Diplomarbeit

DIFFERENTIAL EXPRESSION OF METALLOPROTEINASES AND THEIR SPECIFIC INHIBITORS IN DELAYED MISCARRIAGE VERSUS CONTROLS

A WIDE SPECTRUM SCREENING FOR MMPs, ADAMs, ADAM-TSs AND TIMPs IN FIRST TRIMESTER PLACENTAL TISSUE

eingereicht von

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zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt an der

Universitätsklinik für Frauenheilkunde und Geburtshilfe

unter der Anleitung von

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... Knowledge cannot start from nothing – from the tabula rasa – nor yet from observation. The advance of our knowledge consists in the modification and the correction of earlier knowledge. Of course it is sometimes possible to take a step forward through an observation or through a chance discovery; but the significance of an observation or of a discovery generally depends upon whether it enables us to modify existing theories.

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(Sir Karl Popper: On the So-Called Sources of Knowledge. Lecture delivered to the University of Salsburg 27 July 1979. From *In Search of a Better World*. London: Routledge 1984.)

ACKNOWLEDGMENT

I really want to express my gratitude and appreciation to each of this open-hearted research team I quickly became integrated. I had the chance to take my first steps in a conducive scientific surrounding which was a delightful and challenging experience at all. In particular, I want to thank Prof. Gernot Desoye and Dr. Ursula Hiden for the fabulous mentorship. Further, I want to say thanks to the complete team of the lab which was always there if I had a problem, namely Heidi, Martina, Susanne, Ate and Luciana.

I am deeply indebted to my father, Dr. Johannes Greimel, for putting me through my academic studies and I want to enunciate my admiration, respect and love for him.

ZUSAMMENFASSUNG

Die Entstehung und Aufrechterhaltung einer Schwangerschaft beim Menschen ist ein strikt regulierter und äußerst komplexer Prozess. Lebenswichtig für die werdende Mutter und den Feten ist die regelrechte Entwicklung und das reibungslose Funktionieren der Plazenta. Im Mittelpunkt der meisten kritischen Abläufe in der Plazentaentwicklung steht ein spezifischer Zelltyp, der Trophoblast. Trophoblasten invadieren schon in den ersten Wochen der Schwangerschaft mütterliches Gewebe unter stetigem Abbau der extrazellulären Matrix (ECM). Dieser Vorgang wird durch die proteolytische Eigenschaft einer Enzymgruppe ermöglicht, den Matrix Metalloproteasen. Zu diesen zählen MMPs (matrix metalloproteinases), ADAMs (a disintegrin and metalloproteinases), ADAM-TSs (a disintegrin and metalloproteinases with trombospondin motif) während die spezifische Hemmung durch TIMPs (tissue inhibitors of metalloproteases) vermittelt wird. Ein häufiges Schwangerschaftsereignis in der Frühschwangerschaft ist das Absterben der Frucht *in utero*. Falls das abgestorbene Schwangerschaftsprodukt nicht spontan ausgestoßen wird, spricht man von delayed miscarriage. In unserer Untersuchung möchten wir eine mögliche Verbindung zwischen delayed miscarriage und veränderter Expression von ECM abbauenden Proteasen nachweisen.

Gewebe von delayed miscarriage Schwangerschaften und Kontrollgewebe von Schwangerschaftsabbrüchen aus psychosozialen Gründen wurde zu Studienzwecken gewonnen. Diese Proben stammten alle von Schwangerschaften im ersten Trimester. Nach RNA Isolierung führten wird Reihenuntersuchungen diverser Enzyme mittels PCR Methode durch. Signifikanz wurde mit $p<0.05$ definiert.

Eine auffallende Neigung zu Enzymhochregulierung unter pathologisch veränderten Bedingungen wurde festgestellt. Interessanterweise war durchwegs ein signifikanter Unterschied in der 7. bis 8. Woche zu finden.

Dieses Expressionscreening von Metalloproteasen in der Frühschwangerschaft stellt das erste seiner Art und seines Umfanges dar. Wir konnten die trophoblastäre Expression einiger bis dahin nicht erwähnter Enzyme nachweisen und dürfen die potentielle Wichtigkeit dieser Enzymgruppe in der menschlichen Fortpflanzung herausstellen. Des Weiteren wird in dieser Arbeit der Versuch gewagt, pathophysiologische Zusammenhänge aufgrund der gewonnenen Daten neu zu formulieren.

ABSTRACT

The establishment of pregnancy in humans is a complex and tightly regulated event. Central for fetal and maternal survival is the proper development and functioning of the placenta. Mainly responsible for the development of placental structures and the functional bonding of the two organisms is a specialised cell lineage, the trophoblast. Trophoblastic cells start to invade maternal tissue in early first trimester of pregnancy, thereby degrading extracellular matrix (ECM). This strictly governed process is dependend on ECM degrading metalloproteinases, namely the groups of MMPs (matrix metalloproteinases), ADAMs (a disintegrin and metalloproteinases), ADAM-TSs (a disintegrin and metalloproteinases with trombospondin motif), while specific inhibition is provided by TIMPs (tissue inhibitors of metalloproteases). A common pathology in early pregnancy is the fetal death with absent expulsion from the uterus, which is defined as delayed miscarriage. In our study we aimed to detect a possible association between delayed miscarriage and altered metalloprotease expression.

First trimester placental tissue of women suffering from delayed miscarriage and controls from legal pregnancy interruptions carried out for psycho-social reasons was obtained. RNA was isolated and PCR screening for all relevant enzymes was performed subsequently. Results were regarded as significant at $p<0.05$.

In summary, it can be stated that there is a strong trend of metalloprotease upregulation under pathologically altered conditions. Further, it was possible to detect significant differences particularly in gestational weeks 7 and 8.

This is the first wide spectrum screening for all relevant members of the metalloprotease family in first trimester trophoblasts. We could demonstrate the expression of several previously unknown metalloproteinases in human trophoblasts, thus enabling us to underline the importance of these enzymes involved in crucial developmental events. In consideration of significant protease upregulation in early first trimester, this is a new approach to identify mechanisms of delayed miscarriage.

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1 INTRODUCTION

1.1. The human placenta

1.1.1 Morphology of the mature delivered placenta

The human placenta is a soft discoidal organ with an average thickness between 25 to 30 mm and a diameter at time of delivery from 200 to 220 mm (Huppertz 2008)(Sadler, Langman 2003). The range of weight at delivery depends on the mode of delivery and the content of blood at time of quantification, so data give a mean of about 500 g (Huppertz 2008). Futhermore it is important to point out that the human placenta is an example for the hemochorial type of placentas where maternal blood is circulating around the villious trees composed of fetal tissue to provide the exchange of essential substances (Sadler, Langman 2003) [see figure 1].

Examining the delivered placenta, two surfaces of the organ can be differentiated: the smooth amnion covered fetal surface and the maternal surface. The fetal surface imposes through a big variety of different sized vessels fusing to form the umbilical vein and the two umbilical arteries heading to the fetal abdominal wall. On the maternal surface there are 10 to 40 slightly elevated regions which are defined as cotyledon (Sadler, Langman 2003)(Gray 1918) or placental lobes (Huppertz 2008), further devided through a system of membranes (placental septum in fig. 1).

Regarding the microscopic anatomy of the human placenta, a severalfold branching system of the villous tree can be defined, leading from stem villi to the freely floating villi, subdivided in five groups and responsible for the final diffusion and transport of metabolites. A specialized villous type providing mechanical stability is the achoring villous, which is embedded in the fetal plate as well as in the maternal basal plate. The five different types of floating villi are described on the basis of their qualitative and quantitative characteristics (Huppertz 2008)(Kaufmann, Sen & Schweikhart 1979, Castellucci et al. 1990): Early pregnancy dominating *mesenchymal villous* is the forerunner of the intermediate villous type, mostly consisting of mesenchymal cells and aborning blood vessels, partially even lacking a vessel lumen. The next step of differentiation is the *immature intermediate villous* which is characterised by the formation of matrix-free channels where a large number of placental macrophages can be found. It continues to develop into a *stem villous* by fibrosation of the stroma. The previously mentioned stem

villous meets requirements for a mechanical stabiliser of the villlous tree by mostly consisting of dense fibrous stroma with a few small vessels. Generated by the branching stem villous, rich in vessels and capillaries, *mature intermediate villi* give rise to *terminal villi.* Finally, at the end-branch of the villous tree, large quantities of capillaries and vessels with an extremely thin syncytial layer can be found (Huppertz 2008) due to the need for a short diffusion barrier to fulfill the physiological requirements during pregnancy.

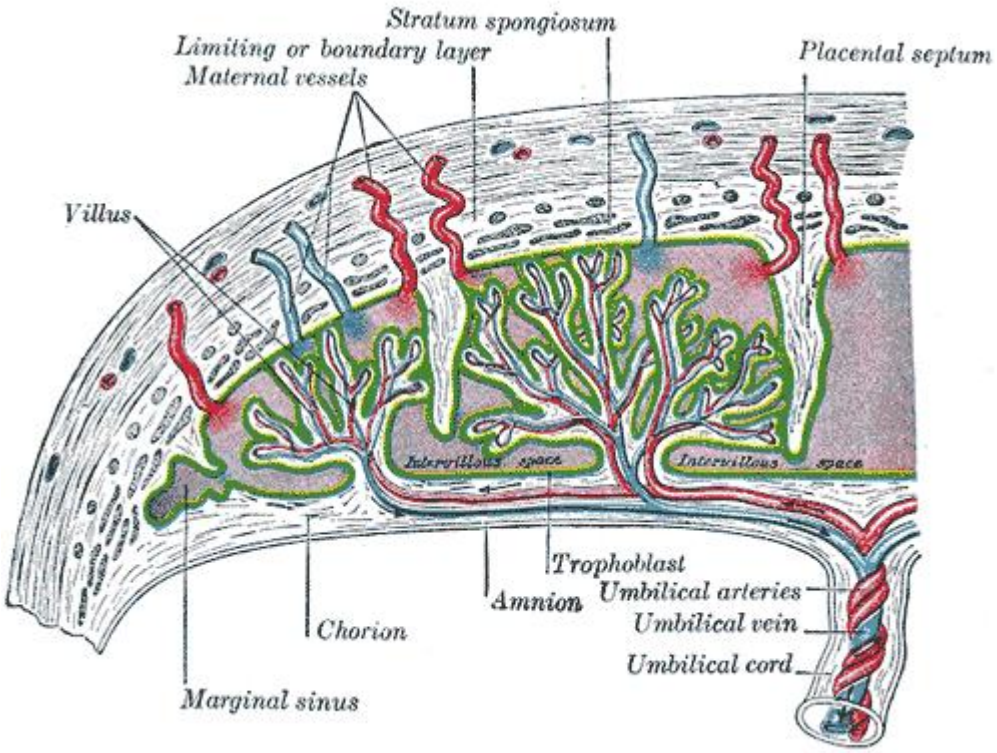


Figure 1: Macroscopic morphology and the way of blood supply. Source: (Gray 1918)**.**

1.1.2. Physiological aspects of the human placenta

The human placenta mechanically connects the mother and fetus although the blood circulations are strictly seperated by the placental barrier. Thus, the maternal blood stream comes from the uterine arteries and reaches the intervillous space through the so called spiral arteries, a special artery type in pregnancy (Robertson 1976), and fills the space between placental villous structures. The outstanding basic function of the placenta is the exchange of metabolites and gases. Oxygen, carbon dioxide, carbon monoxide and electrolytes pass the placenta barrier by diffusion in contrast to nutritients like aminoacids and fatty acids which are dependend on specific transport systems (Sadler, Langman 2003). Concerning carbohydrates we find a form of facilitated transport via hexose transporters.

Also hormones are synthesised and secreted by the placenta. Already in early stages of development relevant amounts of human chorionic gonadotropin (hCG) are produced by the conceptus. This hormone enables the early detection of pregnancy by easily accessible urine tests and it is used by professionals for diagnosis in obstetrics. Evidence exists that hCG and its hyperglycosylated form (hCG-H) could be of potential importance as biomarker for pregnancy outcome at a very early stage of gestation (Norris et al. 2011, Cole 2007, Cole 2010, O'Connor et al. 1998, Canfield et al. 1987). In the end of the 4^{th} month of gravidity the placenta is able to maintain pregnancy even without support by ovary derived hormones, because of the high levels of progesteron produced to signal the needs of the growing conceptus (Sadler, Langman 2003). Another important hormone is the human placental lactogen, also called human chorionic somatomammotropin, which functions as growth hormone (Sadler, Langman 2003).

Futhermore, the placenta plays a crucial role with regard to the immnune system. Transplacental efflux of class IgG antibodies protects the newborn from several infectious diseases (Sadler, Langman 2003)(Chucri et al. 2010). But that is just one aspect of the immunological role of the placenta. The other aspect is the now basically understood immunological cross-talk between mother and conceptus which might be responsible for rejection or establishment of the very early pregnancy and probably plays a fundamental role in the pathophysiology of pre-eclampsia (Veenstra van Nieuwenhoven, Heineman & Faas 2003, Renaud et al. 2011)(Redman, Sargent 2010). It is known that processes like implantation, angiogenesis and invasion of the trophoblast are strictly monitored by the maternal immune cells and that the maternal organism changes the state of immune

response not only locally but also in the peripheral system. This happens in terms of a switch from type 1 T-cell derived cytotoxic and pro-inflammatory response to the type 2 T-cell dominated pathway, which leads to the activation of the humoral immune system. In case of failure of this maternal immune response there is a higher risk for pregnancy loss (Veenstra van Nieuwenhoven, Heineman & Faas 2003). But not only the mother is modulating the state of reactivity, also invading trophoblastic cell lines actively downregulate the maternal rejection by specifically binding to apoptosis inducing receptors of maternal immune cells and by a mechanism called immune escape. Immune escape is accomplished as trophoblasts do not express a specific form of MHC (i.e. MHC Ia) proteins on their cell surface so that maternal immune cells cannot distinguish between self and non-self (Veenstra van Nieuwenhoven, Heineman & Faas 2003, Murphy, Choi & Holtz 2004, Coady et al. 1999).

1.1.3. Developmental biology of the human placenta

To overview the early formation of the placenta, (Huppertz 2008, Chaddha et al. 2004) and partly (Benirschke, Kaufmann & Baergen 2006) are summarised:

Pre-implantation stage. About day 4 to 5 after fusion of the maternal and paternal cells, the conceptus evolves into two distinct cell lines, the embryoblast and the trophoblast. The trophoblastic cells are surrounding the embryoblastic cell convolute and a cavity circularly. This stage is also called the phase of the blastocyst because of the tissue´s cystic character. In the later growth, most parts of the placenta and fetal membranes are derived from the trophoblast while the embryoblastic tissue gives rise to the embryo, umbilical cord as well as the mesenchymal tissue of the placenta.

Prelacunar stage. Only two to three days later, the beginning of the prelacunar stage is defined by the attachement of the blastocyst to the uterine epithelium followed by the event of implantation. The outcome of this process is strongly dependend on proper orientation of the implanting blastocyst. Hence, the embryoblastic cell mass should be located directly beneath the attaching trophoblast layer, therefore called polar trophoblast, playing a leading role in further formation of the placental structures. Once the attachment of the blastocyst is stable, the trophoblast is undergoing the next step of differentiation while more and more trophoblastic cells start to fuse and produce a syncytial layer which is defined as syncytiotrophoblast from now on. This process is sustained by the remaining stem-cell-like trophoblastic cells through dividing and fusion now refered to as cytotrophoblasts.

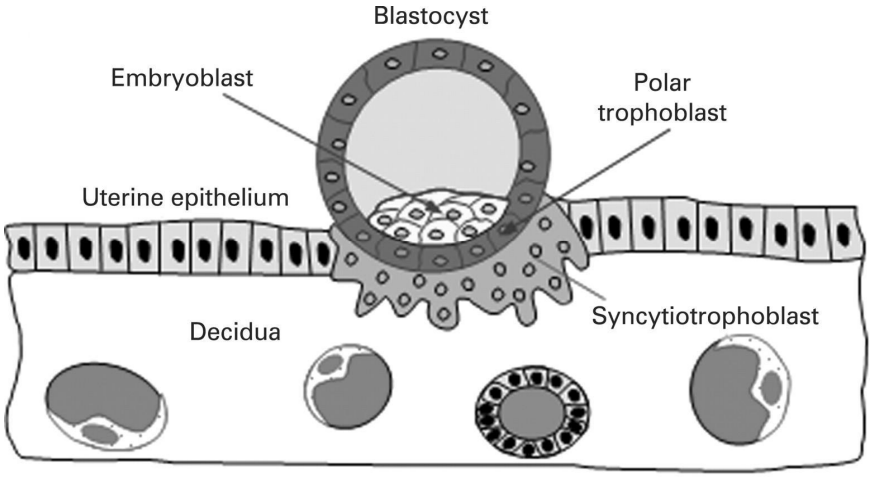


Figure 2: Implantation of the blastocyst. Adopted and modified from (Huppertz 2008)**.**

Two important facts have to be noticed here: First, the syncytiotrophoblast forms a mantle around the conceptus and shows invasive activity, hence, completely embedding the conceptus into the uterine wall. Second, the syncytiotrophoblast is the only tissue coming in contact with maternal cells and fluids which might comprises implications on immunological aspects of pregnancy.

Lacunar stage. Already eight days after conception the emergence of fluid filled areas inside the growing syncytial layer, refered as to lacunae, can be observed. The persisting tissue in between forms the so called trabeculae, later origin of the villous tree. So now, the three distinct zones of the future placenta can be separated: The early chorionic plate facing the embryo, villous tree and intervillous space and the basal plate connecting the organ to the maternal tissue. Further, invading syncytiotrophoblast leads to erosion of superficial capillaries and the venous plexus. Sequentially the first maternal blood cells can be found in the lacunar system. At about day 12 post-conception, cytotrophoblasts start to penetrate the syncytiotrophoblast from the chorionic plate heading forward to reach the maternal side of the developing placenta. There, they leave the placenta proper and invade into the uterine wall. Since the cells are not part of the villous system any more, they have differentiated into extravillous trophoblasts. The extravillous trophoblast plays a pivotal role in angiogenesis and regulation of placental blood supply (Kaufmann, Black & Huppertz 2003). Two main subtypes of the extravillous trophoblast have to be discriminated: The endovascular extravillous trophoblast invades maternal spiral arteries and causes an occlusion of the lumen just at the implantation site. This reduces blood flow and establishes a hypoxic milieu relative to the mother during the first weeks of gestation. It is supposed to be essential for early placental angiogenesis ("hypoxic drive") and protection of the developing fetal structures from oxygen-mediated harm. The interstitial extravillous trophoblast can be found accentuated in the beginning of the second trimester. This seems to be responsible for the remodelling of the spiral arteries into passively dilated tubes, the secretion of vasodilatating signal molecules and even the adaption of the mother´s cardiovascular system. To underline the importance of extravillous trophoblast invasion, the association between the little invasion deepness by extravillous trophoblast cells, the reduced intrauterine trophoblast cell density and the incidence of pregnancy pathologies like pre-eclampsia and intrauterine growth restriction has to be pointed out (Kadyrov et al. 2003, Naicker et al. 2003, Hemberger et al. 2003).

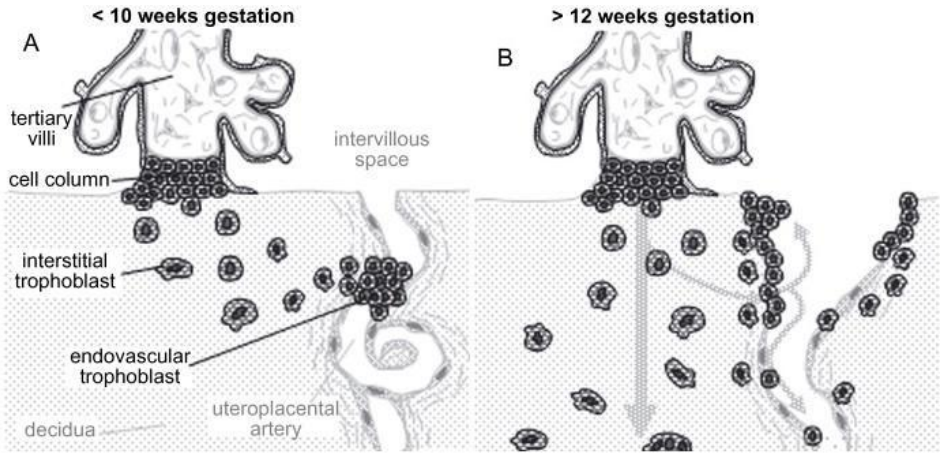


Figure 3: Characteristics of the two invasive trophoblastic subtypes: (A) displays the obliteration of the spiral artery lumen by the endovascular extravillous trophoblast in early first trimester. (B) shows the further development of the spiral artery into a passively dilated tube by the interstitial extravillous trophoblast ensuring supply for the fetus. Modified taken from (Chaddha et al. 2004)**.**

Villous stage. Starting at about day 13 post-conception, the above decribed trabeculae display small protrusions which form the multiple branching villous system in the course of their development, bulging out into the intervillous space. Three steps of villi have to be distinguished: Primary villi consisting of a cytotrophoblastic core and a surrounding syncytial layer. Secondary villi arise through the mesenchymal filling derived from the embryo. Finally, tertiary villi come up with the formation of vessels and blood cells.

1.2. Trophoblast biology

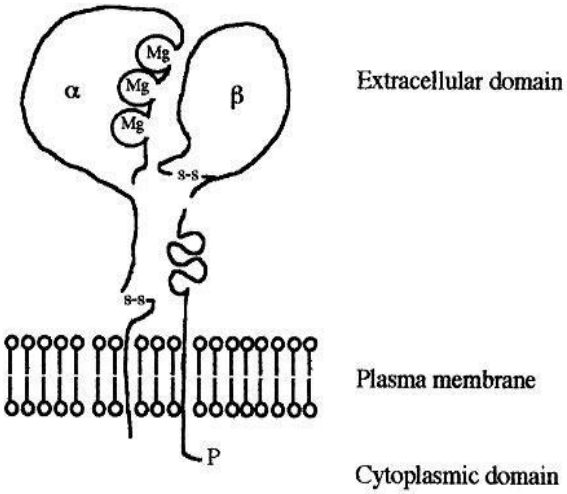
1.2.1. Differentiation of the human trophoblast

The trophectodermal derived cell line of the trophoblast gives rise to several different subtypes which can be found in the villous tree (i.e. villous trophoblast) and beyond the villous structures (i.e. extravillous trophoblast). In the stage of stem cells, villous cytotrophoblasts act as generative units for the above localized layer of syncytially fused cytotrophoblasts named villous syncytiotrophoblast. In addition, specialized cytotrophoblasts form typical cell collums at the distal ends of the villi, anchoring the villous structures firmly to maternal tissue, reffered to as anchoring trophoblasts. In the course of further development as described above (see 1.1.3.), a relevant amount of trophoblastic cells start to detach from the cell collum structures and invade the decidua. There, three patterns of biological behaviour can be differentiated depending on where the migrating cells are located (see also 1.1.3.): Endovascular trophoblasts invade the lumen of spiral arteries, interstitial trophoblasts are spread in the decidual stroma and the inner third of the myometrium while the recently discovered endoglandular trophoblasts could play a significant role in histiotrophic nutrition in the early phase of pregnancy by infiltration of endometrial glands, thus probably ensuring adequate glandular fluid production and secretion (Fitzgerald et al. 2010, Anin, Vince & Quenby 2004, Burrows, King & Loke 1996, Staun-Ram, Shalev 2005, Knofler 2010).

One of the most outstanding features of the invasive phenotype is the capability to detach from the villous basal membranes and maneuver through the maternal tissue by attachment to and degradation of extracellular and cell membrane structures in the decidual environment. The state of motility is achieved through shifts in the spectrum of cell adhesion molecules (CAMs), more precisely the group of integrins, cadherines and immunoglobulin superfamily adhesion receptors (Ferretti et al. 2007).

The most important family of CAMs related to trophoblast cell migration are the integrins, a class of cell surface receptors mediating adhesion to extra cellular matrix (ECM) proteins. Integrins are transmembrane gylcoprotein receptors for ECM molecules such as collagenes, fibronectin and laminin, consisting of two non-covalently associated alpha/beta subunits. There is a large extracellular domain, a transmembrane segment and a cytoplasmatic tail (see fig. 4). Responsible for the ligand specifity of the heterodimere is the specific matching of the manifold alpha and beta subunits of the exracellular domain

(for coupling of subunits and ligand specifity see fig. 4). Abundant evidence exists for the importance of an integrin pattern switch: Villous cytotrophoblasts express the α6β4-laminin receptor while connected to the basal membrane and switch to α5β1-fibronectin receptor as they leave basement sructures followed by α1β1-laminin/collagen receptor expression as fully developed invasive phenotype. The α6β4-laminin receptor is widely spread among epithelial cells to establish a basement anchoring. α1β1-lamin/collagen receptor is interesting in a bifocal perspective: First, it is supposed to facilitate the transmission of several different signals to the migrating trophoblasts, second, for receptor binding a certain degree of matrix proteolysis is required due to the cryptic binding site of this molecule. α5β1-fibronectin receptor is associated with cell motility. (Anin, Vince & Quenby 2004, Burrows, King & Loke 1996, Ferretti et al. 2007, Aplin et al. 1999, Damsky, Fisher 1998, Damsky et al. 1994, Zhou et al. 2003) In pathologically altered pregnancies such as pre-eclampsia and other pregnancy related diseases, the typical integrin switch fails to appear, thus underlining the importance of integrins for migration and cell motility (Zhou et al. 1993).



Integrin heterodimer	Ligand
$\alpha_1\beta_1$	Laminin/collagen
$\alpha_2\beta_1$	Laminin/collagen
$\alpha_3\beta_1$	Laminin/collagen/fibronectin
$\alpha_4\beta_1$	Fibronectin
$\alpha_5\beta_1$	Fibronectin
$\alpha_6\beta_1$	Laminin
$\alpha_v\beta_1$	Vibronectin/fibronectin
$\alpha_6\beta_4$	Laminin

Figure 4: Schematic representation of the integrin structure (left) and listing of diverse subunit couples and their specific ligands (right). Source: (Burrows, King & Loke 1996)**.**

In line with further differentiation, human throphoblastic cells also generate a specific expression pattern for major histocompatibility complex (MHC) molecules. MHC proteins are a highly polymorphic group of membrane complexes that allow the crucial distinction between self and non-self. Three classes exist: MHC Ia, MHC Ib and MHC II. The basic function of MHC Ia molecules is to present antigens to immune competent lymphocytes assuring the detection of foreign cells followed by their disintegration. Interestingly,

trophoblasts do not express MHC Ia proteins. This results in failing rejection of the partly allogenic conceptus by the mother´s immune control, a cue for immune escape. Concerning MHC Ib molecules, HLA-G and HLA-E (human leukocyte antigen) are specifically expressed by invading trophoblasts while villous cytotrophoblasts and villous syncytiotrophoblast do not display these proteins. The ultimate role is not yet clear, but accumulating evidence indicates the potential regulatory functions by rendering the trophoblasts more resistant to lysis by uNK cells (uterine natural killer cells). Moreover, suppression of lymphocyte proliferation and immune response shifting from type 1/cytotoxic to type 2/humoral could be facilitated by above-mentioned MHC Ib molecules. (Veenstra van Nieuwenhoven, Heineman & Faas 2003, Murphy, Choi & Holtz 2004, Coady et al. 1999, Anin, Vince & Quenby 2004, Ferretti et al. 2007)

Because of the importance for further understanding, the main factors initiating trophoblast differentiation should be brought up. The change in phenotype from epithelial to endothelial (e.g. integrin patterns) is maintained by VEGF (vascular endothelial growth factor), PLGF (placental growth factor), HGF (hepatocyte growth factor), EGF (epidermal growth factor), LIF (leukemia inhibitory factor), IGF-1 (insulin like growth factor 1), cytokines (IL-1, IL-6, IL-8, IL-11), TGF-β (transforming growth factor β), TNF-α, GCM1 (glial cell missing factor 1), oxygen levels and hormones like hCG (human chorion gonadotropin) and progesterone. (Anin, Vince & Quenby 2004, Burrows, King & Loke 1996, Staun-Ram, Shalev 2005, Knofler 2010, James, Stone & Chamley 2006, Tuuli, Longtine & Nelson 2011, Lunghi et al. 2007)

1.2.2. Specific features and their regulation

During the implantation process trophoblasts have to perform several idiosyncratic functions enabling the proper establishment of pregnancy. Therefore, I will focus on the interactions between evTBs and decidual blood vessels, thereby evolving questions of oxygen levels and how trophoblasts are dealing with the challenge of changing conditions. Further, importance of tightly regulated proliferation and apoptosis will be reviewed and, finally, ECM adhesion and disintegration will be mentioned.

Blood vessels and the invasive trophoblast. In early first trimester the oxygen tension is physiologically low and evidence suggests that there is no blood flow to the developing placental bed up to the 10^{th} to 12^{th} week of gestation (James, Stone & Chamley 2006, Tuuli, Longtine & Nelson 2011, Jauniaux et al. 1992, Jaffe, Woods 1993). This is constituted via endoluminal plugging of decidual arteries by invading trophoblasts during the first weeks of gestation. This phenomenon is restricted to the site of implantation and enables placental growth through a so called hypoxic drive (Chaddha et al. 2004). Members of the selectin family, in particular L-selectins, E-selectins and P-selectins, a group of molecules that mediate cell-cell and cell-matrix adhesion play a role herein. Selectins are present at the cell surface of vascular endothelial cells in the decidua bordering the placental bed and through binding to them, endoluminal plugging by trophoblasts may be facilitated. One potential ligand of selectins is the sialylated Lewis X ligand, which has been found expressed in endovascular trophoblasts. Furthermore, ICAM-1 (inter cellular adhesion molecule 1) and NCAM (neural cell adhesion molecule) could play a role in cell-cell adhesion during endovascular plugging while ICAM-1 is not specifically expressed at the site of implantation and is likely wide spread. In contrast, NCAM-NCAM homotopic bonding could be responsible for the loosely trophoblast aggregates in the vessel lumen during first weeks of gestation (Burrows, King & Loke 1996, Staun-Ram, Shalev 2005, Burrows, King & Loke 1994).

As the conceptus grows, the need for sufficient supply increases exponentially the blood flow into the placenta. This is achieved by the so called vascular remodelling, a process which includes the degradation and resynthesis of extracellular matrix and replacement of endothelial cells by infiltrating trophoblasts. ECM digesting proteases could be of great importance in this physiological alteration (Tuuli, Longtine & Nelson 2011, Caniggia et al. 2000)(Anin, Vince & Quenby 2004).

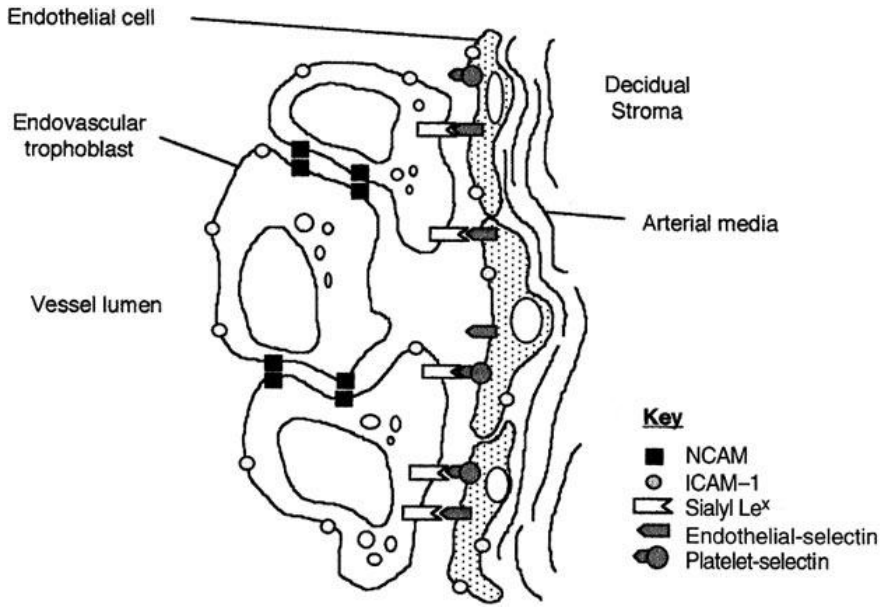


Figure 5: Trophoblast-endothelial and trophoblast-trophoblast cell interaction and supposable cell surface receptors involved. Taken from (Burrows, King & Loke 1996)**.**

Oxygen and trophoblast biology. The oxygen tension in first trimester placentas is very low (pO_2 < 20 mm Hg), thus inducing a steep gradient between mother and fetus while the onset of intervillous space perfusion creates a normoxic environment (pO_2 40-80 mm Hg) starting at early second trimester (see fig. 6). The question of oxygen is essential for early placental development since physiological hypoxia at early stages of placenta formation plays a key role in the regulation of vascularisation, trophoblast invasion and differentiation. Apart from the regulative aspect of oxygen, the early placenta and the arising fetus are not sufficiently protected from oxidative stress by enzyme systems capable of metabolising reactive oxygen species (ROS) harming the cellular integrity. Until approximately 8-9 weeks of gestation protective enzyme systems are not expressed and increase significantly as the placenta becomes exposed gradually to rising levels of oxygen. Copper-zinc superoxide dismutase, gluthation peroxidase and catalase are held to be responsible for averting imminent damage (James, Stone & Chamley 2006, Tuuli, Longtine & Nelson 2011, Watson et al. 1997). Therefore, untimely and excessive perfusion of the intervillous space has been underlined as potential cause of early pregnancy loss (Jauniaux et al. 2000, Caniggia, Winter 2002, Hustin, Jauniaux & Schaaps 1990).

Trophoblast adaptions to changing oxygen conditions are broadly reviewed, but there is still controversial approach to the underlying mechanisms of trophoblast behaviour (James, Stone & Chamley 2006, Tuuli, Longtine & Nelson 2011, Caniggia et al. 2000, Caniggia, Winter 2002, Seeho et al. 2008, Huppertz et al. 2009). Evidence suggests a pivotal role for

the family of hypoxia inducible factors (HIF) leading to hypoxia mediated gene expression, though HIF-1α is in the middle of interests. HIF-1α has been shown to regulate gene expression of more than 20 genes responsible for coping mechanisms due to changing oxygen tension. Under hypoxic conditions stabilized HIF-1α bonds HIF-β, a constitutively expressed subunit, while translocated to the nucleus and binds to DNA in form of HIF-1α-β-dimer in order to induce relevant gene expression (see fig. 6). Expression of HIF-1α can be found first during week 5 of gestation and decreases at about week 9. After the 12th week of pregnancy HIF-1α was hardly detectable by immunostaining performed by Cannigia et al. Factors regulating HIF-1α activity despite oxygen are diverse including stabilization of HIF-1α by phosphorylation, dimerization, translocation, transcriptional co-factors, growth factors and hormones (see fig. 7). Interestingly, HIF-1α up-regulation hinders trophoblasts to differentiate towards an invasive phenotype. Thus, low oxygen and HIF-1α activity are associated with an intermediate trophoblast phenotype expressing α5β1-integrin patterns, showing high proliferative activity and lower invasive behaviour, thereby reduced matrix metalloproteinase 2 (MMP2) activity, and, worthy of mention, inducing transforming growth factor β3 (TGFβ3) expression which seems to be responsible for inhibition of early trophoblast differentiation, outgrowth and invasion.

In contrast to the above statements, inverse results were demonstrated by other scientists, as significant up-regulation of the uPA receptor was found under hypoxic conditions. The rationale is activation of the uPA/plasminogen system that accounts for the conversion of plasminogen into plasmin, capable of degrading ECM components and activating ECM digesting proteases crucial for invasion. The issue is not yet decided, thus, one may conclude that contradictory evidence gives two possible explanations for trophoblast behaviour under low oxygen tension. First, hypoxia is followed by a proliferative phenotype in order to provide a large amount of trophoblasts for later invasion in second trimester. Second, low oxygen promotes differentiation towards the invasive phenotype due to sufficent depth and extent in the process of invasion.

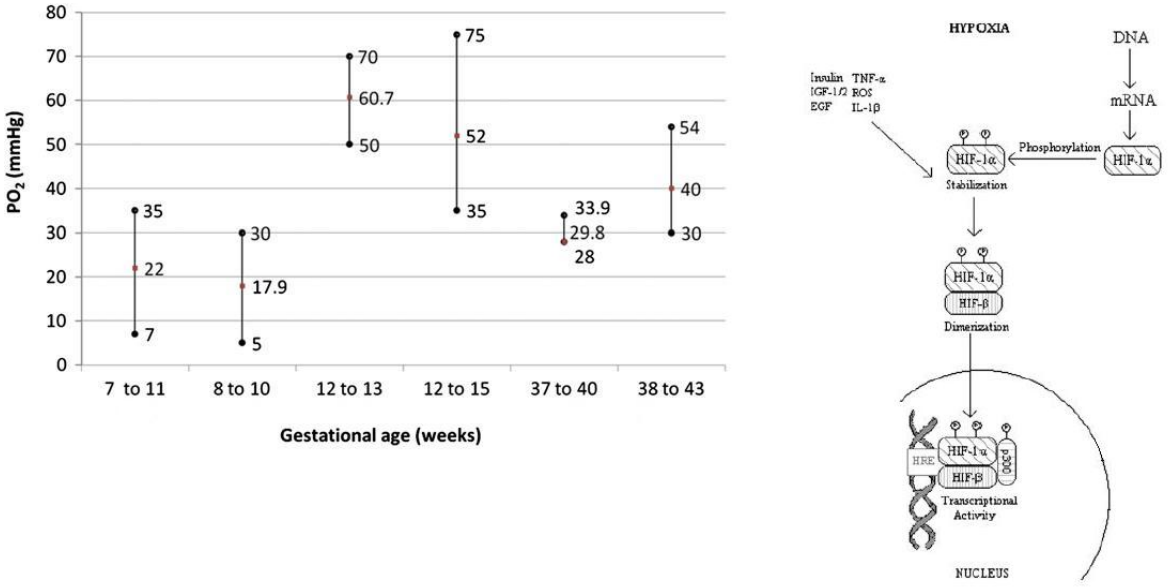


Figure 6: Intervillous oxygen concentrations by *in vivo* measurements depicted as means, minimum and maximum values at differing gestational ages (left). Cascade of HIF-1α in the state of hypoxia and central factors of influence (right). Source left part: (Tuuli, Longtine & Nelson 2011) **and right figure modified from** (James, Stone & Chamley 2006)**.**

Proliferation and apoptosis - maintaining the essential balance. It is known that trophoblastic and malignant cells share comparable molecular mechanisms to obtain their proliferative, migratory and invasive potential. Some authors even understand human trophoblasts as a pseudo-neoplastic cell line, actually not far-fetched. Distinctive is the stringent surveillance of underlying molecular curcuits which ensures the pseudo-malignant capacities of the trophoblast to be temporally and spatially controlled. Trophoblasts are highly proliferative lacking of cell contact inhibition, showing altered proto-oncogen expression and mutual growth stimulation via paracrine as well as autonomous stimuli via autocrine ligand-receptor loops such as EGF/EGFR, VEGF/VEGFR and HGF/HGFR. Interestingly enough, the evasion of apoptosis mediated by IGF-1/IGF-2/IGF-1R signalling could also play a central role during the proliferative burst in first trimester. Others detected the membrane bound Fas ligand (FasL) which leads to induction of apoptosis following interaction with Fas. This is probably one way trophoblasts mutually regulate their expansion at the frontier of invasion. FasL is also involved in immunoregulative functions during implantation, thus initiating apoptosis in maternal leukocytes exposed to invasive trophoblasts (Veenstra van Nieuwenhoven, Heineman & Faas 2003, Anin, Vince & Quenby 2004, Ferretti et al. 2007).

The interplay of trophoblasts and extracellular matrix in view of placentation. Physiological events such as arterial remodelling, basement membrane penetration and migration are dependend on the orchestrated disintegration of various structural proteins, glycoproteins and proteoglycans that contribute to the extracellular matrix (ECM). Regarding trophoblast migration, three functional steps have to be highlighted (Anin, Vince & Quenby 2004): The first step is adherence to ECM molecules and decidual cells mediated by membrane bound integrin patterns and other cell adhesion molecules (CAMs). Second, proteolysis of ECM substrates such as collagenes, elastines and gelatines is initiated, whereas not only a remodelling of the structural environment takes place but also alteration of regulative effects by ECM occurs. Hence, ECM degradation impacts basic cellular functions of surrounding trophoblastic cells such as proliferation, phenotype specific differentiation and migratory potential. Finally, the way is cleared for invasion.

Four groups of ECM converting enzymes exist. The matrix metalloproteinases (MMP), a disintegrin and metalloproteinases (ADAM), a disintegrin and metalloproteinases with trombospondin motifs (ADAMTS) and the plasminogen activator (PA) system of serine proteases are held responsible for most crucial processes of ECM turnover. The two classes most discovered and characterised in literature are the well known MMPs and the PA system, thereby many authors have pointed out the importance of MMP-2 and MMP-9 based on both *in vitro* and *in vivo* studies (Staun-Ram, Shalev 2005, Staun-Ram et al. 2004, Bischof, Meisser & Campana 2001, Bischof et al. 1995, Cohen, Meisser & Bischof 2006). Biological behaviour of trophoblastic cells is regulated in numerous ways by these enzymes, including cell-matrix and cell-cell interference, activation or inactivation of autocrine and paracrine signalling molecules as well as cell surface receptors.
The regulation of MMPs is governed by a complex network of diverse influences. Pregnancy related hormones such as human chorionic gonadotropin (hCG) and progesterone are the main endocrinologic players, thereby late pregnancy dominating progesterone is associated with low invasive potential of the trophoblast and distinctly smaller expression of MMP-9, MMP-1, MMP-3 and little PA system activity (Schatz et al. 1999). This is consistent with the fact of low progesterone levels in first trimester as lytic activity is the highest as well as vanishing proteolysis by MMPs in the third trimester while the progesterone level peaks to protect the fetus from preterm delivery. Progesterone levels also affect the onset of menstruation by triggering degradation of functional endometrium due to progesterone withdrawal in the end of the periodic cycle. *In vitro* findings suggest a

strong correlation between increased hCG concentrations and the secretion of big amounts of MMPs, especially MMP-9 (Cohen, Bischof 2007, Fluhr et al. 2008). This result was supported by another research group which discovered hCG levels in extra uterine pregnancies. There was proteinase upregulation and deeper invasion in women with higher hCG levels (Oktay et al. 1994, Klein et al. 1995, Natale et al. 2003). In addition, the above mentioned integrin repertoire exerts influence on MMP expression. Through acquisition of the invasive phenotype, HLA-G/α1 subunit positive trophoblasts display proteolytic activity (Bischof, Haenggeli & Campana 1995). The effects of growth factors and cytokines on invasive behaviour and proteinase expression have been extensively reviewed. It seems like interleukin-1, interleukin-6, interleukin-15, prostaglandins (in particular PGE2) and tumor necrosis factor-α (TNF-α), that account for the pro-inflammatory activation of the immune system, cause MMP upregulation and stimulation of invasiveness. In contrast, anti-inflammatory cytokines as interleukin-10 and leukemia inhibitory factor (LIF) antagonise invasion and MMP upregulation (Staun-Ram, Shalev 2005, Ferretti et al. 2007, Bischof, Meisser & Campana 2001, Cohen, Meisser & Bischof 2006). ()Interestingly, this interrelation gives a striking cue to pregnancy loss caused by ascending infections where a severe inflammation leads to uterine contractions and the preterm onset of delivery. Further, the artificial labor induction by prostaglandins has to be remarked, thus, the pivotal pro-inflammatory signals are already in clinical use for cervical maturation in early stage of labor. Regarding the influence of growth factors, the importance of EGF (epidermal growth factor), IGF II (insulin-like growth factor II) and IGFBP-1 (insulin-like growth factor binding protein-1) have to be noted which maintain relevant upregulation of several ECM degrading enzymes while transforming growth factor-β (TGF- β) directs to a downregulation of the same (Anin, Vince & Quenby 2004, Burrows, King & Loke 1996, Staun-Ram, Shalev 2005, Knofler 2010, Ferretti et al. 2007, Staun-Ram et al. 2004, Bischof, Meisser & Campana 2001, Cohen, Meisser & Bischof 2006). Interestingly, also p53 could be involved in MMP activation and expression. Some authors found impaired function of p53 in diverse carcinoma cell lines linked with pathologically overexpressed MMP-9. It is hypothesized by the authors that a modified status of p53 could be instrumental in increasing the invasive potential of trophoblasts (Cohen et al. 2007, Cohen et al. 2008).

1.3. Proteinases and their inhibitors

It is well-established that cell behaviour is strictly governed by the surrounding tissue micro-environment comprising ECM and ECM-linked factors such as growth factors, cytokines, chemokines and receptors detached from cell surface. Thus, since the early 1960´s, research is successively detecting proteinases involved in tissue remodelling, particularly in the turnover of ECM macromolecules. The most important members of the metzincin superfamily account for those processes, including the groups of MMPs, ADAMs and ADAMTSs, whereby the characteristic catalytic region is zinc-based. Since substrates of above-mentioned enzymes can be found ubiquitously in almost every human tissue, MMPs, ADAMs and ADAMTSs are supposed to represent central manipulators in physiological ECM turnover during development as well as under pathologically altered conditions like chronic inflamation or neoplasia where it comes to tissue destruction. (Shiomi et al. 2010, Murphy 2008, Chang, Werb 2001, Murphy, Nagase 2008, Malemud 2006).

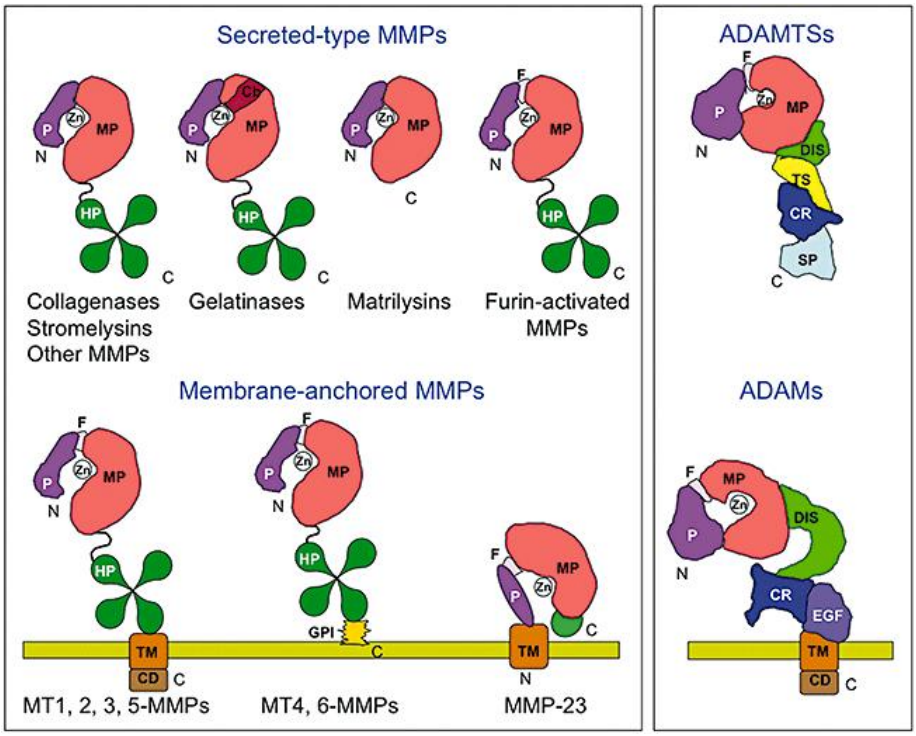


Figure 7: Domain structures of MMPs, ADAMs and ADAMTSs. (P): propeptide domain. (MP): metalloprotease domain. (HP): hemopexin-like domain. (Cb): collagen-binding fibronectin repeats. (F): furin-recognition site. (GPI): glycosylphosphatidylinositol-linked. (DIS): disintegrin domain. (TS): trombospondin motifs. (CR): cysteine-rich domain. (SP): spacer domain. (TM): transmembrane domain. (CD): cytoplasmic domain. Taken from (Shiomi et al. 2010)**.**

1.3.1. Classification and structure characteristics

MMPs.

Today´s literature suggests evidence for more than 20 different MMP molecules, though relevant differences in substrat specificity and structure configuring domains are known. The family members of MMPs are typically characterised by the ability to degrade ECM molecules, the dependence upon Zn^{2+}-binding, hence enabling the catalytic activity of the molecule, and finally, by the criterion of MMP-1/fibroblast collagenase (collagenase 1) sequence homology (Chang, Werb 2001, Murphy, Nagase 2008). We can basically differentiate two groups of enzymes, namely the major group of secreted-type MMPs and the cluster of membrane-anchored MMPs. Generally, MMPs are composed of several different functional domains (Cohen, Meisser & Bischof 2006, Shiomi et al. 2010, Chang, Werb 2001, Murphy, Nagase 2008). After the *pre-prosequence* is removed, the *propeptide* (around 10 kDa) assures the abscence of activation by interacting with the crucial catalytic Zn^{2+} ion. The propeptide is followed by the *catalytic domain* of about 20 kDa. This region displays the representative motif binding a Zn^{2+} ion to facilitate proteolysis. A *hemopexin-like domain* is seperated by a small *hinge region* from the catalytic domain. The hemopexin serum glycoprotein alike domain could play a potential role in substrate recognition and interference with specific endogenous inhibitors of MMPs, the tissue inhibitors of MMPs (TIMPs). The topic of inhibition by those molecules will be alluded later on. Additional regions are present in some MMPs whereas a few MMPs lack specific domains. For example, there is a *fibronectin type II insertion* in the catalytic domain of MMP-2 and -9 and membrane bound MMPs contain additional 75-100 residues to enable the formation of a *transmembrane helix* and a cytoplasmatic tail. In contrast, MMP-7 and -26 fail to include the hemopexin-like domain in their polypeptid chain.

Based on substrate specificity and distinctive structural features, we roughly classify the MMPs into membrane-anchored and secreted-type MMPs, therefrom 6 subclasses are deducted. In this consideration the classification adopted from (Shiomi et al. 2010) is prefered due to its meticulous methodical approach as well as its complemented composition, although there is plenty consistent information available by other reviewers (Bischof, Meisser & Campana 2001, Cohen, Meisser & Bischof 2006, Murphy, Nagase 2008, Flannery 2006).

Secreted-type MMPs.

Collagenases. There are three members of the collagenase group, by name collagenase 1 (MMP-1), collagenase 2 (MMP-8) and collagenase 3 (MMP-13), mainly digesting fibrillar collagen types I, II, III and other ECM structures at physiological pH. The interplay of the hemopexin domain and their catalytic domain is considered very important for collagenolytic activity as absence of the hemopexin domain is linked to failure of catalysis.
Gelatinases. The most extensively specified MMPs are the two gelatinases, gelatinase A (MMP-2) and gelatinase B (MMP-9) (Staun-Ram et al. 2004, Cohen, Bischof 2007, Fluhr et al. 2008, Salamonsen, Nie 2002, Meisser et al. 1999). There is a fibronectin type II motif in their catalytic domain mediating substrat binding. Efficiently cleaving denatured collagens (i.e. gelatines), a big variety of other ECM molecules such as collagen I, III, IV, V, VII, X and XI as well as elastine, fibronectin and laminin can be modified by gelatinases. MMP-9 also activates TGF-β, several cytokines and leads to the conversion of plasminogen into angiostatin, which is an interesting cue to a negative feedback mechanism, given that plasminogen derived plasmin is associated with the activation of relevant proteases and trophoblast invasiveness (Barnea, Hustin & Jauniaux 1992). Further, MMP-9 secretion by trophoblasts has been shown to correlate with surrounding hCG levels (Cohen, Bischof 2007). Activation of proMMP-2 happens through cleavage of the propeptide site by membrane anchored MMPs (MT-MMPs), thus MMP-2 potentially reaches high lytic concentrations in the pericellular compartment (Murphy, Nagase 2008). Both MMP-2 and MMP-9 are supposed to be essential for first trimester placental invasiveness inasmuch as trophoblast invasion can be suppressed by non-specific inhibition of these enzymes in vitro (Bischof et al. 1995).
Stromelysins. Stromelysins 1, 2, and 3, also referred as to MMP-3, MMP-10 and distantly related MMP-11 (see also furine activated MMPs), digest a broad spectrum of ECM molecules. Interestingly, MMP-11 belongs to the group of furin activated MMPs at the same time, which means that it is converted into the active form already intracellularily and secreted thereafter. MMP-3 and MMP-10 contribute to the activation of proMMPs.
Matrilysins. Specified by the absence of both the hinge region and the hemopexin-like domain, the matrilysins MMP-7 and MMP-26 convert a multitude of ECM substrates. MMP-7 not only acts through ECM digestion but also cleaves cell surface structures such as Fas-ligand, proTNF-α and E-cadherin, thus providing soluble forms of these molecules. The term “sheddase” is used in this context (Murphy, Nagase 2008) expressing the process in which above-mentioned molecules detach from the cell membrane.

Furin-activated MMPs. This subgroup consists of two MMPs dependend on activation within the Golgi apparatus, namely MMP-11 (see stromelysins) and MMP-28. The activation is mediated by a subfamily of proprotein convertases, in particular by furin which interacts with the furin-recognition site of the MMPs.

Other secreted-type MMPs. MMP-12, also referred as to metalloelastase, is associated with macrophage function and trophoblast invasion in first trimester. Preliminary data by Hiden, U. (unpublished) demonstrates highly expressed MMP-12 in first trimester and mark the potential role as converter of plasminogen into angiostatin. MMP-19 is capable of degrading basement membranes, thus, it might be important for cell migration during diverse biological events such as woundhealing or trophoblast migration. MMP-20 is tooth specific and not really relevant for our considerations of trophoblast biology. MMP-21 and MMP-27 are not sufficiently studied yet, hence, we do not find reviewed information about their catalytic specificity (Shiomi et al. 2010).

Membrane-type MMPs.

Membrane-type MMPs (MT-MMPs) are structurally anchored on the cell surface, thus altering especially the pericellular micro-environment by lytic activity. Due to their furin-like convertase recognition sequence, activation occurs intracellularly and the molecules are expressed on cell surface. Besides the ECM turnover whereupon mainly collagens, laminins and gelatines are cleaved, MT-MMPs contribute to the shedding of cell membrane proteins and, which has to be pointed out, they are competent to activate proMMP-2. Following MMPs belong to the MT-MMP class: MMP-14, -15, -16, -24 (type 1 transmembrane-type), MMP-17 and -25 (GPI-linked MMPs) and MMP-23 (type 2 transmembrane-type MMP) (Shiomi et al. 2010, Murphy, Nagase 2008, Itoh, Seiki 2006, Takino et al. 1995).

Table 1: Substrates of human matrix metalloproteases. Taken from (Shiomi et al. 2010)**.**

Enzymes	ECM substrates	Non-ECM substrates
Secreted-type MMP		
Collagenases		
Interstitial collagenase (MMP-1)	Collagens I, II, III, VII and X; gelatins; aggrecan; link protein; entactin; tenascin; perlecan	α2-M; α1-PI; α1-antichymotrypsin; IGFBP-2, 3, 5; proIL-1β; CTGF
Neutrophil collagenase (MMP-8)	Collagens I, II and III; gelatins; aggrecan; link protein	α1-PI
Collagenase-3 (MMP-13)	Collagens I, II, III, IV, IX, X and XIV; aggrecan; Fn; tenascin; osteonectin; Ln; Perlecan	CTGF; ProTGF-β; MCP-3; α1-antichymotrypsin
Gelatinases		
Gelatinase A (MMP-2)	Gelatins; collagens IV, V, VII, X and XI; Ln; Fn; elastin; aggrecan; link protein	ProTGF-β; FGF receptor I; MCP-3; IGFBP-5; proIL-1β; galectin-3; plasminogen
Gelatinase B (MMP-9)	Gelatins; collagens III, IV and V; aggrecan; elastin; entactin; link protein, vitronectin; N-telopeptide of collagen I	ProTGF-β; IL-2 receptor α; Kit-L; IGFBP-3; proIL-1β; ICAM-1; α1-PI; galectin-3; plasminogen
Stromelysins		
Stromelysin-1 (MMP-3)	Aggrecan; decorin; gelatins; Fn; Ln; collagens III, IV, IX and X; tenascin; link protein; perlecan	IGFBP-3; proIL-1β; HB-EGF; CTGF; E-cadherin; α1-antichymotrypsin; α1-PI; α2-M; plasminogen; uPA; proMMP-1, 7, 8, 9, 13
Stromelysin-2 (MMP-10)	Aggrecan; Fn; Ln; collagens III, IV and V; link protein	Pro1, 8, 10
Matrilysins		
Matrilysin-1 (MMP-7)	Aggrecan; gelatins; Fn; Ln; elastin; entactin; collagen IV; tenascin; decorin; link protein	Proα-defensin; Fas-L; β4 integrin; E-cadherin; proTNFα; CTGF; HB-EGF; RANKL; IGFBP-3; plasminogen
Matrilysin-2 (MMP-26)	Gelatin; collagen IV; Fn; fibrinogen; vitronectin	ProMMP-9; α1-PI
Furin-activated MMP		
Stromelysin-3 (MMP-11)	Fn; Ln; aggrecan; gelatins	α1-PI; α2-M; IGFBP-1
Epilysin (MMP-28)	Unknown	Casein
Other secreted-type MMP		
Metalloelastase (MMP-12)	Elastin; aggrecan; Fn; collagen IV; osteonectin; Ln; nidogen	Plasminogen; apolipoprotein(a)
RASI-1 (MMP-19)	Collagen IV; gelatin; Fn; tenascin; aggrecan; COMP; Ln; nidogen	IGFBP-3
Enamelysin (MMP-20)	Amelogenin; aggrecan; gelatin; COMP	Unknown
MMP-21	Unknown	Unknown
MMP-27	Unknown	Unknown
Membrane-anchored MMP		
Type I transmembrane-type MMP		
MT1-MMP (MMP-14)	Collagens I, II and III; gelatins; aggrecan; Fn; Ln; fibrin; Ln-5	ProMMP-2; proMMP-13; CD44; MCP-3; tissue transglutaminase
MT2-MMP (MMP-15)	Fn; tenascin; nidogen; aggrecan; perlecan; Ln	ProMMP-2; tissue transglutaminase
MT3-MMP (MMP-16)	Collagen III; Fn; gelatin	ProMMP-2; tissue transglutaminase
MT5-MMP (MMP-24)	PG	ProMMP-2
GPI-linked MMP		
MT4-MMP (MMP-17)	Gelatin; fibrinogen	Unknown
MT6-MMP (MMP-25)	Gelatin; collagen IV; fibrin; Fn; Ln	ProMMP-2
Type II transmembrane-type MMP		
MMP-23	Gelatin	Unknown

α2-M, α2-macroglobulin; α1-PI, α1-proteinase inhibitor; COMP, cartilage oligomeric matrix protein; CTGF, connective tissue growth factor; Fas-L, Fas ligand; FGF, fibroblast growth factor; Fn, fibronectin; HB-EGF, heparin-binding epidermal growth factor like growth factor; IGFBP, insulin-like growth factor binding protein; ICAM-1, inter-cellular adhesion molecule 1; Kit-L, kit ligand; Ln, laminin; MCP-3, monocyte chemotactic protein-3; MMP, matrix metalloproteinases; MT-MMP, membrane-type MMP; PG, proteoglycan; proIL-1β, pro interleukin-1β ; Pro, proteinase type; proTNF-α, pro tumor necrosis factor-α; proTGF-β, pro transforming growth factor β; ProMMP, latent MMP; RASI-1, rheumatoid arthritis synovium inflamed-1; RANKL, receptor activator for nuclear factor κ B ligand; uPA, urokinase plasminogen activator.

ADAMs.

The group of ADAMs (a disintegrin and metalloproteases) has been extensively reviewed in the last decade while the bulk of information emanated from cancer research. However, literature gives various clues for the relevance in trophoblast biology as ADAMs mediated functions like proteolytic processing of transmembrane molecules, cell adhesion, alteration of diverse signalling pathways and ECM turnover is essential to neoplastic cells as well as to trophoblasts during the process of placentation (Ferretti et al. 2007). 13 proteolytic and 8 non-proteolytic ADAMs can be differentiated among the 21 relevant ADAM gene family

members including ADAM-like decysin 1 (ADAMDEC1) (Shiomi et al. 2010). ADAMs are membrane-anchored enzymes and their proteolytic representatives (ADAM-8, -9, -10, -12, -15, -17, -19, -20, -21, -28, -30, -33) display dependency on zinc for catalytic activity, thus bearing resemblance to the catalytic region of MMPs (Shiomi et al. 2010, Mochizuki, Okada 2007). The N-terminal domains correspond to those of the MMPs. Commenced by a pro-sequence, the metalloprotease domain is linked to a disintegrin domain and followed by a cysteine-rich domain. The disintegrin sequence is similar to fibronectin, thus some authors proposed a binding site for integrin receptors (Seals, Courtneidge 2003). The cysteine-rich domain contains a hypervariable region which is held responsible for specific protein interaction. Furthermore, there is an epidermal growth factor-like (EGF-like) domain, a transmembrane domain and the cytoplasmic tail, whereas isoforms of ADAM-9, -11, -12 and -28 have been found to lack the cell membrane connecting domains, thereby contributing to the subtype of secreted ADAMs.

Membrane bound ADAMs facilitate a wide range of biological features. Besides ECM digestion and modulation of cell adhesion, more specific processes such as “RIPping” and “shedding” can be observed. Shedding is the event of membrane-bound protein cleavage adjacent to the cell surface, thus generating solubilsed forms of these structures. It seems to play a pivotal role in both paracrine and autocrine signalling, because a big variety of cytokines, chemokines and growth factors are initially translocated to the cell membrane as pro-forms and released in the form of active messengers subsequently due to “sheddase” activity by ADAMs (Murphy 2008). ADAM-17 has been identified as a major player in this ectodomain shedding of membrane proteins, in particular shedding of proTNF-α. TNF-α, responsible for a wide spectrum of inflammatory responses, is located at the cell surface in the form of a precursor and gets bioavailable after the conversion by ADAM-17, also known as TNF-α converting enzyme (TACE). ProTGF-α (pro-transforming growth factor-α), proNGF (pro-nerve growth factor) and pro-HB-EGF (pro-heparin binding epidemal growth factor) are processed in the same manner by the enzyme. ADAM-10 enables the shedding of the Fas-ligand, which is of outstanding importance for cell survival and and the induction of apoptosis. Interestingly, also cytokine receptors are shed by ADAM activity, notably TNF receptor I and TNF receptor II as well as macrophage colony stimulating factor 1 receptor, hence, potentially modifying ligand-receptor activities in these signalling circuits (Shiomi et al. 2010, Murphy 2008, Mochizuki, Okada 2007). RIPping (i.e. regulated intra-membrane proteolysis) is a very complex phenomenon leading to cleavage of the membrane-embedded portion of a membrane-anchored protein

by a so called γ-secretase complex. Thereby, the cytoplasmatic domain of the cleaved molecule is emitted into the cell plasm, subsequently acts there as a modulator of intracellular events or gets degraded. This just mentioned sequence is probably mediated by ADAMs (especially ADAM-10 and -17) as they have to remove most of the protein´s ectodomain first in order to enable intramembrane cleavage of the remaining protein by γ-secretase (Shiomi et al. 2010, Murphy 2008, Mochizuki, Okada 2007, Selkoe, Wolfe 2007).

Moreover, sheddase activity of ADAMs affects also cell adhesion molecules (CAMs). By solution of CAMs such as VCAM (vascular CAM), L1-CAM, L-selectin, CD44, E-cadherin and N-cadherin, ADAMs possibly change cell-cell and cell-matrix interactions effectively, thus enabling cell migration and triggering downstream signalling pathways. Additionally, ADAMs might interfere with integrins mediated by their non-catalytic domains, namely the disintegrin and cysteine-rich domain (Murphy 2008). Further, several ADAMs haven been described to show ECM digesting potential. ADAM-9, -10, -12 and -15 are known to cleave type IV collagen, gelatine and fibronectin (Shiomi et al. 2010, Murphy 2008, Mochizuki, Okada 2007).

There are two groups of endogenous inhibitors regarding ADAMs activity. Both TIMPs (tissue inhibitors of metalloproteases) and RECK (reversion-inducing cysteine-rich protein with Kazal motifs) are capable of ADAM inhibition. The question of inhibitory potential in vivo is still unclear, while ADAM-10, -12, -17, -28 and -33 can be antagonised by TIMP3 in vitro (Murphy 2008).

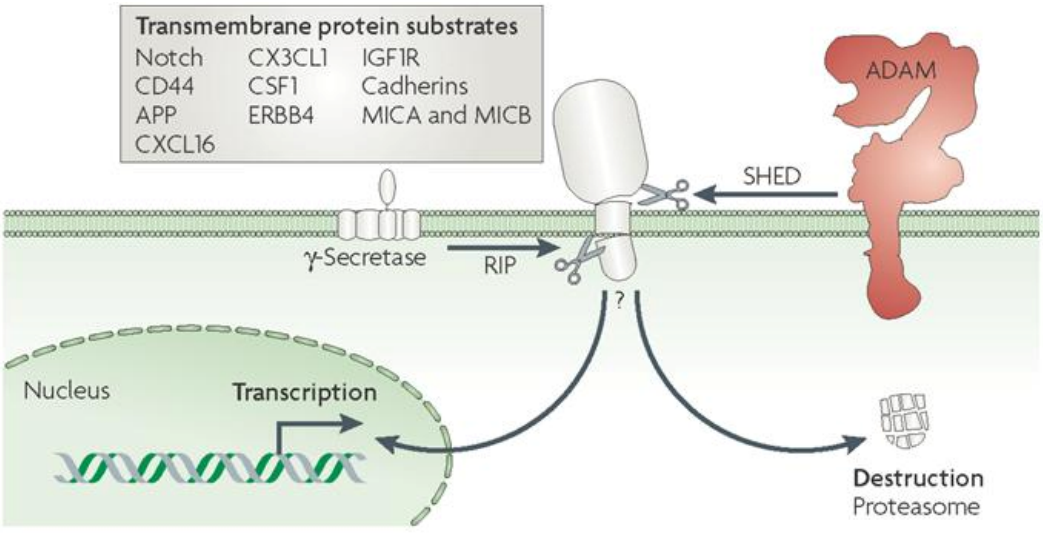


Figure 8: Shedding and RIPping by proteolytic ADAMs. Adopted from (Murphy 2008)**.**

ADAMTSs.

The ADAMTS group (a disintegrin and metalloproteases with trombospondin motifs) is composed of 19 members showing basic structural similarities with the ADAMs group, though lacking the transmembrane domain. In addition, ADAMTSs include the group specific trombospondin motifs. A furin-recognition site bonds the pro-sequence to the catalytic domain while the proximate disintegrin domain is connected to the trombospondin region and a cysteine-rich and spacer domain (Shiomi et al. 2010). Proteolytic ADAMTSs (i.e. ADAMTS-1,-2, ,-3, -4, -5, -8, -9, -15) are well known to cleave ECM components, for example procollagens (ADAMTS-2, -3, -4), aggrecan (especially ADAMTS-4, -5), brevican and versican (ADAMTS-1, -4) (Shiomi et al. 2010, Flannery 2006, Mochizuki, Okada 2007). Furthermore, von Willebrand factor (vWF), which is a cardinal factor in the coagulation cascade, can be modified by ADAMTS-13, thus providing a sufficient degradation of vWF-multimers and anticoagulatory effects. Under ADAMTS-13 deficient conditions TTP (thrombotic thrombocytopenic purpura), a life-threatening coagulopathia, may occur (Flannery 2006).

Interestingly, the general endoprotease inhibitor α2-macroglobulin and the more specific tissue inhibitor of metalloproteases-4 (TIMP-4) can be disrupted in their inhibitory activity by ADAMTS-4, respectively. ADAMTS-5 has been detected to be capable of α2-macroglobulin cleavage as well (Flannery 2006). ADAMTS-5 and ADAMTS-6 could be of major relevancy in the course of trophoblast invasion, since their location in the human placenta and uterus has been verified (Shiomi et al. 2010, Mochizuki, Okada 2007).

TIMPs.

The most important family of endogenous inhibitors regarding metalloproteases are referred as to TIMPs (tissue inhibitors of metalloproteases). Structurally consisting of two domains, namely the N-terminal and the C-terminal domain, TIMPs interact with the zinc containing catalytic region of metalloproteases, mostly MMPs. Thereby the N-terminal domain interferes with the catalytic region of the metalloprotease while the C-terminal half mediates substrate recognition (Nagase, Visse & Murphy 2006, Maskos, Bode 2003, Massova et al. 1998). The TIMP family currently comprises four different inhibitors, TIMP-1 to TIMP-4, that show sequence homology from 41 up to 52 per cent (Maskos, Bode 2003). TIMPs have been found to inhibit all MMPs tested so far with the exception of TIMP-1 which is not capable of MT1-MMP inhibition (Cohen, Meisser & Bischof 2006, Shiomi et al. 2010, Maskos, Bode 2003, Visse, Nagase 2003). In addition, TIMP-3

mediated inhibition of ADAM-10, -12, -17, -28 and -33 has been reported, whereas TIMP-1, -2, and -3 display less inhibitory potential anent ADAMs. The only inhibitor of ADAMTS-4 and -5 is TIMP-3 (Shiomi et al. 2010, Flannery 2006, Visse, Nagase 2003).

TIMPs inhibit metalloprotease activity in a 1:1 stochiometric manner, thus local changes in TIMP levels could directly impact MMP catalytic interactions (Shiomi et al. 2010, Maskos, Bode 2003, Visse, Nagase 2003). In fact, a fine-tuned homeostasis for metalloproteases and their inhibitors could be of great importance in biological processes since TIMPs and MMPs are co-expressed in human trophoblasts (Cohen, Meisser & Bischof 2006). Finally, there is a very interesting interrelation between MT1-MMP (i.e. MMP-14), TIMP-2 and proMMP-2 (pro-gelatinase A) which results in the activation of the latter. ProMMP-2 forms a complex with TIMP-2, hereby binding is facilitated by the hemopexin-like domain of proMMP-2 and the C-terminal domain of the inhibitor, respectively. Subsequently, the N-terminal region of TIMP-2 gets in contact with MT1-MMP, hence, the consequently surface-bound proMMP-2 releases its pro-peptide due to catalytic cleaving by another circumjacent MT1-MMP molecule (Shiomi et al. 2010, Nagase, Visse & Murphy 2006, Maskos, Bode 2003, Visse, Nagase 2003). Further, TIMP-1 and TIMP-2 are involved in cell growth promotion while TIMP-3 could act pro-apoptotic via TNF-α and Fas signalling circuits (Visse, Nagase 2003).

1.3.2. Medical relevance

Recapitulatory, there is plenty evidence for the biological importance of metalloproteases. In essence, almost in every major biological process these enzymes could play a pivotal role given that ECM substrate turnover is central to neoplasia, non-neoplastic diseases and developmental events such as implantation, placentation and embryogenesis.
Several representative biological features of neoplastic cells like cell growth, migratory and invasive potency and angiogenesis are mediated by the catalytic activity of proteases (Ferretti et al. 2007, Murphy 2008, Chang, Werb 2001, Mochizuki, Okada 2007). Thus, big effort is put into the exploration of possible inhibitors to battle cancer effectively. A profound breakthrough is not yet achieved, largely based on the wide spectrum of metalloprotease activities and the complexity of regulation (Murphy, Nagase 2008, Flannery 2006, Morrison et al. 2009, Rundhaug 2005).

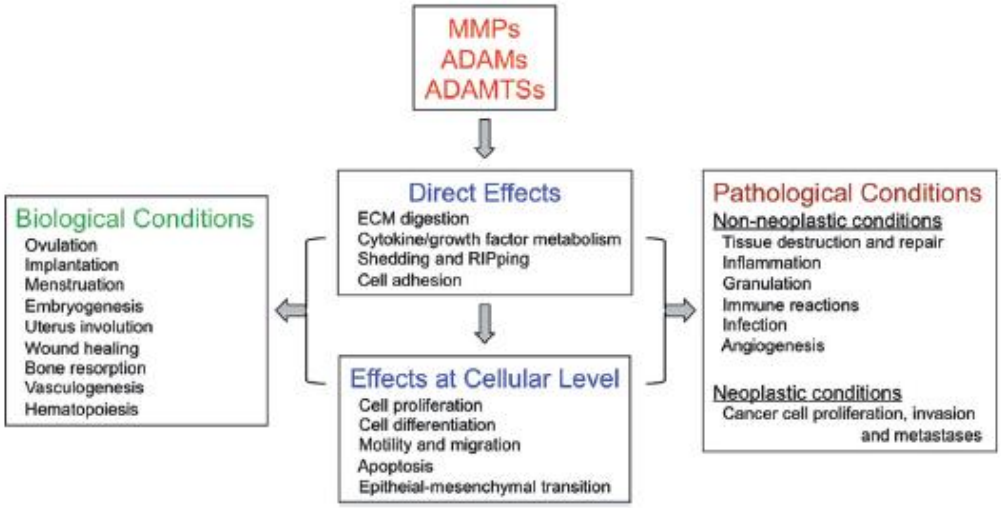


Figure 9: Effects of MMPs, ADAMs and ADAMTSs and their involvement in biological and pathological conditions. Adopted from (Shiomi et al. 2010)**.**

Abundant information aubout the involvement of metalloproteases in non-neoplastic diseases has been published in the last decade. The following diseases should exemplify the relevance while some of them are counted among the "big killers" in medicine: MMP-2 and MMP-9 are held responsible for myocardial remodelling after ischaemia, whereas the formation of atherosclerosis is maintained by the above-mentioned gelatinases and MMP-1, -3, -12, -13, -15, -17, additionally ADAM-9, -15, -17 and ADAMTS-1, -7. Furthermore, chronic obstructive pulmonary disease (COPD) and asthma bronchiale as

well as neurodegenerative and neuroinflammatory diseases are associated with altered local metalloprotease expression. Moreover, ADAMs and ADAMTSs are supposed to be fundamental for pathogenesis in addition to the ubiquitous players of MMPs in the important group of rheumatic joint and muscular diseases (Shiomi et al. 2010, Malemud 2006, Tayebjee, Lip & MacFadyen 2005, Raffetto, Khalil 2008).

ECM degradation is essential for the establishment of pregnancy. While trophoblasts invade through the uterus due to proteolytic activity, decidual cells tightly govern invasiveness and proteolysis at the feto-maternal interface. In particular, MMPs are most extensively studied both *in vivo* and *in vitro*, whereas only limited data about ADAMs and ADAMTSs expression during placentation is available. (Anin, Vince & Quenby 2004, Burrows, King & Loke 1996, Staun-Ram, Shalev 2005, Lunghi et al. 2007, Bischof, Meisser & Campana 2001, Cohen, Meisser & Bischof 2006, Cohen, Bischof 2007, Salamonsen, Nie 2002, Salamonsen 1999, Krussel et al. 2003, Baek et al. 2002).

1.4. Early pregnancy loss

Since the traditional classification of pregnancy events lack distinctiveness, early pregnancy events are described in this diploma according to the updated and revised nomenclature by the Special Interest Group for Early Pregnancy (SIGEP) (Farquharson et al. 2005). As a result of advancing and more accessible ultrasound diagnostics, it is now possible to achieve standardised and thus comparable information in early pregnancy.

Approximately 15-20 % of all pregnancies detected by clinicians end spontaneously in the first trimester (i.e. gestational age < 12 weeks) (Farquharson et al. 2005, Ngoc et al. 2004). Thereafter two potential outcomes are reported. Either the dead conceptus undergoes expulsion in line with uterine contractions or it remains *in utero* with absent fetal cardiac activity detectable by the sonographer. The former is referred as to early pregnancy loss while the latter is defined as delayed early pregnancy loss or delayed miscarriage (Farquharson et al. 2005).

Therapy regime for delayed miscarriage includes three possibilities so as to prevent septic complications, namely surgical dilatation of the cervix plus subsequently curettage, manual vacuum aspiration or the medicamentous way by a prostaglandin E analog (Ngoc et al. 2004).

1.5. Purpose of the study

In light of the strong evidence for proteinases implicated in crucial processes during the formation of the human placenta, it is supposable that they may show different patterns of expression in physiological versus pathologically altered conditions over the first trimester. As physiological expulsion of the conceptus is absent in delayed miscarriage, we hypothesised that abnormal trophoblast invasion and, hence, irregular expression of proteinases may be the underlying reason for the pathology. After having received very promising data from preliminary tests perfomed by Hiden U. (unpublished), the need for a sufficient clarification arose. Thus, my aim was the detection of a possible asssociation between delayed miscarriage and altered MMP, ADAM, ADAM-TS and TIMP expression.

2. MATERIALS AND METHODS

2.1. Subject characteristics

First trimester placental tissue was obtained from 41 women at the Department for Gynecology and Obstetrics/Medical University Graz. Study was approved by the Ethic committee of the Medical University Graz.

Two groups were defined:

- group 1 (cases) encloses samples of delayed miscarriage.
- group 2 (controls) includes samples obtained from normal pregnancy terminations.

For further investigation we formed 3 groups: weeks 7+8 (early first trimester, n = 14), weeks 9+10 (middle first trimester, n = 17) and weeks 11+12 (late first trimester, n = 10). The rationale was the finding of different longitudinal expression of proteases over the first trimester by several authors (Staun-Ram, Shalev 2005, Staun-Ram et al. 2004, Cohen, Meisser & Bischof 2006, Xu et al. 2000). Thus, it would be possible to compare cases and controls dependend on gestational weeks.

Legal pregnancy interruption was carried out for psycho-social reasons in the control group whereas surgical interventions performed in the case group were due to medical reasons. After medical intervention by the clinicians, samples were directly frozen in liquid nitrogen to keep tissue quality as high as possible.

Table 2: Classification.

	Gestational age	Group (1) *Delayed miscarriage*	Group (2) *controls*
early first trimester	*7 weeks*	*3*	*4*
early first trimester	*8 weeks*	*3*	*4*
middle first trimester	*9 weeks*	*4*	*3*
middle first trimester	*10 weeks*	*5*	*5*
late first trimester	*11 weeks*	*4*	*3*
late first trimester	*12 weeks*	*1*	*2*
sum		*20*	*21*

2.2. RNA isolation

Tri-Reagent© RNA-isolation (Cat. No. TR 118) was used to gain total RNA for further experiments:

1. Homogenization: 500 µl Tri-Reagent plus 50 mg tissue. Incubation at room temperature: 5 min.
2. Phase separation: Homogenate plus 1/10 Vol of Tri-Reagent or 50 µl BCP (i.e. bromochloropropan). Shake vigorously for 15 seconds. Incubation at room temperature: 15 min. Centrifugation at 4°C and 12000 g: 15 min.
3. RNA precipitation: Colorless upper aqueous phase plus 0,5 ml isopropanol. Incubation at room temperature: 10 min. Centrifugation at 4°C and 12000 g: 8 min.
4. RNA wash: Remove supernatant. Wash RNA with 75 % ethanol (stored at -20 °C). Centrifugation at 4°C and 7500 g: 5 min.
5. RNA-solubilization: Remove ethanol supernatant. Briefly air-dry RNA pellets for 3-5 min. Disolve with 30-100 µl (dependend on pellet size) RNAse free water. Shake at 50°C for 10 min.

2.3. Spectroscopic RNA quantification

Spectroscopic analysis of the isolated RNA was performed with "eppendorf BioPhotometer plus" at 260 nm (Eppendorf AG, Germany). Thereafter, data was assessed through Microsoft Excel (Microsoft Corporation, USA) to gain a 100 ng/µl target dilution of RNA for sq-RT-PCR.

2.4. sq-RT-PCR

Semi-quantitative-RT-PCR was performed with Quiagen one-step PCR kit according to the recommendations by the manufactors (QIAGEN GmbH, Germany). Primers were obtained from Ghaffari-Tabrizi, N. (Institute for Pathophysiology, Medical University Graz). For primer information see tables 3 to 7.

The trophoblast specific cytoskeletal protein Cytokeratin 7 was used as a housekeeping gene (Blaschitz et al. 2000).

Prior to serial PCR testing, we evaluated optimal cycle number in pilot experiments. 100 ng RNA total were applied for PCRs, thereby 31 cycles were conducted for enzyme

polimerisation and 25 cycles for cytokeratin 7, respectively. Each cycle comprised 30 seconds denaturation at 95°C, 60 seconds annealing at 60°C and a 60 seconds elongation step at 72°C. Pre-PCR steps were performed as follows: step one at 50°C for 30 minutes and step two at 95°C for 15 minutes. PCRs were conducted with "Mastercycler epgradient" (Eppendorf AG Hamburg, Germany) and "MJ Mini Personal Thermal Cycler" (Bio-Rad, CA, USA).

Enzymes and inhibitors tested for differential expression over the first trimester involve MMPs, ADAMs, ADAMTSs and TIMPs. For listing of all relevant proteases and inhibitors see tables 3 to 7. Additional, we performed PCR-testing of human chorionic gonadotropin (hCG) due to its potential implications for protease regulation (Staun-Ram, Shalev 2005, Bischof, Meisser & Campana 2001, Cohen, Bischof 2007, Fluhr et al. 2008).

Table 3: relevant primers TIMPs.

Gene name	forward	reverse
TIMP1	TGACATCCGGTTCGTCTACA	GTTTGCAGGGGATGGATAAA
TIMP2	AAGCGGTCAGTGAGAAGGAA	AGGCTCTTCTTCTGGGTGGT
TIMP3	GGGGAAGAAGCTGGTAAAGG	TGCAGTTACAACCCAGGTGA
TIMP4	CAGACCCTGCTGACACTGAA	AGACTTTCCCTCTGCACCAA

Table 4: relevant primers for standardisation and quality control.

Gene name	forward	reverse
CK7	AGGAGAGCGAGCAGATCAAG	AAGTCCTCCACCACATCCTG
L30	CCTAAGGCAGGAAGATGGTG	CAGTCTGTTCTGGCATGCTT
hCG	GTCAACACCACCATCTGTGC	GGCCTTTGAGGAAGAGGAGT

Table 5: relevant primers MMPs.

Gene name	forward	reverse
MMP1	GGTCTCTGAGGGTCAAGCAG	AGTTCATGAGCTGCAACACG
MMP2	ATGACAGCTGCACCACTGAG	ATTTGTTGCCCAGGAAAGTG
MMP7	TGTATGGGGAACTGCTGACA	TGGGGATCTCCATTTCCATA
MMP9	TTCATCTTCCAAGGCCAATC	CAGAAGCCCCACTTCTTGTC
MM10	CCAGTCTGCTCTGCCTATCC	ACCAACGTCAGGAACTCCAC
MMP11	GCAACCGACAGAAGAGGTTC	ATCCCCTTCTCGGTGAGTCT
MMP12	ACACATTTCGCCTCTCTGCT	CCTTCAGCCAGAAGAACCTG
MMP14	CACTGCCTACGAGAGGAAGG	TTGGGGTACTCGCTATCCAC
MMP15	GGCCGACATCATGGTACTCT	GTCAACGTCCTTCCACTGGT
MMP16	GCTGACCCAAGGAAAAATGA	GCATTGGGTATCCATCCATC
MMP17	GGAGTGGAGTGGCTAAGCA	CGACAGGTTCCTCTTGTTCC
MMP19	GCTTCCTACTCCCCATGACA	GGCCCAGCAACAGGTATTTA
MMP20	TGAGAGGGGCACTGCTTACT	GTCTTCTGTGGCTCCCTGAG
MMP24	TGAAGGCATTGACACAGCTC	CGCTCAGTTTCTGGTTGTCA
MMP26	TGGTCAGCTTCAGACACTGG	TTGGATATCATCGGCACTGA
MMP28	AGAGCGTTTCAGTGGGTGTC	GGTAGGAGAGGTGCTGCTTG

Table 6: relevant primers ADAMs.

Gene name	forward	reverse
ADAM8	TTCCGGCTACACAGAGACCT	GAACTCTGCATTGTCCACGA
ADAM9	TGAATCACGATGATGGGAGA	CCAGCGTCCACCAACTTATT
ADAM10	AGCAACATCTGGGGACAAAC	CCCAGGTTTCAGTTTGCATT
ADAM15	AGCCTCAAAAAGGTGCTTCA	TCTTCCCTGGTAGCAGCAGT
ADAM17	TTGGGTCTGTCCTGGTTTTC	CCATTCTCTGGTGGTCCAGT
ADAM19	CTCTGCTTGCTGGCGTTT	TCCTCTAATTCCTCGGCAAG
ADAM20	AACAGGTTGGTCGTTTTTGC	CACATTCCTCCCCTTCTTCA
ADAM22	GTAACCTGGGAGGCAACAAA	TGTGAAACTCCGCTTCCTCT
ADAM28	ATGGAGGGGGAGTGTTCTCT	GCTCAGTGCTTTGTCCATCA
ADAM33	GTTCACCTAGATGGCCAGGA	CTGGAGCACAGTGGCAGTTA

Table 7: relevant primers ADAMTSs.

Gene name	forward	reverse
ADAMTS-1	CCTCTGTCTGTGTGCAAGGA	GTGGCTCCAGTTGGAATTGT
ADAMTS-2	CGGGTCTCACTGACGTACAA	GAAGCCACGGTGTACCATCT
ADAMTS-3	GTCTTCACACCGATGGACCT	GCTGCGATGGACCATTTTAT
ADAMTS-5	TGTGGAAAGGGGAGAATCTG	CCTCTTCCCTGTGCAGTAGC
ADAMTS-6	AACCTTGGTGGCTGAATGAC	CCTAGGCTGGAATCACGGTA
ADAMTS-7	GGTCGGTCAGCAAAGAGAAG	CCCCTTCATGTTGATGCTTT
ADAMTS-9	CCCAGACAGTGGCTTAGCTC	CACAGGCTCTTTGCTCATCA
ADAMTS-15	CAACATCGTTGTGGTCAAGG	GGTCACACATGGTACCCACA
ADAMTS-19	CCCCTTGTTCACGAACTTGT	GTCCGATGACGTATGCCTTT
ADAMTS-20	TGGAAACAAGCAGTGTGAGC	ACAGGACGTGTTTCCGTTTC

2.5. Electrophoresis

2 % agarose gels were used for electrophoresis. The gels were composed of 100 ml TAE buffer (i.e. Tris-acetate-EDTA) and 2 g agarose (Biozym LE Agarose. Biozym, Hessisch Oldendorf, Germany) mixed and subsequently microwave heated. 2 µl of ethidium bromide were added and the gels were mixed again. Thereafter, the gels were poured in the prepared molds and the combs were positioned in the gels. After 30 min polimerisation time, the gels were used for electrophoresis.

In order to achieve optimal comparability of each gene within the time related groups, they were loaded on the same gel. Gel 1: early first trimester, n = 14; Gel 2: middle first trimester, n = 17; Gel 3: late first trimester, n = 10.

4 µl "6x Loading Dye" (Fermentas International, USA) was added to the PCR products. Gel slots were loaded with 9 µl DNA-Loading Dye mix, respectively, while the first slot was loaded with 5 µl DNA Ladder 50bp in 1:12 dilution. Electrophoresis time was 30 min in TAE buffer agent.

2.6. Quantitative analysis

After gel electrophoresis PCR products were visualised under trans-UV illumination (Universal Hood II, Bio-Rad, CA, USA). Documentation was accomplished by “Quantity One 4.6.5. (Basic) 1-D Analysis Software” (Bio-Rad, CA, USA). DigiDoc 3.2.3. software (Alpha Innotec, CA, USA) was used for final determination of band intensity.

2.7. Statistical evaluation

Raw data was analysed in Microsoft Excel (Microsoft Corporation, USA), thereby differential enzyme expression was normalised to cytokeratin 7 expression. Normalised data were imported to “SigmaPlot 10.0” (Systat Software, Inc. Chicago, USA) and subjected to statistical testing. T-test and, if normality test and equal variance test failed, Mann-Whitney Rank Sum Test were performed. Differences were accepted as statistically significant at $p<0.05$. Evaluation and graphical representation was implemented within the above-mentioned three time-related groups, in particular weeks 7+8 (early first trimester, n = 14), weeks 9+10 (middle first trimester, n = 17) and weeks 11+12 (late first trimester, n = 10).

3. RESULTS

In this first trimester screening for differential expression of proteases and their specific inhibitors, sq-RT-PCR was performed to detect relevant expression and significant differences between delayed miscarriage and controls. The level of significance was set at $p<0,05$ and statistical evaluation was performed within the three time-related groups: weeks 7+8 (early first trimester, n = 14), weeks 9+10 (middle first trimester, n = 17) and weeks 11+12 (late first trimester, n = 10).

Expression of MMP17, -20, -26, -28 and ADAM-TS9 could not be detected, whereas all other proteinases and inhibitors were detectable by sq-RT-PCR (see table 8).

Interestingly, delayed miscarriage placentas showed a constant pattern of higher protease levels, particularly in weeks 7-8 of gestation. This significant overexpression in relation to controls was found for *MMP2* (p=0,0035), *MMP7* (p=0,003), *MMP9* (p=0,005), *MMP10* (p=0,002), *MMP11* (p=0,007), *MMP19* (p<0,001), *ADAM15* (p=0,02), *ADAM17* (p=0,029), *ADAM19* (0,002), *ADAM20* (p=0,005), *ADAM28* (p=0,005), *ADAM33* (p=0,013), *ADAM-TS1* (p=0,029), *ADAM-TS2* (p=0,005), *ADAM-TS3* (p=0,008), *ADAM-TS5* (p<0,001) and *ADAM-TS15* (p=0,003). In later first trimester the interrelation between enzyme upregulation and delayed miscarriage was identifiable, but not statistically significant anymore.

Further, tissue inhibitors of metalloproteases were measured and displayed parallelly upregulated expression, namely *TIMP1* (p=0,005) and *TIMP2* (p=0,005). β-hCG expression was verified in both cases and controls, but no difference was detected.

Table 8: PCR results and significant differences. The first collum shows whether the protease was detected by PCR. Collums 2-4 indicate whether a significant difference was observed between expression levels in placentas from delayed miscarriage vs. controls or not.

	expression	significant differences in weeks 7-8	significant differences in weeks 9-10	significant differences in weeks 11-12
MMP1	+	-	-	-
MMP2	+	+	-	-
MMP7	+	+	-	-
MMP9	+	+	-	-
MM10	+	+	+	-
MMP11	+	+	-	-
MMP12	+	-	-	-
MMP14	+	-	-	+
MMP15	+	-	-	-
MMP16	+	-	-	-
MMP17	-	-	-	-
MMP19	+	+	-	-
MMP20	-	-	-	-
MMP24	+	-	-	+
MMP26	-	-	-	-
MMP28	-	-	-	-
ADAM8	+	-	-	-
ADAM9	+	-	-	-
ADAM10	+	-	-	-
ADAM15	+	+	-	-
ADAM17	+	+	-	-
ADAM19	+	+	-	-
ADAM20	+	+	-	+
ADAM22	+	-	-	-
ADAM28	+	+	-	-
ADAM33	+	+	-	-
ADAMTS-1	+	+	-	-
ADAMTS-2	+	+	-	+
ADAMTS-3	+	+	-	-
ADAMTS-5	+	+	-	+
ADAMTS-6	+	-	-	-
ADAMTS-7	+	-	-	-
ADAMTS-9	-	-	-	-
ADAMTS-15	+	+	-	+
ADAMTS-19	+	-	-	-
ADAMTS-20	+	-	-	-
TIMP1	+	+	-	-
TIMP2	+	+	-	-
TIMP3	+	-	-	-
TIMP4	+	-	-	-
β-hCG	+	-	-	-

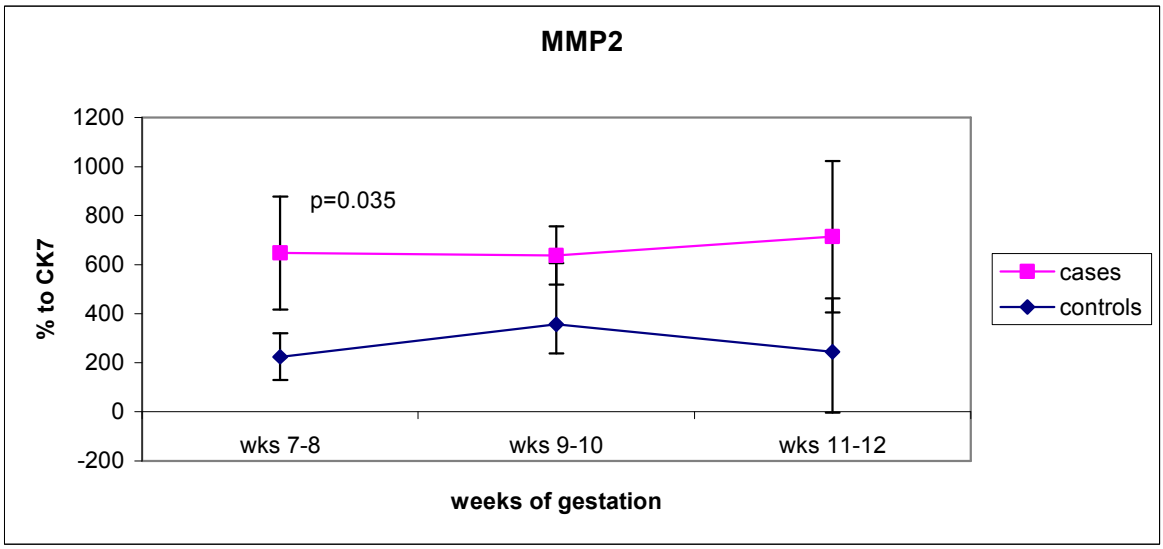


Figure 10: Expression of MMP2 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

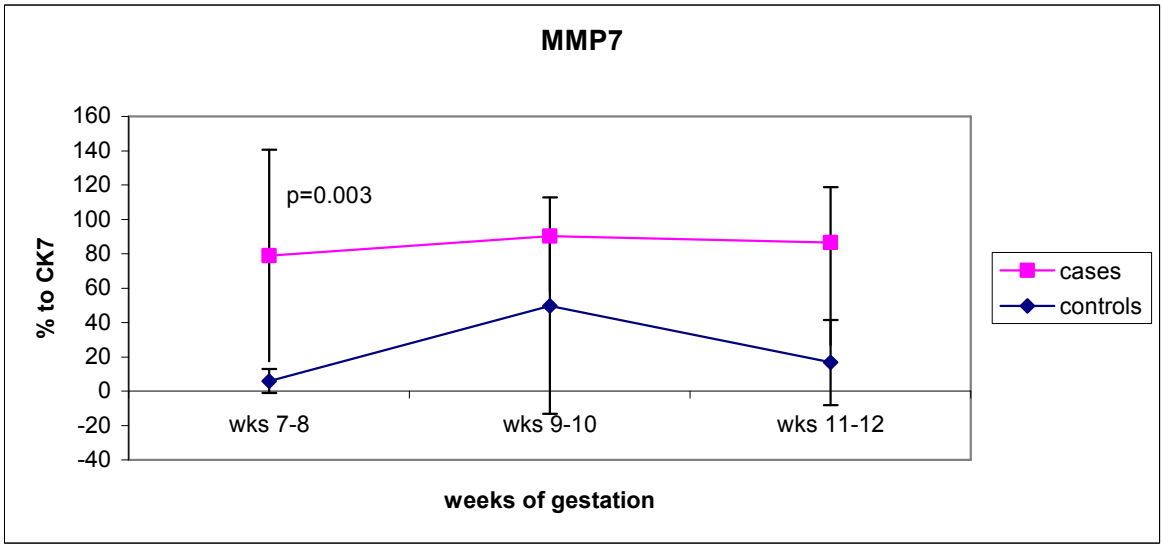


Figure 11: Expression of MMP7 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

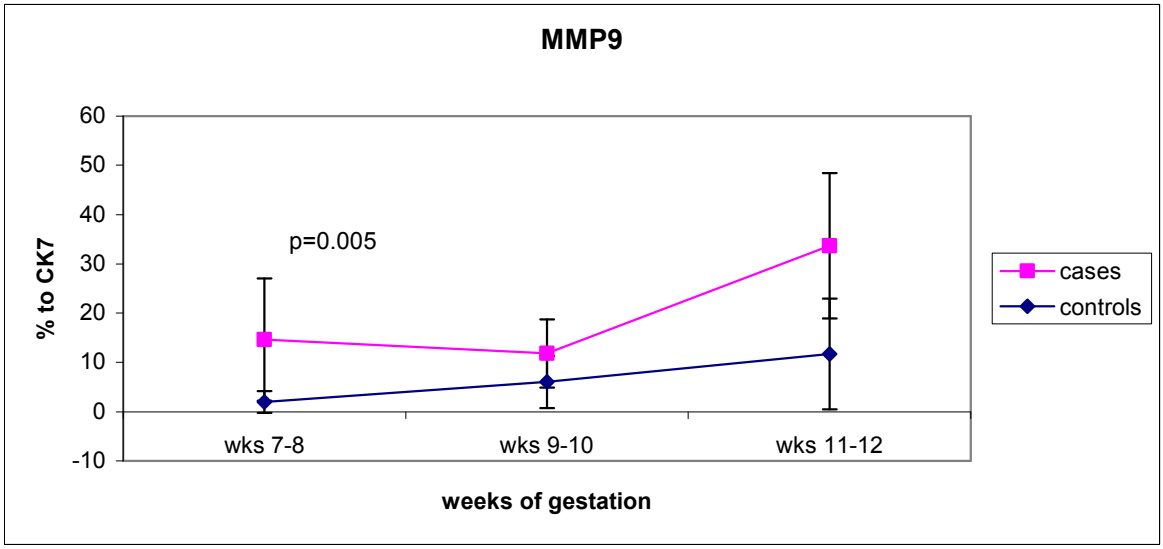


Figure 12: Expression of MMP9 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

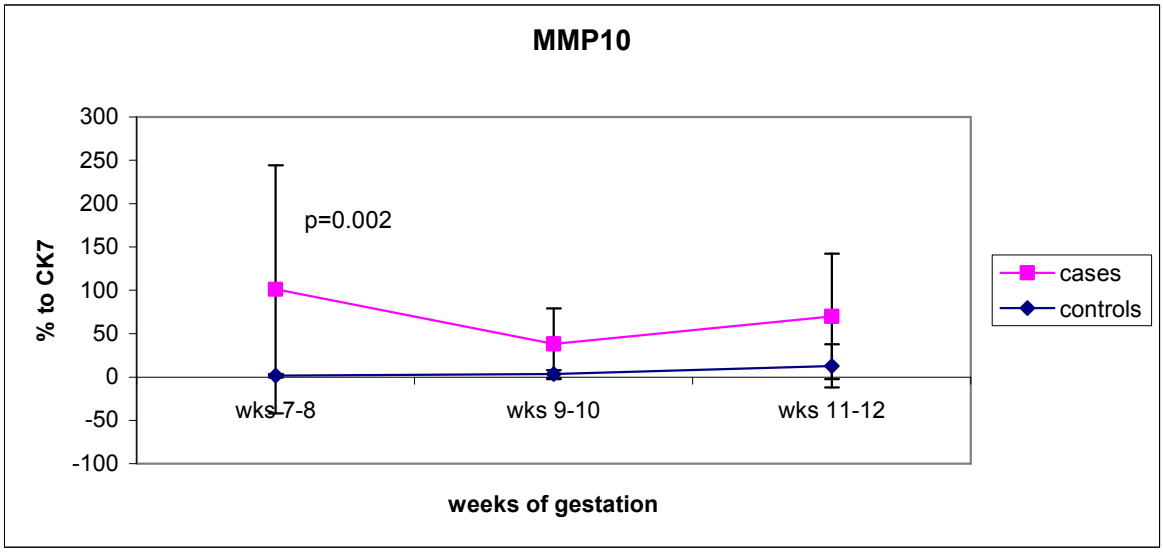


Figure 13: Expression of MMP10 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

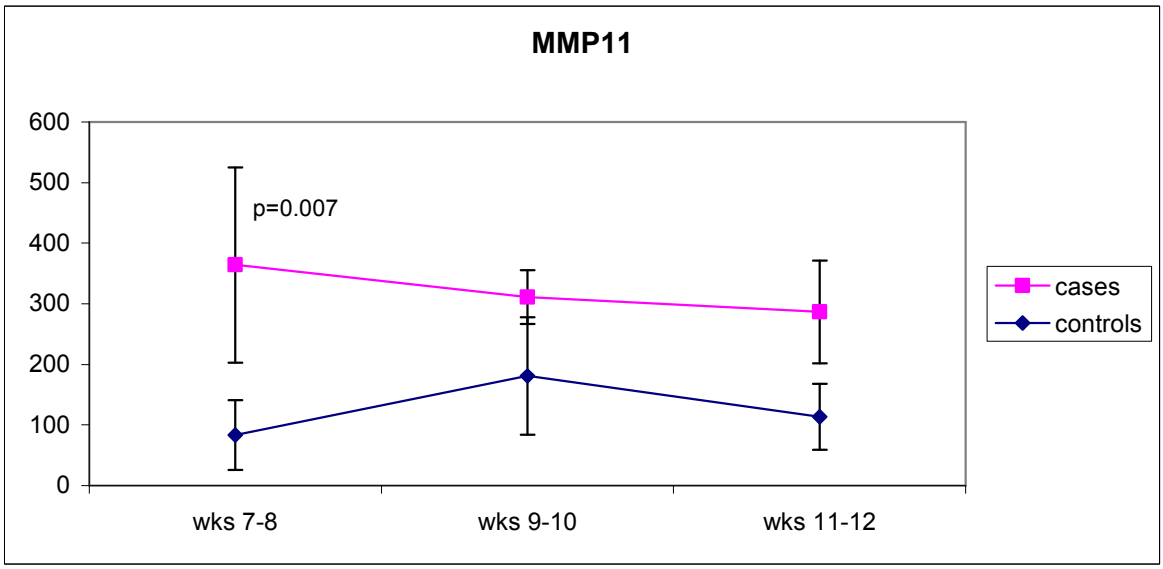


Figure 14: Expression of MMP11 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

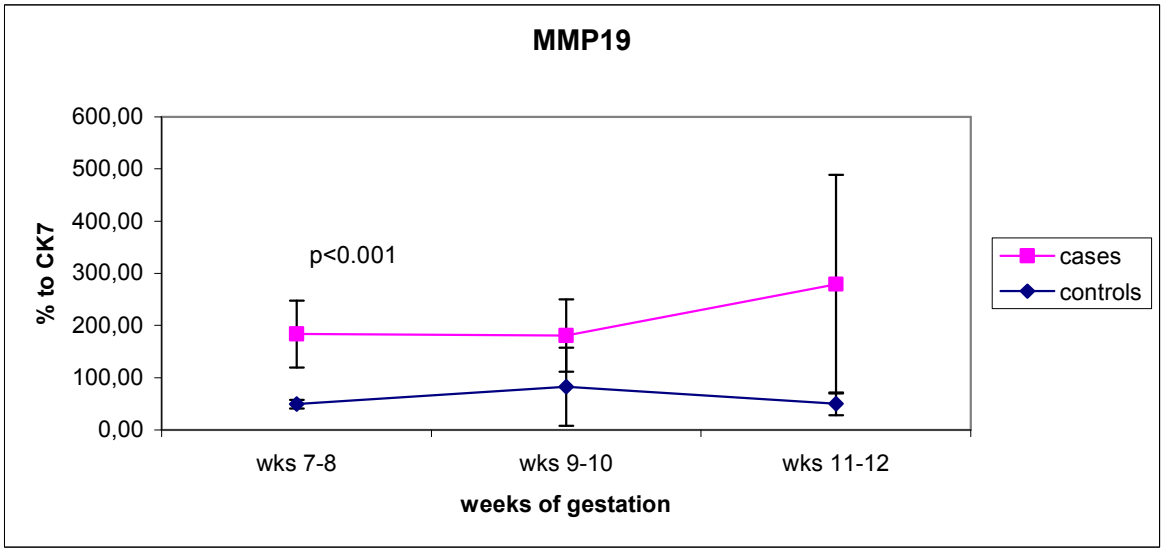


Figure 15: Expression of MMP19 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

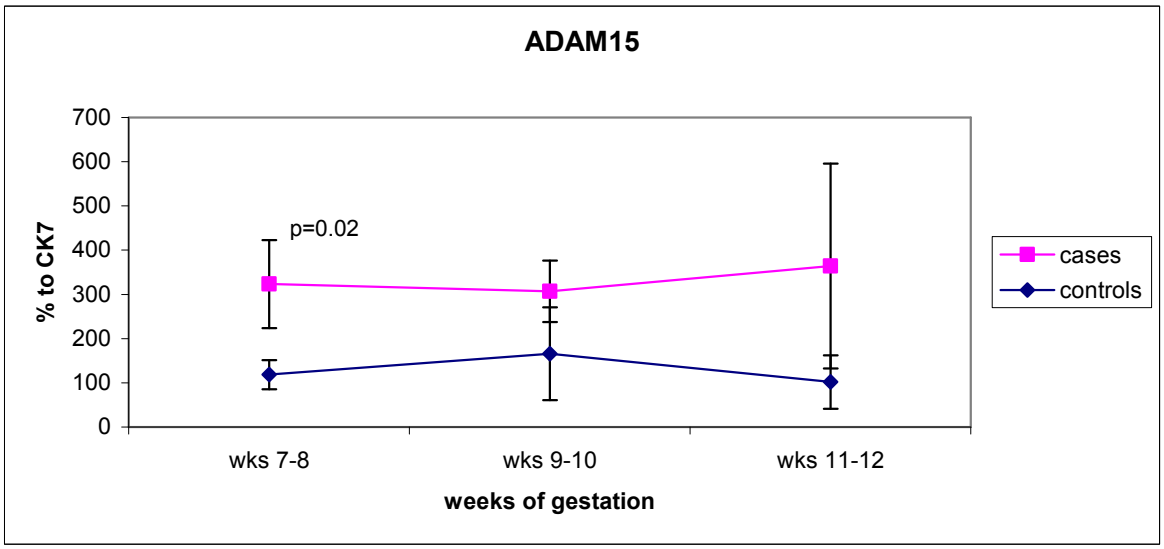


Figure 16: Expression of ADAM15 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

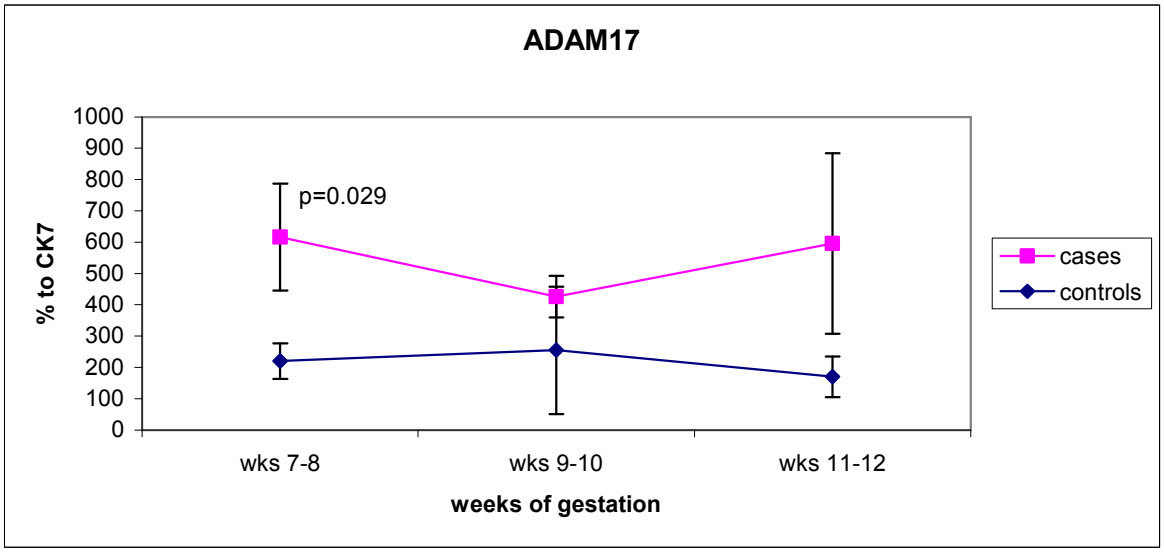


Figure 17: Expression of ADAM17 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

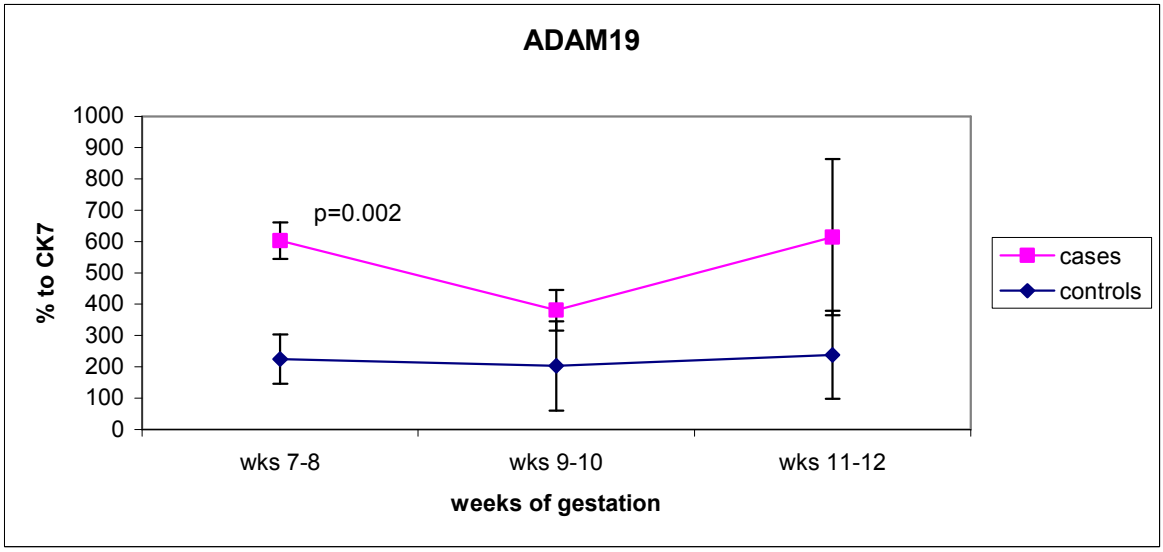


Figure 18: Expression of ADAM19 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

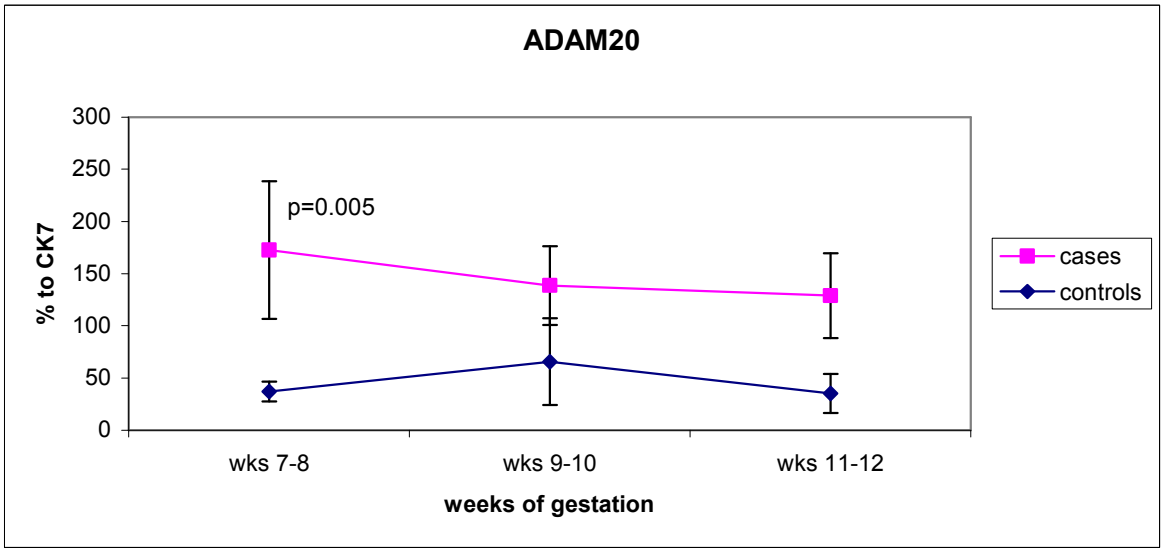


Figure 19: Expression of ADAM20 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

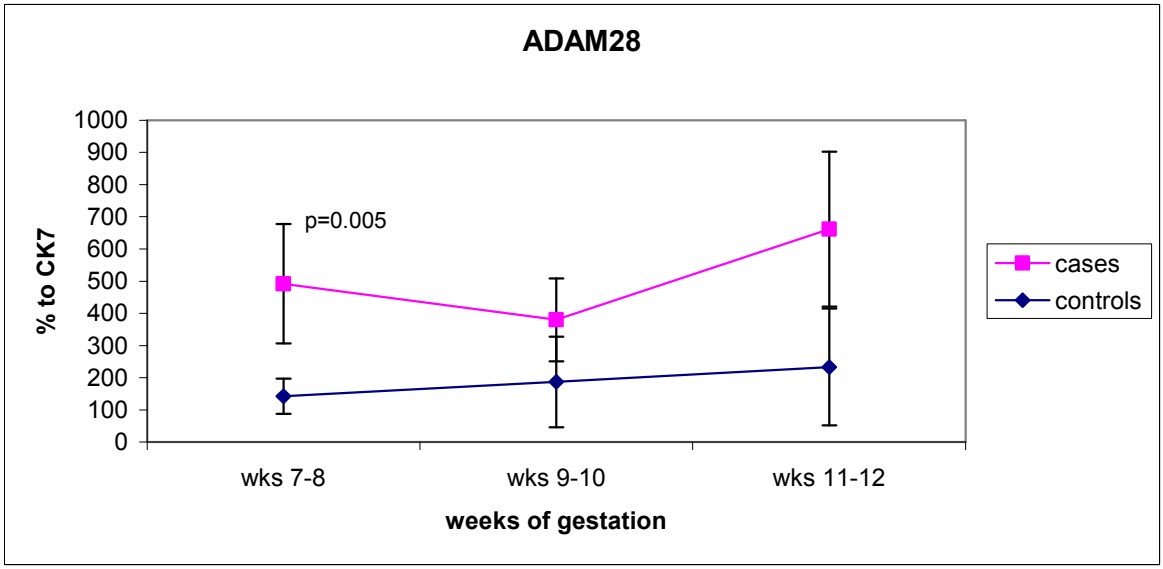


Figure 20: Expression of ADAM28 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

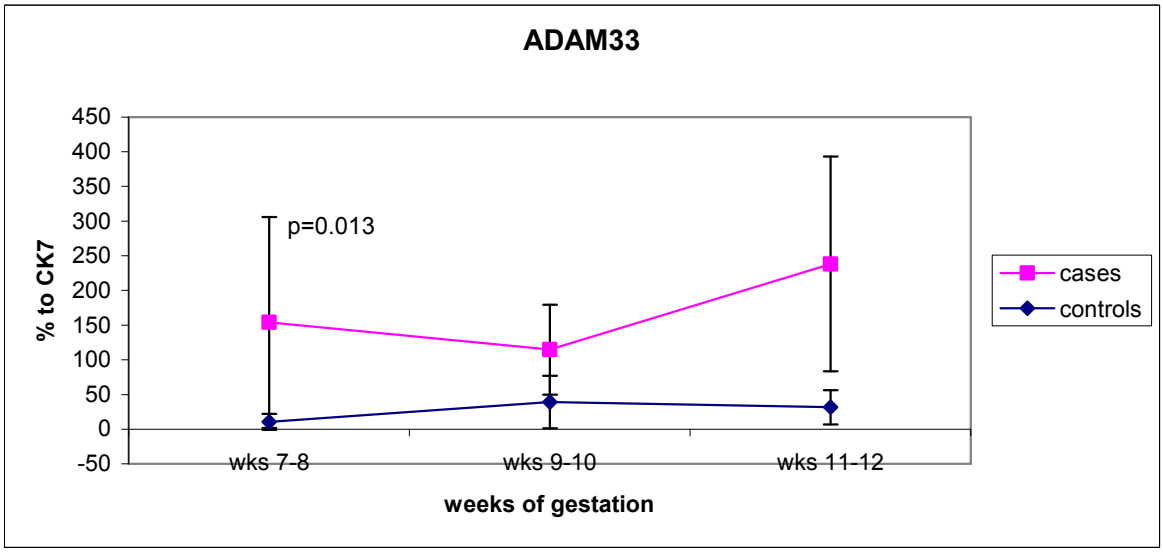


Figure 21: Expression of ADAM33 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

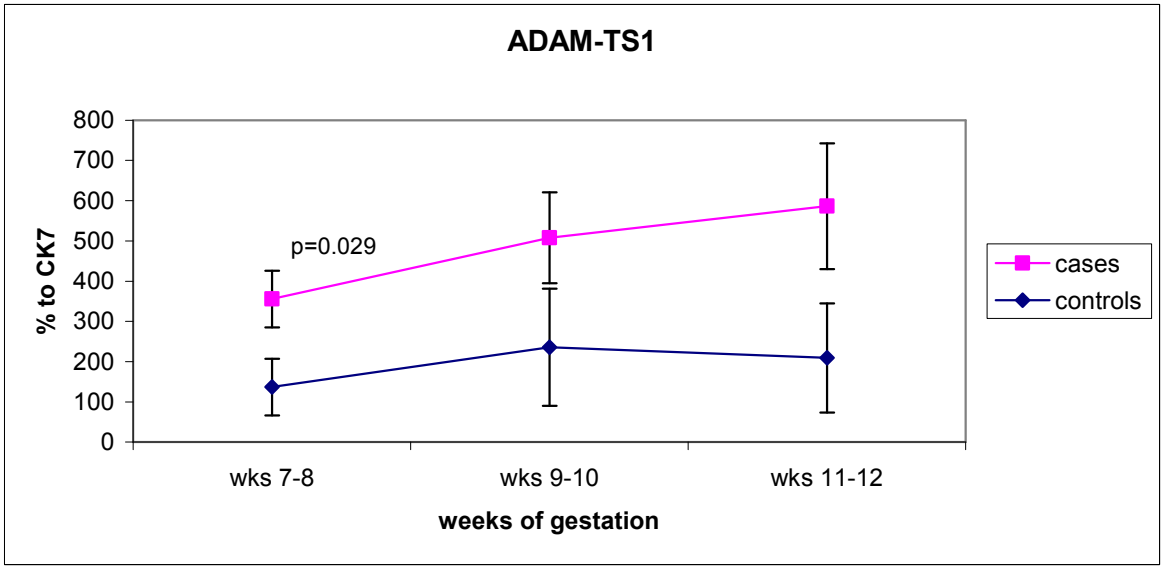


Figure 22: Expression of ADAM-TS1 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

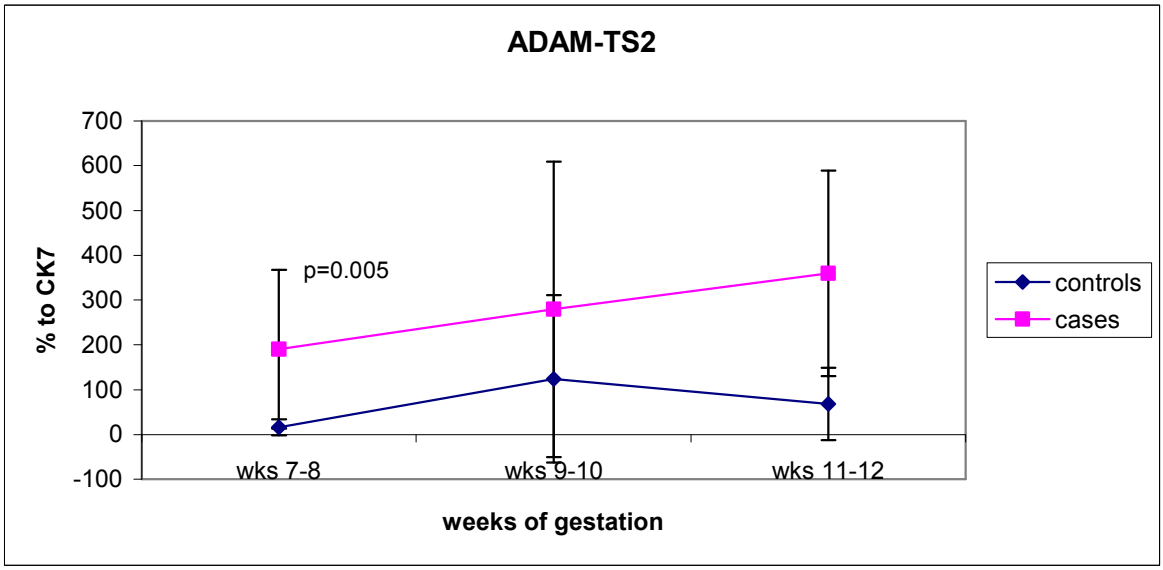


Figure 23: Expression of ADAM-TS2 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

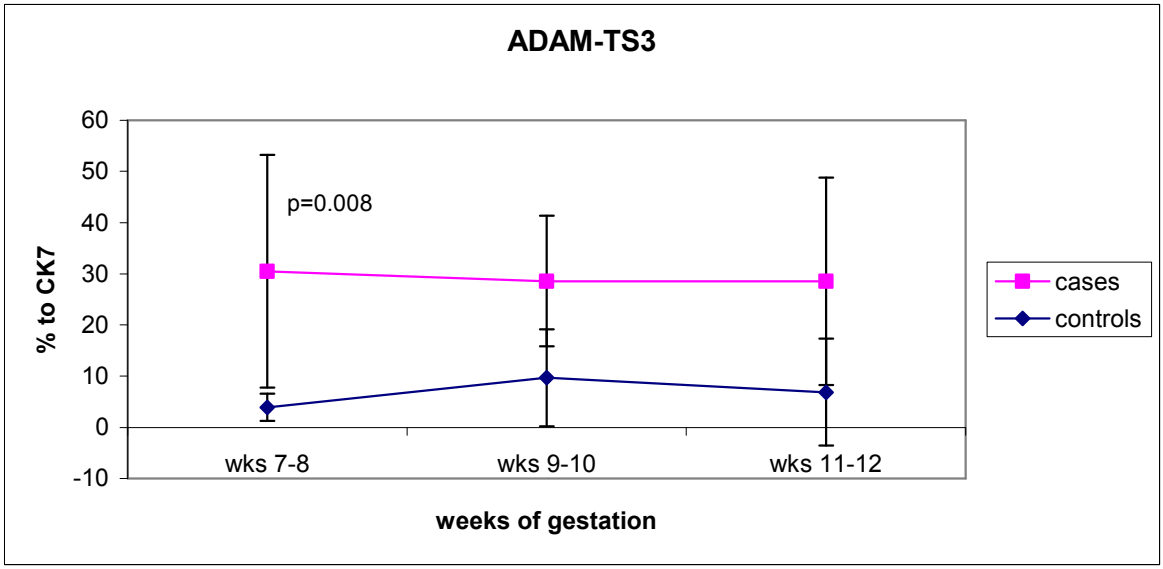


Figure 24: Expression of ADAM-TS3 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

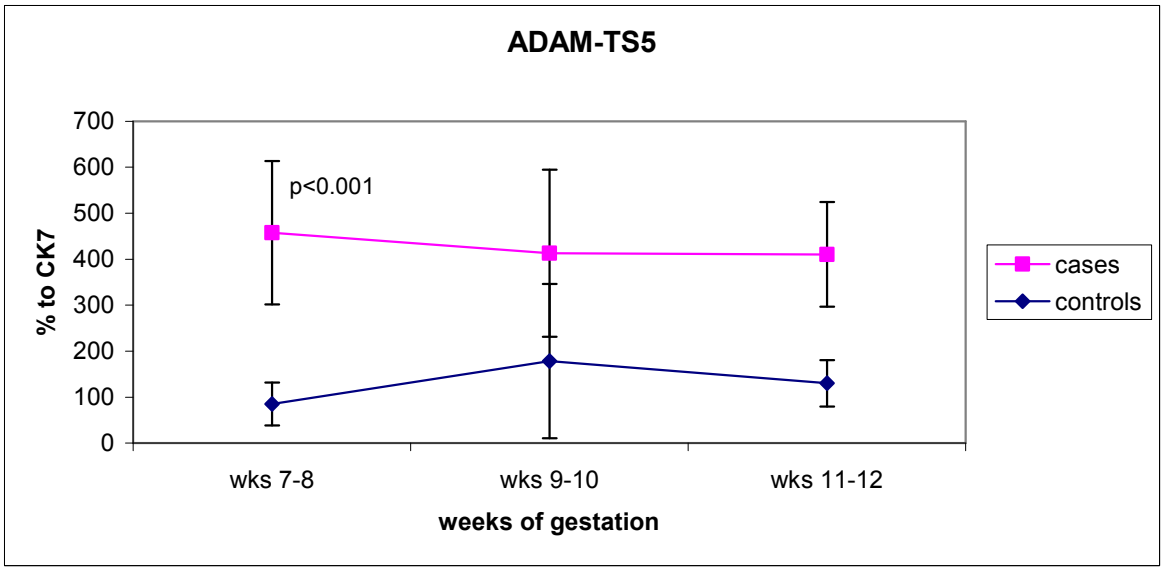


Figure 25: Expression of ADAM-TS5 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

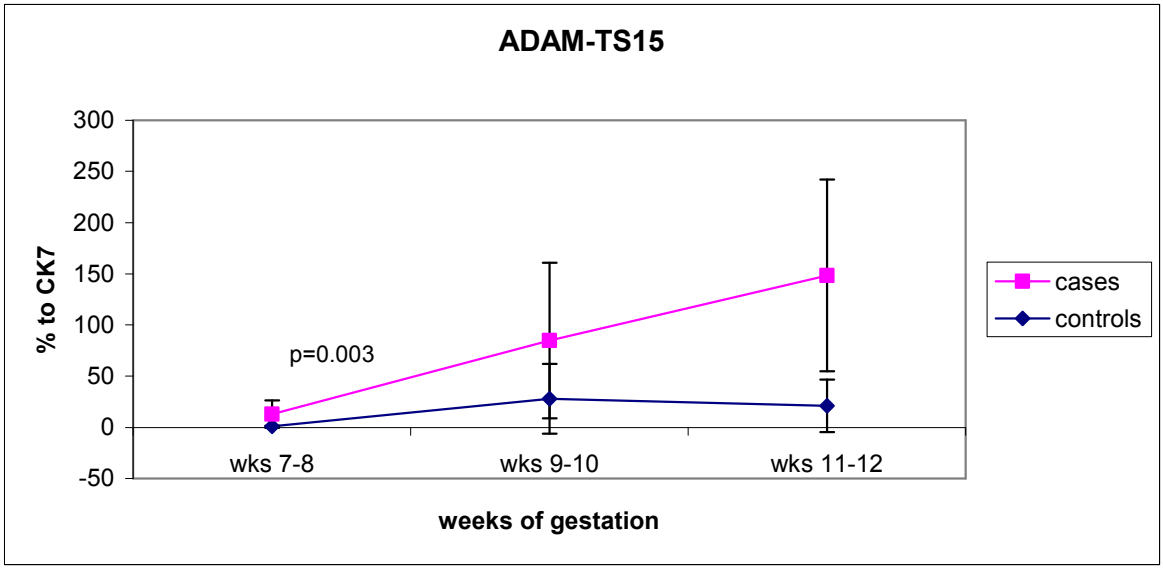


Figure 26: Expression of ADAM-TS15 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

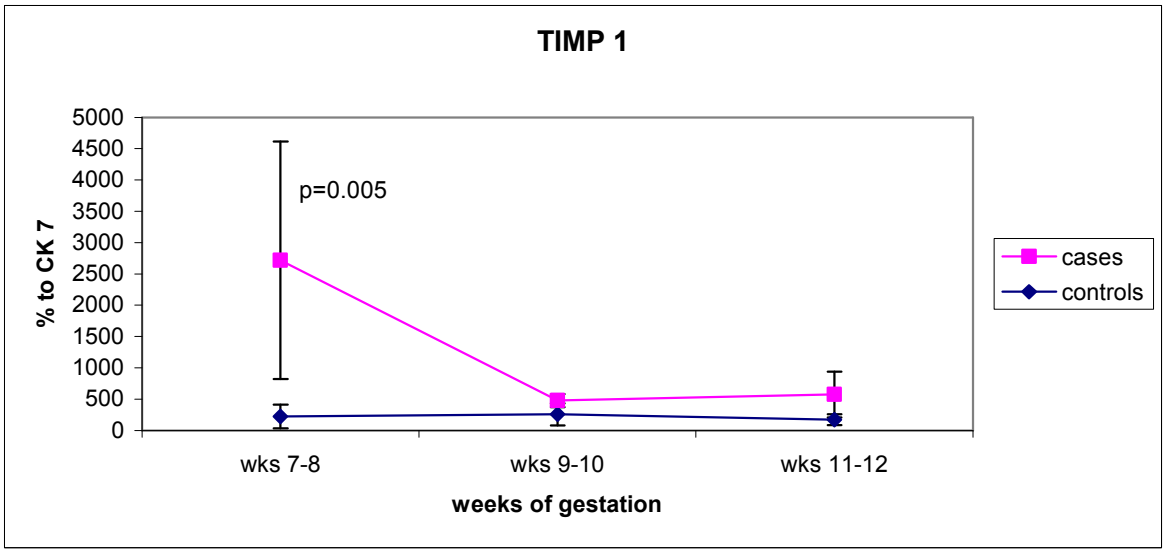


Figure 27: Expression of TIMP1 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

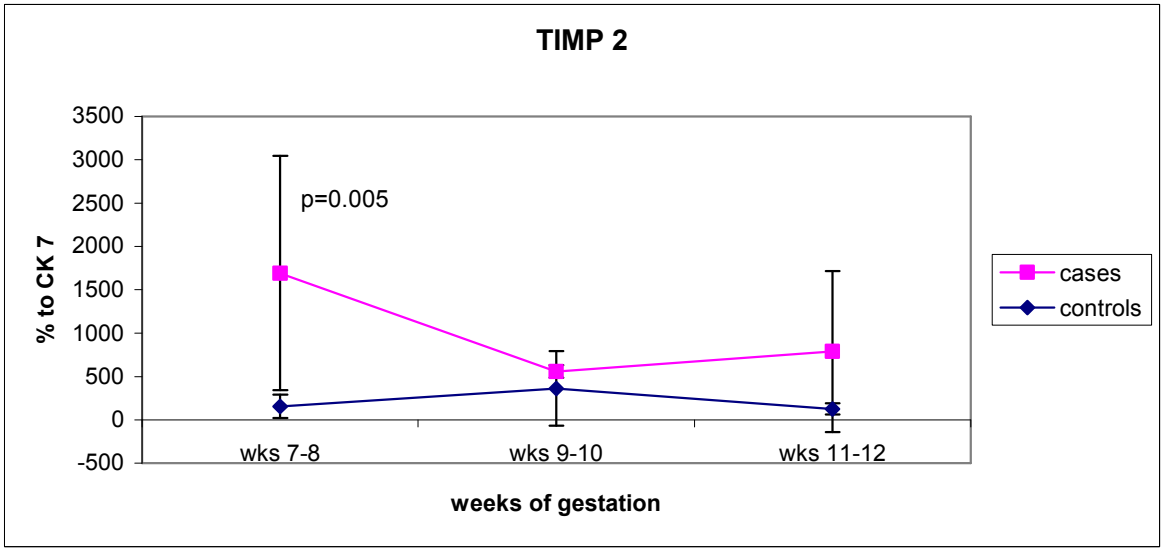


Figure 28: Expression of TIMP2 in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

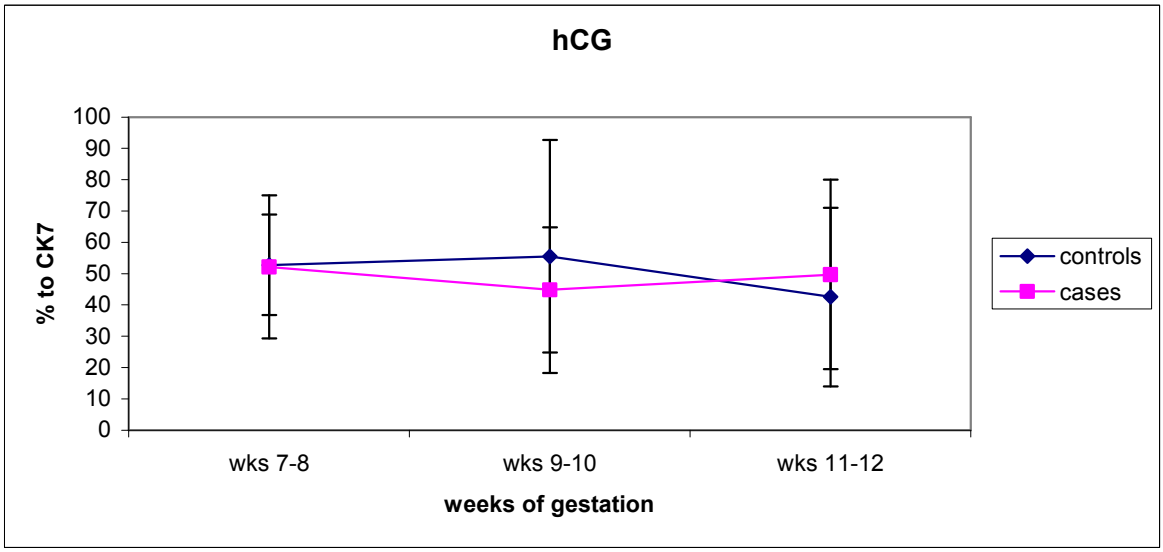


Figure 29: Expression of hCG in delayed miscarriage vs. controls during the first trimester. Illustration of mean longitudinal enzyme expression.

4. DISCUSSION

We are swiftly inveigled to interpret enzyme upregulation in delayed miscarriage as a consequence of exaggerated hypoxic conditions in the placental bed during early first trimester. But the entire information of all available data suggests a vital role for hypoxemia in early first trimester, hence, inducing relevant physiological events in early placental development and suppressing oxygen related damage to the vulnerable fetus. So, hypoxemia seems to be necessary in first trimester and should not be considered as pathology in this special period of pregnancy, though in second and third trimester a hypoxic milieu is defined as pathologic (James, Stone & Chamley 2006, Tuuli, Longtine & Nelson 2011, Caniggia et al. 2000, Caniggia, Winter 2002). Another indication for this statement is the fact that hypoxemia is not associated with protease upregulation, whereas the onset of utero-placental blood flow is linked to trophoblast invasiveness and protease upregulation very well (James, Stone & Chamley 2006, Caniggia et al. 2000). Thus, it is advisable to strike a new path to interpret these results of significant upregulation of metalloproteases in early pregnancy in case of delayed miscarriage.

In contrast to the strikingly simple idea that hypoxia leads to counterregulative protease upregulation to provide adequate blood supply for the fetus, a new approach might be conceived to explain causal conjunctions. Therefore I would like to propose a new conception for the interrelation between metalloproteases and the pathology of delayed miscarriage. As shown in the results, there is a strong trend to upregulation of above-mentioned enzymes in delayed miscarriage and significant difference in weeks 7+8. It is well known, that the event of “endoluminal plugging” of the spiral arteries is linked to the establishment of a hypoxic milieu circumjacent to the placental bed (James, Stone & Chamley 2006, Tuuli, Longtine & Nelson 2011, Jauniaux et al. 2000, Caniggia, Winter 2002). This process peaks about weeks 7-10, which is exactly the period the widest differences between pathology and controls were detected. Therefore a failure in “endoluminal plugging” of the spiral arteries or a similar unknown event followed by disproportionate oxygen levels for gestational age may be proposed legitimately.

Usually, low oxygen tension in early first trimester leads to augmented HIF-1α activity which is associated with an intermediate trophoblast phenotype expressing α5β1-integrin patterns, showing high proliferative activity and lower invasive behaviour. After the onset of placental perfusion in later first trimester and further pregnancy, HIF-1α is suppressed and a switch from the proliferative trophoblast phenotype towards the invasive type can be

observed (James, Stone & Chamley 2006, Tuuli, Longtine & Nelson 2011, Caniggia et al. 2000, Caniggia, Winter 2002). So, I consider it possible that altered oxygen tension in delayed miscarriage causes metalloprotease upregulation and overwhelming infiltration via HIF-1α suppression, thus possibly harming fetal structures by ROS (i.e. reactive oxygen species). The immature fetus, lacking protective enzyme systems to cope with oxidative stress, dies and subsequently experiences no expulsion from the uterus due to excessive infiltration and embedding. This situation would be typical for the pathology of delayed miscarriage. The hypothesis corresponds with evidence received from some authors who proved the association between premature placental blood supply, increased oxygen levels too early for gestational age and loss in placental mass or even spontaneous early pregnancy loss (Jauniaux et al. 2000, Huppertz et al. 2009).

In contrast, several researchers point out basically unlike biological actions of oxygen in first trimester. According to them, low oxygen tension promotes the integrin switch to an invasive phenotype and leads to the activation of metalloproteases via the uPA cascades system (James, Stone & Chamley 2006, Graham et al. 2000, Graham, Fitzpatrick & McCrae 1998). Further studies need to be carried out to verify or falsify our statements and to obtain a clearer view on this controversial issue.

Alternatively, the immune system could also influence protease expression. Several authors detected MMP upregulation due to prostaglandines (in particular prostaglandin E2) and other proinflammatory cytokines involved in activation of proinflammatory cascades (Anin, Vince & Quenby 2004, Burrows, King & Loke 1996, Staun-Ram, Shalev 2005, Knofler 2010, Staun-Ram et al. 2004, Bischof, Meisser & Campana 2001, Cohen, Meisser & Bischof 2006, Schatz et al. 1999, Cohen, Bischof 2007, Bischof, Haenggeli & Campana 1995, Salamonsen 1999). Further, altered immune regulation is considered to be responsible for early pregnancy failures within the meaning of absent switch from type 1 T-cell derived cytotoxic and pro-inflammatory response to the type 2 T-cell dominated pathway which is followed by the activation of the humoral immune system and seems to be beneficial for pregnancy outcome. In case of failure of this maternal immune response there are higher rates of early pregnancy loss (Veenstra van Nieuwenhoven, Heineman & Faas 2003, Renaud et al. 2011). Dysregulation in immunological processes during early pregnancy could be of great importance for vascularisation, invasion and metalloproteinase expression. Thus, it is easily conceivable that the immune system governs the establishment of pregnancy at the highest hierarchical level. Research in pregnancy immunology could probably give answers about key issues in the next decades. The

findings on metalloprotease expression in this study give little clues to ongoing immunological events but do not explain interrelations in this complex network of immune competent cells, soluble messenger molecules, tolerance inducing ligand-receptor couples and the heterogeneous immunological fingerprints of mother and fetus.
Finally, a potential bias in this study should be noted. The exact lenght of time delayed miscarriage samples were *in utero* before medical treatment is not known. So we should also consider reactive inflammatory processes due to devitalised fetal tissue *in utero* as responsible for protease upregulation. Therefore hCG mRNA determination for indication of tissue vitality was performed, but demonstrated no significant differences on mRNA level. Unfortunately, urin samples of women included in our study were not available.

Some proteinases were not described in placental tissue before, namely ADAM20, ADAM22, ADAM28, ADAM33 and ADAM-TS2, ADAM-TS3, ADAM-TS15, ADAM-TS19, ADAM-TS20. So, our enzyme profiling over the first trimester provides an excellent overview of all relevant metalloproteases potentially involved in implantation/invasion, both well-known and newly discovered in this study.
Expression of MMP2 (gelatinase A) and MMP9 (gelatinase B) in placental tissue was mentioned by almost every reviewer (Staun-Ram, Shalev 2005, Ferretti et al. 2007, Staun-Ram et al. 2004, Bischof, Meisser & Campana 2001, Cohen, Bischof 2007, Fluhr et al. 2008, Meisser et al. 1999) and their supposably leading role in the process of invasion was pointed out. In addition to the mainly studied gelatinases, expression of MMP1 (collagenase-1), MMP7 (matrilysin-1), MMP19, MMP14 (MT1-MMP), MMP15 (MT2-MMP) (Cohen, Meisser & Bischof 2006), MMP10 (Goyal et al. 2010, Hess et al. 2007), MMP11 (stromelysin-3) (Maquoi et al. 1997), MMP12 (Hannan, Salamonsen 2008, Harris et al. 2010), MMP16 (MT3-MMP) (Plaisier et al. 2008) and MMP24 (MT5-MMP) (LaMarca et al. 2005) was linked with development of the human placenta. ADAM8, ADAM12, ADAM15 (van Goor et al. 2009), ADAM10 (Bouillot et al. 2011), ADAM19 (Zhao et al. 2009), ADAM9, ADAM17 (Kim et al. 2006), ADAM-TS5, ADAM-TS6 (Shiomi et al. 2010) and ADAM-TS7 (Hurskainen et al. 1999) expression was brought in line with invasive potential of trophoblasts during implantation, while ADAM-TS1 knock-out mice show impaired fertility (Flannery 2006).

Recapitulatory, our study provides the first wide spectrum analysis of all relevant protease families in human trophoblasts during the first trimester of pregnancy. The study

demonstrated that more proteases are expressed in human trophoblasts than known previously. Not only intensively studied MMPs seem to be of great importance in human reproduction, but also the families of ADAMs and ADAM-TSs should be considered as central in processes associated with trophoblast invasiveness and vascularisation of the placental bed.

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