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

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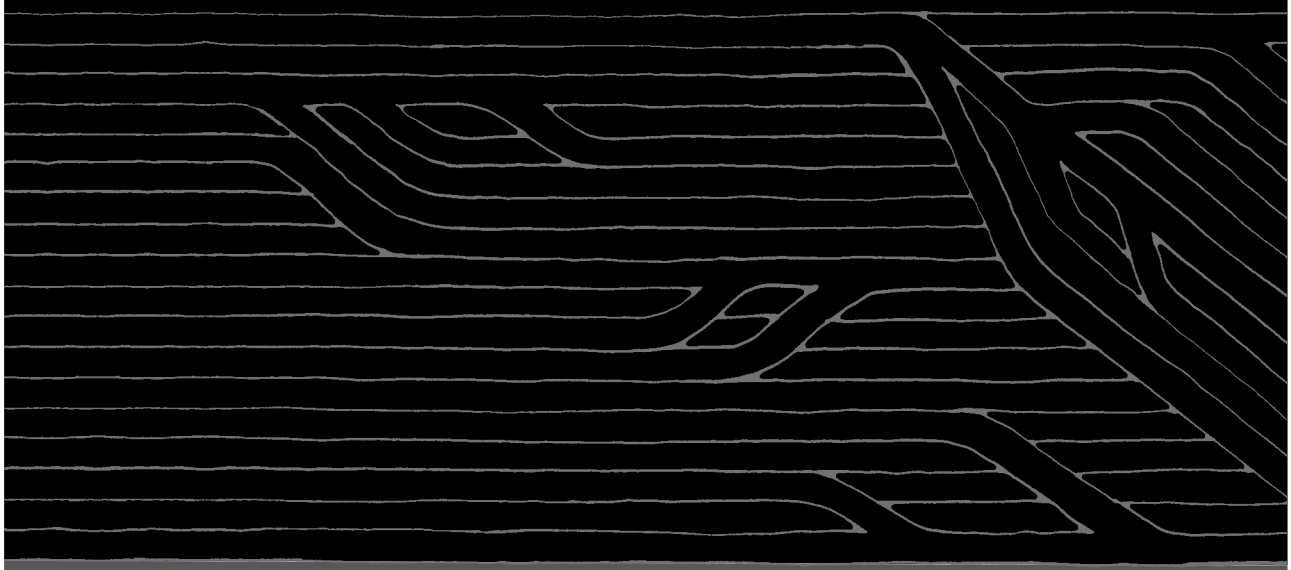
Colour Figures



CHAPTER 1

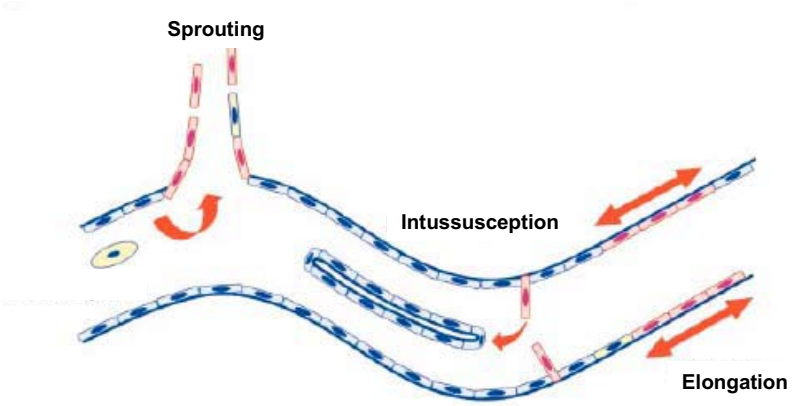


Figure 2. Endometrial angiogenesis proceeds by elongation and intussusception rather than by classical sprout formation. Adapted from Rogers 1998.

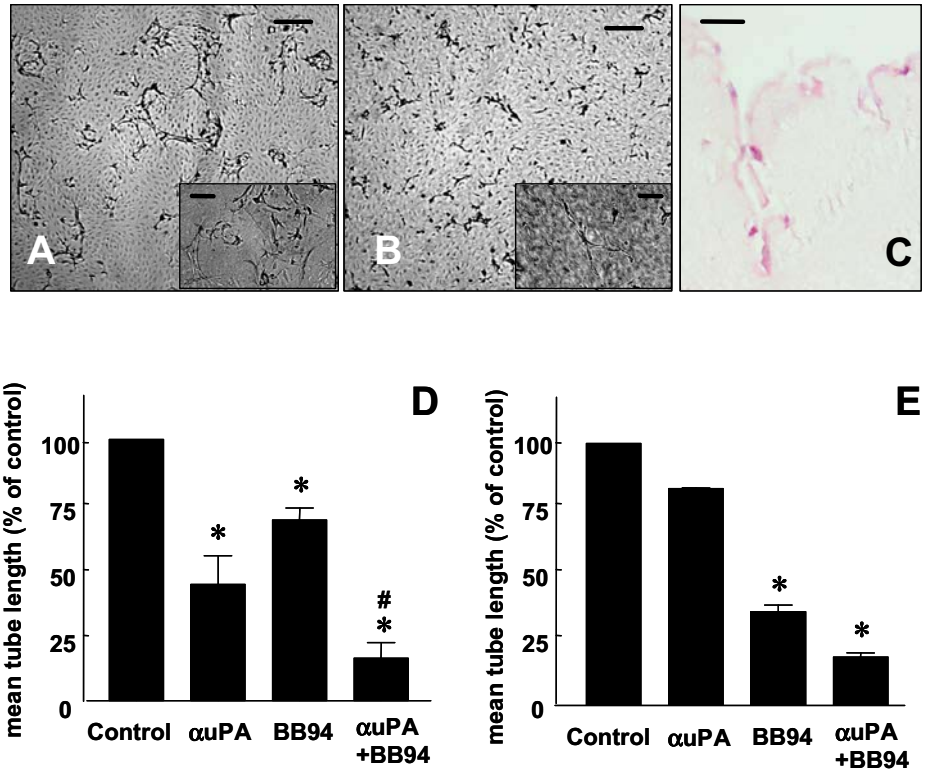


Figure 1. Capillary-like tube formation by hEMVEC in a fibrin or collagen matrix depends on u-PA and MMP activities.

hEMVEC were cultured on top of a three-dimensional fibrin matrix (A,C,D) or 50-50% fibrin/collagen-type-1 matrix (B,E) and stimulated with VEGF-A (10 ng/mL). A and B: Micrographs taken after 4 days of culturing; insets in A and B show details of capillary-like structures. Bar = 300 μm, Bar insets = 100 μm. C: Cross section perpendicular to the matrix surface and stained with Hematoxylin-Phloxine-Safran (bar = 50 μm). D and E: hEMVEC were cultured with 10 ng/mL VEGF-A (control) in the absence or presence of polyclonal anti-u-PA (αuPA, 100 μg/mL), BB94 (5 μg/mL) or a combination of BB94 and anti-u-PA. After 3-5 days of culturing, mean tube length was measured by image analysis. The data in panel D are expressed as a percentage of VEGF-A-induced tube formation ± SEM of 6 independent experiments of duplicate wells performed with 3 different hEMVEC isolations. Panel E represents 3 experiments. ∗: *P<0.05 vs. control, #: P<0.05 vs. αuPA.*

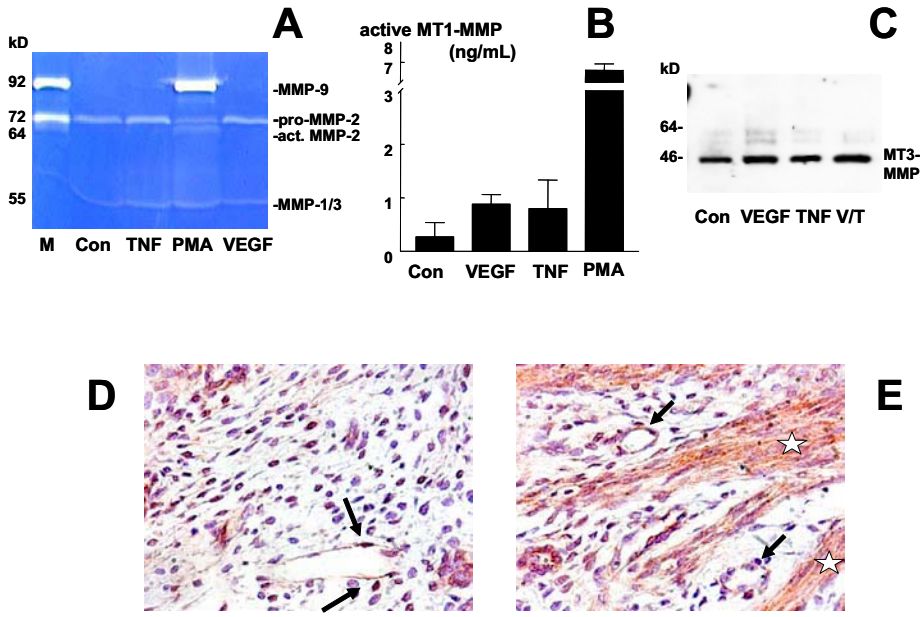


Figure 2. hEMVEC express various MMPs and MT-MMPs.
hEMVEC were cultured for 24 h in M199 supplemented with 0.5% HSA (A) or 20% HS (B,C) and were not stimulated (control) or stimulated with TNFα (2.5 ng/mL), VEGF-A (10 ng/mL) or PMA (10^{-8} M), as indicated. A: Gelatin zymography of 24 h conditioned medium. (M = ladder) B: MT1-MMP activity in cell lysates (mean ± range of two experiments performed in duplicate wells with two different isolations; detection limit of the assay 0.2 ng/mL). C: Western blot of MT3-MMP in 24 h conditioned medium. D and E: Immunohistochemical analysis of MT3-MMP in endometrial tissue shows the presence of MT3-MMP in endothelial cells (D, arrows) and myometrium (E, stars).

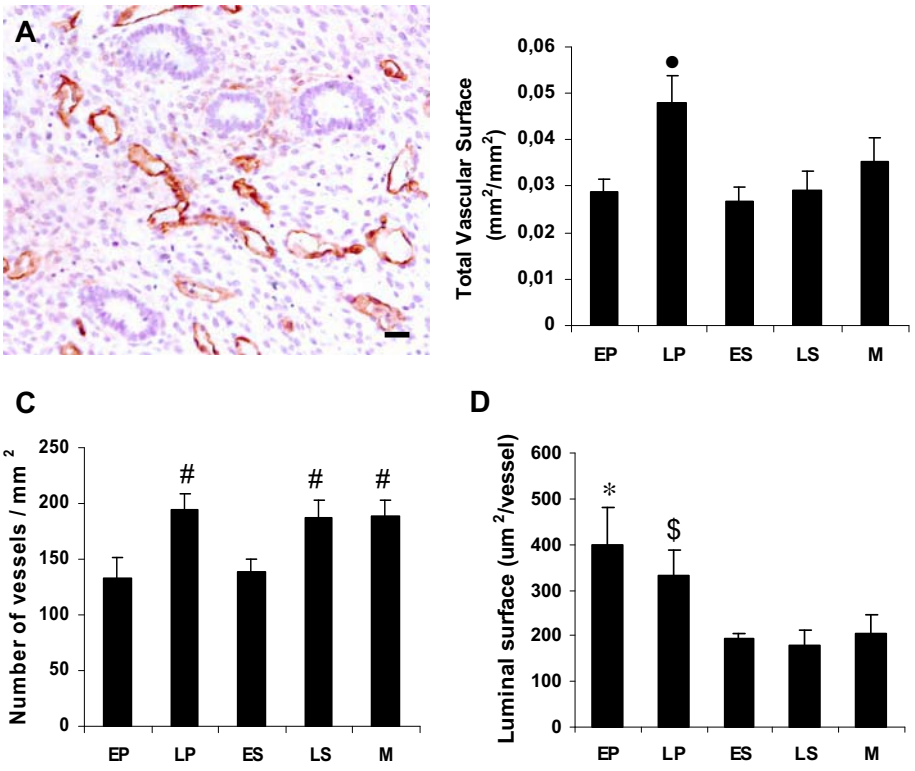


Figure 1. Vascularisation pattern in cycling human endometrium.
The vascularisation pattern in human endometrium was determined by image analysis of anti-CD34-stained sections. Six fields per biopsy were scanned at 100x magnification. **A** CD34 antigen expression in endothelial cells in late proliferative endometrium (bar = 50 μm). **B** The total vascular surface (mm²/mm²), **C** the number of vessels per mm² and **D** the luminal surface (μm²/vessel) were calculated for each sample and expressed as mean ± SEM. EP = Early proliferative phase (n=6), LP = Late proliferative phase (n=7), ES = Early secretory phase (n=6), LS = Late secretory phase (n=6), M = menstrual phase (n=2); • p<0.01 versus EP, ES and LS; # p <0.02 versus EP, ES; ∗ p<0.01 versus ES, LS and M; and $ p<0.05 versus ES, LS and M.

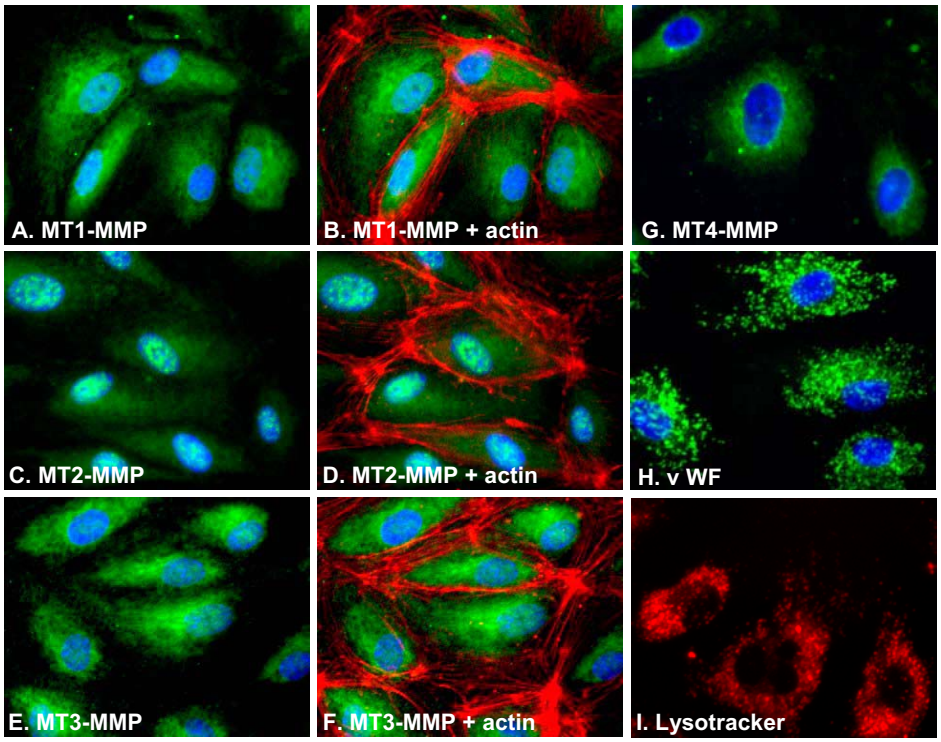


Figure 2. MT-MMP antigen expressions in hEMVEC.
Endothelial MT-MMP expression was determined by fluorescence microscopy (magnification 40x). The expression of MT1-MMP (± actin **A, B**), MT2-MMP (± actin **C,D**), MT3-MMP (± actin **E,F**), MT4-MMP (**G**), von Willebrand Factor in Weibel Palade bodies (**H**) and Lysotracker in lysosomes (**I**) in cultured endometrial endothelial cells (hEMVEC). The negative control only showed blue staining of the nuclei (not shown). Blue = DAPI in nuclei, green = target protein, red = actin or lysotracker.

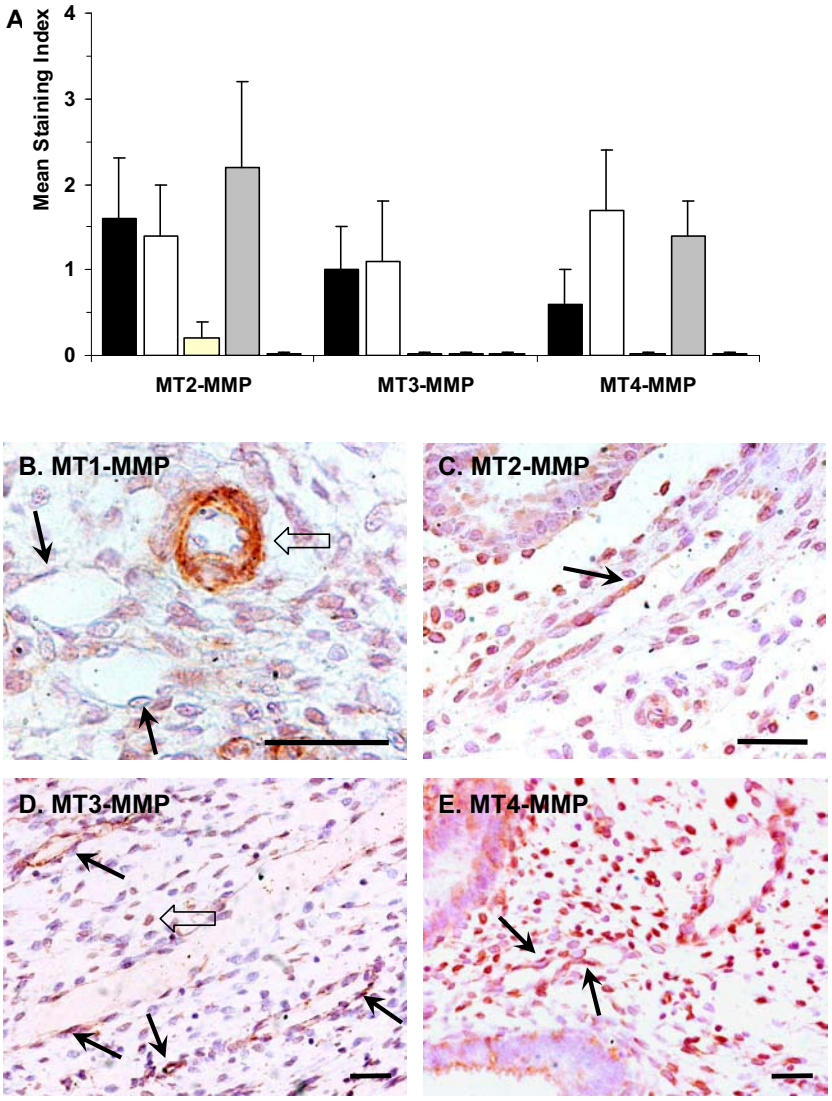


Figure 3. MT-MMP antigen expressions in endometrial endothelium and other cell types.
The expression of MT-MMP antigens by endometrial endothelial cells was determined by immunohistochemistry. **A**. MT-MMP antigen expressions in endothelial cells expressed per cycle phase as mean staining index ± SEM (all $p>0.05$). The staining index is minimally 0 (no stained cells) and maximally 9 (>50% strongly stained cells). **B**. MT1-MMP antigen detected in PSMCs (*open arrow*) but not in endothelial cells (*arrows*) in late proliferative (LP) endometrium. **C**. MT2-MMP antigen expression in endothelium in LP endometrium (arrow). **D.** MT3-MMP expression in endothelial cells (*arrows*) and stromal cells (*open arrow*) in LP endometrium. **E**. MT4-MMP expression in endothelial cells (*arrow*) in LP endometrium. **F.** Negative control. Bar = 50 µm.
■ = Early proliferative phase (n=6), □ = Late proliferative phase (n=7), □ = Early secretory phase (n=6), ▨ = Late secretory phase (n=6), ▥ = menstrual phase (n=6).

CHAPTER 5

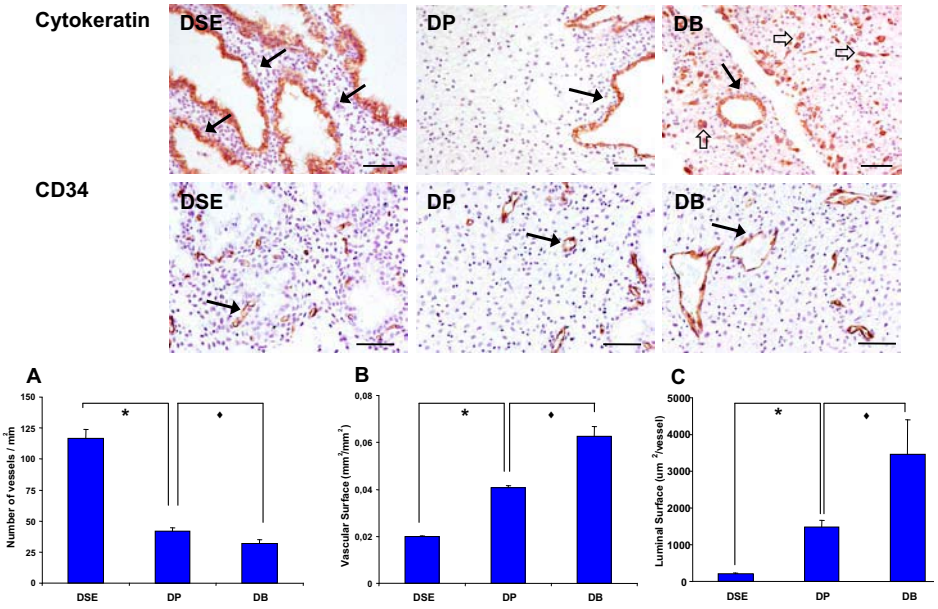


Figure 2. Cytokeratin expression and vascularisation in the three decidual tissues.
Differentiation between three decidual tissues was obtained by HPS and anti-cytokeratin staining (**top panel**). DSE and DP expressed cytokeratin only in glandular epithelial cells (closed arrows), whereas cytokeratin is also expressed in extra-villous trophoblasts of DB (open arrows). The vascularisation pattern in human decidual tissues was determined by image analysis of anti-CD34-stained sections (**middle and bottom panel**). CD34 antigen expression in endothelial cells (arrows) of DSE, DP and DB was compared within subjects. Data were analysed using the repeated measures ANOVA. **A.** The number of vessels per mm², **B.** the vascular surface per area (mm²/mm²) and **C.** the luminal surface (µm²/vessel) were calculated and expressed as mean ± SEM. * p<0.0001; ♦ p< 0.04. Bar =100 µm.

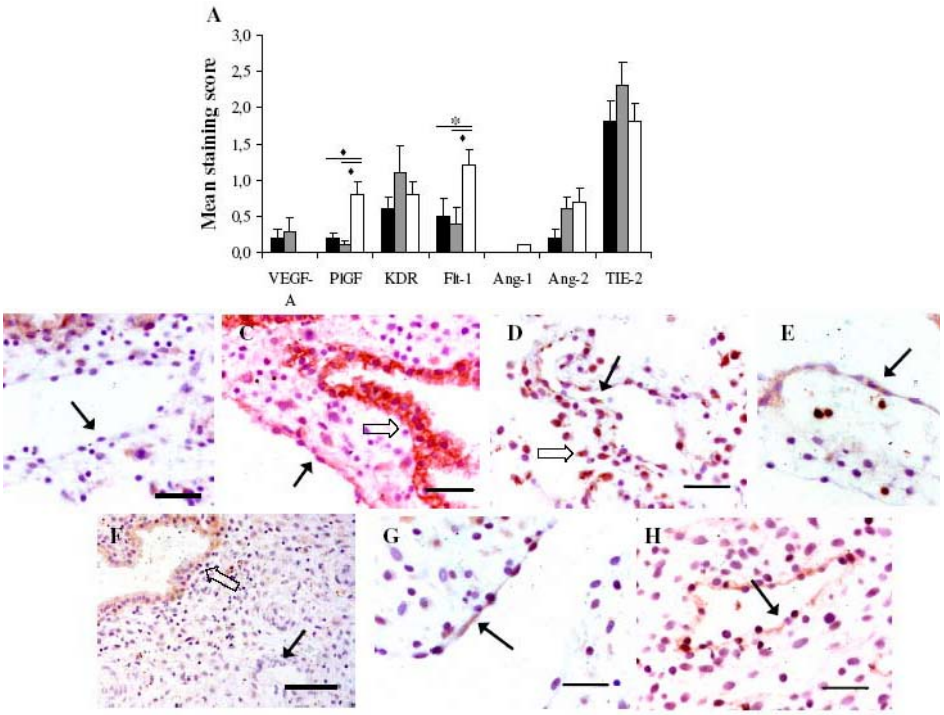


Figure 3. Protein expression of angiogenic factors in endothelium of early first- trimester decidua.
The protein expression of angiogenic factors was determined in DSE, DP and DB. Friedman test, a non-parametrical test for paired samples, and the Wilcoxon test were used for statistical analysis. **A.** Protein expression of each angiogenic factor in endothelium was expressed as the mean staining index ± SEM and **B-H** show examples of these expressions. **B.** Endothelial VEGF expression was not detectable in DB. **C.** PlGF was expressed in epithelium (open arrow) and endothelium (closed arrow) of DB. **D.** Endothelial KDR expression (closed arrow) and stromal KDR expression (open arrow) in DB. **E.** Endothelial Flt-1 expression in DB (arrow) **F.** Angiopoitein-1 in DB was detected in epithelium (open arrow), but not in stromal and endothelial cells (closed arrow). **G.** Endothelial angiopoietin-2 expression in DB (arrow). **H.** Endothelial TIE-2 expression in DB (arrow). ■ DSE, ▒ DP, □ DB. Bar = 100 μm (except F = 50 μm).

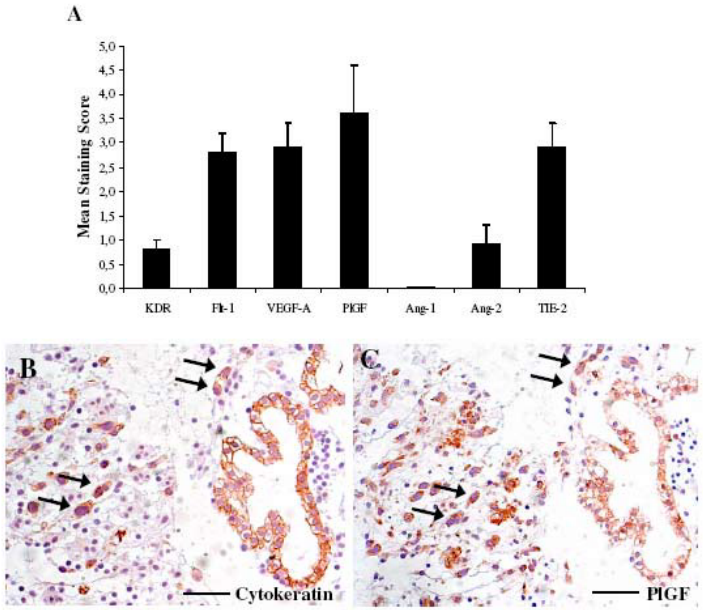


Figure 4. Protein expression of angiogenic factors in extra-villous trophoblasts (EVT) of early first-trimester decidua.

The protein expression of angiogenic factors in EVT in decidua basalis (DB) was studied in serial sections stained against cytokeratin and the target protein. **A.** Protein expression in EVT was expressed as the mean staining index ± SEM. **B.** Cytokeratin (arrows) and **C.** serial PlGF expression (arrows) in EVT. Bar = 50 µm.

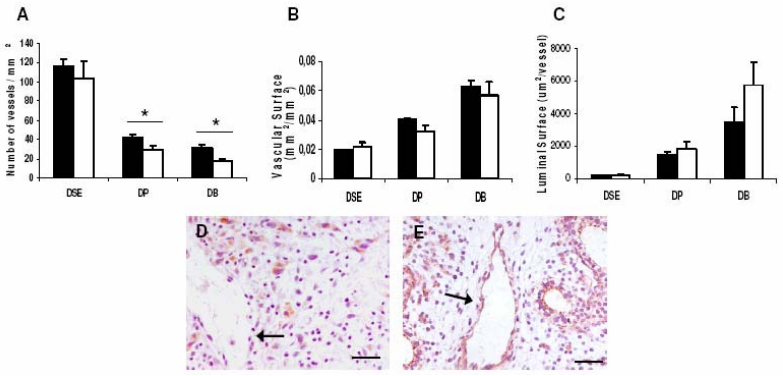


Figure 5. Vascularisation pattern and endothelial protein expression in early versus late first- trimester decidua.

The vascularisation pattern in human early (black bars) and late (white bars) first-trimester decidual tissues was determined by image analysis of anti-CD34-stained sections. Data were analysed using the repeated measures ANOVA. **A.** The number of vessels per mm², **B.** the vascular surface per area (mm²/mm²) and **C.** the luminal surface (µm²/vessel) were expressed as mean ± SEM. VEGF expression was not detectable in early first- trimester endothelium of DB (arrow, **D**), whereas the expression was present in late first- trim. endothelium of DB (arrow, **E**). Early first- trimester and late first- trimester. Bar = 50 µm, * p<0.04.

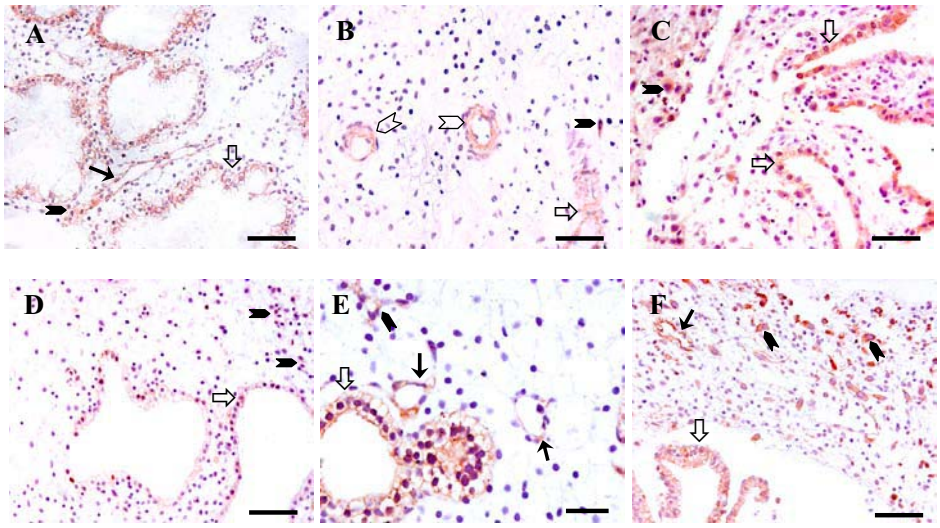


Figure 1. Protein levels of proteases in early first-trimester decidua.
The level of proteases was determined in DSE, DP and DB. **A.** MT1-MMP antigen was detectable in epithelial (open arrow), endothelium (closed arrow) and stromal cells (closed arrowhead) of DSE. **B.** MT2-MMP antigen in epithelium (open arrow), stromal cells (closed arrowhead) and pericytes (open arrowhead) of DB. **C.** Epithelial (open arrow) and stromal (closed arrowhead) MT3-MMP antigen in DB. Pericytes not present in this field. **D.** MT5-MMP antigen in epithelium (open arrow), and only dimly in stromal cells (closed arrowheads) of DP. **E.** uPA antigen in epithelium (open arrow), stromal cells (closed arrowhead) and endothelium (closed arrows) in DB. Pericytes not present in this field. **F.** Epithelial (open arrow), endothelial (closed arrows) and stromal cells (closed arrowheads) uPAR protein in DB. **A-D,F** Bar = 100 μm, **E.** Bar = 50 μm.

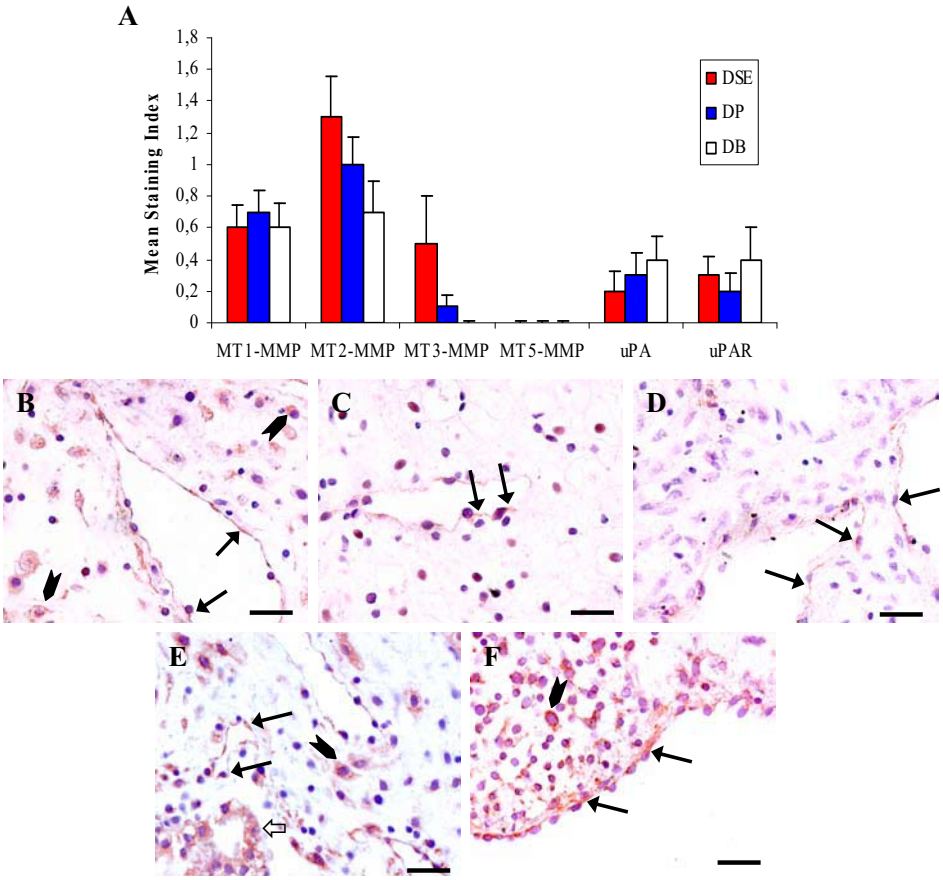


Figure 2. Protein levels of proteases in endothelium of early first-trimester decidua.
A. Presence of protease antigens in endothelium (EC) was expressed as the mean staining index ± SEM and **B-F** show examples of endothelial protease expression. **B.** Endothelial (arrows) and stromal (arrowheads) MT1-MMP was detectable in DB. **C.** MT2-MMP was detected in endothelium (arrows) of DB. **D.** Endothelial MT3-MMP antigen (arrows) in DSE. **E.** uPA antigen in endothelium (closed arrows), epithelium (open arrow) and stromal cells (arrowhead) in DB **F.** uPAR in DB was detected in endothelial cells (arrows) and stromal cells (arrowheads). **B-F.** Bar = 50 μm.

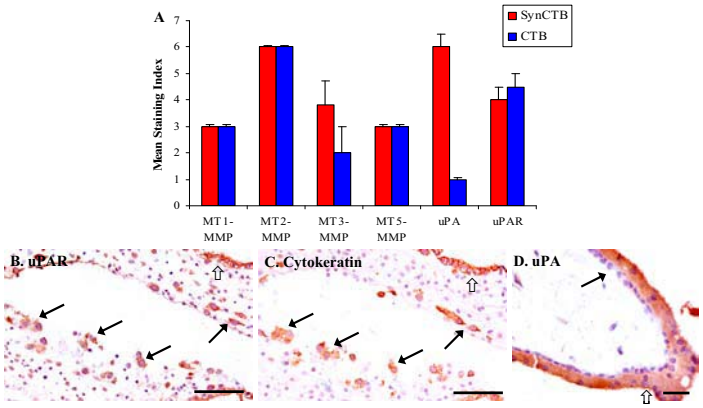


Figure 3. Protein levels of proteases in villous and extra-villous trophoblasts (EVT) of early first-trimester decidua.
The presence of protease antigens in villous and extra-villous trophoblasts in decidua basalis (DB) was studied in serial sections stained against cytokeratin and the target protein. **A.** Immunostaining in syncytio- and cytotrophoblasts (synCTB and CTB) was expressed as the mean staining index ± SEM. **B.** and **C.** uPAR and cytokeratin antigens in serial expression in EVT (closed arrows) and epithelium (open arrows). **D.** uPA antigens in syncytiotrophoblast (open arrow) and not in cytotrophoblast (closed arrow). **B-D.** Bar = 50 μm.

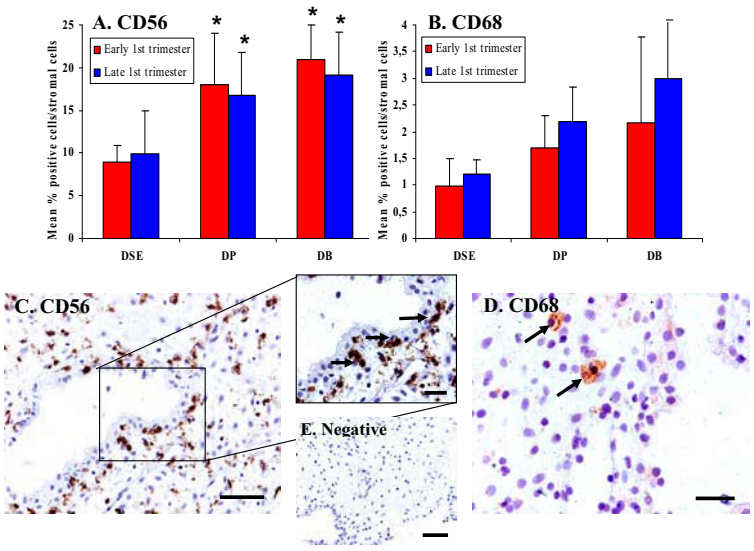


Figure 4. CD56- and CD68- positive cells in early and late first-trimester decidua.
A. CD56-positive cells in decidua, expressed as the mean number of positive cells per total number of stromal cells (mean ± SD) **B.** CD68-positive cells in decidua, expressed as the mean number of positive cells per total number of stromal cells (mean ± SD). **C.** CD56 expression in decidua basalis, bar = 100 μm. Blow up shows CD56+ cells (arrows) surrounding a vessel and gland, bar = 50 μm **D.** CD68-positive cell (arrow) in decidua parietalis, bar = 50 μm. **E.** Negative control, bar = 100 μm. * $p<0.05$ versus DSE

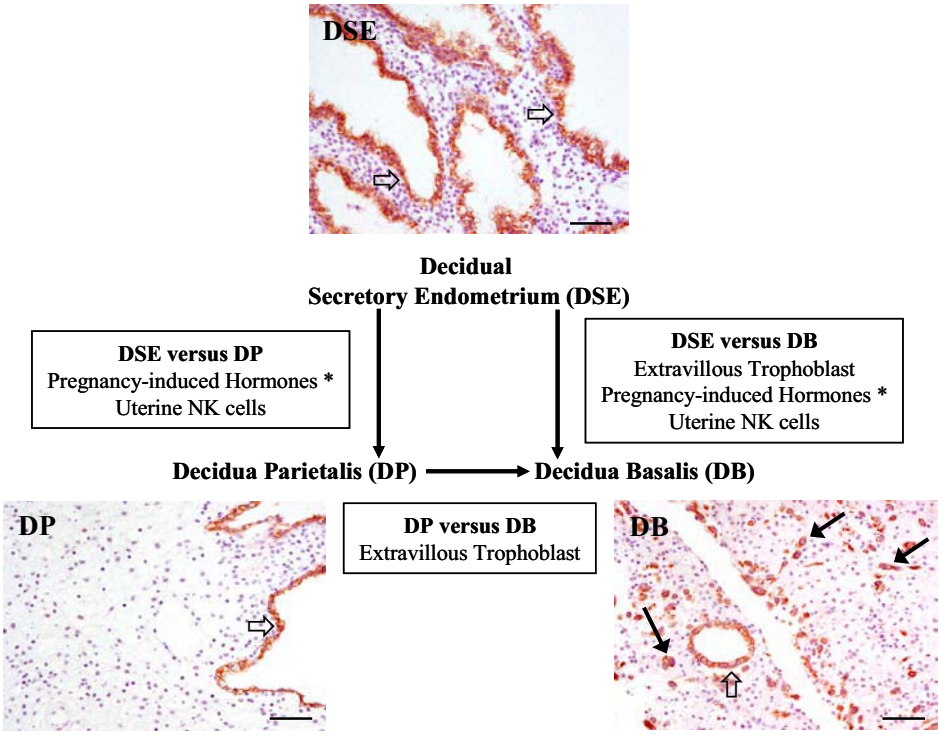


Figure 5. Human first-trimester decidual tissues.
Representation of the influences of immune cells, extra-villous trophoblast and pregnancy-induced hormones (*hCG, oestradiol and progesterone) on first-trimester decidual tissues. Differentiation between decidual secretory endometrium (DSE), decidua parietalis (DP) and basalis (DB), was obtained by HPS and cytokeratin staining. DSE and DP express cytokeratin in glandular epithelium (open arrows), whereas cytokeratin is also expressed in EVT (closed arrows) of DB. Bar =100 µm.

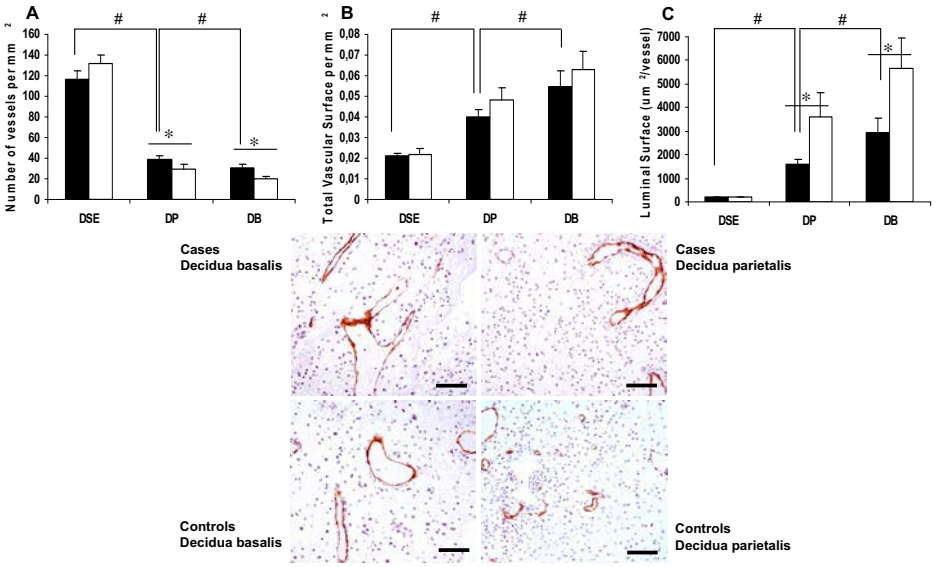


Figure 1. Vascularisation pattern in cases and controls.
The vascularisation pattern in decidual tissues of controls (n=16, black bars) and cases (n=11, white bars) was determined by image analysis of anti-CD34-stained sections. **A.** Vessel density (number of vessels per mm²), **B.** the total vascular 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.

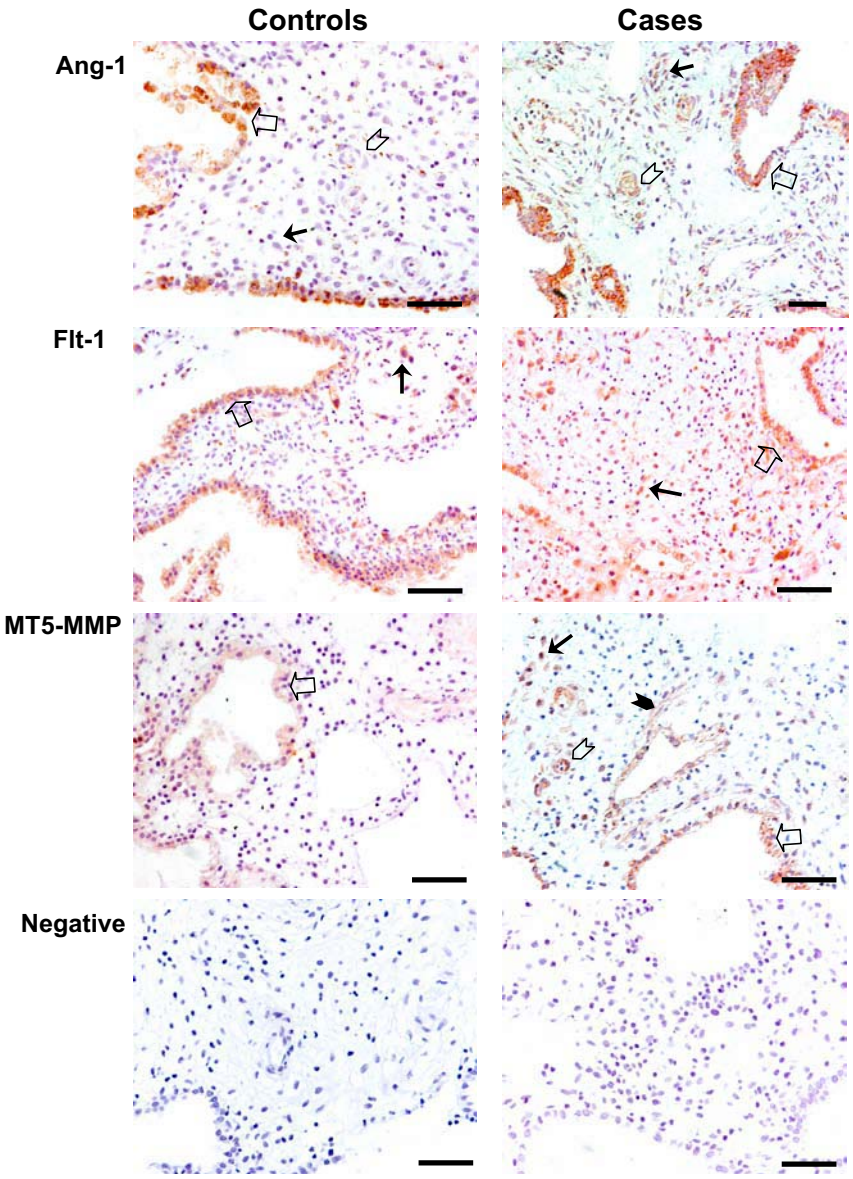


Figure 2. Antigen presence of angiogenic factors and proteases in cases and controls.
Examples of the antigen expression of Ang-1, Flt-1 and MT5-MMP in controls (left site of panel) and cases (right site of panel). **Ang-1** antigen presence in epithelial cells (open arrow) in DB of controls, compared 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. No vasculature present in these fileds. **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.

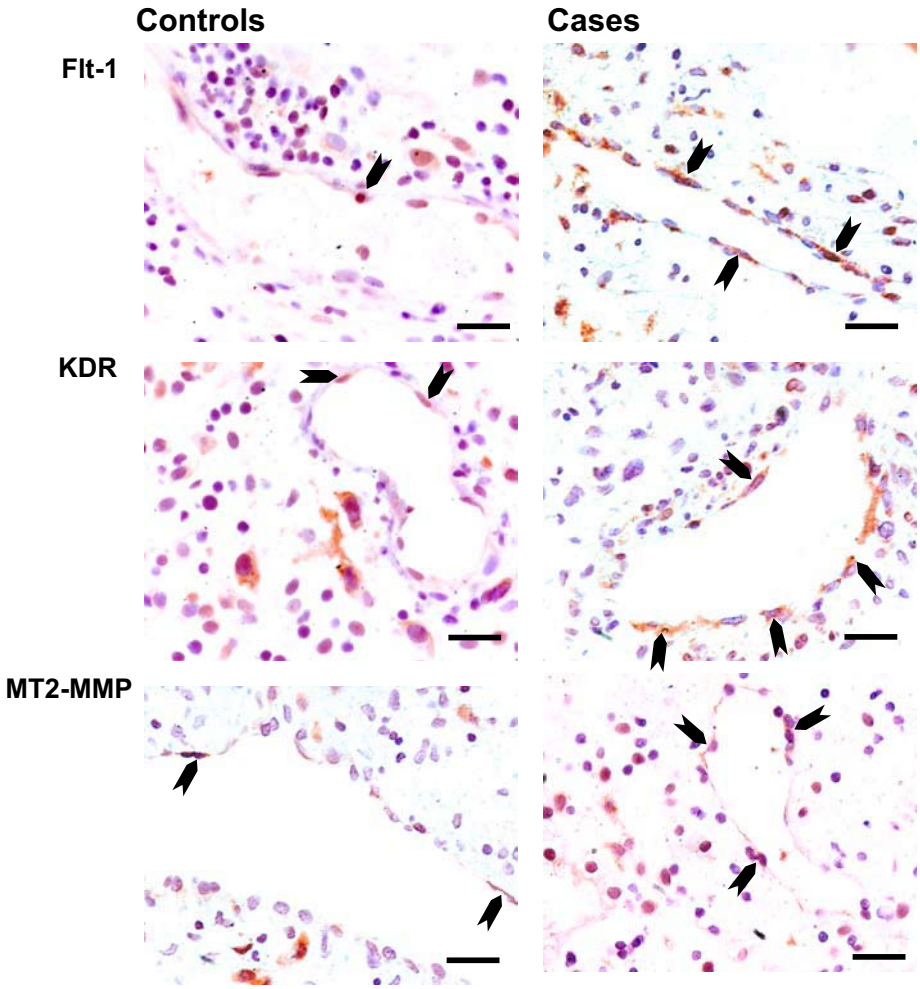


Figure 3. Antigen presence of angiogenic factors and proteases in endothelium in cases and controls. The protein expression of angiogenic factors and proteases was determined in DSE, DP and DB of cases and controls via immunohistochemistry. Examples of the differential endothelial expression (arrowheads) of flt-1, KDR and MT2-MMP between cases (right site of panel) and controls (left site of panel) are given. **Flt-1.** Elevated flt-1 expression in endothelial cells in DB of cases compared to controls. **KDR.** Elevated KDR expression in endothelial cells in DB of cases compared to controls. **MT2-MMP.** Elevated MT2-MMP expression in endothelial cells in DB of cases compared to controls. All panels bar = 50 µm.