Angiogenesis, proteases and angiogenic factors during the inception of pregnancy. Crucial contributors or trivial bystanders?

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Chapter 7

Decidual vascularisation and the expression of angiogenic growth factors and proteases in first-trimester spontaneous abortions.

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Submitted to Human Reproduction

ABSTRACT

Objective: Decidual vascular development is important for implantation. This study analysed decidual vascular adaptation to implantation in correlation with miscarriage in decidual secretory endometrium (DSE), decidua parietalis (DP) and basalis (DB) of miscarriage patients and matched controls.

Methods: Decidua was obtained during first-trimester termination of pregnancy (controls) and vacuum-aspiration in case of missed abortion (cases). Vascularisation and the expression of VEGF-A, PlGF, flt-1, KDR, Angiopoietin-1, Angiopoietin-2, TIE-2, and membrane-type matrix metalloproteinases MT1-, MT2-, MT3- and MT5-MMP were determined at mRNA and protein level. uNK cells (CD56), macrophages (CD68), proliferation (Ki67) and apoptosis (activated-caspase-3) were evaluated in consecutive sections.

Results: Decidual vascularisation showed differences between cases and controls, i.e. fewer vessels with larger circumference in cases. This correlated with the differential expressions of various factors at mRNA/antigen level and with increased endothelial flt1, KDR, MT2- and MT5-MMP expression miscarriage patients. The differences between cases and controls were probably not based on altered proliferation and/or apoptosis, since Ki67 and active caspase-3 showed comparable expression levels in both groups. Although DB of cases and controls showed similar amounts of CD56- and CD68-positive cells, the missed abortion group did show elevated levels of CD56 in DSE and of CD68 in DSE and DP compared to the control group.

Conclusion: The differences in vascularisation and in the expression of angiogenic factors and proteases between both groups suggest a correlation between decidual vascularisation and the occurrence of miscarriages.

INTRODUCTION

Decidual vascular adaptation to implantation is important for the success of pregnancy and already starts during the receptive secretory phase of the menstrual cycle. Decidualisation, vascular remodelling and invasion of immune cells are prominent processes in the first weeks after fertilisation [Bulmer *et al*. 1991; Salamonsen *et al*. 2003; Smith 2000]. Immune cells increase from 8% of total stromal cells during the menstrual cycle up to 30% during the first-trimester. Approximately 70% of these leucocytes are uterine-NK (uNK) cells and 10% are macrophages [Bulmer *et al*. 1991].
Vascular adaptation includes (pseudo-)vasculogenesis, arterial remodelling, and angiogenesis, the formation of new vessels out of existing ones [Burton *et al*. 1999; Pijnenborg *et al*. 1983]. Angiogenesis is characterised by increased vascular permeability, endothelial cell proliferation and migration, and is regulated by various growth factors, e.g. vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and angiopoietins, and proteases like the membrane-type matrix metalloproteinases [Carmeliet 2003; Pepper 2001]. Disturbances in vascular development may play a role in the pathogenesis of miscarriages [Vailhe *et al*. 1999; Vuorela *et al*. 2000; Zygmunt *et al*. 2003].

The angiogenic growth factors VEGF-A and PlGF are extensively studied in the vascular development of the placenta [Plaisier *et al*. 2007; Smith 2000; Vuorela *et al*. 1997]. VEGF is one of the earliest genes activated during pre-implantation embryo development and is produced by maternal decidual cells and by the invading blastocyst and trophoblast [Kapiteijn et al. 2006; Jackson et al. 1994; Plaisier *et al*. 2007]. VEGF is a potent inducer of angiogenesis and interacts with the Flt-1 (VEGFR-1) and KDR (VEGFR-2) to promote endothelial cell proliferation, migration and vascular permeability. KDR is generally recognized as the central VEGF receptor in angiogenesis, while Flt-1 plays a supporting role. During pregnancy, also a soluble variant of Flt-1 (sFlt-1) is formed, which quenches VEGF and thereby limits its activity [Maynard *et al*. 2003].
PlGF shares biochemical and functional features with VEGF, but only interacts with Flt-1. PlGF and VEGF have synergistic effects regarding angiogenesis, however PlGF induced vessels are suggested to be more mature and stable than vessels induced by VEGF alone [Carmeliet *et al*. 2001]. PlGF is abundantly expressed in human placenta and may be an important paracrine regulator of decidual angiogenesis and an autocrine mediator of trophoblast function [Sherer, Abulafia 2001; Plaisier *et al*. 2007].
The angiopoietins and their receptor TIE-2 are also known for their regulating capacities regarding angiogenesis. The two ligands bind with equal affinity to TIE-2 but have different functions. Angiopoietin-1 (Ang-1) maintains vessel integrity and probably plays a role in the later stages of vascular remodelling [Geva *et al*. 2000]. Angiopoietin-2 (Ang-2) is a

functional antagonist of Ang-1 and leads to loosening of cell-cell interactions and allows access to angiogenic inducers like VEGF [Geva *et al*. 2000].
Ang-1, Ang-2 and TIE-2 are detected in various maternal cells, endovascular trophoblasts, and (syn-) cytotrophoblasts during human first-trimester pregnancy. These findings suggest an additional role for angiopoietins in regulating trophoblast behaviour in the development of uteroplacental circulation [Plaisier *et al*. 2007; Dunk *et al*. 2000; Zhang *et al*. 2001]. An association of the angiopoietins with miscarriage has not been described, but reduced endothelial TIE-2 expression has been linked to the occurrence of miscarriage [Vuorela *et al*. 2000].

The membrane-associated localisation of MT-MMPs makes them suited for pericellular proteolysis [Hotary *et al*. 2000; van Hinsbergh *et al*. 2006]. We studied the transmembrane-spanning MT-MMPs, MT1- (MMP-14), MT2- (MMP-15), MT3- (MMP-16) and MT5-MMP (MMP-24), since these MT-MMPs have the proteolytic potential to induce capillary tube formation. The GPI-anchored MT4- and MT6-MMP were unable to do so [Hotary *et al*. 2000].
MT1-MMP is the best known MT-MMP and promotes cell migration, angiogenesis and tumour metastasis [Visse, Nagase 2003]. MT2- and MT3-MMP are less well studied but are also involved in cell migration and invasion. MT1-, MT2- and MT3-MMP induce angiogenesis *in vitro* and MT2- and MT3-MMP may even be potential regulators of endometrial angiogenesis *in vivo* [Hotary *et al.,* 2000; Plaisier *et al*. 2004/2006]]. MT5-MMP is known for the induction of embryonic brain development and axonal growth [Llano *et al.,* 1999].
MT1- and MT2-MMP RNA and protein expression are described in decidual extracts, stromal cells, and the extra-villous trophoblast [Bjorn *et al*. 2000; Nawrocki *et al*. 1996; Curry,Osteen 2003]. These MT-MMPs are assumed to regulate trophoblast invasion during implantation [Salamonsen *et al*. 2003]. To which extent migration of other cell types, e.g. immune and endothelial cells, is also regulated by MT-MMPs remains to be seen.

Vascular adaptation to implantation has mainly been studied with regard to physiology [Craven *et al,* 1999; Kam *et al*. 1999]. Recently, the potential regulation of decidual vascularisation by both the extra-villous trophoblast (EVT) and pregnancy-induced hormones has been described. Moreover, the differential expression of VEGF-A, the angiopoietins, PlGF, Flt-1, MT2- and MT3-MMP suggested their role in regulating the vascular adaptive process in uncomplicated first-trimester pregnancies [Plaisier *et al*. 2007/2008]. Few studies have addressed the question whether vascularisation and its regulatory factors are involved in the pathogenesis of first-trimester complications, like miscarriage. A higher decidual vessel density was correlated with missed abortions [Vailhe *et al*. 1999]. Also, differential antigen expression of VEGF and its receptors has been correlated to re-

current miscarriage [Vuorela *et al*. 2000]. Moreover, elevated uterine MMP and reduced TIMP expression has been linked to failed implantation [Jokimaa *et al*. 2002].
The present study determined decidual vascular adaptation to implantation in correlation with failure of implantation, i.e. missed abortion. Vascularisation pattern and the expressions of the above described angiogenic factors and proteases were determined in three first-trimester decidual tissues, decidual secretory endometrium (DSE), decidua parietalis (DP) and basalis (DB) of miscarriage patients and matched controls. The comparison of these parameters between the two groups enabled hypothesising about their correlation with the occurrence of miscarriages.

MATERIALS AND METHODS

Study group

The study was approved by the ethics committee of the Leiden University Medical Centre and informed consent was provided by all study subjects.
Decidua samples in the case group were obtained during vacuum-aspiration from missed abortion patients. Missed abortion was diagnosed based on ultrasound examination and absence of clinical miscarriage symptoms. Aspirated tissue was analysed by an independent pathologist and samples with pathologic abnormalities were excluded. Patients with pathological conditions known to interfere with implantation, like haemostatic abnormalities, auto-antibodies, etc, were also excluded. Gestational age at foetal death was determined by ultrasound.
Decidua samples in the control group were obtained from women with a healthy, viable intrauterine gravidity, undergoing vacuum aspiration in case of a legal voluntarily abortion. Foetal cardiac activity and gestational age were confirmed by ultrasound. Women with symptoms of miscarriage, with underlying illnesses, and cases with discrepancy between the ultrasound-determined gestational age and last day of menstruation were excluded in both control and case group.
Cases and controls were matched based on maternal and gestational age. These parameters and number of previous pregnancies and miscarriages did not differ significantly between the cases and the controls. Patient characteristics of the study groups are given in Table 1.

Tissue Samples

Decidua samples were obtained from the aspirated tissue (vacuum aspiration), fixed in formaldehyde overnight and embedded in paraffin. Hematoxylin Phloxin Safrane (HPS) and anti-cytokeratin staining were performed. Hematoxylin (50 g potassium aluminium sulfate, 1 g hematox, 500 mg citric acid, 25 g chloralhydrate, 200 mg $NaJO_3$ in 1000 ml

Table 1. Characteristics of the study subjects.

	Controls (*n*=16)	**Missed Abortions** (*n*=11)
Maternal Age (years) [a]	31.0 ± 1.3	33.3 ± 1.6
Gestational age (days) [a]	50.4 ± 2.7	53.8 ± 3.3
Gestational age (weeks) [a]	7.2 ± 0.4	7.7 ± 0.5
Number of previous pregnancies [a]	1.5 ± 0.4	2.6 ± 0.5
Number of previous miscarriages [a]	0.3 ± 0.1	0.8 ± 0.4

[a] All p>0.05.

aqua dest) stains nuclei and calcium blue. Phloxin (0.25 g phloxin in 100 ml aquadest) stains erythrocytes, cytoplasm, fibrin and muscle red. Safrane (3 g safrane in 1000 ml 100% alcohol) stains calcium free bone, cartilage and collagen yellow.
The HPS staining was used to differentiate between decidua and decidual secretory endometrium (DSE), which microscopically resembles secretory endometrium. Decidua basalis (DB) and decidua parietalis (DP) were differentiated by the presence or absence of extra-villous trophoblasts using an anti-cytokeratin staining (see section immunohistochemistry). DSE, DP and DB were obtained from the same curettement and therefore originate from the same depth and area of the uterine wall. Only subjects with at least two complete sets of DSE, DB and DP were included (n=11 in missed abortion group and n=16 in control group). Serial sections of the paraffin embedded tissue samples were used for all experiments and all parameters were compared between tissues within subjects.

RNA isolation and cDNA synthesis

RNA was extracted from paraffin embedded tissue samples [Plaisier *et al.* 2007]. Several samples per tissue per patient were used and these samples contained a proportionate amount of cells and cell types. In short, 5 μm sections were mounted on RNase-free glass slides. The first and last sections were used to verify the presence of the tissues of interest. The other sections were deparaffinised and the tissues of interest, DSE, DP or DB (without villous tissue), were dissected and dissolved in 190 μl lysis buffer (Tris 20 mM pH 7.4, EDTA 1 mM pH 8.0, 2% SDS) and 10 μl proteinase K (20 mg/ml proteinase K, Life-Technologies Gibco BRL, Gaithersburg) for 18 hours at 60°C. Subsequently, 400 μl Solution D (4 M guanidium isothiocyanate, 0.75 M sodium citrate, 10% sarkosyl and 2-mercapto-ethanol) was added and RNA was isolated [Plaisier *et al.* 2007].
RNA quantity and quality were analysed in a spectrophotometer (Nanodrop ND-1000). The OD260/280 of the RNA samples ranged between 1.85 and 2.00. Reverse transcription was performed with 1 μg total RNA, random primers and cDNA synthesis kit according to the manufacturer's protocol and the obtained 32 μl cDNA was diluted 1:3 (Ready-to-go You-Prime first strand beads, Amersham Biosciences, Buckinghamshire, UK).

Real-time RT-PCR

The mRNA levels were quantified according to the Taqman real-time PCR method using validated primer and probe (FAM/TAMRA double-labelled) sets for VEGF-A, VEGF-R1 (Flt-1), VEGF-R2 (KDR), PlGF, Angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2), TIE-2, MT1-MMP, MT2-MMP, MT3-MMP, and MT5-MMP. glycerlylaldehyde'-3-phosphate dehydrogenase (GAPDH, primers/VIC-labelled probe) was used as an endogenous reference gene. Other genes, β-actin, β_2-microglobulin and cyclophilin, were also used as reference genes and showed comparable results (data not shown, all primer/probe sets purchased from Applied Biosystems, Foster City, CA USA).

RT-PCR reactions for target gene/GAPDH pairs were performed in 12.5 µl reactions, containing 2.5 µl cDNA solution, using mastermix (RT-QP2X-03, Eurogentec, Maastricht, The Netherlands), DNAse free water and the above described primer/probe sets. Reactions were performed in duplicate, expressed in cycle times (Ct) and quantified into ng/µl using a standard curve of total RNA. The amount of RNA expression of DSE was set at 100% to compare DSE with DP and DB and the gene expression of DP was set at 100% to compare DP with DB. Water and negative-RT samples, obtained by the omission of the reverse transcriptase enzyme in the cDNA reaction, were used as negative controls.

Immunohistochemistry

Serial sections were deparaffinised, endogenous peroxidase was quenched with 3% H_2O_2/methanol and aspecific binding was reduced by incubation with 5% bovine serum albumin. Antigen retrieval in trypsin was used for the detection of CD34, TIE-2, MT2- and MT5-MMP. Heat retrieval in 0.1 M citrate buffer was used for the detection of cytokeratin, Ang-2, Ki67, CD56 and CD68.

The first antibodies are described in Table 2. Polyclonal rabbit anti-MT1-MMP was produced and characterised as previously described [Plaisier *et al.* 2004]. The following second antibodies were used: biotinylated horse anti-mouse antibody (1:300, BA-2000, Vector), biotinylated donkey anti-rabbit antibody (1:300, RPN1004, Amersham Biosciences), biotinylated rabbit anti-goat (1:300, E-0466, DakoCytomation).

Primary antibodies were applied overnight at 4°C followed by one hour incubation with biotinylated secondary antibody. Antibody binding was visualised using StreptABComplex/HRP, a streptavidin complexed with biotinylated peroxidase (K0377, DakoCytomation, Glostrup, Denmark) and NovaRED™ substrate (SK-4800, Vector, Burlingame, USA) according to the manufacturer's protocol. Only CD56 immunohistochemistry was stained with DAB (diaminobenzidine). All incubations were performed in 1% bovine serum albumin in PBS.

Sections were counterstained with Mayer's hematoxylin. Specificity of the immunohistochemical reaction was verified by the omission of the first antibody as well as using non-immune mouse IgG_1, concentration adjusted to primary IgG_1 concentration, and

Table 2. Specifications of the primary anibiodies.

Antigen	Source	Clone-number	Species	Pre-treatment	Dilution
Cytokeratin	DakoCytomatation	Z0622	Rabbit	Citrate HR	1:2000
CD34	Novocastra	QBEnd/10	Mouse (IgG_1)	0.1% Trypsin	1:1000
CD56	Monosan	MONX 10844	Mouse (IgG_1)	Citrate HR	1:50
CD68	DakoCytomatation	M0814	Mouse (IgG_1)	Citrate HR	1:200
Ki67	Novocastra	Ncl-L-Ki67-MM1	Mouse (IgG_1)	Citrate HR	1:50
Act Casp 3	Cell Signaling	9661	Rabbit	None	1:300
VEGF-A	Santa Cruz Tech	Sc-152 (A-20)	Rabbit	None	1:100
PlGF	Santa Cruz Tech	Sc-1880 (C-20)	Goat	None	1:50
Flt-1	Santa Cruz Tech	Sc-316 (C-17)	Rabbit	None	1:50
KDR	Santa Cruz Tech	Sc-6251 (A-3)	Mouse (IgG_1)	None	1:50
Ang-1	Santa Cruz Tech	Sc-6319 (N-18)	Goat	None	1:100
Ang-2	Santa Cruz Tech	Sc-7016 (N-18)	Goat	Citrate HR	1:150
TIE-2	Santa Cruz Tech	Sc-9026 (H-176)	Rabbit	0.1% Trypsin	1:100
MT1-MMP	TNO QoL		Rabbit	None	1:1000
MT2-MMP	Oncogene	IM48L	Mouse (IgG_1)	0.1% Trypsin	1:250
MT3-MMP	Oncogene	IM50L	Mouse (IgG_1)	None	1:100
MT5-MMP	Sigma	M6684	Rabbit	0.1% Trypsin	1:200

goat and rabbit serum instead of first antibody. To evaluate the expression in extra-villous trophoblast, cytokeratin and target protein staining were performed on serial sections of 3 μm.

Evaluation of the vascularisation pattern

CD34-stained decidual sections were used to scan sequential fields [Vailhe *et al.* 1999]. Two to four samples of each type of tissue of each study subject were analysed and six fields per sample were scanned at 100x magnification. The area within the stained vessels and the number of vessels per field were analysed using an image analysis software (Qwin, Leica Microsystems) and these data were used to calculate the number of vessels per mm^2, total vascular surface per mm^2 (mm^2/mm^2) and the luminal surface (μm^2/vessel).

Evaluation of Immunohistochemical staining

In order to avoid intensity differences in the staining we analysed control samples (16 donors) simultaneously with the samples of 11 cases. Immunostaining of Ki67, active Caspase-3 (aCasp-3), CD56 and CD68 was evaluated by counting the number of positive cells and total stromal cells in ten 16 μm^2 fields per tissue per patient. In this way, the mean percentages of positive cells per stromal cells and per μm2 were determined. In

case of Ki67 and aCasp-3, the expression in endothelial and epithelial cells was also determined similarly.

Immunostaining of the other antigens was evaluated by calculating a staining index (SI): proportion of stained cells x staining intensity. The proportion of stained cells was expressed as 0, 1, 2 or 3, which marks positive staining signal in 0%, <10%, 10-50% or >50% of the cells of a particular cell population. The intensity of staining was expressed as 1, 2 or 3 (weak, moderate or strong staining, respectively). The minimum score was 0 and the maximum score 9 [Plaisier *et al.* 2007]. The average score of two independent observers was used to calculate the mean and total staining index (Mean and Total SI). The inter-observer variability was 6% for VEGF-A, 7% for PlGF, 11% for Flt-1, 10% for KDR, 5% for Ang-1, 6% for Ang-2, 9% for TIE-2, 4% for MT1-MMP, 14 % for MT2-MMP, 8 % for MT3-MMP, 7 % for MT5-MMP (mean variability 8%).

The Mean SI represents the protein expression per studied cell type. The Total SI represents the total protein expression per tissue and was calculated as the sum of the Mean SI of endothelial cells (EC), peri-vascular smooth muscle cells (PSMC), glandular epithelium (GE) and stromal cells (SC) in DSE and DP samples. The Mean SI of EVT was also included in the Total SI of DB samples.

Statistical analysis

Data are expressed as mean ± SEM. All parameters were compared between DSE and DP, between DSE and DB and between DP and DB within subjects. A general linear model for repeated measurements, repeated measures ANOVA, was performed to analyze the double paired data within the case and control group as well as to compare data between the case and control group. Where appropriate, the Friedman's test for non-parametric investigations of correlated observations and the wilcoxon test for paired samples were used to study differences within the two groups (SPSS 11.5). The used statistical analyses are described in legends and table footnotes. P-values of < 0.05 were considered significant.

RESULTS

The vascularisation pattern in cases and controls

Intense CD34 staining of vascular structures was observed in all decidual tissues of controls and missed abortion cases. Their vessel density as well as their total and luminal surfaces were quantified in all controls (n=16) and cases (n=11).

Controls

Vessel density was significantly elevated in DSE (116.1 ± 8.5 vessels/mm²) compared to DP (39.1 ± 3.3) and DB (30.9 ± 3.8); vessel density in DP and DB also differed significantly (all p<0.01, Figure 1A). The total vascular surface was significantly smaller in DSE (0.021 ± 0.002 mm²/mm²) compared to DP (0.040 ± 0.004) and DB (0.055 ± 0.007). The total surface in DP differed significantly from that in DB (all p<0.01, Figure 1B). The luminal surface was significantly smaller in DSE compared to DP (203.8 versus 1576.0 μm²/vessel) and DB (2928.1 μm²/vessel); this parameter was also significantly smaller in DP compared to DB (all p<0.01, Figure 1C). Thus, larger but fewer vessels were present from DSE to DP to DB.

Cases versus Controls

The vessel density was significantly lower in DP and DB of cases compared to the controls; 29.6 vessels/mm² versus 39.1 in DP and 19.8 versus 30.9 in DB respectively (both p<0.05, Figure 1A). The luminal surface of the cases was significantly higher in DP and DB of cases; 3604 μm²/vessel versus 1576 in DP and 5661 versus 2928 in DB respectively

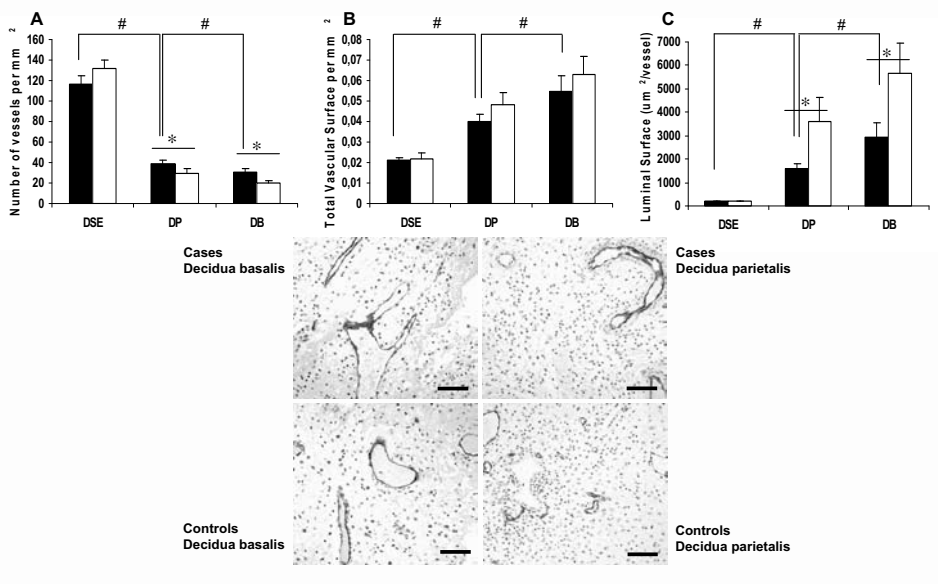

Figure 1. Vascularisation pattern in cases and controls.
The vascularisation pattern in decidual tissues of controls (n=16, black bars) and cases (n=11, white bars) was determined by image analysis of anti-CD34-stained sections. **A.** Vessel density (number of vessels per mm²), **B.** the total vascular surface (mm²/mm²) and **C.** the luminal surface (μm²/vessel) were calculated and expressed as mean±SEM. # p<0.05 within controls, ∗ p<0.05 in cases versus controls. The bottom panels show examples of vascularisation in decidua parietalis and basalis of cases and controls (control; left panels) and cases (missed abortions; MA: right panels).
(see colour figures supplement)

(both p<0.04, Figure 1C). The total vascular surface and all the parameters in DSE did not differ between cases and controls.

Angiogenic factors and proteases in cases and controls: mRNA levels

The expression of various angiogenic growth factors and MT-MMPs were evaluated at the mRNA level in the three decidual tissues in cases and controls. The data of cases and controls are separately described in Table 3a and 3b. Their relative content in cases versus controls is described in Table 4.

The data of the 16 controls on mRNA levels of angiogenic factors and proteases are given in Table 3a. They were highly comparable to previous data on control samples of 25 different women [Plaisier *et al.,* 2007; Plaisier *et al.,* 2008].

Table 3a Differential mRNA content of angiogenic factors and proteases in first-trimester decidua of controls.

	mRNA content (ng/μl) [a]			mRNA (fold Induction)				
	DSE	DP	DB	DSE [b]	DP	DB	DP [c]	DB
PlGF	1.09 ± 0.22	0.69 ± 0.23	10.1 ± 2.1	1.0	0.7 ± 0.3	12.7 ± 2.7*	1.0	35.8 ± 2.8#
VEGF-A	0.18 ± 0.04	0.20 ± 0.04	0.21 ± 0.05	1.0	1.1 ± 0.2	1.2 ± 0.2	1.0	1.5 ± 0.3
KDR	0.85 ± 0.17	1.68 ± 0.56	1.23 ± 0.50	1.0	1.1 ± 0.1	1.2 ± 0.1	1.0	0.9 ± 0.2
Flt-1	0.81 ± 0.12	0.76 ± 0.09	0.94 ± 0.15	1.0	1.1 ± 0.1	1.2 ± 0.1	1.0	1.3 ± 0.2
Ang-1	0.10 ± 0.02	0.09 ± 0.02	0.03 ± 0.01	1.0	0.9 ± 0.1	0.5 ± 0.1*	1.0	0.6 ± 0.1#
Ang-2	0.51 ± 0.13	0.33 ± 0.04	0.16 ± 0.05	1.0	1.1 ± 0.2	0.4 ± 0.1*	1.0	0.6 ± 0.2#
TIE-2	3.58 ± 0.52	1.87 ± 0.39	0.76 ± 0.20	1.0	0.5 ± 0.1$	0.3 ± 0.1*	1.0	0.6 ± 0.1#
MT1-MMP	3.35 ± 0.43	6.05 ± 0.91	5.69 ± 1.11	1.0	2.1 ± 0.4$	1.7 ± 0.5*	1.0	1.4 ± 0.4
MT2-MMP	0.09 ± 0.03	0.06 ± 0.02	1.45 ± 0.39	1.0	3.1 ± 1.1	23.3 ± 4.2*	1.0	19.3 ± 3.2#
MT3-MMP	7.39 ± 1.89	6.20 ± 1.77	16.3 ± 2.5	1.0	1.4 ± 0.3	7.7 ± 1.0*	1.0	11.4 ± 2.4#
MT5-MMP	39.88 ± 4.89	28.75 ± 7.35	5.94 ± 0.80	1.0	0.7 ± 0.2	0.1 ± 0.0*	1.0	0.3 ± 0.1#

mRNA content was determined by RT-PCR in DSE, DP and DB of early first-trimester pregnancies and compared within subjects of the control group. The 'repeated measures ANOVA' was performed to analyse the data.

a Mean cycle threshold (Ct) per gene: GAPDH 25, VEGF-A 26.0, PlGF 29.2, KDR 28.5, Flt-1 28.4, Ang-1 33, Ang-2 27.4, TIE-2 32.1, MT1-MMP 27, MT2-MMP 30, MT3-MMP 30, MT5-MMP 29. Cycle threshold were converted into ng/μl using a standard curve of total RNA. Then values were corrected for RNA input by calculating the ratio (ng/μl (target gene))/ (ng/μl (GAPDH)) and mean ± SD ratios are showed in the columns DSE, DP and DB.

b Fold induction was calculated as the ratio (DP)/ (DSE) and (DB)/ (DSE) to compare mRNA levels in DB and DP with those in DSE (1.0) within subjects. Discrepancies between absolute and percental data might originate from inter-individual differences.

c Fold induction was calculated as the ratio DB)/ (DP) to compare DB with DP (1.0) within subjects.

$ P<0.05 DP vs DSE, * p<0.05 DB vs DSE, # p<0.05 DB vs DP

Cases versus Controls

The mRNA levels in cases are given in Table 3b and the comparison of these levels to the mRNA contents of controls is described in Table 4. The mRNA expression in DSE showed an upregulation of KDR (3.5), Flt-1 (3.5), TIE-2 (2.7), and MT1-MMP (2.4) and a reduced MT3-MMP expression (0.7) in DSE of cases compared to controls (1.0) ($p<0.01$, Table 4). Regarding the mRNA levels in DP, VEGF-A (2.1), KDR (4.4), Flt-1 (3.8), Ang-2 (2.1), and TIE-2 (3.6) expression was significantly elevated and MT3-MMP expression was reduced in the case group (Table 4).

Finally, the mRNA levels in DB showed upregulation of all angiogenic factors, except PlGF, in decidua of the cases. On the other hand, none of the protease were differentially expressed (Table 4).

Angiogenic factors and proteases in cases and controls: immunohistochemistry

The presence and cellular localisation of the various antigens were determined in serial sections of DSE, DP and DB. The studied proteins were detectable in all decidual tissues

Table 3b Differential mRNA content of angiogenic factors and proteases in first-trimester missed abortion decidua.

	mRNA content (ng/µl) [a]			mRNA (fold Induction)				
	DSE	DP	DB	DSE [b]	DP	DB	DP [c]	DB
PlGF	1.81 ± 0.67	0.44 ± 0.06	10.67 ± 3.11	1.0	0.6 ± 0.1	13.4 ± 3.9*	1.0	21.2 ± 4.2#
VEGF-A	0.29 ± 0.04	0.40 ± 0.05	0.84 ± 0.17	1.0	1.7 ± 0.3	3.7 ± 0.9*	1.0	2.3 ± 0.4#
KDR	2.97 ± 0.57	5.74 ± 1.79	3.90 ± 0.99	1.0	2.6 ± 0.7	2.1 ± 0.8	1.0	1.4 ± 0.6
Flt-1	2.68 ± 0.14	2.88 ± 0.52	2.44 ± 0.47	1.0	1.0 ± 0.2	0.9 ± 0.2	1.0	0.9 ± 0.1
Ang-1	0.12 ± 0.02	0.10 ± 0.01	0.09 ± 0.02	1.0	1.3 ± 0.4	1.0 ± 0.3	1.0	1.0 ± 0.1
Ang-2	0.80 ± 0.14	0.68 ± 0.10	1.46 ± 0.41	1.0	1.3 ± 0.3	3.1 ± 1.0	1.0	2.6 ± 0.6
TIE-2	7.97 ± 0.72	6.04 ± 0.94	4.58 ± 1.08	1.0	0.8 ± 0.1	0.6 ± 0.2	1.0	0.9 ± 0.3
MT1-MMP	8.09 ± 1.49	6.73 ± 0.65	8.40 ± 1.48	1.0	1.1 ± 0.2	1.3 ± 0.3	1.0	1.2 ± 0.1
MT2-MMP	0.12 ± 0.03	0.05 ± 0.03	1.21 ± 0.22	1.0	0.6 ± 0.3	18.0 ± 4.4*	1.0	73.5 ± 12.4#
MT3-MMP	1.93 ± 0.35	0.93 ± 0.23	13.3 ± 2.30	1.0	0.8 ± 0.3	10.2 ± 2.1*	1.0	24.3 ± 5.4#
MT5-MMP	26.97 ± 4.32	28.44 ± 5.82	9.54 ± 1.85	1.0	1.2 ± 0.3	0.4 ± 0.1*	1.0	0.6 ± 0.2 #

mRNA content was determined by RT-PCR in DSE, DP and DB of early first-trimester pregnancies and compared within subjects of the Missed Abortion group. The 'repeated measures ANOVA' was performed to analyse the data.

a Mean cycle threshold (Ct) per gene: GAPDH 25, VEGF-A 26.0, PlGF 29.2, KDR 28.5, Flt-1 28.4, Ang-1 33, Ang-2 27.4, TIE-2 32.1, MT1-MMP 27, MT2-MMP 30, MT3-MMP 30, MT5-MMP 29. Cycle threshold were converted into ng/µl using a standard curve of total RNA. Then values were corrected for RNA input by calculating the ratio (ng/µl (target gene))/ (ng/µl (GAPDH)) and mean ± SD ratios are showed in the columns DSE, DP and DB.

b Fold induction was calculated as the ratio (DP)/ (DSE) and (DB)/ (DSE) to compare mRNA levels in DB and DP with those in DSE (1.0) within subjects. Discrepancies between absolute and fold induction data might originate from inter-individual differences.

c Fold induction was calculated as the ratio DB)/ (DP) to compare DB with DP (1.0) within subjects.

$ $P<0.05$ DP vs DSE, * $p<0.05$ DB vs DSE, # $p<0.05$ DB vs DP

and their presence was graded as median staining indices (median SI) per cell type and total staining indices (total SI) based on the sum of the cell types-dependent indices (Table 5a and 5b).

Controls

First, the total expression of the studied antigens in DP and DSE were compared. This showed a more abundant total expression of Flt-1, TIE-2 and MT1-MMP in DP (Table 5a and 5b, $p<0.05$). Then, the staining patterns in DB and DSE were compared; the total SI's of PlGF, Flt-1, TIE-2, and MT1-MMP were increased whereas the total SI of Ang-1 was decreased in DB. Finally, the comparison between DP and DB showed an increased PlGF total SI and a decreased Ang-1 total SI in DB.

The antigen expression was most abundant in glandular epithelial cells, followed by perivascular smooth muscle and stromal cells. Endothelium displayed the expression of all angiogenic factors, except Ang-1 and MT5-MMP. TIE-2 showed the highest expression in endothelium. The endothelial protein expression of PlGF and Flt-1 showed significant differences between the three decidual tissues and MT2-MMP appeared less abundantly expressed in DB compared to DP and DSE (Table 5a and 5b, Figure 3). All angiogenic factors, except Ang-1 and MT5-MMP very dimly, were also detected in extra-villous tro-

Table 4. Fold induction mRNA expression in first-trimester decidua of cases versus controls (set at 1.0).

	DSE	DP	DB
PlGF	1.7	1.5	1.1
VEGF-A	1.6	2.1*	4.9*
KDR	3.5*	4.4#	5.3*
Flt-1	3.5*	3.8*	2.7*
Ang-1	1.2	1.3	2.8#
Ang-2	1.6	2.1*	8.3*
TIE-2	2.7*	3.6*	5.3*
MT1-MMP	2.4*	1.7	1.8
MT2-MMP	1.3	1.7	0.8
MT3-MMP	2.6#	0.3#	0.8
MT5-MMP	0.6	0.8	1.8

mRNA levels of angiogenic factors and proteases in DSE, DP and DB were compared between cases and matched controls (1.0). The 'repeated measures ANOVA' was performed to analyse the data. Mean cycle threshold (Ct) per gene: GAPDH 25-26, MT1-MMP 27, MT2-MMP 30, MT3-MMP 30, MT5-MMP 29. Cycle threshold were converted into ng/μl using a standard curve of total RNA and values were corrected for RNA input by calculating the ratio (ng/μl (target gene))/ (ng/μl (GAPDH)). Fold induction was calculated as the ratio ng/μl (case)/ ng/μl (controls).

* $p<0.01$, # $p<0.05$

Table 5a. Antigen levels of angiogenic factors in early first-trimester decidua of cases versus controls (medians, 25th and 75th percentile)

		EC		PSMC		GE		SC		EVT		Total SI	
		Co	Case	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case
VEGF	DSE	0.0 (0.0-0.0)	0.0 (0.0-0.0)	3.0 (2.3-3.9)	3.0 (3.0-6.0)	6.0 (3.0-6.0)	6.0 (3.8-6.0)	2.0 (0.0-2.0)	0.0 (0.0-1.5)[b]	NP	NP	10.0 (7-16)	9.0 (8-20)
	DP	0.0 (0.0-0.0)	0.2 (0.0-1.6)	2.0 (0.0-5.0)	3.0 (3.0-3.0)	6.0 (6.0-6.0)	6.0 (4.5-6.0)	2.0 (2.0-4.0)	3.8 (3.0-6.0)	NP	NP	12.0 (10-16)	15.1 (8-15)
	DB	0.0 (0.0-2.0)	0.0 (0.0-0.0)	3.0 (2.5-3.0)	3.0 (3.0-3.8)	6.0 (3.4-6.0)	4.3 (3.0-6.0)	2.0 (1.0-3.8)	3.4 (2.3-5.6)	3.3 (3.3-4.0)	2.0 (1.0-6.0)	10.0 (7-16)	14.0 (8-32)
PlGF	DSE	0.0 (0.0-0.1)	0.0 (0.0-0.0)	3.0 (3.0-3.0)	3.0 (2.5-3.0)	6.0 (3.0-6.0)	3.0 (3.0-4.5)	2.0 (0.0-4.0)	0.0 (0.0-0.0)[b]	NP	NP	13.0 (10-13)	7.8 (6-9)[#]
	DP	0.0 (0.0-0.0)	0.0 (0.0-1.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	6.0 (3.0-6.0)	6.0 (3.8-6.0)	2.0 (2.0-3.5)	3.0 (2.0-4.5)	NP	NP	11.0 (9-12)	12.0 (11-17)
	DB	1.0 (0.0-2.0)*	0.0 (0.0-2.0)	3.0 (2.7-3.0)	3.0 (3.0-3.0)	6.0 (6.0-6.0)	3.0 (3.0-4.5)	4.0 (2.3-4.0)	2.5 (2.0-3.0)	2.4 (1.3-3.7)	1.5 (1.5-2.0)	16.0 (9-18)*	10.5 (10-13)
KDR	DSE	0.3 (0.0-1.0)	0.3 (0.0-0.3)	4.0 (3.0-9.0)	3.0 (1.0-3.0)	6.0 (3.8-9.0)	3.0 (3.0-5.2)	0.0 (0.0-1.5)	0.0 (0.0-0.0)	NP	NP	**12.0 (8-20)**	**8.0 (7-9)**
	DP	1.0 (0.0-2.0)	1.0 (0.3-2.0)	3.0 (3.0-5.0)	1.3 (0.0-3.0)	6.0 (3.5-6.0)	3.0 (3.0-6.0)	2.0 (0.5-4.0)	1.5 (1.0-2.4)	NP	NP	14.0 (9-17)	12.0 (10-14)
	DB	**1.0 (0.0-2.0)**	**2.1 (0.0-2.5)***	3.1 (1.5-6.0)	3.0 (3.0-3.0)	6.0 (3.0-6.0)	3.0 (3.0-3.0)	1.0 (1.0-3.0)	1.3 (1.0-2.0)	1.0 (0.0-1.0)	0.6 (0.0-1.0)	10.0 (6-17)	9.0 (6-10)
Flt-1	DSE	0.0 (0.0-0.3)	0.0 (0.0-1.0)[#]	2.5 (1.8-3.0)	3.0 (3.0-3.0)	6.0 (5.3-6.0)	6.0 (4.5-6.0)	1.0 (1.0-2.0)	2.0 (0.0-2.0)[b]	NP	NP	9.0 (8-11)[#]	11.0 (9-15)[#]
	DP	**0.0 (0.0-0.1)**	**1.0 (0.0-2.0)**	2.0 (1.0-3.8)	4.0 (3.0-4.0)	3.0 (2.0-4.0)	6.0 (6.0-6.0)	4.0 (2.0-4.5)	6.0 (3.5-6.0)	NP	NP	**12.0 (10-13)**	**20.0 (16-22)**
	DB	**0.0 (0.0-1.0)***	**1.0 (1.0-1.5)**	3.0 (3.0-3.4)	4.5 (3.0-6.0)	3.0 (3.0-3.0)	6.0 (6.0-6.0)	3.0 (1.0-3.8)	6.0 (4.5-6.0)	2.0 (2.0-2.2)	2.0 (1.3-2.0)	**11.5 (11-14)**	**19.5 (12-22)**

Table 5a. Antigen levels of angiogenic factors in early first-trimester decidua of cases versus controls (medians, 25th and 75th percentile)

		EC		PSMC		GE		SC		EVT		Total SI	
		Co	Case	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case
Ang-1	DSE	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.6 (0.0-3.0)	3.0 (0.8-3.0)	6.0 (3.0-6.0)	3.0 (3.0-5.0)	0.0 (0.0-1.0)	1.0 (0.0-1.0)	NP	NP	6.0 (4-10)	8.5 (5-10)
	DP	0.0 (0.0-0.0)	0.2 (0.0-0.25)	0.0 (0.0-3.0)	3.0 (3.0-3.0)	6.0 (3.0-6.0)	6.0 (3.8-6.0)	1.0 (0.0-1.0)	4.5 (2.3-5.0)	NP	NP	**6.0 (5-10)**	**16.5 (12-22)**
	DB	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	3.0 (3.0-6.0)	0.0 (0.0-0.0)	5.0 (2.0-6.0)	0.0 (0.0-0.0)	1.0 (0.7-1.0)	**3.0 (3-5)***	**12.1 (5-19)**
Ang-2	DSE	0.0 (0.0-1.0)	0.0 (0.0-1.0)	3.0 (0.0-3.0)	0.0 (0.0-1.5)	6.0 (4.5-6.0)	3.0 (3.0-4.5)	0.0 (0.0-0.0)	0.0 (0.0-1.0)	NP	NP	9.0 (6-10)	3.0 (3-12)
	DP	1.0 (0.0-1.0)	0.0 (0.0-0.5)	0.0 (0.0-3.0)	0.8 (0.0-2.1)	6.0 (6.0-6.0)	3.75 (0.0-4.5)	2.0 (0.5-3.0)	0.7 (0.0-3.0)	NP	NP	10 (8-12)	7.0 (0.0-12.0)
	DB	1.0 (0.0-2.0)	0.0 (0.0-0.5)	1.5 (0.0-3.0)	0.0 (0.0-1.3)	6.0 (3.0-6.0)	2.5 (0.0-3.0)	1.0 (0.5-2.0)	1.5 (0.0-2.5)	0.9 (0.5-1.0)	1.3 (0.0-1.9)	9.0 (7-13)	6.0 (0.0-7.0)
TIE-2	DSE	1.0 (0.0-2.0)	1.0 (0.5-2.0)#	3.0 (3.0-3.0)	3.0 (3.0-3.0)	6.0 (5.3-6.0)	3.0 (2.0-3.0)	1.1 (0.0-2.0)	1.0 (0.5-1.0)#	NP	NP	10.0 (9-13)#	9.0 (7.0-13.0)#
	DP	2.0 (0.0-4.0)	1.5 (1.0-2.0)	3.0 (3.0-3.4)	3.0 (3.0-3.0)	6.0 (3.8-6.0)	3.0 (3.0-3.8)	2.5 (1.3-4.0)	3.0 (2.5-3.0)	NP	NP	13.0 (11-17)	12.5 (11-19)
	DB	1.0 (1.0-2.0)	2.0 (1.0-3.0)	2.1 (1.1-3.0)	3.0 (3.0-3.0)	3.0 (3.0-6.0)	3.0 (3.0-6.0)	3.0 (1.5-5.0)	3.0 (2.0-3.8)	2.8 (2.0-4.0)	3.0 (3.0-3.0)	14.0 (9-16)	15.0 (12-20)

The antigen levels of angiogenic factors in DSE, DP and DB were compared between cases (case) and controls (co). Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis within groups and the general linear model was used to analyse differences between the two groups. The staining was expressed as median staining indices (Median SI) per cell type and as the total staining indices (total SI), which is the sum of the mean staining indices of endothelial cells (EC), peri-vascular smooth muscle cells (PSMC), glandular epithelium (GE), stromal cells (SC) and extra-villous trophoblasts (EVT) per decidual tissue. NP = not present.

Within groups: *$p<0.05$ in DB vs DP and DSE, # $p<0.05$ in DSE vs DP and DB.

Between groups: **bold** $p<0.05$ in MA versus AP.

Table 5b. Antigen levels of proteases in early first-trimester decidua of cases versus controls (medians, 25th and 75th percentile)

		EC		PSMC		GE		SC		EVT		Total SI	
		Co	Case	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case
MT1	DSE	0.0 (0.0-1.0)	0.0 (0.0-0.0)	2.0 (1.0-3.0)	3.0 (0.0-3.0)	3.0 (3.0-3.0)	3.0 (3.0-3.8)	1.5 (1.0-2.0)	2.0 (2.0-2.0)	NP	NP	6.5 (6.0-9.0)#	8.0 (7.0-8.0)
	DP	0.0 (0.0-1.0)	0.0 (0.0-1.0)	2.0 (0.0-3.0)	2.0 (0.0-3.0)	2.0 (1.0-3.0)	3.0 (2.5-3.0)	2.0 (1.0-2.0)	2.0 (2.0-2.0)	NP	NP	9.0 (6.5-10.0)	7.0 (4.5-9.0)
	DB	1.0 (0.0-1.0)	0.0 (0.0-1.0)	1.0 (0.0-2.0)	0.0 (0.0-3.0)	3.0 (2.0-3.0)	2.0 (2.0-3.0)	2.0 (2.0-2.0)	2.0 (1.5-2.0)	1.0 (0.5-1.0)	1.0 (0.0-1.0)	9.0 (8.0-10.0)	5.0 (3.5-10.0)
MT2	DSE	2.0 (0.3-2.0)	1.0 (1.0-1.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	4.5 (3.0-6.0)	4.5 (3.0-6.0)	1.5 (1.0-2.0)	2.0 (2.0-4.5)	NP	NP	11.0 (8.0-13.8)	10.5 (8.8-12.5)
	DP	1.0 (1.0-1.0)	1.0 (1.0-1.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	4.0 (3.0-6.0)	3.0 (3.0-6.0)	2.5 (1.5-3.5)	2.0 (2.0-3.8)	NP	NP	13.0 (8.5-14.5)	10.0 (9.0-13.8)
	DB	**1.0 (0.5-1.0)**	**1.7 (1.0-2.0)**	3.0 (3.0-3.0)	3.0 (3.0-3.0)	2.5 (2.0-4.0)	3.0 (3.0-3.0)	2.5 (2.0-3.5)	2.0 (2.0-4.0)	1.5 (1.0-2.0)	2.0 (1.0-2.0)	**9.5 (8.3-11.8)**	**12.0 (10.0-15.0)***
MT3	DSE	0.0 (0.0-0.0)	0.0 (0.0-0.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	3.5] (2.0-4.0)	3.0 (3.0-3.0)	0.0 (0.0-0.5)	0.0 (0.0-0.0)	NP	NP	9.0 (6.0-11.0)	6.0 (6.0-6.0)

Table 5b. Antigen levels of proteases in early first-trimester decidua of cases versus controls (medians, 25th and 75th percentile)

		EC		PSMC		GE		SC		EVT		Total SI	
		Co	Case	Co	Case	Co	Case	Co	Case	Co	Case	Co	Case
	DP	0.0 (0.0-0.0)	0.0 (0.0-0.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	4.0 (2.0-4.0)	3.0 (3.0-3.0)	1.0 (0.0-1.0)	0.0 (0.0-0.8)	NP	NP	**10.0 (8.0-11.0)**	**6.0 (6.0-6.0)**
	DB	0.0 (0.0-0.0)	0.0 (0.0-0.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	1.5 (1.0-2.5)	2.0 (1.0-2.0)	0.0 (0.0-1.0)	0.0 (0.0-0.0)	**8.0 (5.5-13.0)**	**4.0 (4.0-8.0)**
MT5	DSE	**0.0 (0.0-0.0)**	**1.0 (0.0-1.0)**	2.0 (0.0-2.0)	3.0 (0.0-3.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	0.5 (0.0-2.0)	1.0 (0.8-2.0)	NP	NP	**2.5 (0.0-3.0)**	**8.0 (6.5-8.5)**
	DP	**0.0 (0.0-0.0)**	**1.0 (0.0-1.0)**	0.0 (0.0-1.0)	0.0 (0.0-2.0)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	0.5 (0.0-1.5)	0.5 (0.0-1.0)	NP	NP	**2.5 (0.0-3.5)**	**4.5 (4.0-6.5)**
	DB	**0.0 (0.0-0.0)**	**1.0 (0.0-1.0)**	0.0 (0.0-1.5)	0.0 (0.0-2.3)	3.0 (3.0-3.0)	3.0 (3.0-3.0)	0.5 (0.0-1.0)	0.5 (0.0-1.3)	0.5 (0.0-1.0)	0.0 (0.0-0.0)	**2.5 (0.0-3.8)**	**5.0 (4.0-6.5)**

The antigen levels of proteases in DSE, DP and DB were compared between cases (case) and controls (co). Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis within groups and the general linear model was used to analyse differences between the two groups. The staining was expressed as median staining indices (Median SI) per cell type and as the total staining indices (total SI), which is the sum of the mean staining indices of endothelial cells (EC), peri-vascular smooth muscle cells (PSMC), glandular epithelium (GE), stromal cells (SC) and extra-villous trophoblasts (EVT) per decidual tissue. NP = not present.

Within groups: * $p<0.05$ in DB vs DP and DSE, # $p<0.05$ in DSE vs DP and DB.

Between groups: bold $p<0.05$ in MA versus AP.

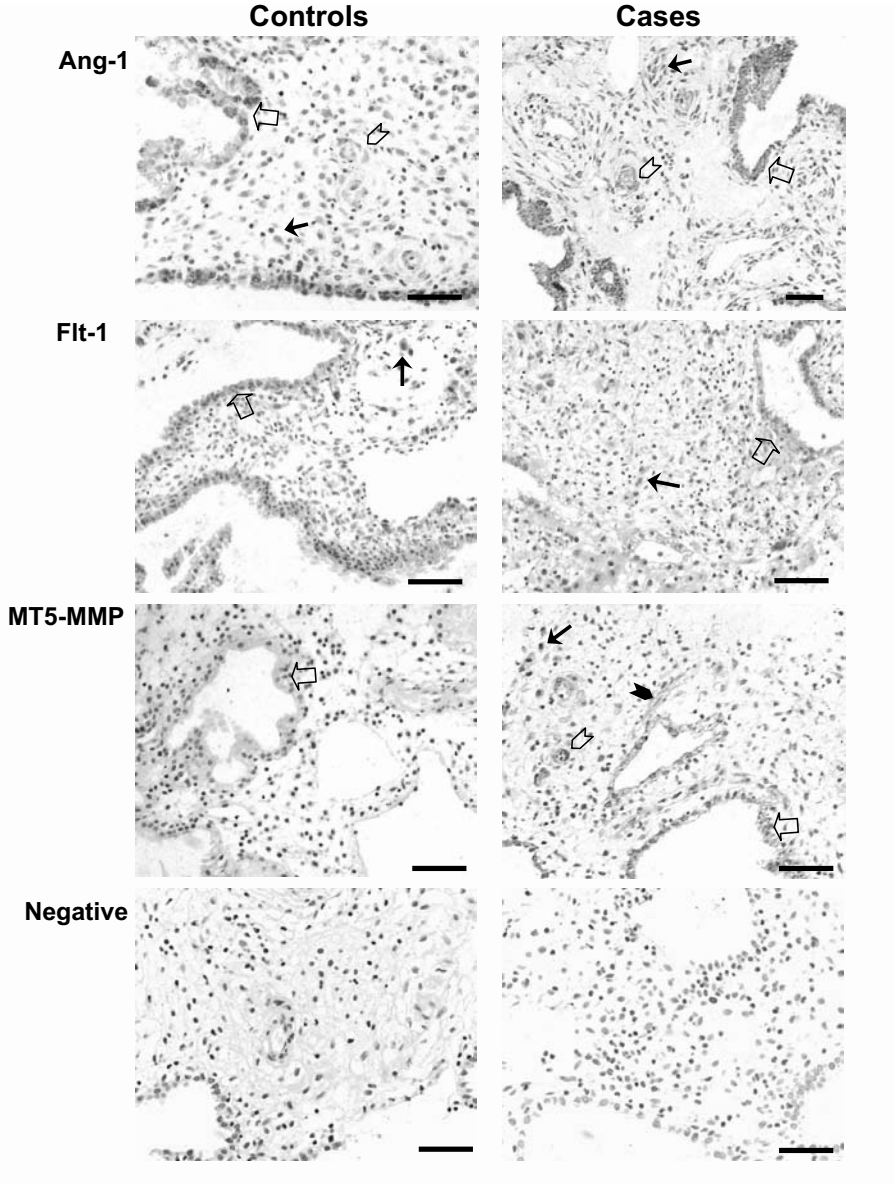

Figure 2. Antigen presence of angiogenic factors and proteases in cases and controls.
Examples of the antigen expression of Ang-1, Flt-1 and MT5-MMP in controls (left site of panel) and cases (right site of panel). **Ang-1** antigen presence in epithelial cells (open arrow) in DB of controls, compared to their presence in stromal (closed arrow), epithelial cells (open arrow) and PSMC (open arrowhead) in DB of cases. **Flt-1** antigen presence in epithelial cells (open arrow) and stromal cells (closed arrow) in DB of controls, compared to their elevated presence in DB of cases. (compare figure 3 for endothelial expression). **M5-MMP** antigens in epithelial cells (open arrow) in DB of controls, compared to their presence in PSMC (open arrowhead), endothelial (closed arrowhead), stromal (closed arrow) in DB of cases. **Negative** controls using non-immune mouse IgG_1 (left) and omission of the first antibody (right). All panels bar = 100 μm. (see colour figures supplement)

phoblasts; PlGF, VEGF, Flt-1 and TIE-2 were moderately expressed, whereas KDR and Angiopoietin-2 expressions were weak (Table 5a and 5b).

Cases versus Controls

The comparison of the antigen expression between cases and controls is described in Table 5a and 5b and Figure 2 and 3. Firstly, comparing protein expressions in DSE of cases and controls showed a reduced total staining index for KDR and increased total SI for MT5-MMP in DSE of cases (Table 5a and 5b, $p<0.05$).

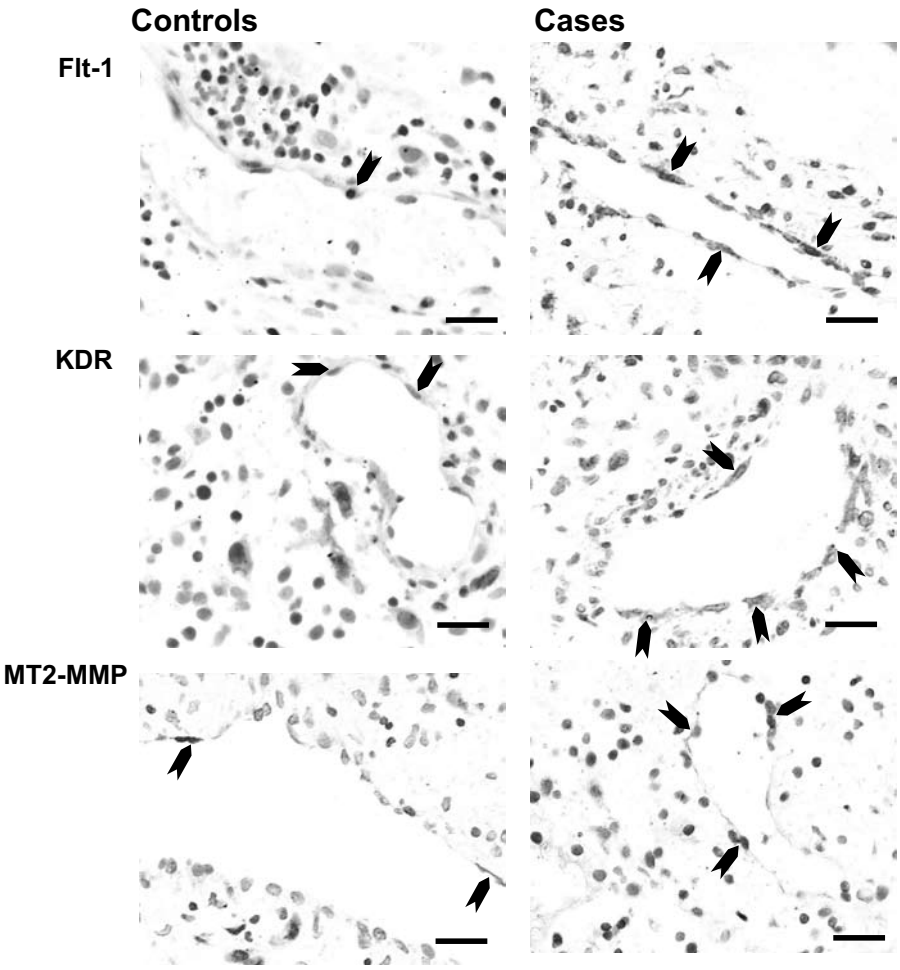

Figure 3. Antigen presence of angiogenic factors and proteases in endothelium in cases and controls. Examples of the differential endothelial expression (arrowheads) of flt-1, KDR and MT2-MMP between DB specimens of cases (right site of panel) and controls (left site of panel) are given. All panels: bar = 50 μm. (see colour figures supplement)

With regard to DP, the total expression of flt-1 was elevated in the case group (Table 5a and 5b, $p<0.05$). The proteases showed differential expression as well, reflected by a reduced total MT3-MMP and MT5-MMP expression in missed abortion decidua.
The angiogenic factors in DB were differentially expressed; the total SI of flt-1 and Ang-1 expression were elevated in missed abortion DB ($p<0.05$), in particular in perivascular smooth muscle cells and stromal cells. In addition, reduced total MT3-MMP and elevated total MT2- and MT5-MMP were detected in DB of cases ($p<0.05$, Figure 2).
The endothelial protein expression of KDR, Flt-1, MT2- and MT5-MMP showed differential expression between cases and controls (Table 5a and 5b, Figure 3). Finally, the Ang-1 expression was significantly elevated and the Ang-2 expression appeared decreased in DP and DB of cases compared to controls (Figure 2). This trend was also detected in DSE tissues. Subsequently, the Ang-2/Ang-1 ratio was decreased in all tissues of cases compared to controls ($p<0.02$ for DB and DP, $p<0.05$ for DSE).

The expression of proliferation (Ki67) and apoptosis (active Caspase 3) markers

To evaluate whether cell proliferation and apoptosis contributed to the observed changes, we evaluated the presence of active caspase-3 and the proliferation marker Ki67. The expression of active Caspase-3 (aCasp-3) in epithelial (range 17-25%), endothelial (range

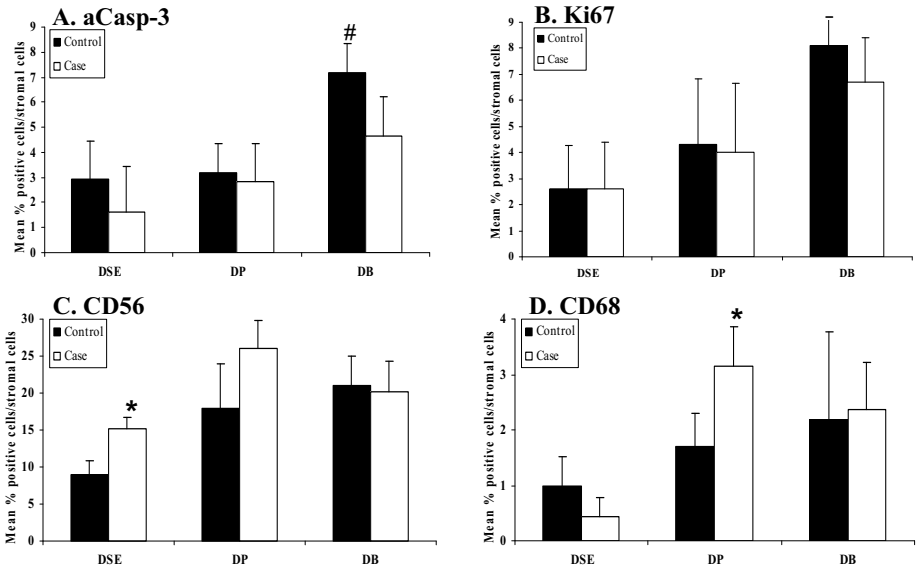

Figure 4. Proliferation, apoptosis, and immune cells in cases and controls.
The number of positive cells per total stromal cells for **A.** active Caspase-3 (apoptosis marker), **B**. Ki67 (proliferation marker), **C**. CD56 (uNK cell marker), and **D.** CD68 (macrophage marker) were determined via immunohistochemistry and expressed in graphs as mean ± SEM. #P<0.05 in DB versus DSE and DP of controls. * P<0.05 in cases versus controls.

0.0-0.1%) and stromal (range 1.6-7.2%) cells was comparable in both groups. The differences between the decidual tissues within each group were similar, except for the expression in stromal cells. The controls showed a significantly decreased percentage of stromal aCasp-3 expression in DSE and DP compared to DB and the cases displayed the same trend (Figure 4A).
The expression of proliferation marker Ki67 in epithelial (range 0.3-1.9%), endothelial (range 0.3-2.3%) and stromal (range 2.6-8.1%) cells was comparable in cases and controls. Strikingly, the endothelial and stromal expression appeared to be decreased in DSE and DP compared to DB in both cases and controls (Figure 4B).

The expression of uNK cell (CD56) and macrophage (CD68), markers

Because decidualisation is accompanied by a large influx of immune cells, which can influence angiogenesis, we determined the number of uNK cells and macrophages in the tissues of controls and cases. The CD56 staining showed inhomogeneous staining, mainly situated surrounding vessels and glands, which was corrected for by analysing various samples per patient. DSE showed a significantly increased number of uNK cells in the case group of 15±2% positive cells per total number of stromal cells versus 9±2% in the control group. No significant differences were detected in between the two groups DP (18±6% vs 26±4%) and DB (21±4% vs 20±4%, Figure 4C).
The CD68 staining showed scattered positive cells throughout the tissues (range 0.4-3.2%). No significant differences were detected in DSE and DB between the two groups. The number of macrophages was significantly increased in DP of cases compared to controls (Figure 4D).

DISCUSSION

The present study showed that the vascularisation pattern varied in decidual secretory endometrium (DSE), decidua parietalis (DP) and decidua basalis (DB) of missed abortion cases and matched controls, i.e. fewer vessels with larger circumference, were present in DP and DB of cases. Strikingly, these differences correlated with the differential expression of several angiogenic molecules at mRNA and antigen level, including the endothelial protein expression of Flt1, KDR and MT2-, MT3- and MT5-MMP. These findings might indicate a correlation between decidual vascularisation and the occurrence of miscarriages.

Maternal vascular adaptation to implantation and its regulation has been subject to discussion, but has mainly been studied with regard to arterial remodelling and villous vascularisation [Craven *et al.,* 1999; Kam *et al.,* 1999]. Recently the potential regulation

of decidual vascularisation by the extra-villous trophoblast (EVT), immune cells and pregnancy-induced hormones has been described [Plaisier *et al.* 2007/2008]. The present study compared the vascularisation pattern and the expression of angiogenic factors and proteases in miscarriage cases and matched controls, which enabled hypothesising about their correlation with the occurrence of miscarriages. All methods were performed on serial paraffin sections, which allowed studying all parameters in the same tissue. Karyotyping was not allowed. However, no differences in the number of leucocytes in general, in uNK cells and macrophages, and in decidual architecture were previously detected between chromosomally normal and abnormal pregnancies [Quack *et al.* 2001; Greenwold *et al.* 2003; Shimada et al. 2006]. Thus, the lack of karyotyping will probably not significantly bias our observations.

The vascularisation pattern showed increasing vascular and luminal surfaces and decreasing vessel density from DSE to DP to DB in both uncomplicated and complicated first-trimester pregnancies. Vailhe *et al* reported a decreased vessel density in DB compared to DP, but did not study DSE [Vailhe *et al.* 1999]. The authors described a vessel density ranging between 20 and 30 vessels/mm^2, which is comparable to our findings. The changes in vascular pattern from DSE to DP to DB may be related to mechanical factors and/or proteins regulating angiogenesis. For instance, the lower vessel density and the enlargement of the vascular surface in DB may be associated with the increased blood flow in the proximity of the implantation site [Gibbons *et al.* 1994; Kam *et al.* 1999; Plaisier *et al.* 2007]. Increased luminal size might also be induced by a relatively low oxygen level [Charnock-Jones *et al.* 2002].

The decreased vessel density and increased vascular surface in DB and DP might also be due to an altered balance between angiogenesis regulating factors. Changes in the relative content of VEGF, angiopoietins and their receptors have been associated with the formation of dilated or enlarged microvessels [Adams, Alitalo, 2007; Nagy et al, 2003[. Whether the altered contents of angiopoietin-1 (higher) and MT-MMPs (lower) in smooth muscle cells of cases reflect an altered interaction between pericytes and endothelial cells contributing to changes in vasculature is yet unknown and requires further studies.

Comparison of cases and controls showed that the total vascular surface did not differ between the two groups. However, the arrangement of the vessels did differ in DB and DP, i.e. a significant decreased vessel density and increased luminal surface in DB and DP of cases. This is not in concordance with the previously described increased vessel density in DP of missed abortions tissue specimens (Vailhe *et al.* 1999).

The differences in vascularisation patterns between controls and cases resemble the differences between early and late first-trimester vascularisation, respectively, suggesting that a too fast maturation of the vasculature is associated with the pathogenesis of mis-

carriages [Plaisier *et al* 2007]. The pre-maturely ripening may allow access of maternal blood in the intervillous space too early in the development of pregnancy, which was also demonstrated in vivo by Doppler ultrasound imaging [Greenwold *et al.* 2003; Jauniaux, Burton 2005]. This might result in increased oxygen levels with subsequent excessive oxidative stress, which is able to modulate the architecture of vasculature and the expression of peri-cellular proteases and angiogenic factors [Burton *et al.*1999, Sharkey *et al.*2000, Kingdom, Kaufman 1997, Zhang *et al*. 2001].
The role of smooth muscle cells has been described regarding vascular adaptation to pregnancy. The quantification of smooth muscle cells in the studied samples may provide interesting information in the future.

Steroidal hormones, the EVT, and immune cells are able to modulate vascularisation directly or indirectly via angiogenic factors [Hanna *et al.* 2006; Hyder, Stancel 1999; Simoncini *et al.* 2003; Zygmunt *et al.* 2002]. We confirm the previously described potential role for VEGF-A (via KDR), the angiopoietins (via TIE-2) and PlGF (via Flt-1) in the regulation of decidual vascularisation [Plaisier *et al.* 2007]. Additionally, their possible association with the differential vascularisation pattern in the miscarriage group is evaluated. The closer the tissue is situated near the implanting embryo, the more angiogenic factors show increased expressions in cases compared to controls. PlGF, the most abundantly regulated factor in uncomplicated first-trimester decidua [Plaisier *et al.* 2007], is not differentially expressed and appears not to be involved in the pathogenesis of missed abortions. In which way the increased expression of angiogenic factors are related to the fewer but larger vessels remains to be elucidated in future research.
In contrast with the mRNA results, the antigen expression of the angiogenic factors showed remarkable little differences between cases and controls. Apparently, the regulation at transcriptional level could not be detected at the translational level. Reasons for this might be an increased cytoplasma storage of the antigens, reduced bio-availability or the higher sensitivity of RT-PCR. Only the endothelial and total expression of Flt-1 and KDR antigens and the total expression of Flt-1 and Ang-1 were differentially expressed. Thus, the receptors of VEGF and PlGF, and not the growth factors themselves, seem to be regulated at the protein level. It should be noted that our assays did not differentiate between the expression of Flt-1 and the soluble form of Flt-1, which is known to have a counter regulatory role in VEGF signal transduction during in early pregnancy [Maynard *et al.* 2003].
Decidual tissues of controls showed an Ang-2/Ang-1 ratio in favour of Ang-2, whereas decidual tissues of cases showed a ratio in favour of Ang-1. This altered ratio might be based on different oxygen levels in both groups, since hypoxia enhances Ang-2 transcription and destabilises Ang-1 [Zhang *et al* 2001]. The favour of Ang-1 in cases might in part explain the observed decreased vessel density with increased circumference, since Ang-1

promotes the generation of a more complex vascular network and vessels of increased luminal size [Geva *et al.* 2000; Asahara *et al.* 1998]. In addition, less complex and fewer large arteries are reported in Ang-1 -/- mice [Suri *et al.* 1996].

The expression of pericellular acting proteases was assessed in decidua of both groups. MT1-MMP did not show differential expression between cases and controls. The differential expression at protein but not at RNA level suggests that MT2- and MT5-MMP are regulated at translation and not at transcription level. The differential endothelial antigen levels of MT2, MT3- and MT5-MMP may be partially responsible for the differences in vascular pattern in DB and DP. Especially, since we know that MT2- and MT3-MMP are likely to be regulators of decidual angiogenesis [Hotary *et al.* 2000; Plaisier *et al.* 2004/2006]. In which way MT5-MMP is involved in decidual vascularisation and possibly other decidual remodelling processes remains a question.

Causal conclusions from histo-pathological studies of abortion tissue are often questioned since foetal death may occur days before evacuation, allowing post-mortem inflammatory or apoptotic reactions during this "retention time". No effect has been described of retention time on the outcome of vascular parameters [Meegdes *et al.* 1988; Nelen *et al.* 2000; Lisman *et al.* 2004]. This most likely excludes a contribution of "retention time" to the observed vascularisation differences between cases and controls. Nevertheless, inflammatory, proliferation, and apoptotic processes were analysed.

The expression of proliferation and apoptosis markers suggests an increased cellular turnover from DSE to DP to DB in both cases and controls. The level of proliferation and apoptosis was comparable in cases and controls, suggesting that our data were not seriously biased by differences in proliferation and apoptosis in response to foetal loss. The amount and distribution of apoptosis is comparable with earlier reports and the elevated apoptosis level in DB compared to DP has been described in uncomplicated pregnancies [Joswig *et al.* 2003; Marx *et al.* 1999; Von Rango *et al.* 2003]. No references data were available regarding proliferation in the various decidual tissues, although moderate Ki67 expression has been described in DB [Korgun *et al.* 2006].

Similar numbers of uNK cells were demonstrated in DP and DB of cases and controls, but their number was significantly increased in DSE of cases. Reports on uNK cells and miscarriages describe increased uNK cells in receptive endometrium of patients with recurrent miscarriages (RM) [Quenby *et al.* 1999; Tuckerman *et al.* 2007]. The abundant presence of uNK cells in DSE samples may reflect these observations. The difference between cases and controls were levelled in DP and DB, possibly by hormone regulated uNK cell migration to normal values [Van den Heuvel *et al.*, 2005b].

The number of macrophages has been reported not to be related to miscarriages [Hill *et al.* 1995; Quack *et al.* 2001]. We detected increased amounts of macrophages in DP of

cases and not in DSE and DB. Strikingly, the number of uNK cells and macrophages at the actual implantation site did not differ between the two groups

Conclusion

Vascular adaptation to implantation is important in the development of a healthy pregnancy. Here, the involvement of decidual vascularisation was studied in relation to miscarriages. Differences were observed in vascularisation pattern between decidual tissues of cases and controls. Strikingly, the vascular differences correlated with the differential expressions of several angiogenic molecules at mRNA and antigen level. Furthermore, the endothelial antigen levels of MT3-MMP were decreased and of Flt1, KDR and MT2- and MT5-MMP were enhanced in DP and DB of cases. These findings might indicate a correlation between decidual vascularisation and the occurrence of miscarriages.