I. Supplemental Methods.

a) Flowchart of enrolled women and biological samples analyzed in the study

b) Indication for the 2nd and 3rd trimester amniocentesis procedures, exclusion criteria and definition of preterm labor and preterm premature rupture of membranes (PPROM).

c) Biochemical and microbiological studies of amniotic fluid.

d) Umbilical cord blood collection and storage.

e) The MR score.

f) Ang-1, Ang-2, sTie2, IL-6 and IL-8 immunoassays.

g) Immunohistochemistry on placental villous tissue for Ang-1 and Ang-2.

h) Quantitative real-time PCR procedures and primer sequences.

i) Placental explant culture experiments

j) Statistical analysis.

II. Supplemental Results.

a) Clinical, laboratory and outcome characteristics of the patients (Table 1 and Table 2).

b) Ang-1, Ang-2 and sTie2 amniotic fluid levels for 2nd and 3rd trimester groups (Table 3).

c) Ang-1, Ang-2 and sTie2 amniotic fluid levels for the preterm labor group (Table 4).

d) Amniotic fluid Ang-1, Ang-2 and sTie2 levels in intra-amniotic infection by type of bacteria.

e) Amniotic fluid Ang-1, Ang-2 and sTie2 levels and amniotic fluid white blood cell (WBC) count.

III. Supplemental Discussion.

a) General discussion about placental vasculogenesis and angiogenesis.

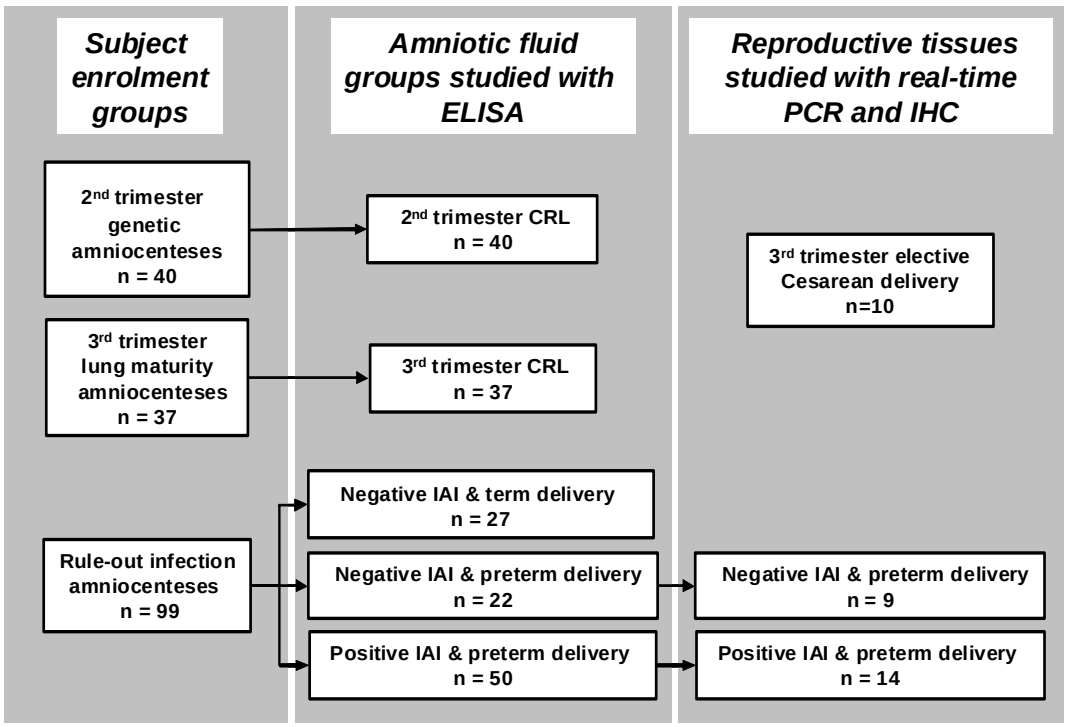
b) The origin of Ang-1, Ang-2 and their inhibitor sTie2 in amniotic fluid.

c) Critical evaluation of our results regarding the presence, level and regulation of Ang-1, Ang-2 and sTie2 in physiologic pregnancy.

IV. Literature cited in Supplemental appendix.

I. SUPPLEMENTAL METHODS

a) Flowchart of enrolled women and biological samples analyzed in this study.



Abbreviations: IAI: intra-amniotic inflammation, CRL: control, IHC: immunohistochemistry.

Our study was approved by the Yale Human-Investigation-Committee. All patients provided written informed consent. Amniocentesis procedures were indicated, independent of our research protocol. Gestational age (GA) was established based on ultrasonographic examination prior to 20 weeks. A cut-off of 27 completed weeks GA was chosen to delineate the 2nd (<28 weeks GA) from 3rd trimester (≥28 weeks GA).

b) Indications for 2nd and 3rd trimester amniocentesis procedures, exclusion criteria and definitions of preterm labor and PPROM. Second and 3rd trimester control groups were designed to identify possible gestational age (GA) mediated changes in Ang-1, Ang-2 and sTie2 AF levels. The indications for 2nd trimester amniocentesis included: advanced maternal age, abnormal serum screening, or presence of minor ultrasound markers suggestive of aneuploidy (choroid plexus cyst, pyelectasis, intra-cardiac echogenic foci). Amniocentesis results for all patients included in this group showed normal fetal karyotypes. The 3rd trimester group consisted of healthy women where confirmation of fetal lung maturity was clinically indicated prior to induction of labor or elective Cesarean delivery.

Preterm labor was defined as the presence of regular uterine contractions and documented cervical effacement and/or dilatation in patients <37 wks GA (1). PPROM was confirmed by visualization of vaginal "pooling" at sterile speculum examination, and positive "nitrazine" or "ferning" tests. Exclusion criteria for the

study population included: chromosomal aneuploidy, fetal structural abnormalities, history of preterm labor during the current gestation, multiple gestation, uterine contractions for the 2nd and 3rd trimester controls, known maternal medical conditions, anhydramnios, viral infection (human immunodeficiency or hepatitis virus), and fetal heart rate abnormalities at enrollment (bradycardia, or prolonged/repeated variable decelerations).

c) Biochemical and microbiological studies of amniotic fluid. Following retrieval, amniotic fluid was analyzed for glucose concentration, lactate dehydrogenase (LDH) activity, white blood cell (WBC) count and Gram stain. Standard culturing methods for aerobic and anaerobic bacteria, including *Ureaplasma* and *Mycoplasma* species were employed. Positive Gram stain and/or culture results were considered suggestive of intra-amniotic infection. Once the clinical requirements were satisfied, the remaining fluid was centrifuged at 3,000g, 4ºC for 10 min., aliquoted and stored at -80ºC for research purposes.

d) Umbilical cord blood collection and storage. Cord blood was obtained by aseptic puncture of the umbilical vein immediately after delivery. The cord blood was centrifuged at 1,000g for 15 minutes. Serum was aliquoted and stored at –80°C until assayed for interleukin-6 (IL-6) levels. The umbilical cord IL-6 levels for these cases were used in a previous study to investigate the fetal inflammatory response in women with IAI (2).

e) The MR score. The SELDI-TOF protocol has been previously described in detail (3). Briefly, the MR score is comprised of 4 proteomic biomarkers: defensin-2, defensin-1, S100A12 (calgranulin C) and S100A8 (calgranulin A) and ranges from 0 to 4, depending upon the presence or absence of each of the 4 protein biomarkers. A value of 1 was assigned if a biomarker peak was present and 0 if absent. An MR score of 3 or 4 indicates the presence of IAI. All SELDI-TOF tracings were scored blindly by one of the investigators (IAB).

f) Ang-1, Ang-2, sTie2, IL-6 and IL-8 immunoassays. Ang-1, Ang-2, and sTie2 ELISA assays for human Ang-1, Ang-2 and sTie2 were performed in duplicate according to the manufacturer's protocol (R&D systems, Minneapolis, MN, USA). Samples were diluted from 1:2 to 1:50 to fall within the range of the standard curves. The reported intra- and inter-assay coefficients of variation were for 3.4%, 6.4% (for Ang-1) 6.9%, 10.4% (for Ang-2) and 5.0%, 8.3% (for sTie2). The minimal detectable concentrations for Ang-1, Ang-2 and sTie2 were: 3.45 pg/mL, 8.3 pg/mL and 0.02 ng/mL, respectively. An IL-6 ELISA system (Pierce-Endogen, Rockford, IL) was used to measure levels in amniotic fluid and umbilical vein serum with a minimal detectable concentration of 1-pg/mL. Interleukin-8 was measured in the placental explant culture media by ELISA (R&D Systems, Minneapolis, MN, USA). The minimal detectable concentration was 1.5-7.5 pg/mL. All measurements were performed according to manufacturers' instructions and in duplicate. The inter- and intra-assay coefficients of variation was <10% for both cytokines.

g) Immunohistochemistry on placental villous tissue for Ang-1 and Ang-2. Using well-established histological criteria, sections of the chorionic plate, extraplacental membranes and umbilical cord were examined by a perinatal pathologist (EZ) (4). Five μm paraffin sections of placental villous tissue from the same groups studied by real time PCR were deparaffinized in xylene and rehydrated with graded ethanol to potassium-phosphate-buffered saline solution, pH 7.2. Following antigen retrieval with citrate buffer, the sections were pretreated with 1% hydrogen peroxide for 15 min. followed by overnight incubation (at 4°C) with goat anti-human Ang-1 (Santa Cruz Tech, Santa Cruz, CA, http://www.scbt.com, diluted 1:100 to 1μg/mL) or goat anti-human Ang-2 (Santa Cruz Tech, diluted 1:200 to 1μg/mL) antibodies and then a 1-hour incubation at room temperature with biotinylated donkey anti-goat IgG (Jackson Immunochemicals, West Grove, PA, diluted 1:600). Immunohistochemical staining was performed with avidin-biotin staining (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA) with 3,3´-diaminobenzidine/nickel sulfate (Ni-DAB) as the chromogen solution. The tissue sections were dehydrated in graded ethanols, cleared, and mounted. Slides where the first antibody was omitted, served as negative control. Specific staining was evaluated semiquantitatively and a median score was computed for each patient. In blinded fashion, 2 independent investigators examined 6 random fields/slide. Scoring of the intensity of the chromogen deposited in the amnion epithelium, chorio-decidua, placental villous trophoblast, villous stroma, and endothelial cells as well as chorionic plate and maternal decidua was performed on a scale from 0 (no staining) to 5 (intense blue-black staining).

h) Quantitative real-time PCR procedures and primer sequences. Immediately after delivery, tissues (placenta and amniochorion membranes) from women who delivered preterm in the setting of negative IAI (n=9) and positive IAI (n=14) were frozen in liquid nitrogen and kept at -80°C for mRNA studies. Examination of the negative IAI tissues showed no evidence of histological chorioamnionitis. There was no significant difference in GA at delivery between the negative and the positive IAI groups (P=0.088). Additionally, tissues were obtained from a group of healthy, term, non-laboring women (n=10, GA: 38-40 weeks), undergoing scheduled elective cesarean delivery for indications such as fetal malpresentation or prior Cesarean birth. All term cases had reassuring fetal heart rate patterns prior to surgery. Total RNA was isolated using Tri 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). The following primers that have been previously reported were synthesized and gel-purified at the Yale DNA Synthesis Laboratory (Critical Technologies, New Haven, CT): *Ang-1* forward primer 5'–CAGCGCCGAAGTCCAGAAAAC–3'; reverse primer 5'–CACATGTTCCAGATGT TGAAG–3' (204 bp), (5) *Ang-2* forward primer 5'–GTCCACCTGAGGAACTGTCT–3'; reverse primer 5'–TTGTGACAGCAGCGTCTGTA–3' (289 bp), (6) Tie2 forward primer 5´–TAATGAGACAATGCTGGC–3'; reverse primer 5'–CATGTTTTCTCAGCAGTTG–3' (241 bp), (7) 18SRNA: forward primer 5'–GATATGCTCATGTGGTGTTG–3'; reverse primer 5'–AATCTTCTTCAGTCGCTCCA–3' (236 bp) (8).

cDNA (500 ng) was amplified in real time PCR using the FastStart Taq DNA Polymerase master mix (Roche, Indianapolis, IN) in the presence of the specific primer pair for each target LightCycler® 2.0 System (Roche).

i) Placental explant culture experiments. Placental cotyledons from the central part of the placenta were removed under sterile conditions and chorionic villi were dissected within 30 min of delivery as previously described (9). The villous tissue was cut into pieces of similar weight, washed thoroughly with ice-cold saline and four pieces [approximately 100 milligrams (mg) wet weight] were cultured as freely suspended villi in 24-well plates in 1.5 mL RPMI 1640 medium (Gibco, Grand Island, NY) containing 100 U/mL penicillin and 100 micrograms/mL streptomycin (Gibco). Cultures were maintained at 37°C in a humidified gas mixture of 5% CO2-95% air. After 24 hours the supernatants were collected, centrifuged to remove cellular debris and stored at –80ºC. The incubated tissue was immediately homogenized in 1-mL cell extraction buffer (20-mmol/L Tris-HCl, 150-mmol/L NaCl, 1% Triton X-100, 1-mmol/L phenylmethylsulfonyl fluoride, and Complete protease inhibitor cocktail [Roche, Indianapolis, IN]). Specimens were spun at 1,000 g at 4°C for 15 min. and protein quantification in incubated tissue performed using Bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) according to the manufacturer's instructions. The analytes' explant medium concentration was normalized to total protein in tissue extract to correct for variations in tissue incubated per each well. For each experimental condition, values were derived by averaging normalized values from duplicate wells either without (untreated) or with the various treatments. Pro-inflammatory stimuli were represented by lipopolysaccharide (LPS, 1µg/mL, a component of Gram negative bacteria and ligand for toll-like receptor [TLR]-4) and Pam3Cys-Ser-(Lys)4 hydrocholoride [Pam3Cys], 1µg/mL, Calbiochem, LaJolla, CA, a synthetic mimic of Gram positive bacteria and TRL-2 ligand) (9). Cycloheximide (10 µg/mL. inhibitor of protein synthesis at ribosomal level), brefeldin A (5µg/mL) and monensin (2µM) (disruptors of protein secretion) were dissolved first in dimethyl sulfoxide (DMSO) and then further diluted in culture medium (10). Parallel wells were also treated with the equivalent dose of DMSO alone. All drugs and chemicals were from Sigma Chemical Co. (St Louis, MO) unless specified. To assess tissue viability, the release of the intracellular enzyme LDH into the incubation medium was determined in explant medium and tissue extract using the LDH Liqui-UV® Assay (Stanbio, Boerne, TX). The inter-assay and intra-assay coefficients of variation were <5%. In preliminary experiments we determined that the maximum unstimulated assayable level of Ang-2 and sTie2 were at 24 hours while the LDH activity in explant medium was minimal. Beyond 24 hours the LDH levels began to increase which was suggestive of loss in tissue viability. Ang-1 remained under the level of the lowest standard even at 24 hours.

j) Statistical analysis. We noted a non-linear variation in the levels of amniotic fluid Ang-1, Ang-2 and sTie2 across human gestation. A two-step clustering method complemented by receiver operating curve (ROC) analysis was undertaken to identify an unbiased GA separation point that partitioned the levels of each angiogenic cytokine into two clusters ("low" vs. "high") (11). A P value of <0.05 was considered statistically significant in all analyses. Normality testing was performed using the Kolmogorov-Smirnov test. Data were compared with 1-way ANOVA followed by Student Newman Keuls tests (parametric), Kruskal-Wallis on ranks

followed by Dunn's tests (non-parametric), Fisher's exact or Chi square tests as appropriate. Statistical analysis was completed before or after logarithmic transformation of data. Data were presented as median with inter-quartile range. Pearson correlations, stepwise multivariable linear and logistic regression analyses were used to measure co-linearity and concurrent relationships between variables and to correct for possible influences of GA on angiogenic cytokine levels. The best fit equation was chosen based on the highest coefficient of correlation (R). Comparison between correlations was achieved based on z statistic (12). Explant data was analyzed using 1-way ANOVA followed by Dunnett's tests with the untreated or vehicle (DMSO) treated as control. Statistical analysis was performed using SPSS Statistics v. 17.0 (SPSS Inc, Chicago IL), MedCalc (Broekstraat, Belgium), SigmaStat (RockWare, Golden, CO) and TableCurve 2D (Systat Software, Inc. Point Richmond, CA) softwares.

II. SUPPLEMENTAL RESULTS

a) Demographic, clinical, laboratory and outcome characteristics of the patients. Demographic and outcome characteristics of the women at amniocentesis are presented in Table 1 (next page). We determined that women who had a genetic amniocentesis were of more advanced age compared to the other two groups. Third trimester control women were of higher parity and as expected, were evaluated at a more advanced GA compared to the other groups. Women with preterm labor had a higher frequency of uterine contractions, advanced cervical dilatation, membrane rupture and clinical signs or symptoms of chorioamnionitis. They experienced more frequently preterm birth (73/99), delivered at an earlier GA, had shorter amniocentesis to delivery intervals and delivered babies of lower birthweight compared to the 2nd trimester group. The 3rd trimester control group had a clinically indicated amniocentesis at a GA median [range] of 36 [34-36] wks. Given that 88% of the patients included in this group had a lecithin/sphingomyelin (L/S) ratio >2.5 (suggestive of fetal lung maturity), the 3rd trimester group experienced a higher frequency of medically indicated preterm birth.

Table 1 Variable	2nd trimester genetic amniocenteses n = 40	3rd trimester lung maturity amniocenteses n = 37	Preterm labor amniocenteses n = 99
Characteristics at amniocentesis			
Age, *years*, †	33 [28 – 37]	28 [24 – 32]	27 [22 – 32] *
Parity †	1 [0 – 1]	1 [1 – 2]	1 [0 – 2] *
Gravidity †	2 [1 – 3]	3 [2 – 5]	2 [1 – 4]
Gestational age, *weeks* †	18 [17 – 20]	36 [35 – 37]	27 [24 – 31] **
Uterine contractions ‡	0 (0)	0 (0)	50 (51) **
Cervical dilatation > 3 cm ‡	0 (0)	0 (0)	19 (19) *
Ruptured membranes ‡	0 (0)	0 (0)	39 (39) *
Clinical chorioamnionitis ‡	0 (0)	0 (0)	12 (12)
Outcome characteristics			
Preterm birth (< 37 weeks) ‡	0 (0)	14 (38)	72 (73) *
Gestational age at delivery, *weeks* †	39 [38 – 40]	37 [36 – 37]	31 [25 – 37] **
Amniocentesis-to-delivery interval, *days* †	143 [134 – 152]	2 [1 – 3]	3 [0.4 – 41] **
Birthweight (grams) †	3,180 [2,841 – 3,601]	3,055 [2,722 – 3,297]	1,675 [890 – 2,543]

† Data presented as median [interquartile range] and analyzed by Kruskal-Wallis ANOVA
‡ Data presented as n (%) and analyzed by Chi square test; * $P<0.05$; ** $P<0.001$

The clinical, amniotic fluid and placental histological characteristics of the preterm labor group are presented in the Table 2 (next page). Women with positive intra-amniotic inflammation (IAI, by MR scores 3 or 4) were recruited and delivered at an earlier GA compared to women with negative IAI. In addition, the positive IAI group had a shorter amniocentesis-to-delivery interval, higher frequency of PPROM and delivered babies of lower birthweight compared to the negative IAI group. The results of the chemical and microbiological studies of the amniotic fluid showed a lower glucose, higher lactate dehydrogenase (LDH) activity, higher white blood cell (WBC) counts, higher incidence of positive Gram stain, positive microbial cultures and elevated interleukin-6 (IL-6) levels in the positive IAI group. These demographical, clinical and laboratory characteristics are consistent with our prior studies and with what would be expected based on the inclusion/exclusion criteria for enrolment (2,13).

Table 2 Variable	**Rule-out infection amniocenteses (n = 99)**	
	Negative intra-amniotic inflammation; n = 49	**Positive intra-amniotic inflammation; n = 50**
Clinical characteristics		
Gestational age at amniocentesis, *weeks* †	30 [25 – 32]	26 [24 – 29] *
Gestational age at delivery, *weeks* †	37 [32 – 38]	26 [24 – 30] **
Amniocentesis-delivery interval, *days* †	36 [14 – 71]	0.6 [0.2 – 2] **
Ruptured membranes ‡	10 (20)	29 (58) **
Smoker §	3 (6)	11 (22) *
Birthweight (grams) †	2,515 [1,750 – 3,087]	955 [712 – 1,507] **
Placental weight (grams) †	430 [328 – 538]	300 [228 – 363] **
Amniotic fluid		
Glucose, *mg/dL* †	34 [25 - 41]	6 [2 - 15] **
Lactate dehydrogenase (LDH) activity, *U/L* †	135 [98 - 174]	639 [442 – 1,102] **
White blood cell (WBC) count, *cells/mm*^{3} †	3 [1 - 6]	713 [200 – 1,513] **
Positive Gram stain §	0 (0)	23 (46) **
Positive cultures §	0 (0)	35 (70) **
Interleukin 6, *ng/mL* †	0.2 [0.1 - 0.4]	12 [6.5 - 28.8] **
Histological examination of the placenta	***n = 26***	***n = 47***
Chorionic plate inflammation, stage ‡	0 [0 - 0]	3 [2 - 3] **
Amnionitis, grade ‡	0 [0 - 0]	3 [2 - 3] **
Chroriodeciduitis, grade ‡	0 [0 - 2]	3 [3 - 4] **
Funisitis, grade ‡	0 [0 - 0]	3 [0 - 4] **

† Data presented as median [interquartile range] and analyzed with Mann-Whitney test
§ Data presented as n (%) and analyzed by Fisher's exact test; * P<0.05; ** P<0.001

b) Amniotic fluid Ang-1, Ang-2 and sTie2 levels for 2nd and 3rd trimester groups are presented in Table 3.

Table 3 Variable	**Controls (n = 77)**	
	2nd trimester genetic amniocenteses n = 40	**3rd trimester lung maturity amniocenteses n = 37**
Angiopoietin-1, *pg/mL* †	26 [17 - 48]	967 [753 – 1,230] **
Angiopoietin-2, *pg/mL* †	1,539 [908 – 2,851]	1,057 [669 – 1,976] **
soluble Tie2 *pg/mL* †	869 [750 – 1,057]	311 [256 – 428] **

† Data presented as median [interquartile range] and analyzed with Mann-Whitney test. Values were not corrected for gestational age, for clinical relevance. * P<0.05; ** P<0.001

c) Amniotic fluid Ang-1, Ang-2 and sTie2 levels for the preterm labor groups are presented in Table 4.

Table 4 Variable	**Rule-out infection amniocenteses (n = 99)**	
	Negative intra-amniotic inflammation; n = 49	**Positive intra-amniotic inflammation; n = 50**
Angiopoietin-1, *pg/mL* †	423 [231 – 799]	473 [242 – 646]
Angiopoietin-2, *pg/mL* †	1,441 [676 – 2,588]	2,881 [2,009 – 3,900] **
soluble Tie2 *pg/mL* †	606 [400 – 937]	1,053 [708 – 1,451] **

† Data presented as median [interquartile range] and analyzed with Mann-Whitney test. Values were not corrected for gestational age, for clinical relevance.* P<0.05; ** P<0.001.

d) Amniotic fluid Ang-1, Ang-2 and sTie2 levels in intra-amniotic infection by type of bacteria.

Thirty-five women had positive amniotic fluid cultures. All these cases had intra-amniotic inflammation as depicted by the MR score. Most frequently encountered infections were with Gram negative microorganisms (n=10) and with *Ureaplasma Urealyticum* (n=10), followed by Gram positive bacteria (n=6) and Mycoplasma hominis (n=2). The remainder of women (n=7) grew a combination of Gram positive, Gram negative and/or *Mycoplasma/Ureaplasma* spp. A list with the isolated species is presented in Table 5.

Table 5

Cultivated microorganism	Number of cases
Ureaplasma urealyticum	14
Bacteroides spp.	5
Escherichia coli	4
Streptococcus viridans	4
Fusobacterium nucleatum	3
Mycoplasma hominis	3
Peptostreptococcus spp.	3
Prevotella spp.	2
Gardenella vaginalis	1
Citrobacter koseri	1
Streptococcus Group B	1
Haemophilus influenzae	1
Klebsiella oxytoca	1
Staphylococcus coagulase neg	1

When we restricted our analysis to cases where a single class of microorganism was cultivated (either Gram negative, Gram positive bacteria or *Ureaplasma*), we did not find significant differences in amniotic fluid Ang-1, Ang-2 and sTie2 levels among the three groups after correction for gestational age at amniocentesis (Ang-1: P=0.102; Ang-2: P=0.725 and sTie2: P=0.255). Moreover, among women with positive cultures there was no relationship between the levels of amniotic fluid angiogenic cytokines and the intensity of intra-amniotic inflammation as reflected by the amniotic fluid IL-6 concentration, after correcting for gestational age. However, it is important to note that these cultivated bacteria represent a minor fraction of the etiological agents responsible for intra-amniotic inflammation and that the diversity of etiological agents linked to preterm birth is underestimated by routine culturing methods (14). Using 16S rRNA sequencing and phylogenetic analysis, we demonstrated in a recent study that all specimens with MR score 3-4 and negative cultures showed presence of bacterial DNA. Furthermore, most samples of amniotic fluid with MR score 3-4 and positive microbial cultures contained DNA of additional bacterial species compared to those found by cultures alone. In fact, 60% of species detected by culture-independent methods were missed by general laboratory cultures. The missed prokaryotes belonged to the class of uncultivated and difficult-to-cultivate species, such as *Fusobacterium nucleatum, Leptotrichia/Sneathia*, *Bergeyella*, *Peptostreptococcus*, *Ureaplasma parvum*, *Bacteroides* and *Clostridiales* spp. (14).

e) Amniotic fluid Ang-1, Ang-2 and sTie2 levels and amniotic fluid white blood cell (WBC) count.

Since Ang-2 could potentially be stored in neutrophils and released upon activation, we asked whether the amniotic fluid WBC count could be a determinant of Ang-2 levels. After correcting for differences in gestational age and for presence of inflammation by the MR score, we did not find a significant correlation between Ang-2 and the number of amniotic fluid neutrophils (P=0.138). Moreover, this lack of correlation remained when analyzing the groups with and without inflammation individually (MR score 0-2: P=0.892 and MR score 3-4: P=0.197). Although women with intra-amniotic inflammation had overall an elevated amniotic fluid neutrophil count (P<0.001, Table 2), the number of WBCs does not appear to be a good marker of WBC activation *per se*. The hypo-osmolar environment of the amniotic cavity, engulfment of bacteria and subsequent cell lysis as well as contamination of the amniotic fluid with blood are just few of the factors responsible for the non-linear relationship between the above two parameters and for the reason why WBC activation markers (such as MR score) are better indicators of outcome than the WBC count (13).

III. SUPPLEMENTAL DISCUSSION

a) General discussion about placental vasculogenesis and angiogenesis. Development of the feto-placental vascular network is a stepwise event and occurs through two distinct processes: vasculogenesis and angiogenesis (15). During vasculogenesis, formation of the primitive vascular system entails the presence and activity of the embryonic progenitor cells (angioblasts) normally recruited from the bone marrow and peripheral blood in response to a variety of stimuli including ischemia and inflammation (16,17). Following vasculogenesis, which occurs primarily in the first part of gestation, the circulatory network of the villi expands by sprouting, remodeling, regression and maturation in a process which characterizes angiogenesis and encompasses a later stage of placental development (15,24,18). Ang-1, Ang-2 and their Tie2 receptor play a central role in blood vessel formation induced by VEGF-A but also in the vascular maturational process attendant angiogenesis (24). Evidence for this assertion was provided through experimental models of VEGF-induced neovascularization and studies in Ang-1, Ang-2 and Tie2 deficient or over-expressing mice (15,19,20).

b) The origin of Ang-1, Ang-2 and their inhibitor sTie2 in amniotic fluid. In this report we determined that the pattern of placental expression of Ang-1 and Ang-2 mimics the levels of these two angiogenic factors in amniotic fluid. A dynamic transfer of angiopoietins at the vascular surface of the placenta may be responsible for our findings. Besides the placenta, we have detected Ang-1, Ang-2 and Tie2 mRNA expression in the amniochorion. However, our *in-vitro* data suggest that the origin of Ang-1 Ang-2 and sTie2 in the amniotic fluid may be the result of a complex process. *Ex-vivo*, Ang-1 does not appear to be a significant secretory product of neither placenta nor amniochorion. This observation argues that the fetus which is known to be an active site of vascular genesis and remodeling is probably the source of amniotic fluid Ang-1. In a recent study, Ang-1 was detected in tracheobronhial aspirates of preterm newborns and appears to protect the lung integrity at birth (21).

Given that in the mouse Ang-2 and Tie2 are expressed in the fetal lung and the kidney, the amniotic fluid Ang-2 and sTie2 could also derive from the fetus as well as the placenta and amniochorion (22,23).

c) Critical evaluation of our results regarding the presence, level and regulation of Ang-1, Ang-2 and sTie2 in physiologic pregnancy. Our results are consistent with previous studies of the morphologic and functional development of the human placental vascular system which is continuously refined until term (15,24). Specifically, we found rising levels of Ang-1 in the amniotic fluid across gestation as well as increased placental Ang-1 mRNA levels at term compared to preterm. Our interpretation of these results is that at the beginning of pregnancy the Ang-1-Tie2 unit works in concert with VEGF-A to potentiate and sustain the placental vascular ingrowth (24). This view is supported by Ang-1's role in recruitment of periendothelial support cells that provide a sealing and stabilizing effect on blood vessels (19). The observed non-linear increase in amniotic fluid levels of Ang-1 across gestation may imply that Ang-1 plays a vital role in angiogenesis even after vasculogenesis has ceased (25).

Ang-2 opposes blood vessel formation, destabilizes blood vessels and enhances vascular leakage by antagonizing Ang-1 (19). The increased vascularization observed in transgenic mice over-expressing Ang-1, unopposed by Ang-2 supports this view. We found that amniotic fluid Ang-2 levels and Ang-2 expression in the placenta were higher in preterm compared to term gestation. Consistent with previous reports, our findings indicate that in the early phase of placental development high levels of Ang-2 are protective owing to its ability to control the vascular growth induced by VEGF and Ang-1 (6,26). The progressive decrease in amniotic fluid level of Ang-2 in the second part of pregnancy and the low placental expression at term suggest that this conversion may be critical to prevent the villous vessels from undergoing regression, destabilization and leakage during angiogenesis.

Using the two-step clustering method we demonstrated for the first time that during human pregnancy the transition point between the "low" vs. "high" amniotic fluid levels of Ang-1 and Ang-2 was different for each factor and occurred at a later GA for Ang-2. Furthermore, we examined the relationship between GA and the Ang-1/Ang-2 ratio in the amniotic fluid and the placenta. Our results suggest that during human pregnancy the Ang-1/Ang-2 ratio is balanced in favor of the vascular pro-stabilizing signal, Ang-1. Based on morphologic studies, the process of vasculogenesis in the human placenta should be completed by the middle of the 2nd trimester (15). Interestingly, the amniotic fluid Ang-1/Ang-2 ratio was significantly increased in favor of Ang-1 after 25 weeks GA. Therefore, this GA may represent the point when Ang-1 switches from a primarily pro-vasculogenic to a pro-stabilizing factor. The molecular mechanism responsible for the distinct gene program which characterizes the GA regulation in the placental expression of Ang-1 and Ang-2 genes remains unknown. However, the divergent effect of Ang-1 and Ang-2 supports the model that the ratio more than the absolute levels of either ligand is a key determinant of the placental vessel fate (27).

Prior to this study the presence and levels of sTie2 in amniotic fluid have not been described. A novel observation is that neither the placental nor the amniochorion expression of the Tie2 receptor mirrors in any way

the amniotic fluid level of sTie2. This suggests that although the amniotic fluid level of sTie2 is GA dependent, the cleavage and processing of the extracellular domain of Tie2 may not be transcriptionally regulated (28). A critical question which still awaits an answer is related to the biological function of sTie2 in the amniotic fluid. *In-vivo,* angiopoietins control vascular permeability (29). Due to its antagonistic effect of both Ang-1 and Ang-2, sTie2 may serve as a fine regulator of water transfer across the placenta and fetal membranes which is an important determinant of the amniotic fluid volume during human gestation.

IV. LITERATURE CITED IN SUPPLEMENTAL APPENDIX

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