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Early Pregnancy Prediction of Spontaneous Preterm Birth Prior to 32 Completed Weeks of Pregnancy Using Plasma RNA:

Transcriptome Discovery and Initial Validation of an RNA Panel of Markers

Carl P. Weiner[1,2], **Yafeng Dong**[2], **Helen Zhou**[1], **Howard Cuckle**[3], **Risa Ramsey**[4], **Robert Egerman**[5], **Irina Buhimschi**[6], **Catalin Buhimschi**[6]

[1]Department of Obstetrics and Gynecology, University of Kansas School of Medicine, Kansas City, KS USA

[2]Rosetta Signaling Laboratory, Mission Hills, KS USA

[3]Faculty of Medicine, Tel Aviv University, Ramat Aviv, Israel

[4]Office of Clinical Research, University of Tennessee Health Science Center in Memphis, TN USA

[5]Department of Obstetrics and Gynecology, University of Florida, Gainesville FL, USA

[6]Department of Obstetrics and Gynecology, University of Illinois Chicago, Chicago, IL, USA

Carl P Weiner MD MBA, Department Obstetrics and Gynecology, KUMC, 3901 Rainbow Blvd, Kansas City, KS, 66160. USA, cweiner@kumc.edu and cpweiner@gmail.com.

Contribution to Authorship:

Carl P. Weiner MD MBA: Principle Investigator on the supporting grants, designed the studies, coordinated the analyses and was the primary author of the manuscript.

Yafeng Dong PhD: Designed molecular studies and analyzed data. Helped edited the manuscript.

Helen Zhou MD: Designed molecular studies and analyzed the data. Authored sections of the manuscript and helped edit the remainder.

Howard Cuckle DPhil: Conducted the analysis of the validation study; wrote sections of the manuscript and helped edit the remainder.

Risa Ramsey PhD, EMBA, BSN, RN, CCRC: Co investigator of the CDC grant and site coordinator; wrote sections of the manuscript and helped edit the remainder.

Robert Egerman MD: Co investigator of the CDC grant and site coordinator; wrote sections of the manuscript and helped edit the remainder.

Irina Buhimschi MD: Co investigator of the CDC grant; wrote sections of the manuscript and helped edit the remainder.

Catalin Buhimschi MD: Co investigator of the CDC grant; wrote sections of the manuscript and helped edit the remainder.

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Carl P. Weiner MD MBA: Founder of Rosetta Signaling Laboratory which controls the intellectual property related to plasma nucleic acid extraction and the RNA markers described including EU Patent No. 2646554, US patent No. 10,954,564 and European and US trademarks on the name FutureBIRTH™ (Serial No. 87/110459).

Yafeng Dong PhD: Co-founder of Rosetta Signaling Laboratory which controls the intellectual property related to plasma nucleic acid extraction and the RNA markers described including EU Patent No. 2646554, US patent No. 10,954,564 and European and US trademarks on the name FutureBIRTH™ (Serial No. 87/110459).

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Details of Ethics Approval:

The blood samples materials were collected, and the research described performed with the signed written informed consent of the participants and with IRB approval under the following protocol numbers:

University of Tennessee, Memphis- 05-08318-FB (Approval 1/6/2006)

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Abstract

Objective: Compare the second trimester plasma cell-free (PCF) transcriptome of women who delivered at term to women with spontaneous preterm birth (sPTB)≦32weeks and identify/validate PCF ribonucleic acid (RNA) markers present by 16weeks.

Design: Prospective case-control study.

Setting: Academic tertiary care centre.

Population: Pregnant women with known outcomes prospectively sampled.

Methods: PCF RNA extracted from women at 22–24weeks (5 sPTB≦32weeks and 5 term) was hybridised to gene expression arrays. Differentially regulated RNAs for sPTB≦32weeks were initially selected based on p-value compared to control ($p<0.01$) and fold change (≥1.5x). Potential markers were then reordered by narrowness of distribution. Final marker selection was made by searching the Metacore™ database to determine whether the PCF RNA interacted with a reported set of myometrial Preterm Initiator genes. RNAs were confirmed by qRT-PCR and tested in a second group of 40 women (20 with sPTB ≦32weeks (mean gestation 26.5, standard deviation ±2.6weeks), 20 with spontaneous term delivery (40.1±0.9weeks) sampled 16–19+5weeks.

Main outcome measure: Identification of PCF RNAs predictive of sPTB≦32weeks.

Results: 297 PCR RNAs were differentially expressed in sPTB≦32weeks. Further selection retained 99 RNAs (86 mRNAs and 13 miRs) and 5 of these interacted *in silica* with 7 Preterm Initiator genes. Four of 5 RNAs were confirmed and tested on the validation group. The expression of each confirmed PCF RNA was significantly higher in sPTB≦32weeks. *In vitro* study of the 4 mRNAs revealed higher expression in placenta of women with sPTB≦32weeks and the potential to interfere with myometrial quiescence.

Conclusions: The PCF RNA markers are highly associated with sPTB≦32weeks by 16weeks.

Tweetable abstract:

Women destined for spontaneous preterm birth can be identified by 16 weeks with a panel of maternal plasma RNAs.

Keywords

pregnancy; preterm birth; transcriptome; plasma RNA; precision medicine

INTRODUCTION

Over 15 million preterm births (PTBs) annually result in >1 million deaths[1] and lifelong sequelae for many survivors[2]. Very (28–32 weeks of gestation) and extreme (<28 weeks) preterm newborns have the highest long-term complication rates.[3, 4, 5]

Proposed and clinically deployed predictors of PTB include historical, biological and mechanical markers.[6, 7, 8] Test studies are often modest in size, and metrics such as negative and positive predictive values are altered by disease prevalence. The area under the curve (AUC) is not affected by disease prevalence and arguably the most useful predictor of

a model's prognostication.[9] An AUC between 90–100% is considered excellent, 80–90% good, 70–80% fair, 60–70% poor, and 50–60% a test failure[10]. Good to excellent PTB screening tests have been elusive. By example, cervical fetal fibronectin at 16–22 weeks yields AUCs of 51–54%, and transvaginal sonographic cervical length yields AUCs of 51–58%[11] although AUC's for sPTB≦32 weeks are higher[12]; both are similar to clinical history[13].

Plasma cell free (PCF) RNAs include coding and noncoding RNAs able to modulate angiogenesis, cell proliferation/death, tumor cell invasion, and cell-to-cell communication[14,15,16]. The plasma transcriptome is altered by disease suggesting the potential for prognostication[17,18,19]. Poon et al[20] reported PCF fetal RNA in maternal blood two decades ago, and since then others have described pregnancy derived RNAs in maternal blood and suggested a screening role[21,22,23,24,25,26,27,28,29].

The myometrial transcriptome of women with sPTB≦32 weeks is uniquely altered compared to nonlabouring women≦32 weeks or term nonlaboring or spontaneously laboring women, suggesting the origin of labor differs at these timepoints[17, 30]. PCF RNAs are relatively stable within their transport vehicle[31, 32, 33, 34, 35] that can enable uptake by specific cell types. We hypothesize that some maternal PCF RNAs are actually 'words' 'read' by cells remote from the cells synthesizing the RNAs providing pathway of communication between conceptus and mother. We further hypothesize that specific PCF RNAs modulate critical adaptive responses for a successful pregnancy and 'miscommunication' can trigger disease. If true, changes in the maternal PCF transcriptome might predict events leading to sPTB. Two corollaries:

1. The earlier the wrong 'message' is sent, the more likely it is to be part of the underlying disease mechanism; and
2. Translating the 'message' will provide new understanding of disease mechanisms and reveal potential therapeutic targets.

Our objective was to test aspects of this hypothesis.

METHODS

Funded by the US Centers for Disease Control, we collected blood samples with the goal of identifying biomarkers for sPTB≦32 weeks, defined as labor and / or preterm premature rupture of the membranes. We reasoned that if the majority of prematurity related health costs are explained by these births [1, 2], their accurate prediction and ultimate prevention would have maximal impact. We further suspected that sPTB between 33–36 weeks would include a mix of term and preterm mechanisms, potentially obscuring unique markers of early sPTB ≦ 32 weeks.

Cohort

Enrollment of women took place in Memphis TN 2005–2010 with informed written consent supervised by two co-investigators (RE and RR) under IRB approval from both the University of Tennessee Health Science Center in Memphis and the University of Kansas

School of Medicine. All clinical records were extracted contemporaneously and entered into a computerized database. Entries were checked/rechecked for accuracy on a randomized basis. Investigators could request a review of the source records should an entry be suspect or a parameter missing. The records were finalized within 6 months of the last subject's delivery and all patient identifiers removed from the working database. The identifying linkage and original files are maintained on a secure server at the University of Tennessee Health Science Center in Memphis under the control of one of the onsite investigators (RR). Further details on the full cohort will be part of a separate report.

Additional participants, whose deidentified samples of myometrium and plasma included in one or more of the studies presented, were obtained after informed written consent for biobank collection under protocols approved by the local IRB at either the University of Kansas School of Medicine or Yale University School of Medicine. The deidentified samples were transferred to and stored in the University of Kansas Pregnancy Biobank (PI CPW). There was no patient, funder or public involvement in the subsequently described research.

Overall, 305 pregnant women were enrolled at 18.3±1.4 weeks (range 16.0–20.9 weeks) and provided blood (serum, plasma [4mL vacuum tubes coated with 7.2mg K2EDTA], and buffy coat fractions), saliva, urine and cervical / buccal swabs. Thereafter, blood and urine samples were obtained every two weeks until delivery, when an umbilical cord blood sample was obtained. The final cohort included 289 participants (305 enrolled; 6 (1.9%) never sampled, 6 (1.9%) lost to follow-up and 4 (1.3%) clinical exclusions (1 "spontaneous" chorioamnionitis at 19 weeks, 1 PPROM 48 hours after elective cerclage placement at 24 weeks, 1 placental abruption at 28 weeks and 1 appropriately grown IUFD at 35 weeks).

Pregnancy Dating

Pregnancy dating was based on a reliable last menstrual period (LMP) when available, confirmed by a dating ultrasound conducted ≤20 weeks of gestation. If the two parameters differed by more than 11 days or there was no LMP, the ultrasound-derived gestational age was used.

Laboratory Methods

No laboratory personnel were aware of pregnancy outcomes.

RNA extraction—PCF RNA was extracted by Rosetta Signaling Laboratory using a proprietary method (Rosetta Signaling Laboratory, Mission Hills, KS). The EDTA sample volume extracted was 500μl. The mean total RNA extracted was 15.9 ± 2.2μg/mL. RNA yield was assessed by Nano spectrometer (NanoDrop Technologies, Wilmington DE) and RNA integrity confirmed by the Agilent Bio-analyzer (Agilent, Santa Clara CA).

Total RNA was extracted from immortalized human pregnant myometrial cells (IHPM), placenta and myometrium using Trizol™ (Life Technologies, Carlsbad CA) according to the manufacturer's instructions.

Microarrays—Affymetrix Human Exon 1.0 ST Array and the Affymetrix GeneChip miRNA array (847 human miRNAs) were used. All microarrays were processed and read in 2010 by the KUMC Genomics Core according to manufacturer's instructions using a GeneChip Microfluidics 450 Center and GeneChip 3000 scanner with 7G upgrade (Applied Biosystems) with Affymetrix GeneChip Command Console Software. Microarray RNA quality control evaluation was performed before each microarray (see Supporting Information). Primary data analysis was conducted by the KUMC Bioinformatics Core following MIAME (minimum information about a microarray experiment) guidelines[1] who conducted gene expression quality control (see Supporting Information).

qRT-PCR assays

mRNA RT: The RNA samples were diluted, and a master mix prepared including dNTP mix, Omniscript Reverse Transcriptase and Random Primer (Invitrogen, Carlsbad CA). The mRNA of each sample was converted into cDNA at 37°C for 60min per manufacturer instructions.

miRNA RT: The miRs were polyadenylated using reagents from the Invitrogen NCode miRNA First-Strand cDNA Synthesis Kit (ThermoFisher). The polyadenylated microRNA was reverse transcribed to generate the first strand of cDNA according to the manufactory's protocol.

Preamplification and qPCR: Multiplex qPCR reactions were performed by SYBR green using the ViiA 7 Real-Time PCR System. The primers for the gene panels were custom designed and synthesized by Integrated DNA Technologies (IDT, Coralville, IA). The probe sets in each reaction well included primers for the biomarker, normalization, and spike genes so that all three genes were run in the same reaction well to minimize assay variation. Information about the primer sequences used is available from the authors. Preamplification was performed, 1ul RT samples were prepared for the preamplification Mix Reaction and underwent 12 cycles. Two ul preamplification cDNA samples were diluted into 10ul PCR reaction mix, then followed by RT PCR using SYBR Green Supermix (ThermoFisher). Threshold cycles (Ct values) of qPCR reactions were extracted using QuantStudio™ Software V1.3 (Applied Biosystems, Foster City CA). Delta-delta CT method is used to calculate the RNAs expression levels and then normalized.

In Vitro Studies-: see Supporting Information.

Discovery

The 24 week plasma samples were used. Discovery subjects were selected at random from the prospectively assembled cohort. Criteria included a clear diagnosis and no reported maternal or pregnancy co-morbidities. Sixty percent were of White and 40% of Black ethnicity. Five sPTB≦32w subjects were selected in chronologic order of enrollment until the target sample size was achieved. Five control subjects were selected by the same process except they delivered >36 weeks and zero days. Prior preterm birth was not considered. PCF cDNA was run on the microarrays as described.

Confirmation

A primer for each selected miR was designed for quantitative (q)RT PCR and tested on the same cDNA samples used for Discovery. Two confirmed miRs were also quantitated across gestation in samples from the same Discovery subjects.

The as yet unconfirmed potential mRNA markers and confirmed miRs were compared to a list of myometrial Preterm Initiator genes that are differentially expressed in sPTB≦32 weeks[17]. The Metacore™ database (Thomson Reuters, release 2010) was then queried to identify whether any selected potential RNA marker was known to interact with Preterm Initiator genes in any reported cell system. The mRNAs so identified were confirmed using qRT-PCR.

Validation

The plasma samples tested were obtained 16.0 to 19+5 weeks. Samples from women participating in validation were selected at random from the cohort after excluding Discovery subjects. Requirements included a clear diagnosis and no reported maternal or pregnancy co-morbidities. Ten subjects (50%) of each outcome group were of Black and 10 subjects of White ethnicity. Twenty (20) sPTB≦32 weeks women were selected in chronologic order of enrollment until the target sample size was achieved. The 20 control subjects were selected by the same process except they delivered >36 weeks and 0 days. Prior PTB was not a consideration. qRT-PCR was performed with the same primers used for Confirmation and expression normalized.

Statistics-

Discovery Study—Differentially regulated RNAs for sPTB≦32 weeks were identified based on p-values adjusted for multiple hypotheses testing (<0.01 compared to control) using the method of Benjamini - Hochberg to establish a false discovery rate (FDR) for each differentially expressed RNA. A FDR cutoff of <0.05 with an absolute fold change greater than or equal to 1.5 was used as the minimal criteria for selecting significantly differentially expressed RNAs. The list of differentially expressed RNAs was then reordered by narrowness of distribution (Ingenuity Systems Pathway Analysis software) so that the RNAs with the smallest variance in expression across the cases were ranked highest. Biological functional and pathway analysis utilized software from Ingenuity Systems. Microarray results were also used to identify ‘normalization’ RNAs, i.e., consistently unaffected by PTB, gestational age, maternal race, fetal gender, and robustly expressed.

Validation Study—The discriminatory power of each marker was assessed in relation to the median values among cases and controls combined. The proportion of cases above the median was compared with the proportion of controls. RNAs with no amplification were classified as missing. The non-parametric Wilcoxon Rank Sum Test (2-tail) was used to determine statistical significance.

In Vitro Studies-—see Supporting Information.

RESULTS

Discovery and Confirmation

Of the 25,934 mRNA and the 847 miR sought, 296 mRNA and 14 miR were differentially expressed in women destined for sPTB≦32 weeks (Table s1). After reordering the 310 RNAs by narrowness of distribution (i.e., lowest variances among group subjects) and fold change, 99 RNAs (86 mRNAs and 13 miRs) were retained. Twenty-two mRNAs were increased and 64 decreased. Three miRs were increased and 10 decreased. None had previously been associated with pregnancy.

Four mRNA and 4 miR, whose expression on microarray was similar in both sPTB≦32 weeks and control groups, were tested across gestational age as potential normalization sequences. One mRNA (PPIA) (Figure s1d) and one miR (snRNA U6) (Figure s2d) fulfilled criteria.

Pathway analysis revealed the top two diseases associated with the retained 99 RNAs (Figure s3) were Hypertension and Neurologic / Neurobehavioral Manifestations. Obesity and Chorioamnionitis ranked 5th and 6th. The number one Map was DNA damage and the number one Network was the O-dexadecanoyl-(L)- carnitine pathway.

Ten of the 13 selected miRs were confirmed (Figure s4a) (confirmation rate 76.9%). The expression of miR 99a was increased by 16 weeks in sPTB≦32 weeks ($p<0.01$); miR 548L expression did not rise until after 20 weeks (consistent with microarrays) (Figure s4b).

The 10 confirmed miRs and 86 potential mRNA markers were then compared to a set of myometrial genes differentially regulated in women with sPTB≦32 weeks[17]. There were no commonalities between lists. However, the Metacore™ query identified five (5) PCF RNAs (PSME2, NAMPT, APOA1, APOA4, and Hsa-Let-7g) that *in silica* interacted directly or indirectly with 7 Preterm Initiator genes (Figure 1a, b). The five RNAs originate from genes on autosomes 3, 7, 11 and 14. Figure 1c illustrates the mean RNA expression measured by qRT-PCR in Discovery samples. APOA1 approached but did not reach significance (confirmation rate 80%).

PCF RNA Panel Validation—The 4 confirmed PCF RNAs were then subject to a preliminary validation study. The 40 women delivered at 26.5±2.6 weeks (average ± SD) for sPTB≦32 weeks and 40.1 ± 0.9 weeks for control. Individual marker expression levels per subject are illustrated in Figure s5. The non-parametric Wilcoxon Rank Sum Test (2-tail) revealed sPTB≦32 weeks was associated increased expression of each of the four confirmed RNAs at 16–20w. (Table 1)

mRNA Marker Origin—The 4 mRNAs were overexpressed in the plasma and placenta of subjects with sPTB≦32w (Figure 2). APOA4 and APOA1 were undetectable in myometrium whether term or sPTB≦32w (Figure 2). Myometrial PSME2 and NAMPT were overexpressed in sPTB≦32w versus term (Figure 2). APOA4 protein was detected only in placenta of sPTB≦32w (Figure s6).

Biologic Plausibility—*In silica,* APOA4 interacts with 5 myometrial Preterm Initiator genes (Figure 1 top); IFNG (interferon gamma) provides a branch point. Transfection of APOA4 containing plasmid into immortalized human pregnant myometrial (IHPM) cells[37] Figure s7a and b) doubled the APOA4 protein level (Figure s7c) and increased intracellular calcium five times (Figure s7d). APOA4 overexpression doubled IHPM contraction frequency (Figure 3b and d)[38]. Transfection with siRNAs targeting IFNGR significantly decreased APOA4 mediated increase in contraction frequency (Figure 3e) suggesting the increase in contraction frequency caused by APOA4 overexpression was at least partially through INFG. While APOA4 and APOA1 were undetectable in myometrium, IFNGR expression was increased in sPTB≦32w myometrium (Figure 3f).

DISCUSSION

Main findings

This is the first plasma transcriptome wide discovery / validation study for sPTB≦32w in the second trimester conducted without prior assumptions. The result was hundreds of potential PCF RNA markers whose expressions were significantly altered by both P value and fold change in women destined for sPTB≦32w. Candidate RNAs were progressively narrowed to a panel of 5 PCF RNA selected after *in silica* study revealed an interaction between them and 7 myometrial genes also differentially expressed in women with sPTB≦32 weeks.[17] Four PCF RNAs were confirmed by qRT-PCR, and their expression levels measured in a 40 subject case-control validation using plasma obtained 16.0 to 19+5 weeks. sPTB≦32 weeks was associated with significant overexpression of the 4 confirmed RNA markers, suggesting the potential for a strong performance in future cohort studies.

We found after that after panel selection that the RNAs were highly expressed in placenta from women with sPTB≦32 weeks and associated with molecular pathways not previously linked to sPTB. Each marker *in silica* had the potential to increase intracellular calcium either directly or indirectly. Overexpression of APOA4 mRNA in IPHM increased APOA4 protein, intracellular calcium concentration, and cell contraction frequency. The increased contraction frequency was reduced by a siRNA blocking IFNGR, a predicted intracellular target of APOA4 (see Supporting Information, Discussion). While the remaining markers require similar testing, it may be that sPTB is preceded by myometrial quiescence dysfunction.

Strengths and limitations

The present study has several strengths. 1. The samples were derived from a prospectively assembled cohort enrolled and first sampled at 16–20 weeks followed by sampling every 2 weeks. Patient follow-up was excellent. 2. It is the first study to utilize Rosetta Signaling Laboratory's proprietary plasma RNA extraction which increases total RNA yield per milliliter of plasma to microgram amounts compared to the nanograms achieved with commercial kits[39]. 3. We focused on sPTB≦32w as these neonates are at highest risk for mortality, and with survival, at highest risk for near and long-term complications that account for the majority of health care costs in the first 12 months of life[1,2]. Further, selecting a gestational age cutoff ≦32 weeks should have reduced mixing of subjects

whose labor was triggered by ill-timed 'term' labor mechanisms. 4. The discovery study specifically included PTB≦32 weeks preceded by PPROM rather than assuming PPROM prior 32 weeks is an independent event. 5. PCF RNA marker selection was unaffected by prior assumptions of previously proposed mechanisms (e.g. inflammation, progesterone metabolism or oxytocin regulation). 6. Our objective was to identify potential markers present before 20 weeks for sPTB≦32 weeks regardless of race. However, other potential PCF RNAs confirmed and validated could prove of future interest specifically in terms of racial differences in rates of sPTB≦32 weeks. 7. The last step in the selection of the PCF RNAs for validation was the result of a novel strategy- identification *in-silica* of potential PCF markers that interact with one or more myometrial genes differentially regulated in women who suffered sPTB≦32 weeks.[17]

There are several limitations to the investigation. 1. The studies were conducted in 2010–2012 and many of the discovery tools widely available today were not widely available then. The delay in publication is unfortunate, but does not undermine the well-studied validity of microarrays. It is possible that newer tools like RNAseq could identify other, relevant RNAs not part of the arrays used, but that in no way diminishes the potential relevance of the PCF RNA markers identified. 2. The discovery sample size is small, certainly based on the cumulative experience with genomics. Yet, the parameters for the bioinformatic analysis were strict and achieved. Further, the plasma transcriptome is a direct reflection of genome activity rather than genomic potential and our markers were selected for narrow variance among cases. Ngo[40] et al described the application of RNAseq to 15 women sampled at 24–30 weeks, 8 of whom experienced sPTB (26.4 ± 2.3 weeks) and all sampled ≦7 days from delivery. More recently, Del Veccio[41] et al conducted a plasma transcriptome discovery study on 26 pregnant women. Perhaps just as important as sample size is the mechanism of disease. In addition, the final markers selected were ultimately based on their potential to interact with a group of myometrial RNAs differentially expressed in women with sPTB≦32 weeks and survived their initial validation. While conventional wisdom holds that sPTB≦32 weeks is a syndrome with multiple causes[42], the fact it occurs at similar rates worldwide[2] suggests the number of causes may be limited. Been[43] et al examined the impact of COVID-19 mitigation on preterm birth in the Netherlands. In a million plus births during 4 months in 2020, they found a significant drop in PTB between 32 and <37 weeks within 2 months of initiating mitigating measures that lasted at least 4 months. Yet there was no significant change in the rate of PTB<32 weeks suggesting early and late PTB have different causes. 3. The case-control initial validation study was subject to selection bias inherent to this study design. However, the analytic approach avoids the risk of overfitting with regression analysis and the strong association of increased PCF RNA expression with sPTB≦32w by 16w supports our working hypotheses. 4. The cohort was enriched with subjects at risk for PTB either because of obstetric history or race, and the identified markers may not be applicable to the 'general' population. Subsequent cohort studies are needed to resolve this concern.

Interpretation

A large body of work confirms labor and cervical softening/shortening are associated with inflammation[41]. Yet, the molecular pathways leading to inflammation differ in term and

preterm labors[17]. The association of PTB with the increased expression levels of the four PCF RNAs begins by 16 weeks and suggests a relationship with implantation or aberrant placental maturation, and that the inflammation characteristic of labor at any gestational age is a terminal event generated along multiple pathways. Our first corollary proposes the earlier in disease development a marker is present, the more likely the marker is to be part of the underlying disease and not a byproduct of secondary events. The association of the differentially expressed PCF RNA panel in this initial validation study suggests sPTB≦ 32 weeks is a placental disease potentially detectable by 16 weeks. If true, sPTB≦32 weeks may be amenable to early screening and ultimately to therapeutic intervention.

We note the work of Ngo[39] et al above. In that same paper, they measured 140 preselected PCF RNAs weekly in 31 Danish women from <12 weeks until delivery. Multiple PCF RNAs were differentially expressed across gestation, enabling them to predict with good accuracy the number of weeks between sampling and delivery. They then applied the Danish model to two USA groups of women at high risk for sPTB. The first group was described above: 15 Black women from Pennsylvania sampled 24–30 weeks, 8 delivered prematurely. The second group consisted of 23 Black women from Alabama sampled at 24 weeks, 5 delivering prematurely (30.6 ± 2.4 weeks) and 18 at term (38.7 ± 0.5 weeks). In support of our hypothesis on the origin of sPTB≦32 weeks, the Danish model correctly predicted the term deliveries but not the PTBs, suggesting the mechanisms of labor differ. Ngo et al then performed RNA-seq on the Pennsylvania samples. Thirty-eight RNAs were differentially expressed and a panel of 7 selected RNAs tested in the Alabama samples in a case control model and obtained a regression derived AUC 81%. As their PTB samples were obtained within 7 days of delivery, their markers may reflect elements of advanced disease and explain why none of their identified RNAs overlapped with the RNAs identified in the current study.

CONCLUSION

The plasma transcriptome has great potential as a source of pathologic, prognostic and diagnostic information. Our results suggest pathways leading to sPTB are established by 16 weeks and may converge on the regulation of myometrial quiescence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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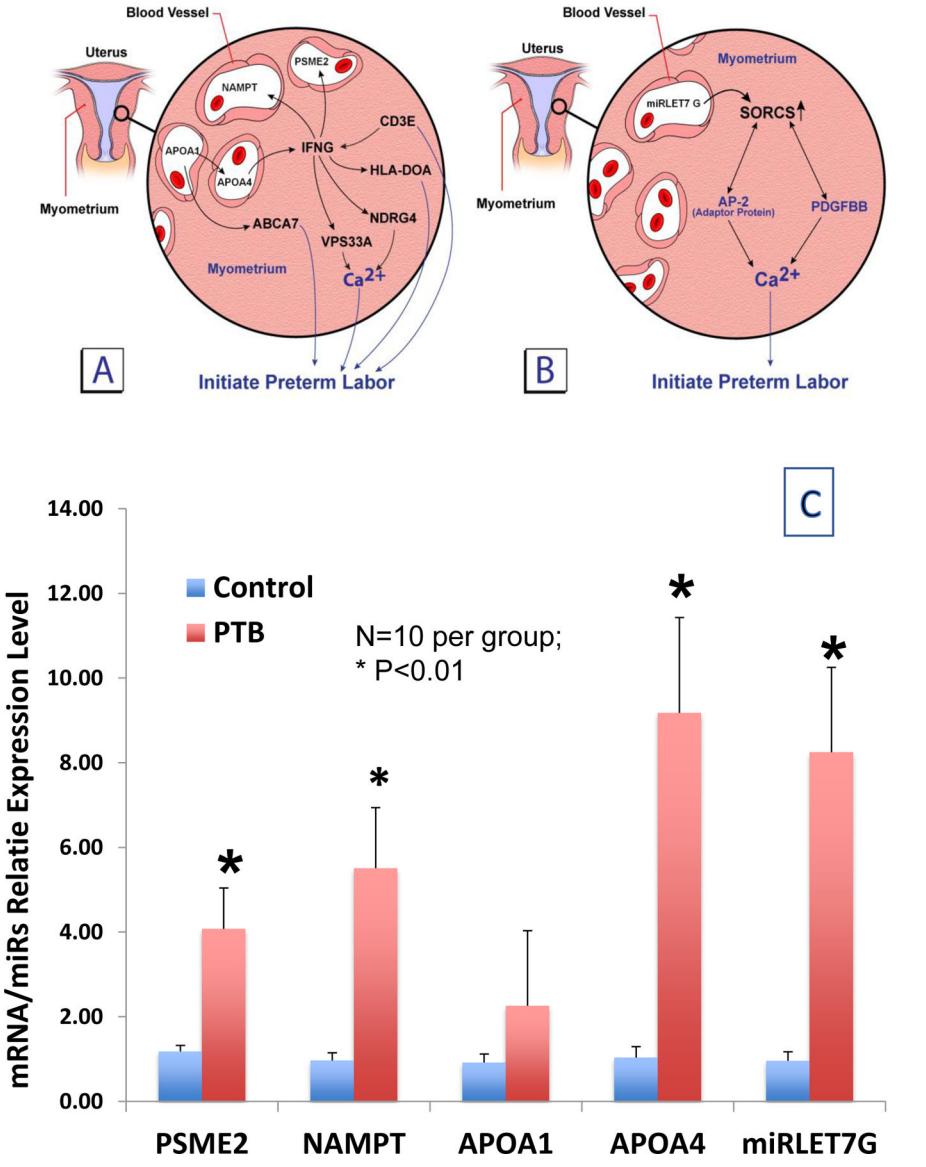

Figure 1:

A and B: The 5 potential sPTB markers selected when an *in-silica* query identified them as interacting either directly or indirectly with 7 preterm initiator genes in tissue other than myometrium[17].

C: Confirmation qRT-PCR performed for the 5 RNA markers using the same 10 Discovery patient samples. Four of the 5 potential sPTB markers were confirmed. APOA1 just missed significance and was not included in the remainder of the studies.

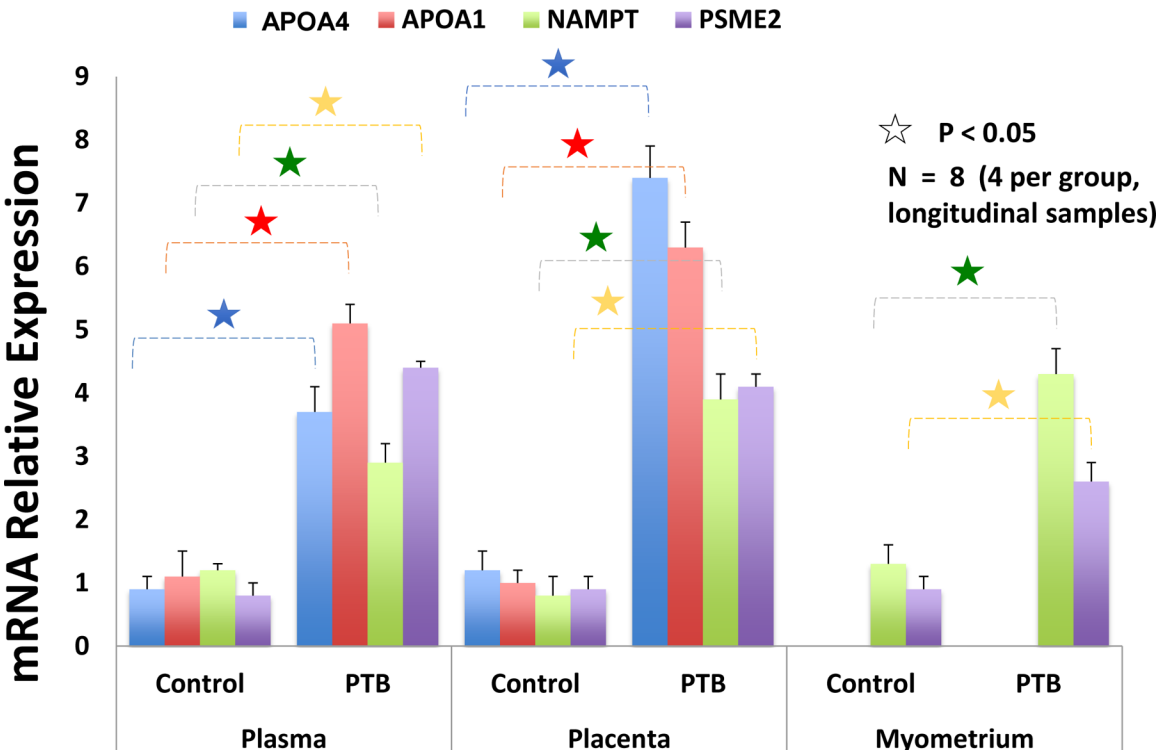

Figure 2:

Origin of PCF mRNA markers for sPTB ≦32 weeks: Expression levels of PSME2, NAMPT, APOA1 and APOA4 were quantified in plasma, placenta and myometrium obtained from 8 women at delivery (4 after term labor, 4 after sPTB ≦32 weeks). Each marker was significantly elevated in both the plasma and placenta of women with sPTB≦32 weeks versus term. APOA4 and APOA1 were undetectable in myometrium. PSME2 and NAMPT were significantly increased in myometrium from women with sPTB≦32-week compared to term.

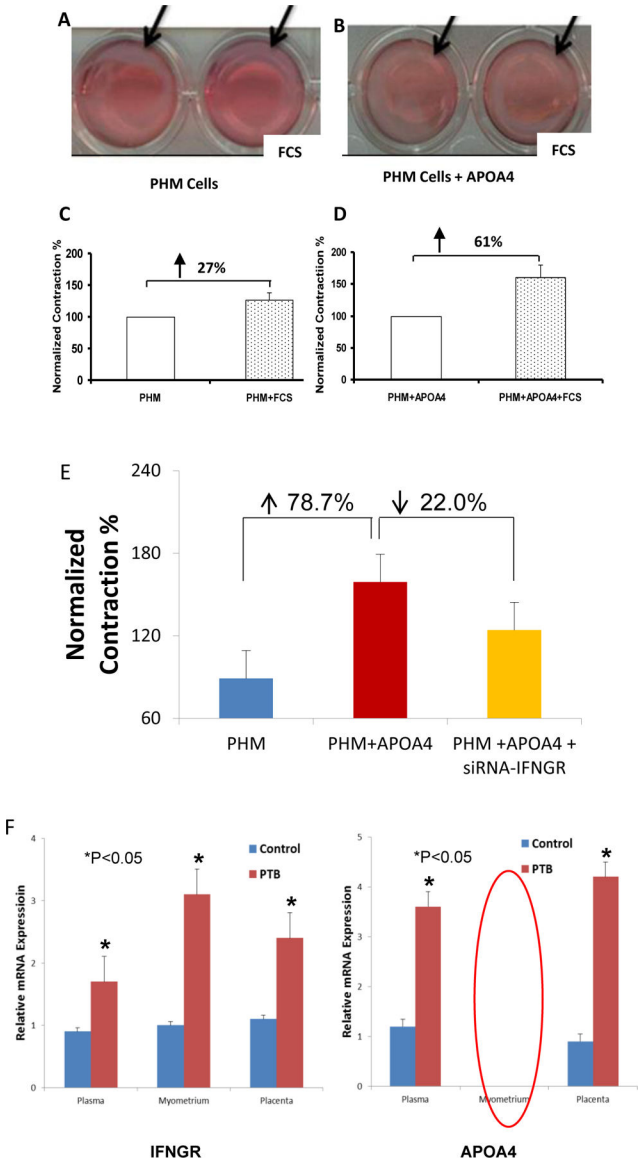

Figure 3:

APOA4 mRNA overexpression doubled (**B-D**) the contraction frequency of immortalized human pregnant myometrial cells (IHMCs) compared to baseline (**A-C**). Transfection with empty phages had no effect on contraction frequency (not shown). **E:** Transfected IHMCs with APOA4 mRNA alone (blue bar) or with siRNA-IFNGR (yellow bar) to determine whether the increase in IHMCs contraction frequency with APOA4 overexpression occurred via IFNG. siRNA-IFNGR significantly reduced the contraction frequency after APOA4 overexpression. Transfection with ghost phage had no effect on contraction frequency. **F:** IFNGR mRNA in plasma, placenta and myometrium of 8 pregnancies (samples same as Figure 3). The expression of IFNGR mRNA was dramatically increased in all samples from women suffering sPTB ≦32 weeks compared to term control. APOA4 was not detected in myometrium.

Table 1.

Validation study – overlap between sPTB cases and term controls 40 women sampled at 16.0–19.7 weeks' gestation. Twenty (20) women suffered sPTB≦32 weeks and 20 delivered at term after spontaneous labor.

Marker	Cases	Controls	Values ≥ median*		P-value**
			Cases	Controls	
PSME2	20	20	19 (95%)	1 (5%)	<0.0001
NAMPT	13	11	12 (92%)	0 (0%)	<0.0005
APOA4	18	20	18 (100%)	1 (5%)	<0.0001
LET-7g	20	20	19 (95%)	1 (5%)	<0.0001

*Cases and controls combined

**Wilcoxon Rank Sum Test (2-tail)