Supplemental Methods

1. **Assessment of the human plasma and placental explant medium anti-Factor Xa activity.**

Anti-Factor Xa activity was measured in citrated plasma from the blood used to measure levels of placental angiogenic factors. Heparin standards were prepared from pooled normal plasma spiked with known low molecular weight heparin (LMWH) concentrations (Sigma-Aldrich, St Louis, MO). Heparin levels in all research samples were then interpreted as chromogenic levels read at 405 nm against a prepared blank. This assay was also used to indirectly measure the residual heparin in placental explants following incubation with either LMWH or with unfractionated heparin (UFH), as previously described.¹

2. **Immunoassays procedures for sFlt-1, sEng, VEGF, PlGF and heparanase.** Samples were assayed in duplicate in a 96-well plate pre-coated with a capture antibody directed against free sFlt-1, sEng, VEGF, PlGF or heparanase. Incubation protocols were performed followed by washings and reading at 450 nm in accordance with the procedure summary. The inter-assay and intra-assay coefficients of variation varied from 3 to 10%. Plates were read at 450 nm with 570 nm wavelength correction. The Softmax Pro 3.1.1 software (Molecular Devices, Sunnyvale, CA) reports a positive value if the optical density of the sample wells is above that of the zero standard (blank wells). If the optical density of a sample well is below that of the zero standard, a negative value is reported and automatically converted to zero. VEGF was the only analyte that
had instances of undetectable levels (values lower than zero standards) in any of the assays. Preliminary spike and recovery experiments validated the use of sFlt-1, PI GF and VEGF assays in biological matrices (serum and explant medium) containing UFH and LMWH and found no significant interference.

3. Western blotting. Samples were mixed 1:2 with reducing sample buffer (Bio-Rad), boiled and applied to SDS-PAGE gels. After electrophoretic transfer, polyvinylidene fluoride (PVDF) membranes were blocked with 5% BSA and then incubated overnight at 4°C with two primary antibodies (“Sigma” mouse monoclonal anti-Flt-1; “Zymed” rabbit polyclonal anti-sFlt-1). Detection was performed using appropriate biotinylated secondary antibodies (1:5,000, Jackson ImmunoResearch, West Grove, PA) followed by streptavidin-linked horseradish peroxidise, (1:8000, Amersham Biosystems, Piscataway, NJ) and chemiluminescence (ECL-Plus, Amersham). Optical density of the bands of interest was analyzed with Image J software (NIH, http://rsb.info.nih.gov).

4. Quantitative real time RT-PCR procedures and primer sequences. Total RNA was isolated using Trizol Reagent (Sigma-Aldrich, St Louis, MO). Reverse transcription was carried out with avian myeloblastosis virus reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) using oligo (deoxythymidine) primers to synthesize first strand complementary DNA (cDNA). sFlt-1 primers were designed using the Light Cycler Probe Design Software (Roche, Indianapolis, IN), synthesized and gel-purified at the Yale DNA Synthesis Laboratory (Critical Technologies, New Haven, CT): sFlt-1 forward primer 5’–TGGGACTGTGGGAAGAAAC–3’; reverse primer 5’–TTTTTGTTGCAGTGCTCACCT–3’ (366 bp). This primer pair amplifies soluble, alternatively spliced variants of sFlt-1 (sFlt-1: NM_00159920.1 and U01134) but not the transmembrane form of Flt-1 (Flt-1: NM_002019). sFlt-1 mRNA levels were normalized to
18S rRNA amplified using the following primers 5’–GATATGCTCATGTGGTTG–3’; reverse primer 5’–AATCTTCTTCAGTCGCTCCA–3’ (236 bp). cDNA (500 ng) was amplified using the FastStart Taq DNA Polymerase master mix (Roche, Indianapolis, IN) in the presence of the specific primer pair for each target using the LightCycler® 2.0 System (Roche). To verify that the correct targets were amplified, PCR products were run on an agarose gel and bands of interest cut out and sequenced to confirm their identity.

5. Placental villous explant culture. Placental cotyledons from the central portion of the placenta were removed randomly from the placental disk. Villi were dissected free and then washed thoroughly to remove the blood, all within 30 minutes of delivery. Equal amounts of tissue (~100 mg wet weight) were suspended into each well of a 24-well plate containing 1.5 mL RPMI (1640 medium Gibco, Grand Island, NY), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco). Cultures were maintained at 37°C in a humidified gas mixture of 5% CO2-95% air. Following incubation, with UFN, LMWH, monensin and amiloride the supernatants were collected, centrifuged to remove cellular debris and stored at –80°C until the investigated analytes were measured by ELISA. Duplicate experiments were conducted for each condition. The incubated tissue was blotted on filter paper and homogenized in 1-mL extraction buffer (20 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Triton X-100 and complete protease inhibitor cocktail (Roche, Indianapolis, IN). Homogenates were spun at 1,000g at 4°C for 15 min. and total protein of the incubated tissue was quantified using Bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). The concentration of the analytes in the explant medium was normalized to total protein in tissue extract, to correct for variations in amount of tissue incubated per well. For each experimental condition, values were derived by averaging normalized values from duplicate wells either without (untreated controls) or with the
various heparin treatments. Tissue viability during in vitro incubations, was assessed through the release of lactate dehydrogenase (LDH) into the incubation medium (LDH Liqui-UV® Assay; Stanbio, Boerne, TX). All drugs and chemicals were from Sigma Chemical Co. (St Louis, MO) unless otherwise specified.

6. Evaluation of placental apoptotic activity. Tissues were homogenized in 70% ethanol. Following extraction the DNA purity and yield were determined by spectrophotometry at 260 and 280 nm. After addition of 6 µl of loading buffer [2.5 g/L Bromophenol Blue and 30% (v/v) glycerol in water], samples were loaded on a 2.2% (w/v) agarose gel in 0.5× TBE (45 mmol/L Tris base, 45 mmol/L borate, 1 mmol/L EDTA) containing 300 µg/L ethidium bromide. Agarose-gel electrophoresis was performed at 10 V/cm for 2.5 h. The ethidium bromide-stained characteristic DNA laddering patterns in the gel were visualized under UV light and photographed. Densitometry was performed on the negative image using the NIH ImageJ software (http://rsbweb.nih.gov/ij/). For each lane, a predetermined area was analyzed between the positions corresponding to 1198 and 179 bp of the pGem markers (Promega, Madison, WI) run on the same gel. Apoptotic activity was also assessed using the Cell Death Detection ELISA (Roche) which measures the release of histone-associated DNA fragments (mono- and oligonucleosomes). Both the incubation medium and homogenate supernatant were analyzed.

7. In-vitro angiogenesis. The tube formation assay was performed as previously described. Briefly, HUVECs (10⁴ cells/well), were plated on growth factor-reduced Matrigel precoated 96-well plates (BD Biosciences, Oxford, UK), and incubated at 37°C in a CO2 incubator for 4-6 hours containing either 20% human serum samples from heparin-treated women (n=5) or gestational-age matched healthy controls (n=5) in the presence or absence of 20ng/mL VEGF
(PeproTech, Rocky Hill, NJ). Total tube length was quantified by analysing 10 fields/well using Image Pro Plus (Media Cybernetics, Bethesda, MD).

8. **Statistical analysis.** Comparisons between 2 groups were performed using Student *t* tests or Mann-Whitney rank-sum tests as appropriate. Multiple comparison procedures were performed using one-way or Kruskal-Wallis analysis of variance (ANOVA). *In-vivo* data, time course and dose response results were analyzed by two-way repeated measures ANOVA and Holm-Sidak method for post-hoc comparisons. Multiple stepwise regression analysis was used to explore concurrent relationships between circulating sFlt-1 level as dependent variable and gestational age, factor Xa activity, heparanase, sEng, PlGF as independent variables. Variables were entered in the model based on *P* < 0.05 and removed if *P* > 0.1. Proportions were compared with chi-square of Fischer exact tests. Relationships between variables (correlations) were explored using Pearson’s product moment rank-order correlations. Med-Calc (Broekstraat, Belgium) and SigmaPlot statistical softwares (v.11, Systat software, San Jose, CA) were used for analysis. We used TableCurve 2D (Systat software) for linear and non-linear curve fitting. We compared the evolution of curves in Cartesian spaces by using the F-test.

**Supplemental Figures and Figure Legends**

Heparin and heparan-sulfate have a high affinity for pro-angiogenic factors such as VEGF, as well as for angiogenic inhibitors such as thrombospondin, platelet factor-4 and sFlt-1. Key points of control may include capture of VEGF on heparin sulfate proteoglycans or modulation of the interaction between VEGF and its soluble or cell surface receptors in a dose-dependent manner. In preliminary experiments we found that incubation *ex-vivo* of the serum of heparin-treated women with heparinase I (from *Flavobacterium heparinum*, Sigma-Aldrich), left
unchanged the basal levels of sFlt-1 and VEGF immunoreactivity. This suggests that circulatory heparanase may play a limited role in up-regulating the systemic levels of sFlt-1 through a displacement mechanism in human pregnancy (Figure 1 Supplemental Material). Furthermore, in heparin-treated women, the maternal circulatory concentration of heparanase remained unchanged. This phenomenon appears to be in contrast with observations in mice and does not exclude a possible role for local tissue heparanase.\textsuperscript{5} Further studies are necessary, given that in multivariate analysis, heparanase impacted on the maternal circulating level of sFlt-1.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure1}
\caption{Figure 1 Supplemental Material. Sera from control and heparin-treated women (n=6-9) were treated \textit{in-vitro} with heparinase I. Free sFlt-1 (A) and VEGF (B) levels were measured by ELISA before and after incubation; ns: P>0.05 vs before incubation (paired t-test). Error bars depict SEM.}
\end{figure}

\section*{Supplemental References}


