Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
C M B Association Publisher

Original Research

Molecular analysis of endometrial inflammation in preterm birth

M. Cecati[1#*], D. Sartini[1#], R. Campagna[1], A. Biagini[1], A. Ciavattini[1], M. Emanuelli[1,2], S. R. Giannubilo[1]

[1] Department of Clinical Sciences, Polytechnic University of Marche, Via Ranieri 65, I-60131 Ancona, Italy
[2] New York-Marche Structural Biology Center (NY-MaSBiC), Polytechnic University of Marche, Via Ranieri 65, I-60131, Ancona, Italy

Correspondence to: moniacecati@gmail.com

Received August 4, 2016; **Accepted** March 25, 2017; **Published** March 31, 2017

[#] These authors contributed equally to this work

This paper is dedicated to the memory of my husband, Andrea L. Tranquilli
Monica Emanuelli

Doi: http://dx.doi.org/10.14715/cmb/2017.63.3.10

Abstract: Spontaneous preterm birth (sPTB) represents the 35%–45% of all preterm birth (PTB) cases and its etiology is unknown. We investigated if the expression level of endometrial cytokines and angiogenetic factors is related to the onset of sPTB.Endometrial tissues from non-pregnant women who experienced sPTB and from non-pregnant women who did not experience sPTB were collected and examined for their expression profile. With this aim, the PCR Array analysis was performed and data were confirmed by Real-Time PCR. Differential gene expression measurements (pathological vs control tissues) showed a significant up-regulation for genes codifying for two angiogenetic factors known as connective tissue growth factor (CTGF), and coagulation factor III (F3). An increased level of expression was detected both for tyrosine kinase endothelial (TEK) and for transforming growth factor beta 2 (TGF-β2) genes but without reaching the statistical significance. The expression level of interleukin 10 receptor alpha (IL10RA) gene was slightly decreased in pathological group compared to control one but, as well as forTEK and TGF-β2 measurements, without reaching the statistical significance. Our work is the first to correlate the imbalance in endometrial district of non -pregnant women with sPTB. These data could suggest a new point of view whence to read sPTB. We need additional clinical and biological studies to clarify sPTB pathogenesis.

Key words: Angiogenetic factors; Endotelial district; Deregulated expression level; Preterm birth; Real-Time PCR.

Introduction

Preterm birth (PTB) occurs in around 10% of all deliveries and is the most significant problem encountered in obstetrics including neonatal morbidity and mortality. This disorder is a complex cluster of problems associated with socioeconomic, sociodemographic, sociobehavioral, environmental, medical, biological, and genetic risk factors. Infection and inflammation are important etiological factors in the development of PTB, since nearly 30% of preterm deliveries are associated with intrauterine infection. Maternal infection (e.g., chorioamnionitis) is often followed by a systemic fetal inflammatory response characterized by elevated levels of pro-inflammatory cytokines in the fetal circulation. A comprehensive mapping of the proteome and microarray analysis was provided by several investigators (1, 2). Normal pregnancy has been proposed to involve physiological activation of the innate limbo of the immune response. Preterm parturition with intact or ruptured membranes is associated with phenotypic and metabolic changes in maternal monocytes and granulocytes that are consistent with the presence of maternal inflammation (3, 4). Human endometrium is a dynamic steroid-responsive tissue that undergoes repeat cycles involving sequential proliferation, differentiation, breakdown and repair. The function of the endometrium is to allow the implantation of a blastocyst and to support any resultant pregnancy. These cycles of tissue remodeling ensure that the endometrium is in a receptive state for the putative "implantation window", the few days of each menstrual cycle when an appropriately developed blastocyst may be available in the uterus to implant. Both implantation and menstruation occur under the control of steroid hormones and are regarded as inflammatory events characterized by leukocyte infiltration and increased inflammatorymediator expression in endometrium.The association of endometrial alteration with decidual formation has been reported previously in rodents. These early observations in rodents, suggest that local injury ofthe endometrium facilitates successful implantation. A high level of several proinflammatory cytokines, characterizes early implantation. These cytokines can be secreted by the endometrial cells as well as by cells of the immune system that are recruited to the site of implantation.In humans and mice,large populations of decidual leukocytes infiltrate the implantation site. Of these cells, 65% to 70% are uterin-specific natural killer cells, which have been shown to be essential for the establishment of an adequate decidua. A role for selected cytokines in preterm labor is based upon the following observations: elevated amniotic fluid concentrations of cytokines and prostaglandins are found in patients with intra-amniotic infection and PTB; in-vitro, bacterial products stimulate production of pro-inflammatory cytokines by human decidua; these cytokines, in turn, stimulate production of prostaglandins by amnion and decidua; administration of interleukin-1 to pregnant

mice or non-human primates induces PTB which can be prevented by administration of IL-1 receptor antagonist protein.

Normal angiogenesis modulated by angiogenic factors and circulating hormones (steroids and gonadotrophins) plays a critical role in endometrial development and differentiation during the normal menstrual cycle. Disruption of the balance between angiogenic factors and their inhibitors at the time of implantation may result in first-trimester miscarriage or, alternatively, defective placentation and thereby increased risk of pregnancy-related disorders (5).

Several histopathological studies carried on cases of spontaneous preterm birth (sPTB) highlighted evidences of abnormal placentation (6, 7). An altered placentation combined to an insufficient vascular development could compromise the placental vascularization and reduce the blood flow in the intervillous space (7, 8). As a consequence, the nutrient delivery to the fetus is reduced and the placenta can not support the fetal growth across the third trimester contributing to PTB recurrence (7,9).

In this research, in endometrial tissues of non-pregnant women who experienced preterm birth, we aimed to study inflammatory pathways because preterm birth may reflect early activation of the normal parturition cascade, in which proinflammatory mediators are typically induced, and angiogenetic pathways because the uterine endometrial implantation of pregnancy may affect vascular nutrition of embryo/fetus and the parturition timing.

Materials and Methods

Patients and tissue collection

This was a prospective study that examined the birth outcomes of women who underwent endometrial biopsy at the Department of Clinical Sciences of Polytechnic University of Marche in the period August 2012 and December 2015. The research was limited for pathology records of reproductive-aged women (18-45 years) who had biopsies in the same period. A written informed consent was obtained from all patients recruited before initiation of the study. The study was approved by the Institutional Review Board. Ten women with previous singleton pregnancy who experienced a spontaneous labor or pPROM that ultimately resulted in a PTB between 16 weeks 0 days' and 33 weeks 6 days' gestation were recruited as the PTB group. As control group were recruited 10 women with a previous singleton pregnancy with the spontaneous onset of labor who delivered at or beyond 37 weeks' gestational age in the same period and in the same institution. All women recruited delivered within 2 years of biopsy and the two groups were matched for age, parity and body mass index. Exclusion criteria at sample were: uterine malformations, hormonal or steroid therapy; exclusion criteria in previous pregnancy were: maternal uterine anomalies, multifetal gestation, fetal aneuploidy or lethal fetal anomalies, polyhydramnios , and serious maternal medical conditions (eg, renal disease, chronic liver disease, severe pulmonary or heart disease, antiphospholipid syndrome, genetic thrombophilia, diabetes mellitus, hemoglobinopathy, chronic conditions requiring medication for control, and maternal chronic infections). The hysteroscopies were scheduled in the follicular phase of the menstrual cycle (days 3–15) and were performed on an outpatient basis with the use of a 5-mm (outer diameter) continuous-flow Bettocchi hysteroscope (Stopler Medical Instruments,Utrecht, the Netherlands & Olympus Belgium N.V., Aartselaar, Belgium).

Avaginoscopic approach was used and an endometrial biopsy was obtained from the uterine fundus with the use of grasping forceps (Karl Storz Endoscopie; UMC-U).The tissues were taken and immediately snap frozen in liquid nitrogen and stored at -80°C until use. This procedure has required less than 5 minutes.

RNA extraction

An aliquot of the frozen tissue (5-10mg) was homogenized in a lysis buffer, and the total RNA was then extracted using a RNeasy Micro Kit (Qiagen, Hiden, Germany). RNA samples were tested by ultraviolet absorption at 260nm in order to determine the RNA concentration. To assess the integrity of total RNA, we analyzed an aliquot of the RNA sample on a denaturing gel stained with ethidium bromide looking for the presence of 28S and 18S rRNAbands.Wecheckedthat the 28S rRNA band was approximately twice as intense as the 18S rRNA band. The 2:1 ratio (28S:18S) is a good indication that the RNA iscompletely intact.

Synthesis of cDNA for PCR Array

Total RNA was used as a template for cDNA synthesis using the RT^2 First Strand kit (SuperArray Bioscience Corporation, Frederick, MD). A 1.5µg of total RNA was pre-warmed with Genomic DNA Elimination Mixture in a final volume of 10µl in a thermal cycler at 42°C for 5min. It was then chilled on ice immediately for at least one minute and added to RT cocktail in a final volume of 20µl. The RT cocktail contains specific RT buffer, primer mix, and the reverse transcriptase enzyme. The reaction was incubated at 42°C for 15min, heated at 95°C for 5min, and then added to 91µl RNase-free H_2O (Diluited First Strand cDNA Synthesis Reaction). It was kept on ice until the following PCR assay step.

PCR Array analysis

For each Array, 102µl of the Diluited First Strand cDNA Synthesis Reaction were added to 1280µl of the 2X SABiosciences RT^2 qPCR Master Mix. Twenty-five microliters of the experimental cocktail were added to each well of the PCR Array. For our study we used RT^2 Profiler PCR Array System purchased from SuperArray Bioscience Corporation (Frederick, MD). We verified the correlation of endometrial inflammation cytokine (RT^2 Profiler PCR Array System- Human Inflammatory Cytokines & Receptors; PAHS-011Z) and angiogenetic factors (RT^2 Profiler PCR Array System- Human Angiogenesis; PAHS-024Z) with sPTB. For the PCR assay, the thermal cycler was programmed as follows: 95°C, 10min; 40 cycles of (95°C, 15sec; 60°C, 1min). A melting curve program ran immediately after the above cycling program. Procedure for melting curve analysis was followed as recommended by the manufacturer. We used a single PCR Array plate to investigate the expression profile of each single sample. ThePCR Array plate has been designed and optimized to have one replicate

Table 1. Sequences of primers used for expression analysis of selected genes.

Symbol	GeneBank	Description	Sequence (Fw) 5'- 3'	Sequence (Rv) 5'- 3'
CTGF	NM_001901	Connective tissue growth factor	GGTTACCAATGACAACGCCT	GCCCTTCTTAATGTTCTCTTCCA
F3	NM_001993	Coagulation factor III	AGTTCAGGAAAGAAAACAGCCA	CGGGAGGGAATCACTGCTT
IL10RA	NM_001558	Interleukin 10 receptor, alpha	CACAATGGCTTCATCCTCGG	ACTTTCTTGTGTGTGAACGTGA
TEK	NM_000459	Tyrosine kinase, endothelial	CAATGAAGCATGCCACCCTG	GCAGAGACATCCTTGGAAGC
TGF-β2	NM_003238	Transforming growth factor, beta 2	CCTTCTTCCCCTCCGAAACT	ATGGCATCAAGGTACCCACA
ACTB	NM_001101.3	Actin, beta	CTCTTCCAGCCTTCCTTCCT	AGCACTGTGTTGGCGTACAG

for each investigated gene.Following evidences present in literature (10), we chose β-actin as housekeeping gene for data normalization.

Relative mRNAexpression was calculated by $2^{-\Delta(\Delta Ct)}$ where $\Delta C_t = C_t$ (Gene of Interest) - C_t (β-actin) and $\Delta\Delta C_t$ = mean-ΔC_t (Pathological samples) - mean-ΔC_t (Normal samples). The parameter Ct (threshold cycle) was defined as the cycle number at which the first detectable increase above the threshold in fluorescence was observed. Genes with a ratio of 0.5 or less and 2 or greater were defined as down-regulated and up-regulated, respectively.Using the RT^2 Profiler PCR Array data analysis tool supplied by SuperArray Bioscience Corporation, we examined in Real-Time PCR assay only those genes whose difference was statically significant ($p<0.05$) between pathological and control samples.

Synthesis of cDNA for Real-Time PCR

Total RNA was isolated from the tissue samples as described above. Two micrograms of RNA were reverse transcribed in a total volume of 25µl for 60min at 37°C with M-MLV Reverse Transcriptase (Promega, Madison, WI) using random nonamers in order to obtain cDNA.

Real-Time quantitative PCR

Genes listed below (Table 1) have been selected for further evaluation. cDNA was used for Real-Time quantitative PCR and generated as described previously. To avoid false-positive results attributable to the amplification of contaminating genomic DNA in the cDNA preparation, the primers were selected to flank an intron, and PCR efficiencies were tested and found to be close to 1. Primer sequences used for expression analysis of selected genes are reported below (Table 1).

The genes were run in duplicate using SYBR Green chemistry. All samples were tested in triplicate using β-actin as the reference gene (Table 1) for data normalization to correct for variations in RNA quality and quantity (10). Direct detection of PCR products was monitored by measuring the fluorescence produced by SYBR Green I dye binding to double-stranded DNA after every cycle. Relative mRNA expression value was calculated as above described.

Statistical analysis

All statistical analyses were performed by using the GraphPad Prism software version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). All values were expressed as mean Relative mRNA expression± Standard Deviation (SD). Differences in gene expression levels between the pathological groups and control group were determined using the Mann-Whitney test. Differences were considered to be significant at $p<0.05$.

Results

PCR Array

To explore whether tissue PCR Array data can be used to identify new markers for PTB, we first determined the expression level of 168 key genes, 84 of them mediating the inflammatory response and the remaining 84 genes modulating the biological processes of angiogenesis in endometrial tissues obtained by biopsy from non-pregnant women who experienced sPTB and non-pregnant women who did not experience sPTB.

A commercially available PCR Array designed for exploring changes in the expression of genes important for inflammatory response and angiogenesis was used on ten samples of control group and on ten women with

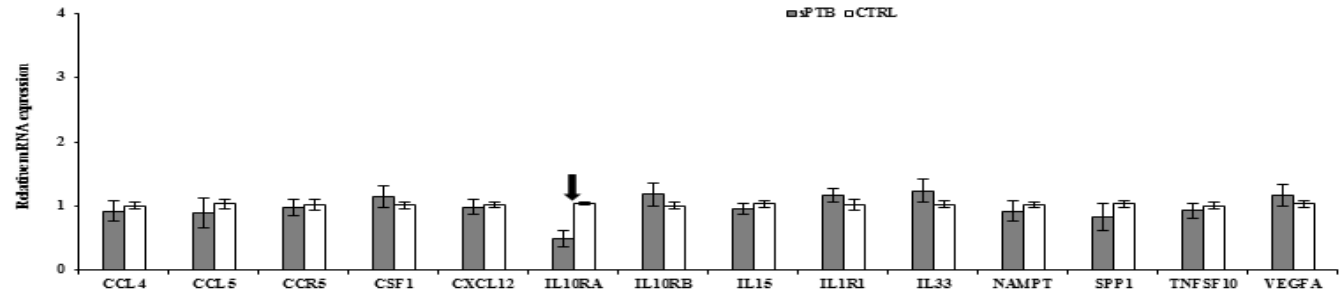

Figure 1. PCR Array analysis of normal and pathological endometrial tissues. Expression profile of genes modulating the inflammatory process. Down-pointing arrow indicates gene showing a significant down-regulation. This gene has been chosen for further measurements by Real-Time PCR. Expression levels are measured as mean Relative mRNA expression ± Standard Deviation. Mean Relative mRNA expression was calculated as the average of all of Relative mRNA expression values within pathological and control samples, respectively. sPTB: spontaneous preterm birth; CTRL: control.

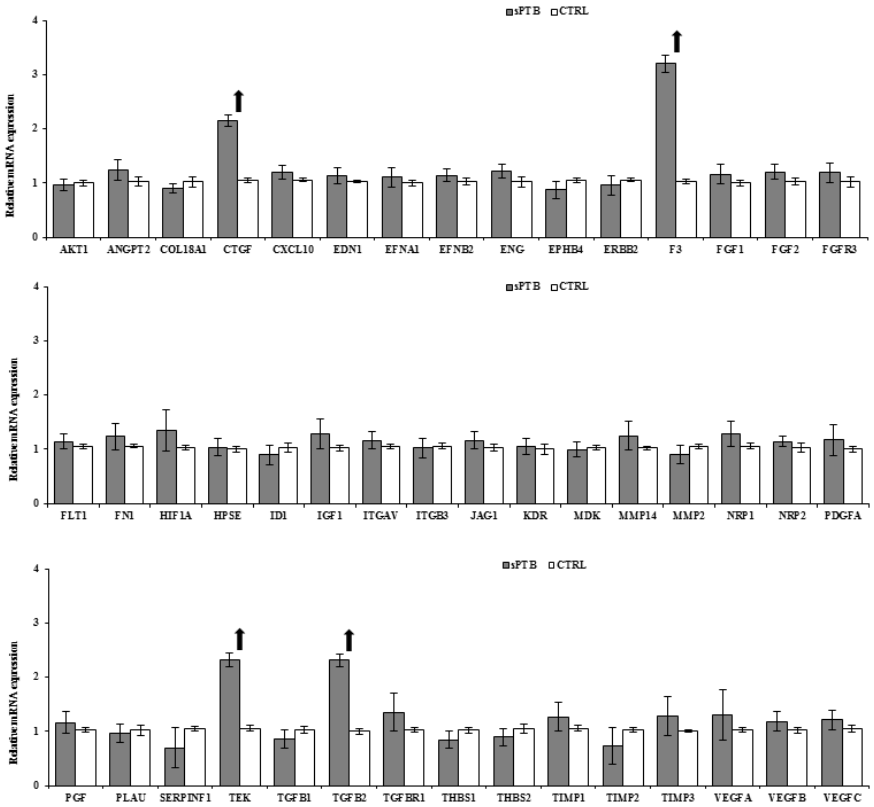

Figure 2. PCR Array analysis of normal and pathological endometrial tissues. Expression profile of genes modulating the biological processes of angiogenesis. Up- and down-pointing arrows indicate genes showing a significant up- or down-regulation, respectively. These genes have been chosen for further measurements by Real-Time PCR. Expression levels are measured as mean Relative mRNA expression ± Standard Deviation. Mean Relative mRNA expression was calculated as the average of all of Relative mRNA expression values within pathological and control samples, respectively. sPTB: spontaneous preterm birth; CTRL: control.

previouss PTB.

PCR Array analysis showed 14 genes coding for cytokine and 46 genes coding for angiogenetic factors having a different expression between pathological and control samples.

Specifically, 5 genes coding for cytokines (Fig. 1) and 32 genes coding for angiogenetic factors are up-regulated (Fig. 2).

Real-Time PCR

To validate PCR Array data and examine gene expression more quantitatively, Real-Time PCR analysis was performed on ten control samples and on ten samples obtained from women with previoussPTB.

As above described, we selected for Real-Time PCR measurements those genes showing, in PCR Array data, a statically significant difference ($p<0.05$) between pathological and control samples. We measured the expression level of CTGF, F3, TEK, TGF-β2 (known as to be some of genes involved in biological process of angiogenesis), and IL10RA (known as to be a gene mediating the inflammatory response) genes.

The Real-Time PCR confirmed the data obtained by PCR Array and allowed us to quantify differential gene expression detected through PCR Array analysis (Table 2).

Differential gene expression measurements (pathological versus normal samples), performed by Real-Time PCR assay, revealed increased levels of expression both for TEK (2.51-fold increase) and TGF-β2 (3.47-fold increase) but without reaching the statistical significance. The expression of IL10RA was slightly decreased in pathological group compared to control one (0.98-fold reduction) but, as well as for TEK and TGF-β2 measurements, without reaching the statistical significance.

A significant up-regulation for CTGF, and F3 (2.53-fold increase and 3.17-fold increase, respectively) was

Table 2. Gene expression levels of genes selected through PCR Array data analysis.

Gene	Relative mRNA expression in pathological samples (mean ± SD)	Relative mRNA expression in normal samples (mean ± SD)	p value[a]	Pathological/normal(-fold)[b]
CTGF	2.58 ± 0.66	1.02 ± 0.36	<0.05	2.53
F3	3.21 ± 0.93	1.01 ± 0.19	<0.05	3.17
IL10 RA	1.08 ± 0.57	1.10 ± 0.64	n.s.	0.98
TEK	2.56 ± 1.85	1.02 ± 0.32	n.s.	2.51
TGF-β2	3.54 ± 3.52	1.02 ± 0.28	n.s.	3.47

[a] n.s., not significant.

[b] Differential gene expression measurements were performed by Real-Time PCR, as above described.

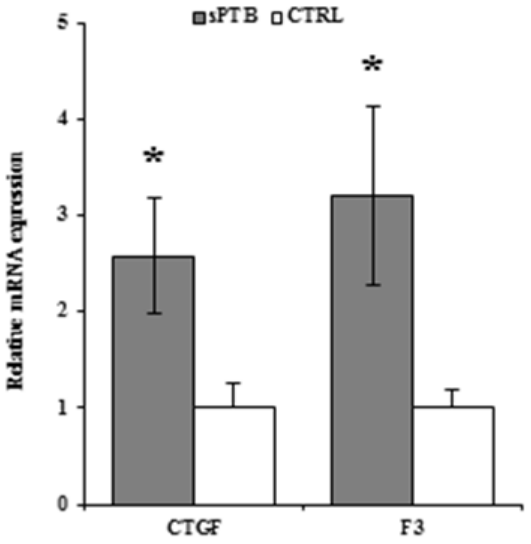

Figure 3. Expression levels of differentially regulated genes in pathological versus control samples (*p < 0.05). Expression levels are measured as mean Relative mRNA expression ± Standard Deviation. Mean Relative mRNA expression was calculated as the average of all of Relative mRNA expression values within pathological and control samples, respectively. sPTB: spontaneous preterm birth; CTRL: control.

detected (Fig. 3).

Discussion

PTB (<37 weeks of gestation) is a complex disorder, whose etiology is influenced by a variety of factors. Age, race, and current smoking status of a woman are known as a risk factors increasing her likelihood to experience a PTB. Moreover, several studies demonstrated women who have had a previous preterm delivery are at higher risk of subsequent preterm deliveries (11, 12).

Clinically, PTB cases are divided into three different categories. Approximately 30%–35% of all PTB are due to a specific condition such as preeclampsia or fetal growth restriction affecting the fetus (11). The 25%–30% of PTB cases depends on pPROMs and, generally, it occurs in cases of infection, placental abruption, or if the mother has any anatomical abnormalities (11). sPTB represents the remaining 35%–45% of PTB cases and its etiology is unknown.

At date, against sPTB there are no reliable predictors, specific preventative measures or treatments and often interventions dispensed after initiation of labor are only intermittently successful.

This inability to act comes, in part, from a reduced understanding of the mechanisms responsibles for sPTB and, as a consequence,from the lack of defined panel of clinical aspects to be investigated early in gestation to identify high-risk pregnancies.

Different studies have been realized selecting several candidate genes involved into those specific biological pathways believed responsible for the sPTB phenotype. Despite the efforts, candidate gene association studies didn't get a reproducibility in maternal or fetal samples, and they didn't give a substantial contribution to the etiology of sPTB.

In this project, we decided to investigate the expression profile of endometrial samples from non-pregnant women who experienced sPTB compared to non-pregnant women who did not experience sPTB.We're willing to hypothesize that sPTB cases could arise from a "state of imbalance" in expression levels of maternal endometrial district, a kind of "burden" which compromises the placental development and the pregnancy outcome.

We decided, in our research, to be focused not on specific candidate gene but on two different biological pathways: we investigated 84 genes involved into inflammatory and angiogenetic process using the PCR Array technology.PCR Array data, confirmed by Real-Time quantitative PCR analysis, revealed an up-regulation for angiogenetic factors such as CTGF (2.53-fold increase), F3 (3.17-fold increase), TEK (2.51-fold incfease) and, TGF-β2 (3.47-fold increase).Conversely, the expression of cytokines, as IL10 RA, wasn't impaired in pathological group compared to control one. The TGF-β2 protein belongs to the TGF-β superfamily which includes different proteins involved in embryonic development, homeostasis, wound healing, chemotaxis, and cell cycle control (13, 14). Several studies in TGF-β field highlighted, not only,the complexity of TGF-β signaling pathways but also the important anti-inflammatory and profibrotic properties of TGF-β elements (15, 16).

TGF-β has long been considered as, probably, the most important key to regulate the deposition of extracellular matrix (ECM) in fibrotic process. In vascular smooth muscular cells, endothelial cells, and also fibroblasts, TGF-β increases the synthesis of ECM proteins (i.e.: fibronectin, collagens and PAI-1), even if TGF-β is present at low concentrations (17). Equally, TGF-β inhibits the ECM degradation and leads to excessive matrix accumulation reducing the collagenase production and stimulating the expression of tissue inhibitor of metalloproteinases (TIMP) (18).TGF-β binds to its receptors and activates the Smad pathway. Smad proteins are citoplasmatic proteins which, once activated, translocate into the nucleus where determine the transcription of different genes involved in fibrosis, including CTGF (19). CTGF, discovered more than a decade ago, is a member of the CCN family of proteins that promote angiogenesis, cell migration, and cell adhesion (20). CTGF is thought as a novel profibrotic factor involving in some TGF-β responses, including apoptosis and fibrosis. Data describe the interaction between TGF-β and CTGF as a mechanism based on a chaperon function of CTGF which binds itself to TGF-β in order to increase the affinity of TGF-β to its different receptors: the result is a TGF-β activity more intense and more prolonged. To support this cooperation, a recent study demonstrated as, a subcutaneous co-injection of CTGF and TGF-β in mice, determined a sustained and persistent fibrotic status (21). In the same way, the blockade of CTGF synthesis or activity reduces TGF-β-induced collagen synthesis and contributes to fibrotic disorders. Actually, CTGF knockout mice exhibited multiple skeletal dysmorphisms because of a reduced expression of bone-specific matrix genes (22). TEK is a receptor essential for angiogenesis belonging to the protein tyrosine kinase Tie-2 family. Angiogenesis consists of blood vessels growing from existing ones. Angiogenesis is a biological process fundamental for embryonic development but also involved in pathogenesis of several fi-

broproliferative diseases (23- 25). A critical role in the process of angiogenesis is played by the biological axis of Angiopoietin-1 and -2 (Ang-1 and -2) and their endothelium-specific receptor tyrosine kinase TEK. Ang-2 acts as a natural antagonist of Ang-1 by binding to TEK competitively and destabilising blood vessels while Ang-1 induces a blood vessels stabilization by promoting the interaction between endothelial cells and the surrounding extracellular matrix (26). TGF-β cytokine has long been proposed as a cytokine able to initiate and maintain fibrosis but a recent study has suggested, also, a TGF-β enrollment into the activation of coagulation cascade. Actually, data show that TGF-β induces the expression of F3 in human lung fibroblasts (27). The F3 is a 263-residue membrane-bound glycoprotein composed of a 219-residue extracellular domain, a single transmembrane sequence and a short cytoplasmic domain (28). Under normal conditions, the F3 is synthesized at the subendothelial level by smooth muscle cells in the tunica media and, specially, by fibroblasts in the adventitia surrounding the vessels (29). Following vascular injury, F3 initiates the coagulation cascade (30). Furthermore, in response to a variety of stimuli, F3 gene expression is induced in different cell-types such as monocytes and macrophages (31, 32), vascular smooth muscle cells (33) and, endothelial cells (34, 35). Fibrotic process and coagulation cascade are strictly related.Bearing in mind the data got from experiments, we have suggested a possibile activation of coagulation cascade mediated by TGF-β which induces the F3 gene expression. In the same way, it's known thatcoagulation factors, as thrombin, modulate the production of profibrotic mediators, such as CTGF (36). Actually, it is still controversial whether angiogenesis has a negative or a positive regulatory role in the pathogenesis of fibrosis. We're aware that our study does not clarify any aspects or doesn't add more informations about the connection among fibrosis process, angiogenesis, and coagulation cascade but our study has been conceived with the first purpose to investigate the possible presence of a "state of imbalance" in endometrial expression levels of women who experienced sPTB.Our data suggest that endometrium of non-pregnant women who experienced sPTB is characterized by an inappropriate expression of some coagulation factors, profibrotic mediators and angiogenetic elements. As a consequence, a relatively hypoxic environment should occur and lead the "hypothetic" trophoblast to work hard to find oxygen by invading the endometrium down to the deciduomyometrial junction looking for arteries and oxygen supply. Furthermore, to do that the "hypothetic" trophoblast promotes angiogenesis to achieve enough oxygen from the surrounding tissue (37).So, the starting imbalance observed in the endometrium should promote the pregnancy at the beginning, but later, should modulate the onset of several biological process leading to sPTB. As known, the expression of F3 in districts different than non-vascular and perivascular cells represents only a small percentage (± 20%) of total amount (38). Probably, this is an adaptive mechanism for limiting the procoagulant potential of the vessel wall. In pathological pregnancies, placenta enhances the release, into the maternal circulation, of syncytiotrophoblast microvesicles containinghigh levels of active F3 (39). In pregnant women who experienced already previous sPTB, the quantity of F3 receptor circulating into maternal vessels might be sufficient to trigger the coagulation cascade and induce the sPTB onset.

In conclusion, our work is the first to demonstrate the presence of an unphysiological expression profile in endometrial tissue of sPTB compared to non-pregnant women.These data could suggest a new point of view whence to read this pregnancy disease that remains an unanswered challenge and an important subject for future research.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Written consent

Written consent was obtained from all individual participants included in the study.

References

1. Buhimschi CS, Bhandari V, Han YW, Dulay AT, Baumbusch MA, Madri JA, et al. Using proteomics in perinatal and neonatal sepsis: hopes and challenges for the future. Curr Opin Infect Dis. 2009; 22: 235-43.

2. Tsangaris G, Weitzdörfer R, Pollak D, Lubec G, Fountoulakis M. The amniotic fluid cell proteome. Electrophoresis. 2005;26:1168-73.

3. Gervasi MT, Chaiworapongsa T, Naccasha N, Blackwell S, Yoon BH, Maymon E, et al. Phenotypic and metabolic characteristics of maternal monocytes and granulocytes in preterm labor with intact membranes. Am J Obstet Gynecol. 2001;185:1124-9.

4. Gervasi MT, Chaiworapongsa T, Naccasha N, Pacora P, Berman S, Maymon E, et al. Maternal intravascular inflammation in preterm premature rupture of membranes. J Matern Fetal Neonatal Med. 2002;11:171-5.

5. Nardo LG. Vascular endothelial growth factor expression in the endometrium during the menstrual cycle, implantation window and early pregnancy. Curr Opin Obstet Gynecol 2005; 17: 419-23.

6. Arias F, Victoria A, Cho K, Kraus F. Placental histology and clinical characteristics of patients with preterm premature rupture of membranes. Obstet Gynecol. 1997;89:265–71.

7. Kim YM, Bujold E, Chaiworapongsa T, Gomez R, Yoon BH, Thaler HT, et al. Failure of physiologic transformation of the spiral arteries in patients with preterm labor and intact membranes. Am J Obstet Gynecol. 2003;189:1063–9.

8. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, et al. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med. 2004;350:672–83.

9. Conroy AL, Silver KL, Zhong K, Rennie M, Ward P, Sarma JV, et al. Complement activation and the resulting placental vascular insufficiency drives fetal growth restriction associated with placental malaria. Cell Host Microbe. 2013;13:215–26.

10. Sadek KH, Cagampang FR, Bruce KD, Shreeve N, Macklon N, Cheong Y.Variation in stability of housekeeping genes in endometrium of healthy and polycystic ovarian syndrome women. Hum Reprod. 2012;27:251-6.

11. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. Lancet. 2008;371:75–84.

12. Cnattingius S, Granath F, Petersson G, Harlow BL. The influence of gestational age and smoking habits on the risk of subsequent

preterm deliveries. N Eng J Med. 1999;341:943–8.
13. Saika S. TGF-beta signal transduction in corneal wound healing as a therapeutic target. Cornea. 2004;23:S25-30.
14. Zhao HW, Li YW, Feng R, Yu JB, Li J, Zhang Y, et al. TGF-β/Smad2/3 signal pathway involves in U251 cell proliferation and apoptosis. Gene. 2015;562:76-82.
15. Grainger DJ. Transforming growth factor-b and atherosclerosis: so far, so good for the protective cytokine hypothesis. Arterioscler Thromb Vasc Biol 2004;24:399–404.
16. Evrard SM, d'Audigier C, Mauge L, Israël-Biet D, Guerin CL, Bieche I, et al. The profibrotic cytokine transforming growth factor-β1 increases endothelial progenitor cell angiogenic properties. J Thromb Haemost. 2012;10:670-9.
17. Leask A, Abraham DJ. TGF-β signaling and the fibrotic response. FASEB J 2004;18:816–7.
18. Verrecchia F, Mauviel A. Transforming growth factor-beta signaling through the Smad pathway: role in extracellular matrix gene expression and regulation. J Invest Dermatol 2002;118:211–5.
19. Leask A, Holmes A, Black CM, Abraham DJ. Connective tissue growth factor gene regulation. Requirements for its induction by transforming growth factor-beta 2 in fibroblasts. J Biol Chem. 2003;278:13008-15.
20. Brigstock DR. The connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed (CCN) family. Endocr Rev. 1999;20:189-206.
21. Wang Q, Usinger W, Nichols B, Gray J, Xu L, Seeley TW, et al. Cooperative interaction of CTGF and TGF-β in animal models of fibrotic disease. Fibrogenesis Tissue Repair. 2011;4:4.
22. Arnott JA, Lambi AG, Mundy C, Hendesi H, Pixley RA, Owen TA, et al. The role of connective tissue growth factor (CTGF/CCN2) in skeletogenesis. Crit Rev Eukaryot Gene Expr. 2011;21:43-69.
23. Keane MP, Strieter RM, Lynch JP 3rd, Belperio JA. Inflammation and angiogenesis in fibrotic lung disease. Semin Respir Crit Care Med. 2006;27:589-99.
24. Elpek GÖ. Angiogenesis and liver fibrosis. World J Hepatol. 2015;7:377-91.
25. Glaser SS, Gaudio E, Alpini G. Vascular factors, angiogenesis and biliary tract disease. Curr Opin Gastroenterol. 2010;26:246-50.
26. Economidou F, Margaritopoulos G, Antoniou KM, Siafakas NM. The angiogenetic pathway in malignant pleural effusions: Pathogenetic and therapeutic implications. Exp Ther Med 2010;1: 3-7.
27. Wygrecka M, Zakrzewicz D, Taborski B, Didiasova M, Kwapiszewska G, Preissner KT, et al. TGF-β1 induces tissue factor expression in human lung fibroblasts in a PI3K/JNK/Akt-dependent and AP-1-dependent manner. Am J Respir Cell Mol Biol. 2012;47:614-27.
28. Scarpati EM, Wen D, Broze GJ Jr, Miletich JP, Flandermeyer RR, Siegel NR, et al. Human tissue factor: cDNA sequence and chromosome localization of the gene. Biochemistry 1987;26:5234-8.
29. Wilcox JN, Smith KM, Schwartz SM, Gordon D. Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. Proc Natl Acad Sci USA 1989;86:2839–43.
30. Mackman N. The many faces of tissue factor. J Thromb Haemost. 2009; Suppl 1:136-9.
31. Mach F, Schonbeck U, Bonnefoy JY, Pober JS, Libby P. Activation of monocyte/macrophage functions related to acute atheroma complication by ligation of CD40: induction of collagenase, stromelysin, and tissue factor. Circulation 1997;96:396–99.
32. Pradier O, Willems F, Abramowicz D, Schandené L, de Boer M, Thielemans K, et al. CD40 engagement induces monocyte procoagulant activity through an interleukin-10 resistant pathway. Eur J Immunol. 1996;26:3048-54.
33. Taubman MB, Marmur JD, Rosenfield CL, Guha A, Nichtberger S, Nemerson Y. Agonist-mediated tissue factor expression in cultured vascular smooth muscle cells. Role of Ca^{2+} mobilization and protein kinase C activation. J Clin Invest 1993;91:547–52.
34. Mesri M, Altieri DC. Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway. J Biol Chem. 1999;274:23111-8.
35. Brox JH, Osterud B, Bjørklid E, Fenton JW 2nd. Production and availability of thromboplastin in endothelial cells: the effects of thrombin, endotoxin and platelets. Br J Haematol. 1984;57:239-46.
36. Chambers RC, Leoni P, Blanc-Brude OP, Wembridge DE, Laurent GJ. Thrombin is a potent inducer of connective tissue growth factor production via proteolytic activation of protease-activated receptor-1. J Biol Chem. 2000;275:35584-91.
37. Tranquilli AL, Landi B. The origin of pre-eclampsia: from decidual "hyperoxia" to late hypoxia. Med Hypotheses. 2010;75:38-46.
38. Schecter AD, Giesen PL, Taby O, Rosenfield CL, Rossikhina M, Fyfe BS, et al. Tissue factor expression in human arterial smooth muscle cells. TF is present in three cellular pools after growth factor stimulation. J Clin Invest. 1997;100:2276-85.
Gardiner C, Tannetta DS, Simms CA, Harrison P, Redman CW, Sargent IL. Syncytiotrophoblast microvesicles released from pre-eclampsia placentae exhibit increased tissue factor activity. PLoS One. 2011;6:e26313.