Chapter 1

General introduction
The maternal organism during pregnancy

Pregnancy is not only a very exciting time in the lives of the parents-to-be, but is also associated with tremendous changes of the maternal organism to establish and maintain a pregnancy-promoting environment. The maternal organism has to adjust to the semi-allogenic fetus and to cope with increased demand of energy and nutrients. In this scenario, the placenta represents the contact surface of the maternal blood with the semi-allogenic antigens and fulfills important functions, such as exchange of nutrients, gases and waste products between the maternal and fetal organism, and also (at least partially) adaptation of the maternal physiology to the pregnancy.[1,2] The fetus and also the placenta are semi-allogenic to the maternal organism since it evolves from early embryonic trophoblast cells.

Already in the blastocyst, the trophoblast and the embryoblast differentiate, and immediately upon implantation of the blastocyst, extravillous trophoblasts start invading into the maternal decidua. At the implantation side, the extravillous trophoblasts invades the already decidualized endometrium. When invading the decidua, the extravillous trophoblasts get into contact with local immune cells, predominantly uterine natural killer (NK) cells. A specific combination of the MHC class I molecules HLA-G, HLA-C and HLA-E protects them from attack by the maternal immune cells.[2–4] The extravillous/interstitial trophoblasts engage in remodeling of deep maternal spiral arteries even until the superficial myometrium and partially become endovascular trophoblast.[5] Meanwhile, the trophoblast lacunae in the syncytiotrophoblast (STB) grow and fuse together, forming the intervillous space. Until around the 9th week of gestation, the embryo is nurtured by diffusion processes, but with increasing size of the embryo diffusion is not sufficient anymore to transport enough oxygen and nutrients. Approximately in the 9th to 12th week of gestation, the remodeled maternal arteries are unplugged from trophoblast plugs and maternal blood fills the meanwhile evolved intervillous space. Fetal villous structures traverse the intervillous space, which are covered with a syncytial, multinucleated cell, the STB. The STB is a constantly renewing cell and is being stocked up by fusion with cytotrophoblasts from the layer below the STB. In contrast to the extravillous trophoblasts, villous trophoblasts such as the STB exhibit no MHC class I molecules which would be expected to confer major protection from attacks by the maternal immune cells, once the intervillous space is filled with maternal blood. The STB is believed to secrete a plethora of factors to protect itself from the maternal immune system and to mediate pregnancy-supportive changes in the maternal body. These are, amongst others, syncytiotrophoblast extracellular vesicles (STB EV), which are secreted into the maternal blood and may target several compartments of the maternal body, such as the hemostatic system or the immune system.
Extracellular vesicles

Both prokaryotic and eukaryotic cells release small membrane-coated particles, so called extracellular vesicles (EV), into their environment, which indicates that this evolutionary conserved process is of great importance for a cell. In the human body, diverse cells, like platelets, monocytes, endothelial cells, tumor cells but also the STB, secrete EV during health and disease and EV are present in all body fluids.[6–9]

Classification and formation of extracellular vesicles

According to their size, mode of formation, function and originating cell, EV can be subdivided into several classes, like macrovesicles/apoptotic bodies, microvesicles (MV), exosomes, dextrasomes or prostasomes (as reviewed by [10]), but the definitions vary between researchers.[11] Most commonly, EV are subdivided into macrovesicles/apoptotic bodies, MV and exosomes. Macrovesicles/apoptotic bodies are relatively large vesicles of 1 to 5 µm in size, which are formed by blebbing from the plasma membrane or cellular fragmentation of apoptotic cells.[10,12] MV are 100 to 1000 nm in size and are formed by cells which are activated, e.g. by renewal or damage. MV are budding directly from the apical side of the plasma membrane (Figure 1).[13] During this process, a translocation of phosphatidylserines from the intracellular side of the plasma membrane to the extracellular leaflet of the MV membrane may occur.[13–15] Phosphatidylserines are often used as a specific MV marker, although MV populations have been described which do not expose phosphatidylserines on their surface.[16] Exosomes are small particles of 30 to 100 nm in size, which are formed in the cell inside early endosomes turning into intracellular multivesicular bodies. The exosomes are released by fusion of the membrane of these intracellular multivesicular bodies with the plasma membrane (Figure 1).[6,10] Additionally it is described, that exosome-like nanovesicles are released by budding from the cell membrane (Figure 1). It is not clear yet, how comparable these nanoparticles are to exosomes or if they even can be differentiated from each other.[6,10] In contrast to MV, exosomes are not expected to expose phosphatidylserines on their surface. However, due to the isolation procedure and freeze-thaw-cycles, also exosome populations have been described to expose phosphatidylserines under certain circumstances.[13]

Function of extracellular vesicles

It has been shown that macrovesicles are shed from the placenta and they are believed to predominantly target endothelial cells [17]. However, they are thought to be cleared from the maternal blood relatively fast in the maternal lungs. [18] Therefore, they will not be further object of this thesis.
While the molecular mechanisms of the release of EV remain largely unknown, the functions of EV have been investigated into more depth and shown to be manifold. It has been suggested that cells secret EV to fulfill diverse functions related to angiogenesis, cell survival, coagulation, waste management, cell communication and immune adaptation.[8,6,7,10] MV and exosomes can originate from the same cell and share a certain amount of the origin-specific molecular loads, e.g. human placental alkaline phosphate in the case of placenta-derived vesicles.[10] However, specific markers for either vesicle type also exist and this suggests that the molecular load is at least partially specific to either MV or exosomes.[6,10] On this basis, it is expected that MV and exosomes have individual functions, differing from each other.

Many studies have focused on the effects of EV on coagulation and immune responses. MV stimulate the coagulation by exposing negatively-charged phosphatidylserines to which coagulation factors may bind.[14,15] Furthermore, anticoagulant factors have been described to be exposed on MV [19,20]. Also active tissue factor was shown to be exposed by MV in several studies, especially in pathologic conditions.[21–24] Here, a high number of MV and a high exposure of phosphatidylserines and tissue factor on these MV induced coagulation.[19,25,26] A reduced MV number in combination to a reduced phosphatidylserines turnover is associated with a bleeding disorder, the Scott syndrome.[27] Mostly, exosomes do not expose phosphatidylserines on their surface and are therefore not expected to engage in the activation of coagulation. However, a minimal part of the exosomes does expose surface phosphatidylserines [13] and a study discovered tissue factor-exposing exosomes in urine and saliva [28]. Thus, there is evidence that exosomes also may promote coagulation.

Next to their coagulation-related function, EV also engage in immune-regulatory processes. As reviewed by Lundy et al., B cells are producing exosomes which expose Fas ligand to mediate a self-tolerant state to avoid for example autoimmunity.[29] However, other studies identified autoantigens in the molecular load of thymocyte- or synovial fluid-derived MV and exosomes, thus proposing that these EV are involved in pathologic autoimmunity processes.[30–32] Furthermore, tumor cells are misusing the tolerance-inducing function of MV and exosomes to protect themselves from their host’s immune system by e.g. induction of Treg cells and killing of effector cells.[9,33,34] Additionally, the tolerance-mediating function of exosomes is increasingly recognized also for therapeutic use. As mentioned before, exomes expose autoantigens which seem to improve allograft tolerance in recipients of organ transplants.[35]
General introduction

Microvesicles and exosomes are formed in separate ways from their originating cell. While microvesicles bud from the plasma membrane, exosomes are built in endosomes during the formation of multivesicular bodies and released by the fusion of the membrane of the multivesicular bodies with the cell membrane. Microvesicles and exosomes may be secreted from the same originating cell and may have a similar molecular load, representing surface markers (shown by orange rectangles) and endoplasmatic molecules (shown by green triangles) from the originating cells. However, microvesicles and exosomes may also carry surface markers (blue and red ovals) and endoplasmatic markers (blue and red circles) which are specific for the respective type of vesicles.

Extracellular vesicles and pregnancy

The semi-allogenicity of a fetus often lead to comparison of fetuses to allografts (reviewed in [36]), while the establishment of the placenta often was compared to the aggressive invasive behavior of tumors (reviewed in [37]). In both allograft rejection and tumor development, the function of EV may be of great importance.[9,34,35] However, too strong comparisons with either of these conditions have been critically discussed over the last years. It is suggested, for instance, that the placenta, or more precisely the STB, secretes EV (STB EV) into the maternal circulation. In general, EV of diverse origin can be found in the blood during pregnancy. MV have been described to originate mostly from platelets, erythrocytes, T helper (Th) cells, monocytes, B cells and endothelial cells, but also STB MV were detected in the plasma from pregnant women.[38,39] Plasma concentrations of total EV were reduced [38], reflecting the disproportional increase of plasma volume compared to blood cell volume during pregnancy [40]. However, total MV concentrations recovered quickly again and subpopulations of MV secreted by monocytes, erythrocytes and STB even increased during pregnancy. Especially STB EV are believed to be associated with several pregnancy-related adaptations of the maternal body, e.g. the hemostatic system or the immune system.
The hemostatic system in pregnancy

Nearly throughout the whole pregnancy, the total blood volume of the mother increases due to an increase of both plasma volume and erythrocytes. Despite this increase in total blood volume, the blood pressure even decreases due to changes in sympathetic tone and the partially vasodilatory effect of hormones, like progesterone. To prevent premature bleeding or excessive post-partum bleeding, also the hemostasis is modified by an increase in pro-coagulant factors like thrombin, fibrin, von Willebrand factor and factors V, VII, VIII, IX, X, and XII in the blood of pregnant women. The increased coagulant potential is also supported by a decreased fibrinolytic activity during pregnancy which quickly returns to non-pregnant levels postpartum. In contrast, peripheral anticoagulant levels, like anti-thrombin III, do not compensate for the high coagulation capacities during pregnancy. Instead, they remain at non-pregnant levels and rise only postpartum to balance the increased coagulation potential. Since the increased coagulation potential normalizes quickly postpartum, pregnancy-associated circumstances such as the presence of the placenta might support the increased coagulation potential during pregnancy. In fact, the placenta is known to secrete several pro-coagulant factors, such as tissue factor, plasminogen activator inhibitor-2 but also STB EV.

The immune system in pregnancy

Pregnancy is characterized by a unique immunologic state which ensures the successful establishment and maintenance of pregnancy by preventing an immune attack of the semi-allogenic fetus while at the same time preserving the protection of the maternal body from external influences. Pregnancy-related changes have been shown in both, the adaptive and the innate immune response and their magnitude differs between peripheral and local uterine immune cells. As mentioned earlier, extravillous trophoblasts are invading the maternal decidua and support spiral artery remodeling. But also local uterine immune cells are of great importance for these mechanisms. Uterine NK cells represent about 70 % of the decidual leukocytes in early pregnancy and gather at the later implantation side already before implantation occurs. Uterine NK cells feature a high CD56 expression as also approximately 10 % of peripheral NK cells do, but other than that they show a very distinct expression profile from peripheral NK cells. They perform a tolerance-inducing function rather than the cytotoxic function of peripheral NK cells. About 20 % of the decidual leukocytes are represented by immune-modulatory M2 macrophages and 10 % by T cells, especially regulatory T (Treg) and memory T cells.

In the periphery, leukocytes support the slight systematic inflammatory state which characterizes normal pregnancy. The number of
monocytes, granulocytes and Treg cells are increased, while the number of NK cells is slightly decreased. The monocytes also show a maturation characteristics by shifting from CD16- classical monocytes to CD16+ intermediate monocytes while the overall immunity experiences a shift towards type 2 immunity. This is based on a decrease of the Th1/Th2 ratio and an increased expression of type 2 cytokines (e.g. interleukin (IL)-4, IL-5, IL-10) from NK and natural killer T (NKT) cells.[57–60] Though activity of Th1 and Th17 cells is strongly regulated during pregnancy, also Th1 and Th17 function is necessary for a successful pregnancy.[12,16,60,61]

Evidence is emerging, that STB EV released by the placenta might drive the described immunologic changes during pregnancy [8] and thus STB EV may represent (one of) the “missing” tolerance-inducing factors of villous trophoblasts. Next to the function of STB EV in healthy pregnancy, STB EV are also thought to play a role in the pathophysiology of the pregnancy-complication preeclampsia (PE).[62]

**Preeclampsia**

Pre-eclampsia (PE) is a severe pregnancy-complication which affects 2-8 % percent of all pregnancies worldwide and accounts for the death of approximately 76,000 women and 500,000 fetuses per year.[63–65] PE is mainly characterized by its major symptoms, which are new-onset hypertension and proteinuria in the second half of pregnancy.[66,67] The development of PE symptoms is based on the presence of the placenta during pregnancy, since also the development of hydatidiiform moles may induce PE symptoms [68,69] and delivery of the placenta relieves the symptoms [67]. The only known cure for PE is the termination of pregnancy which additionally increases the rate of preterm birth.[70,71]

**Pathogenesis of preeclampsia**

Although the overall etiology of PE remains widely elusive, the pathogenesis of this severe pregnancy-complication has been investigated in many studies. Originally, the pathogenesis of PE has been described as a two-stage process starting with an impaired placentation as the first stage which leads to the developments of the symptoms hypertension and proteinuria in the second half of pregnancy as the second stage.[72] However, based on extensive PE research in the past decades, this model has been refined and lately even been extended to six stages, starting already preconceptional and ending in the clinical symptoms and sometimes even development of an arteriosclerosis-like acute arterosis.[73]

According to the state of pregnancy in which PE occurs, it can be subdivided into early onset (<34 weeks of gestation) or late onset (>34 weeks of gestation). Although not fully clarified, it is believed that early and late onset PE feature different etiologies. According to that, early onset origins from impaired placentation due to an inadequate invasion of extravillous
trophoblasts after implantation and reduced remodeling of maternal deep spiral arteries. The reasons for this inadequate trophoblast invasion remains unclear and diverse options are being discussed. An impaired expression of stem cell/pluripotency marker of preeclamptic placentae compared to normal placentae suggest that the differentiation state of the trophoblast hinders the invasion directly. (Weber et al., manuscript submitted for publication, see publication list of the PhD candidate) On the other hand, a changed immune-cellular composition of preeclamptic placentae compared to normal placentae may interfere with the deep trophoblast invasion. As reviewed by Faas et al., the normal placental leukocyte pool predominantly comprises, next to uterine NK cells, immune-modulatory tolerance-inducing M2-type macrophages, which are localized close to the decidual spiral arteries but also to invading (HLA+) trophoblast cells spread over the decidual stroma.[74] In contrast, the preeclamptic placenta shows a shift to pro-inflammatory M1-type macrophages establishing a wall between in the invading trophoblasts and the spiral arteries and a reduced amount of uterine NK cells, which potentially disturbs the contact of trophoblasts with the spiral arteries and consequently their remodeling.[74–76] In either way, the poor remodeling of the maternal spiral arteries causes a reduced blood flow to the placenta and thus a shortage of oxygen and nutrients, and oxidative stress in the placenta. This is thought to increase the secretion of diverse placental factors, such as fms-like tyrosine kinase-1, placental growth factor or STB EV, into the maternal circulation.[38,77] STB EV have been shown to be increased during PE.[38,77] This increased STB EV release may foster inflammatory processes that ultimately lead to endothelial dysfunction which in turn promotes proteinuria, and, in combination with the increased release of other factors, hypertension.[4,78] Furthermore, also the molecular load of preeclamptic STB EV differs partially from that of normal STB EV, indicating an altered functionality of STB EV during PE.[79] Based on this altered functionality, STB EV may promote the disturbed immune reactions [80] and endothelial dysfunction [66] during PE. Additionally, especially early onset PE can be complicated by intrauterine growth retardation (IUGR) of the fetus or can evolve to the hemolysis, elevated liver enzymes, and low platelet count syndrome (HELLP-syndrome) or eclampsia.[78,81]

In contrast to early onset PE, late onset PE seems to origin rather in difficulties of the maternal body to cope with the changes of pregnancy and especially the presence of placental factors. While early onset PE often features very small placentae and as mentioned sometimes IUGR, late onset PE cannot only show small placentae and IUGR but even the opposite placental hyperplasia and increased fetal growth.[4,82] However, although probably not related to an impairment in early placentation, also in late onset PE, the presence of placental factors, such as STB EV, promote endothelial dysfunction, deregulation of the hemostasis and an exaggerated inflammatory state compared to normal pregnancy.

Maternal inflammatory state and coagulation in preeclampsia

Compared to normal pregnancy, PE is associated with an exaggeration of the
systemic inflammatory state in the maternal organisms characterized by endothelial and leukocyte dysfunction.[66] It has been shown, that numbers and activation state of monocytes and granulocytes are increased compared to normal pregnancy and that a stronger shift towards CD14++CD16+ intermediate monocytes occurs.[55,49] Furthermore, peripheral Treg cells are reduced. The Th1/Th2-ratio is increased compared to pregnancy favoring a type 1 immunity, and indeed Th1 but also Th17 are upregulated and more active. Also NK and NKT cells tend to produce more type-1 cytokines during PE compared to normal pregnancy.[49,57,76] On a local uterine/placental level, immune-modulatory tolerance-inducing M2-type macrophages are replaced by pro-inflammatory M1-type and uterine NK cells and Treg cells are reduced. Additionally, PE is connected to a strong fall in platelet counts but also an increased production of tissue factor, which together with the increased secretion of placental factors paves the way for coagulopathies during pregnancy.[67,83]

Aim and outline of this thesis

Although STB EV have already been the focus of several studies, their function still remains widely unclear. Based on the differential formation of MV and exosomes, STB MV and exosomes are expected to perform different functions, with STB MV being rather activating and inflammation-associated while exosomes being rather suppressive/tolerance-inducing.[8] However, the difference in the functionality of STB MV and exosomes has not been sufficiently elucidated so far. The aim of this thesis was to systematically compare the function of STB MV and exosomes on two physiological aspects with extreme importance for pregnancy – the immune system and the hemostatic system. Furthermore, the function of normal and pre-eclamptic STB EV was compared to better align their impact on physiologic processes during normal pregnancy and PE, as one of the major pregnancy-complications.

The quantification of STB EV is a critical step in their analysis. Several approaches (flow cytometry [84,85], whole protein quantification [9,86], nanoparticle tracking analysis [87], ELISA [77]) have been described as suitable methods for the quantification of STB EV. However, all of them have advantages and disadvantages. In the last years, flow cytometry has been discussed very critically because of its limits in detectable particle size.[85] While MV constitute the smaller size limit, exosomes are basically too small to be detected by flow cytometers.[85] For the other methods, there is no consensus about which method to prefer. In chapter 2, we aimed to develop a reliable, but also fast and simple quantification method for STB EV in suspension, such as plasma or suspension from ex vivo placenta perfusion. STB EV are believed to be associated with the pathophysiology of PE and have been shown to be increased in PE compared to normal pregnancy.[77] As described in chapter 3, we performed a multi-center, prospective, blinded, prognostic marker study exploring the usability of the peripheral plasma STB EV concentration as an accessory marker to mid-gestational uterine artery Doppler velocimetry in the prediction of the
development of PE in a high risk population. Currently, diagnosis of PE depends mainly on its major symptoms hypertension and proteinuria and PE prediction is highly limited due to a lack of reliable methods. Uterine artery Doppler velocimetry is a readily available, non-invasive, broadly utilized and accepted technique for the detection of pregnancies at risk and shows a high sensitivity, but rather low specificity for the prediction of PE.[88,89] Attempts have been made, to complement uterine artery Doppler velocimetry by plasma biomarkers like soluble vascular endothelial growth factor receptor-1, placental growth factor or fms-like tyrosine kinase-1, but have not been very successful so far.[90–92] Thus, a complementation of uterine artery Doppler velocimetry by plasma STB EV concentration might be a promising approach.

The chapters 4 and 5 focus on the immunologic functionality of STB MV and exosomes from normal and preeclamptic placentae, regarding peripheral monocytes and granulocytes (chapter 4) and peripheral lymphocyte subpopulations (T cells, NK cells and NKT cells, chapter 5). Since STB MV and exosomes may have different functions, we studied the effect of STB MV and exosomes separately. In chapter 6, we analyzed the pro-coagulant capacity of placental factors. The function of STB MV and exosomes was compared based on a set of samples, in which cell debris/macrovesicles, STB MV and exosomes have been stepwise excluded from the experiments.

In chapter 7, the achieved findings of this thesis and future recommendations are discussed.
References


[57] A.M. Borzychowski, B.A. Croy, W.L. Chan, C.W.G. Redman, I.L. Sargent, Changes in systemic type 1


General introduction


