, Oepkes D. Fetal and neonatal alloimmune thrombocytopenia: prenatal interventions. Prenat Diagn. 2011;31(7):712-9.
13. Ahlen MT, Husebekk A, Killie MK, Skogen B, Stuge TB. T-cell responses associated with neonatal alloimmune thrombocytopenia: isolation of HPA-1a–specific, HLA-DRB3*0101–restricted CD4+ T cells. Blood. 2009;113(16):3838-44.
14. Williamson LM, Hackett G, Rennie J, Palmer CR, Maciver C, Hadfield R, et al. The natural history of fetomaternal alloimmunization to the platelet-specific antigen HPA-1a (PlA1, Zwa) as determined by antenatal screening. Blood. 1998;92(7):2280-7.
15. Kjeldsen-Kragh J, Killie MK, Tomter G, Golebiowska E, Randen I, Hauge R, et al. A screening and intervention program aimed to reduce mortality and serious morbidity associated with severe neonatal alloimmune thrombocytopenia. Blood. 2007;110(3):833-9.
16. Eksteen M. Anti-human platelet antigen (HPA)-1a antibodies: For better or for worse [Dissertation]. Munin: UiT – The Arctic University of Tromsø; 2015.
17. Killie MK, Husebekk A, Kjeldsen-Kragh J, Skogen B. A prospective study of maternal anti-HPA 1a antibody level as a potential predictor of alloimmune thrombocytopenia in the newborn. Haematologica. 2008;93(6):870-7.
18. Ghevaert C, Campbell K, Stafford P, Metcalfe P, Casbard A, Smith GA, et al. HPA-1a antibody potency and bioactivity do not predict severity of fetomaternal alloimmune thrombocytopenia. Transfusion. 2007;47(7):1296-305.

19. Kjær M, Bertrand G, Bakchoul T, Massey E, Baker JM, Lieberman L, et al. Maternal HPA-1a antibody level and its role in predicting the severity of Fetal/Neonatal Alloimmune Thrombocytopenia: a systematic review. Vox Sang. 2019;114(1):79-94.
20. Ni H, Chen P, Spring CM, Sayeh E, Semple JW, Lazarus AH, et al. A novel murine model of fetal and neonatal alloimmune thrombocytopenia: response to intravenous IgG therapy. Blood. 2006;107(7):2976-83.
21. Tiller H, Kahrs B, Haugen G, Bakken K, Akkök Ç. Alloimmunisering mot trombocytt-antigener (inkl FNAIT) ePub: Norsk gynekologisk forening Veileder i fødselshjelp (2020); 2020 [updated Feb 17 2020; cited 2022 May 3]. Available from: https://www.legeforeningen.no/foreningsledd/fagmed/norsk-gynekologisk-forening/veiledere/veileder-i-fodselshjelp/Allomimmunisering-mot-trombocyttantigener/.
22. Williamson DR, Albert M, Heels-Ansdell D, Arnold DM, Lauzier F, Zarychanski R, et al. Thrombocytopenia in Critically Ill Patients Receiving Thromboprophylaxis: Frequency, Risk Factors, and Outcomes. Chest. 2013;144(4):1207-15.
23. Winkelhorst D, Kamphuis MM, de Kloet LC, Zwaginga JJ, Oepkes D, Lopriore E. Severe bleeding complications other than intracranial hemorrhage in neonatal alloimmune thrombocytopenia: a case series and review of the literature. Transfusion. 2016;56(5):1230-5.
24. Chaoying M, Junwu G, Chituwo B. Intraventricular haemorrhage and its prognosis, prevention and treatment in term infants. J Trop Pediatr. 1999;45(4):237-40.
25. Husebekk A, Killie MK, Kjeldsen-Kragh J, Skogen B. Is it time to implement HPA-1 screening in pregnancy? Curr Opin Hematol. 2009;16(6):497-502.
26. Nogués N. Recent advances in non-invasive fetal HPA-1a typing. Transfus Apher Sci. 2020;59(1):102708.
27. Kamphuis M, Paridaans N, Porcelijn L, De Haas M, van der Schoot C, Brand A, et al. Screening in pregnancy for fetal or neonatal alloimmune thrombocytopenia: systematic review. BJOG. 2010;117(11):1335-43.
28. Dębska M, Uhrynowska M, Guz K, Kopeć I, Lachert E, Orzińska A, et al. Identification and follow-up of pregnant women with platelet-type human platelet antigen (HPA)-1bb alloimmunized with fetal HPA-1a. Arch Med Sci. 2018;14(5):1041-7.
29. Winkelhorst D, de Vos TW, Kamphuis MM, Porcelijn L, Lopriore E, Oepkes D, et al. HIP (HPA-screening in pregnancy) study: protocol of a nationwide, prospective and observational study to assess incidence and natural history of fetal/neonatal alloimmune thrombocytopenia and identifying pregnancies at risk. BMJ Open. 2020;10(7):e034071.
30. Tiller H, Kamphuis MM, Flodmark O, Papadogiannakis N, David AL, Sainio S, et al. Fetal intracranial haemorrhages caused by fetal and neonatal alloimmune thrombocytopenia: an observational cohort study of 43 cases from an international multicentre registry. BMJ Open. 2013;3(3):e002490.
31. Kjær M, Geisen C, Akkök ÇA, Wikman A, Sachs U, Bussel JB, et al. Strategies to develop a prophylaxis for the prevention of HPA-1a immunization and fetal and neonatal alloimmune thrombocytopenia. Transfus Apher Sci. 2020;59(1):102712.
32. Tiller H, Killie MK, Chen P, Eksteen M, Husebekk A, Skogen B, et al. Toward a prophylaxis against fetal and neonatal alloimmune thrombocytopenia: induction of antibody-mediated immune suppression and prevention of severe clinical complications in a murine model. Transfusion. 2012;52(7):1446-57.
33. Kjeldsen-Kragh J, Bengtsson J. Fetal and Neonatal Alloimmune Thrombocytopenia—New Prospects for Fetal Risk Assessment of HPA-1a–Negative Pregnant Women. Transfus Med Rev. 2020;34(4):270-6.
34. Van Den Akker E, Oepkes D, Lopriore E, Brand A, Kanhai H. Noninvasive antenatal management of fetal and neonatal alloimmune thrombocytopenia: safe and effective. BJOG. 2007;114(4):469-73.

35. Berkowitz R, Bussel J, McFarland J. Alloimmune thrombocytopenia: State of the art 2006. AJOB. 2006;195(4):907-13.
36. Tiller H, Ahlen M, Akkök Ç, Husebekk A. Fetal and neonatal alloimmune thrombocytopenia - The Norwegian management model. Transfus Apher Sci. 2020;59(1):102711.
37. Ernstsen S, Ahlen M, Johansen T, Bertelsen E, Kjeldsen-Kragh J, Tiller H. Antenatal intravenous immunoglobulins in pregnancies at risk of fetal and neonatal alloimmune thrombocytopenia: Comparison of neonatal outcome in treated and non-treated pregnancies. AJOG. 2022:Advance online publication.
38. Kanhai HHH, Porcelijn L, Engelfriet CP, Reesink HW, Panzer S, Ulm B, et al. Management of alloimmune thrombocytopenia. Vox Sang. 2007;93(4):370-85.
39. Sadler TW. Langman's Medical Embryology. 13th ed: Wolters Kluwer; 2015.
40. Lunghi L, Ferretti ME, Medici S, Biondi C, Vesce F. Control of human trophoblast function. Reprod Biol Endocrinol. 2007;5(1):6.
41. Turco MY, Moffett A. Development of the human placenta. Development. 2019;146(22).
42. Roberts RM, Ezashi T, Schulz LC, Sugimoto J, Schust DJ, Khan T, et al. Syncytins expressed in human placental trophoblast. Placenta. 2021;113:8-14.
43. James JL, Hurley DG, Gamage TKJB, Zhang T, Vather R, Pantham P, et al. Isolation and characterisation of a novel trophoblast side-population from first trimester placentae. REPRODUCTION. 2015;150(5):449-62.
44. Zeldovich VB, Robbins JR, Kapidzic M, Lauer P, Bakardjiev AI. Invasive Extravillous Trophoblasts Restrict Intracellular Growth and Spread of Listeria monocytogenes. PLoS Pathog. 2011;7(3):e1002005.
45. Moffett A, Loke C. Immunology of placentation in eutherian mammals. Nat Rev Immunol. 2006;6(8):584-94.
46. Apps R, Sharkey A, Gardner L, Male V, Trotter M, Miller N, et al. Genome-wide expression profile of first trimester villous and extravillous human trophoblast cells. Placenta. 2010;32(1):33-43.
47. Moffett-King A. Natural killer cells and pregnancy. Nat Rev Immunol. 2002;2(9):656-63.
48. Eksteen M, Heide G, Tiller H, Zhou Y, Nedberg NH, Martinez-Zubiaurre I, et al. Anti-human platelet antigen (HPA)-1a antibodies may affect trophoblast functions crucial for placental development: a laboratory study using an in vitro model. Reprod Biol Endocrinol. 2017;15(1):28.
49. Dubruc E, Lebreton F, Giannoli C, Rabilloud M, Huissoud C, Devouassoux-Shisheboran M, et al. Placental histological lesions in fetal and neonatal alloimmune thrombocytopenia: A retrospective cohort study of 21 cases. Placenta. 2016;48:104-9.
50. Li C, Chen P, Vadasz B, Ma L, Zhou H, Lang S, et al. Co-stimulation with LPS or Poly I:C markedly enhances the anti-platelet immune response and severity of fetal and neonatal alloimmune thrombocytopenia. Thromb Haemost. 2013;110(6):1250-8.
51. Li C, Piran S, Chen P, Lang S, Zarpellon A, Jin J, et al. Maternal immune response to fetal platelet GPIbalpha causes frequent miscarriage in mice that can be prevented by intravenous IgG and anti-FcRn therapies. J Clin Invest. 2011;121(11):4537–47.
52. Kaur G, Dufour JM. Cell lines. Spermatogenesis. 2012;2(1):1-5.
53. Male V, Gardner L, Moffett A. Isolation of Cells from the Feto-Maternal Interface. Curr Protoc Immunol. 2012;97(1):7.40.1-7..11.
54. James JL, Stone PR, Chamley LW. The isolation and characterization of a population of extravillous trophoblast progenitors from first trimester human placenta. Hum Reprod. 2007;22(8):2111-9.
55. Breman A, Patel A. Preparation of Chorionic Villus Samples for Metaphase Chromosome Analysis and Chromosomal Microarray Analysis. Current Protocols in Human Genetics. 2012;75(1):8.3.1-8.3.9.

56. Williams 3rd J. Amniotic fluid and chorionic villus culture and harvest. In: Dunn B, Mouchrani P, Keagle M, editors. The cytogenetic symposia. 2nd ed. ed. San Antonio, TX: Association of Genetic Technologists; 2005.
57. Aboagye-Mathiesen G, Laugesen J, Zdravkovic M, Ebbesen P. Isolation and characterization of human placental trophoblast subpopulations from first-trimester chorionic villi. Clinical Diagnostic Laboratory Immunology. 1996;3(1):14-22.

Appendix

Appendix 1 – Regional Ethics Committee approval (REK-approval)

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK nord			30.11.2015	2015/2192/REK nord
			Deres dato:	Deres referanse:
			27.10.2015	

Vår referanse må oppgis ved alle henvendelser

Heidi Tiller

2015/2192 Plater og placenta - samspill for livet

Forskningsansvarlig: UiT Norges Arktiske Universitet
Prosjektleder: Heidi Tiller

Vi viser til søknad om forhåndsgodkjenning av ovennevnte forskningsprosjekt. Søknaden ble behandlet av Regional komité for medisinsk og helsefaglig forskningsetikk (REK nord) i møtet 19.11.2015. Vurderingen er gjort med hjemmel i helseforskningsloven (hfl.) § 10, jf. forskningsetikkloven § 4.

Prosjektleders prosjektomtale
Sykdommen føtal og neonatal alloimmun trombocytopeni (FNAIT) kan føre til alvorlig blodplatemangel hos foster/ nyfødt og i alvorlig tilfeller forekommer også hjerneblødning. FNAIT skyldes at mor danner blodplateantistoffer (typisk anti-HPA-1a) som kan destruere fosterets blodplater. Immunologisk forskningsgruppe ved IMB, UiT publiserte i 2011 en studie som viste en sterk assosiasjon mellom blodplateantistoffer hos mor og lav fødselsvekt hos barnet. Forskningsgruppen har også nylig funnet at slike blodplateantistoffer ser ut til å påvirke utvikling av tidlig placenta (Eksteen et al, manuskript). Vår hypotese er derfor at mors anti-HPA-1a antistoffer under graviditet påvirker placentautvikling og kan føre til redusert placenta funksjon, noe som igjen kan være årsaken til at barnet får lav fødselsvekt. Vi vil studere hvordan disse antistoffene påvirker både tidlig placentautvikling, placenta funksjon gjennom svangerskap samt placenta ved termin/ etter fødsel.

Vurdering

Design
Det fremgår av søknaden at man nylig har funnet at blodplateantistoffer ser ut til å påvirke utvikling av tidlig placenta. Hypotesen er at mors anti-HPA-1a antistoffer under graviditet påvirker placentautvikling og kan føre til redusert placentafunksjon, noe som igjen kan være årsaken til at barnet får lav fødselsvekt. I dette prosjektet vil man studere hvordan disse antistoffene påvirker både tidlig placentautvikling, placentafunksjon gjennom svangerskap samt placenta ved termin/etter fødsel.

Slik REK forstår søknaden skal det både gjøres nye analyser av biologisk materiale innsamlet i tidligere godkjent FNAIT prosjekt i Polen og gjøres analyser av materialet som innsamles prospektivt og som skal oppbevares i den generelle biobanken i Norge.

Opplysninger som skal hentes fra mor er: fødselsdato, opplysninger om tidligere graviditeter (antall og utfall). Videre opplysninger om aktuell graviditet (termindato, forløp av svangerskapet, evt. komplikasjoner, evt. UL-funn) og forløsning (forløsningsmåte, årsak til evt. keisersnitt, placentavekt og makroskopisk beskrivelse placenta og histopatologisk beskrivelse hvis finnes). Barn: Fødselsdato, kjønn, fødselsvekt, APGAR score, blodplatetall ved fødsel (navlesnorsblod helst), blodplatetall senere i nyfødtperioden hvis

Besøksadresse:
MH-bygget UiT Norges arktiske universitet 9037 Tromsø

Telefon: 77646140
E-post: rek-nord@asp.uit.no
Web: http://helseforskning.etikkom.no/

finnes, evt. blødningstegn (hudblødninger, hjerneblødning). Ved hjerneblødning: radiologisk beskrivelse, kliniske symptomer og utfall.

Mors HPA-1 genotype, påvisning av blodplateantistoffer under/etter graviditet. Mors HLA DR B3 haplotype (DR B3*0101 haplotypen er hyppigst assosiert med FNAIT) Barnets HPA-1 genotype, evt. barnets trombocyttall ved fødsel.

Vurdering av om samtykkene for den norske populasjonen er dekkende
I formålet for informasjonsskrivet merket *«plater og placenta»* oppgis det at
«Vi ønsker å studere effekten av immunresponser på morkake vev i tidlig svangerskap»

I informasjonsskrivet merket generell forskningsbiobank oppgis det at *«Formålet med biobanken er å drive forskning på immunresponser og svangerskapsforhold i forbindelse med blodplateantistoffer og FNAIT, som i sin tur kan bidra til å bedre forebygging, diagnostikk og behandling.»*

REK anser at formålet for det omsøkte prosjektet ligger innenfor de formål som fremgår av de vedlagte samtykkeskrivene.

Vurdering av om forespørsel fra polsk populasjon er dekkende
Den 19.11.15 er det sendt mail til prosjektleder der de bes om å legge ved sist reviderte samstykkeskriv tilhørende prosjekt 2014/83, som omhandler utførsel av biologisk materiale til Tromsø. Denne forespørsel er nå innsendt, og REK vurdere denne som dekkende for det som skal skje i omsøkte prosjekt.

Genetiske undersøkelser
Det beskrives at *«Resultater fra de genetiske undersøkelsene vil kunne være prediktive i den forstand at vi kanskje bedre kan forutsi risiko for alvorlig FNAIT i senere svangerskap dersom vi finner genetiske markører som er assosiert med økt risiko.»* Ettersom dette er pasienter som allerede kjenner til at de i forrige svangerskap hadde blodplater som ikke var kompatible med barnets, vil ikke kunnskap om risiko ved neste svangerskap fremstå som prediktivt for disse deltagerne. Med bakgrunn i dette regnes ikke genetiske undersøkelser inn under definisjonene i biobankloven.

Vedtak
Med hjemmel i helseforskningsloven §§ 2,9 og 10 godkjennes prosjektet.

Sluttmelding og søknad om prosjektendring
Prosjektleder skal sende sluttmelding til REK nord på eget skjema senest 01.05.2027, jf. hfl. § 12. Prosjektleder skal sende søknad om prosjektendring til REK nord dersom det skal gjøres vesentlige endringer i forhold til de opplysninger som er gitt i søknaden, jf. hfl. § 11.

Klageadgang
Du kan klage på komiteens vedtak, jf. forvaltningsloven § 28 flg. Klagen sendes til REK nord. Klagefristen er tre uker fra du mottar dette brevet. Dersom vedtaket opprettholdes av REK nord, sendes klagen videre til Den nasjonale forskningsetiske komité for medisin og helsefag for endelig vurdering.

Med vennlig hilsen

May Britt Rossvoll
sekretariatsleder

Kopi til: terje.larsen@uit.no

Appendix 2 – Data Protection Official Approval (PVO-approval)

UNIVERSITETSSYKEHUSET NORD-NORGE
DAVVI-NORGGA UNIVERSITEHTABUOHCCEVIESSU

Heidi Tiller
Kvinneklinikken

Deres ref.:	Vår ref.:	Saksbehandler/dir.tlf.:	Dato:
	2022/2826	Kristin Andersen / 776 26506	5.4.2022

ANBEFALING – BEHANDLING AV PERSONOPPLYSNINGER

Det vises til Meldeskjema for forsknings- og kvalitetsprosjekt og annen aktivitet som medfører behandling av personopplysninger mottatt 5.4, samt tidligere kommunikasjon og avklaringer i mars-april 2022.

Meldingen gjelder prosjektet:

> ***Nr. 02961***
> ***Navn på prosjektet:*** *Metodeutviklingsprosjekt for å studere morkakeceller*
> ***Prosjektperiode: 5.4.2022 – 1.7.2022***

Prosjektet er et ***kvalitetsprosjekt*** hvor Universitetssykehuset Nord-Norge HF er dataansvarlig.

> ***Formål:*** *«Formålet med prosjektet er å utvikle en metode for å kunne studere morkakeceller fra tidlig svangerskap. Når vi får etablert denne metoden vil vi bruke den i fremtidige forskningsprosjekter om morkakefunksjon.»*

Prosjektet er samtykkebasert. Personvernombudet (PVO) har vurdert prosjektet, og finner at behandlingen av personopplysningene har hjemmel i personvernforordningen artikkel 6 nr. 1 bokstav a) / 6 nr. 1 bokstav e), artikkel 9 nr.2 bokstav a) og h) og artikkel 9 nr. 3. Nasjonalt rettsgrunnlag er pasientjournalloven § 6 andre ledd og helsepersonelloven § 26.

PVO har registrert prosjektet på bakgrunn av tilsendte meldeskjema med vedlegg.

Forespørsel om deltakelse i prosjektet skjer ved at sykepleiere på KK informerer kvinnen om kvalitetsstudiet og spør om hun kan tenke seg å bli med i kvalitetsprosjektet. Prosjektmedarbeiderne vil aldri se samtykkeskrivene men vil få utlevert materialet anonymt. De signerte samtykkeskrivene oppbevares i perm i låst skap til de slettes ved prosjektslutt 1.7.2022

PVO gjør oppmerksom på at prosjektet endrer formål, må dette meldes særskilt.

PVO skal ha melding når prosjektet er avsluttet og samtykkeskrivene er slettet.

Med hjemmel i personvernforordningens artikkel 39, anbefaler PVO at behandlingen kan iverksettes.

PVOs anbefaling forutsetter at prosjektet gjennomføres i tråd med de opplysningene som er gitt, samt i henhold til personopplysningsloven og helseregisterloven med forskrifter. Videre forutsettes det at data anonymiseres etter prosjektavslutning ved at samtykkeskrivene slettes.

Med vennlig hilsen

UNIVERSITETSSYKEHUSET NORD-NORGE HF

for Personvernombudet

Kristin Andersen
personvernrådgiver

Kopi: Klinikksjef Fredrik Sund

Om personvernombud
Personvernombudet er utpekt av Universitetssykehuset Nord-Norge HF (UNN) og meldt til Datatilsynet. Personvernombudet har som oppgave å bidra til at UNN følger gjeldende regelverk for behandling av personopplysninger. Oppgaven innebærer blant annet å kontrollere overholdelsen av regelverket, informere og gi råd til virksomheten og de ansatte, og gi råd i vurdering av personverskonsekvenser. Personvernombudet er uavhengig og kan ikke instrueres av UNN i gjennomføring av sine oppgaver.

Om uttalelsen
Personvernombudets uttalelse er ikke selvstendig juridisk bindende og du kan selv velge hvordan du ønsker å forholde deg til denne. Du er imidlertid selv ansvarlig for at du følger gjeldende personvernregler innenfor ditt ansvarsområde. Velger du å avvike fra personvernombudets uttalelse bør du begrunne dette skriftlig i ditt arbeid.

Klageadgang
Personvernombudets uttalelse er har ingen selvstendig juridisk virkning og det finnes ingen adgang til å klage på uttalelsen. Dersom uttalelsen konkluderte på annen måte enn du ønsket kan personvernombudet bistå.

Taushetsplikt
Personvernombudet har taushetsplikt ovenfor opplysninger om personlige forhold, enkeltpersoners varsling om mulige brudd på personvernlovgivningen, forretningshemmeligheter eller sikkerhetstiltak som det får kjennskap til i utførelsen av sitt arbeid. Dersom slike opplysninger er nødvendig for å gjennomføre lovpålagte oppgaver kan den registrerte bli bedd om samtykke til å gi nødvendige opplysninger videre.

For mer informasjon om personvernombud se Datatilsynets sider om personvernombud
For mer informasjon om pasientens rettigheter se Dine rettigheter på Datatilsynets sider
For mer informasjon om virksomheten (UNN) sine plikter se Virksomhetenes plikter

Appendix 3 – Consent form

UNIVERSITETSSYKEHUSET NORD-NORGE
DAVVI-NORGGA UNIVERSITEHTABUOHCCEVIESSU

Forespørsel om deltakelse i det interne kvalitetssikringsprosjektet *«Metodeutviklingsprosjekt for å studere morkakeceller"*

Bakgrunn og hensikt
Dette er et spørsmål til deg om å delta i et internt kvalitetsprosjekt. Formålet med prosjektet er å utvikle en metode for å kunne studere morkakeceller fra tidlig svangerskap. Når vi får etablert denne metoden vil vi bruke den i fremtidige forskningsprosjekter om morkakefunksjon. Universitetssykehuset Nord-Norge HF er ansvarlig for databehandlingen i prosjektet.

Hva innebærer prosjektet?
Prosjektet innebærer at du gir tillatelse til at det kan tas prøver av abortmaterialet etter svangerskapsavbrudd. Deltakelse i prosjektet vil ikke medføre endring i oppfølging eller behandlingen av deg. Analyser av abortmaterialet medfører ingen risiko.

Hva skjer med prøvene og informasjonen om deg?
Vi vil kun bruke de cellene/ den delen av vevet fra abortmaterialet som inneholder morkakeceller/ morkakevev til prosjektet. Prøvene tatt av abortmaterialet og informasjonen utledet av dette materialet skal kun brukes slik som beskrevet i hensikten med prosjektet. Prøvematerialet vil destrueres når metodetestingen er avsluttet, det betyr innen 1 uke fra vi mottar materialet. Overlege Heidi Tiller er prosjektleder. Alle opplysninger og prøver vil bli behandlet uten navn og fødselsnummer eller andre direkte gjenkjennende opplysninger. Det vil kun være sykepleier ved gynekologisk poliklinikk som kjenner din identitet. Prosjektmedarbeiderne vil ikke kjenne din identitet. Samtykkeskjemaet vil destrueres ved prosjektets slutt innen 1/7-22.

Frivillig deltakelse
Det er frivillig å delta i prosjektet. Dersom du ikke ønsker å delta, trenger du ikke å oppgi noen grunn, og det får ingen konsekvenser for den videre behandlingen du får ved sykehuset. Dersom du ønsker å delta, undertegner du samtykkeerklæringen nederst på siden. Dersom du har spørsmål til prosjektet, kan du kontakte Heidi Tiller på telefon 776 26656.

Personvernombudet ved Universitetssykehuset Nord Norge kan bistå med generelle spørsmål knyttet til bruk og behandling av personopplysninger i forbindelse med opptak og dersom man er usikker på om opplysningene behandles i tråd med det som er beskrevet i dette samtykket. Personvernombudet kan kontaktes via sentralbordet, på telefon 776 26 000.

Oversikt over regelverk, rettigheter og klagemuligheter ved innsamling og bruk av personopplysninger finnes også på Datatilsynets nettside:
https://www.datatilsynet.no/rettigheter-og-plikter/den-registrertes-rettigheter.

Samtykke til deltakelse i prosjektet

Jeg er villig til å delta i *Metodeutviklingsprosjektet for å studere morkakeceller* og samtykker til at mitt materiale brukes slik som beskrevet ovenfor.

(Signert av prosjektdeltaker, dato) Navn med blokkbokstaver

Jeg bekrefter å ha gitt informasjon om studien

(Signert, rolle i studien, dato)

Appendix 4 – Unpublished, preliminary protocol for isolation of trophoblast cells by NH. Nedberg and M. Hofer

List of materials:

- 35 mm and 150 mm diameter petri dishes, sterile
- sterile disposable scalpels
- sterile forceps (fine tip)
- incubator
- centrifuge
- plastic funnel
- 6-well-plate
- 5 ml/10 ml/ 25 ml sterile serological pipettes
- cell strainer (100 µm)

Reagents	**Supplier**	**Order no.**	**Storage**
Trypsin-EDTA (1X) liquid, 0,05 %	Thermo Fischer Scientific	25300-054	Stamløsning i fryser, bruksløsning à 37,5 ml i -20° C fryser.
PBS	Central storage		sterile, at, +4°C
Ham's F12 medium	Sigma	51651C	sterile, at + 4°C
FBS	Sigma		sterile, at + 4°C
Lymphoprep	Axis-Shield		sterile
Fibronectin	Sigma	F4759)	Dilute in 1 ml sterile H_20. Leave to stand for 30 min (do not mix). Store in 20 µl aliquots at -20 °C.
DNase I (1 mg/ml)	Stemcell Technologies	#07900	-20° C

Preparation

Pre-warm 75 ml of trypsin solution to 37°C.

→ Culture Medium: Ham's F12 + 10% FBS + 1% Penicillin/Streptomycin

- *17,8 ml Ham's F12 + 2 ml FBS + 200 µl Penicillin/Streptomycin*
- *44,5 ml Ham's F12 + 5 ml FBS + 500 µl Penicillin/Streptomycin*

→ Fibronectin coating

Thaw 20 µl (one aliquot) of fibronectin and dilute it in 1 ml Ham's F12 medium. Place the diluted fibronectin in a 35 mm petri dish/one well of a 6 well plate and incubate for 45 min at RT. Discard the remaining fibronectin solution using a pipette *and either use immediately or store the dish up to 2 weeks at -20° C.*

Procedure

1. Prepare an ice box with aluminium foil and collect the placenta in a suitable container.
2. Place the tissue in a sterile petri dish with PBS for washing and examination.
3. Gently disaggregate the placenta, separating the villous tissue (feathery, light pink) from the decidua. Remove large blood clots with sterile forceps and scalpel and thoroughly wash the tissue, using multiple petri dishes if needed.
4. Carefully transfer the tissue pieces (using forceps or a 1000 µl pipette) to a 50 ml bottle with 20 ml of **Ham's F12**. Centrifuge for 5 min, RT at 300 × *g*. Aspirate supernatant.
5. Prepare 3 50-ml tubes with 3 ml **FBS** in each (**FBS** to stop enzymatic digestion). Label them 1, 2 and 3.
6. Add 25 ml of the pre-warmed **trypsin** solution to the tube with cells. Allow to digest in the incubator for 10 minutes on a rotator. Take out the tube and let the tissue sediment. Aspirate supernatant, filter the supernatant through a 100 µm cell strainer into the first 50 ml tube. Repeat twice on remaining cells but add 0,2 mg/ml **DNase I** in addition to trypsin on the last round (and put the tubes with filtrate in incubator (37˚C) while repeating). After the third incubation, filter the supernatant together with

the remaining tissue (*change strainer if necessary*). Centrifuge the 3 filtrates for 10 min at 300 × *g*.

7. Resuspend each cell pellet in 4 ml **Ham's F12** (12 ml in total). Prepare two 15 ml tubes with 5 ml **Lymphoprep** (RT!) and carefully layer 6 ml of the cell suspension onto each of the tubes. Centrifuge for 20 min at 800 × *g*, RT, with no brake.
8. After centrifugation, collect the cells at the interface and wash (twice) in ± 10 ml of **Ham's F12** or **PBS**.
9. To remove placental macrophages (Hofbauer cells), resuspend the cell pellet in 3 ml **Ham's F12** medium and incubate in a 35 mm petri dish for 20 min at 37° C.
10. Resuspend cells in 5 ml of **culture medium** and perform cell counting.
11. Seed in a fibronectin-coated cell culture dish. Incubate at 37°C overnight.

Appendix 5 – Full description of strategy 1

List of equipment (supplier and item number/model number are named in parenthesis):

- Styrofoam box lined with aluminium foil
- Ice
- 150 mm diameter petri dishes, sterile (Nunclon Delta surface, 157150)
- 60 mm diameter petri dishes, sterile (Nunclon Delta surface, 150288)
- Sterile forceps
- Sterile scalpels
- Scalpel blade (Swann-Morton, #10 blade, ref. 0201)
- 50 ml centrifugation tube (VWR, 525-1109)
- 15 ml centrifugation tube (VWR, 525-0309)
- Cell strainer (100 µm) (Falcon, 352360)
- 6 well plate (Falcon, Multiwell, 353046)
- 5 ml sterile plastic pipettes
- Pipette Eppendorf (0.1-1 ml)
- Pipetboy (1-25 ml)
- 37°C incubator (Termaks B8133)
- 37°C incubator with CO_2 regulation (Forma Scientific, 3110 Water Jacketed CO_2 incubator)
- Tube rotator (VWR, 444-0500)
- Centrifuge (Eppendorf® Centrifuge 5810/5810R)
- Stereo microscope (Leica MZ6)
- Microscope with camera (Nikon Eclipse Ts2)

Reagents, listed in chronological order of use:

- Dulbecco's Phosphate Buffered Saline (PBS)
- Roswell Park Memorial Institute medium 1640 (RPMI 1640)
- Ham's F12 medium (without calcium and magnesium)

- Fetal bovine serum (FBS)
- Penicillin/Streptomycin (P/S)
- Trypsin-EDTA (1X) liquid, 0.05%, with phenol red. 500 ml
- Collagenase I (350 U/ml)
- Lymphoprep
- Fibronectin (type I)

Preparations:

- **Fibronectin:** Thaw a 20 µl aliquot of fibronectin and dilute it in 1 ml Ham's F12 medium. Place the diluted fibronectin in a well of a 6 well plate and incubate for 45 min at RT. Discard the remaining fibronectin solution using a pipette. Either use immediately or store the plate up to 2 weeks at -20˚C (without the solution, the plate will dry out).
- **Ham's F12 cell culture medium:** Ham's F12 + 10% FBS + 1% Penicillin/Streptomycin
- **Enzymes:** Pre-warm the needed amount of **0.05% trypsin-EDTA** solution and **collagenase** to 37°C.

Procedure:

1. Prepare a box with aluminium foil covering the inside. Fill it up with ice and collect the placenta in a petri dish. Make sure all the reagents are at room temperature.
2. Place the placenta in a sterile petri dish with **PBS** for washing and examination. Rinse thoroughly, using plastic pipettes to aspirate blood.
3. Gently disaggregate the placenta, separating the villous tissue (feathery, light pink) from the decidua.
4. Transfer villous tissue to a new petri dish with **RPMI 1640** and place it under a magnifying glass. Cut the tissue into smaller pieces using two scalpel blades. By using a 1000 µl pipette, transfer the villous tissue to a 6-well plate with **RPMI 1640**. Remove large blood clots with sterile forceps. Estimate the amount of tissue.

5. <u>If you do not have time to proceed with the procedure,</u> collect the tissue suspension in a 50 ml tube and **add 10% FBS and 1% P/S**. Place the tube in 4˚C overnight.
6. <u>Continue the same day:</u> Place the tissue in a 50 ml tube and add equal amounts of **RPMI 1640**. Centrifuge the tissue at 300 × *g* for 5 minutes.
7. Remove the supernatant and add 5 mg/ml of **pre-warmed 0.05% trypsin-EDTA** solution. Place the tube on a rotator in 37˚C and allow to digest in the incubator for approximately 1 hour (check regularly). The tissue should become slimy.
8. Centrifuge the tube at 300 × *g* for 5 minutes at room temperature.
9. Remove the supernatant (time consuming) and add 5 mg/ml **collagenase** (350 U/ml). Place the tube on a rotator in 37˚C and allow to digest in the incubator for approximately 1 hour until the tissue is homogenized (check regularly).
10. Add equal amounts of **Ham's F12** as the collagenase in the tube to stop the enzymatic digestion and filter the supernatant through a 100 µm cell strainer into a new 50 ml tube.
11. Centrifuge the filtrate at 450 × *g* for 5 minutes, room temperature.
12. Aspirate the supernatant with a pipette and discard. The pellet is soft, so care must be taken not to disturb it. Resuspend the pellet in 10 ml **Ham's F12**.
13. Prepare two 8 ml aliquots of Lymphoprep in 15 ml tubes and layer 5 ml cell suspension onto each of the tubes. Centrifuge the tubes at 710 × *g* for 20 minutes, with no brake and at room temperature. While centrifuging, prepare the fibronectin coated 6-well plate.
14. After centrifugation, collect the cells at the interface and wash them with 20 ml of **Ham's F12** and centrifuge at 500 × *g* for 5 minutes, room temperature.
15. To remove placental villous macrophages (Hofbauer cells), resuspend the pellet in 3 ml **Ham's with 10% FBS and 1% P/S**, and incubate in a 35mm petri dish for 20 minutes at 37˚C.
16. Collect the non-adherent cells, transfer and plate them onto a fibronectin coated 6-well plate. Incubate the cell culture at 5% CO_2 in 37˚C (Forma Scientific incubator) overnight.

Appendix 6 – Full description of strategy 2

List of equipment (supplier and item number/model number are named in parenthesis):

- Styrofoam box lined with aluminium foil
- Ice
- 150 mm diameter petri dishes, sterile (Nunclon Delta surface, 157150)
- 60 mm diameter petri dishes, sterile (Nunclon Delta surface, 150288)
- Sterile forceps
- Sterile scalpels
- Scalpel blade (Swann-Morton, #10 blade, ref. 0201)
- 50 ml centrifugation tube (VWR, 525-1109)
- 15 ml centrifugation tube (VWR, 525-0309)
- Cell strainer (100 µm) (Falcon, 352360)
- 6 well plate (Falcon, Multiwell, 353046)
- 5 ml sterile plastic pipettes
- Pipette Eppendorf (0.1-1 ml)
- Pipetboy (1-25 ml)
- 37°C incubator (Termaks B8133)
- 37°C incubator with CO_2 regulation (Forma Scientific, 3110 Water Jacketed CO_2 incubator)
- Tube rotator (VWR, 444-0500)
- Centrifuge (Eppendorf® Centrifuge 5810/5810R)
- Stereo microscope (Leica MZ6)
- Microscope with camera (Nikon Eclipse Ts2)

Reagents, listed in chronological order of use:

- Dulbecco's Phosphate Buffered Saline (PBS)
- Roswell Park Memorial Institute medium 1640 (RPMI 1640)
- Ham's F12 medium (without calcium and magnesium)

- Fetal bovine serum (FBS)
- Penicillin/Streptomycin (P/S)
- Trypsin-EDTA (1X) liquid, 0.05%, with phenol red. 500 ml
- DNase I (1 mg/ml)
- Lymphoprep
- Fibronectin (type I)

Preparation

- Pre-warm 75 ml of 0.05% trypsin-EDTA solution to 37°C.
- **Cell culture Medium:** Ham's F12 + 10% FBS + 1% Penicillin/Streptomycin
- **Fibronectin coating:** Thaw 20 µl (one aliquot) of fibronectin and dilute it in 1 ml Ham's F12 medium. Place the diluted fibronectin in a 35 mm petri dish/one well of a 6 well plate and incubate for 45 min at RT. Discard the remaining fibronectin solution using a pipette *and either use immediately or store the dish up to 2 weeks at -20° C.*

Procedure

1. Prepare an ice box with aluminium foil and collect the placenta in a petri dish.
2. Place the tissue in a sterile petri dish with PBS for washing and examination.
3. Gently disaggregate the placenta, separating the villous tissue (feathery, light pink) from the decidua. Remove large blood clots with sterile forceps and scalpel and thoroughly wash the tissue, using multiple petri dishes if needed.
4. Carefully transfer the tissue pieces (using forceps or a 1000 µl pipette) to a 50 ml bottle with 20 ml of **Ham's F12**. Centrifuge for 5 min, RT at 300 × *g*. Aspirate supernatant.
5. Prepare 3 50-ml tubes with 3 ml **FBS** in each (**FBS** to stop enzymatic digestion). Label them 1, 2 and 3.
6. Add 25 ml of the pre-warmed **0.05% trypsin-EDTA** solution to the tube with cells. Allow to digest in the incubator for 10 minutes on a rotator. Take out the tube and let the tissue sediment. Aspirate supernatant, filter the supernatant through a 100 µm cell

strainer into the first 50 ml tube. Repeat twice on remaining cells but add 60 µl of **DNase I** in addition to trypsin-EDTA on the last round (and put the tubes with filtrate in incubator (37˚C) while repeating). After the third incubation, filter the supernatant together with the remaining tissue (*change strainer if necessary*). Centrifuge the 3 filtrates for 10 min at 300 × *g*.

7. Resuspend each cell pellet in 4 ml **Ham's F12** (12 ml in total). Prepare two 15 ml tubes with 5 ml **Lymphoprep** and carefully layer 6 ml of the cell suspension onto each of the tubes. Centrifuge for 20 min at 800 × *g*, RT, with no brake.
8. After centrifugation, collect the cells at the interface and wash (twice) in ± 10 ml of **Ham's F12** or **PBS**.
9. To remove placental macrophages (Hofbauer cells), resuspend the cell pellet in 3 ml **Ham's F12** medium and incubate in a 35 mm petri dish for 20 min at 37° C.
10. Resuspend cells in 5 ml of **culture medium** and perform cell counting.
11. Seed in a fibronectin-coated cell culture dish. Incubate the cell culture at 5% CO_2 in 37˚C (Forma Scientific incubator) overnight.