PRENATAL BEGINNINGS FOR BETTER HEALTH

EDITED BY: Irina Burd, Ahmet Baschat and Maged Costantine
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PRENATAL BEGINNINGS FOR BETTER HEALTH

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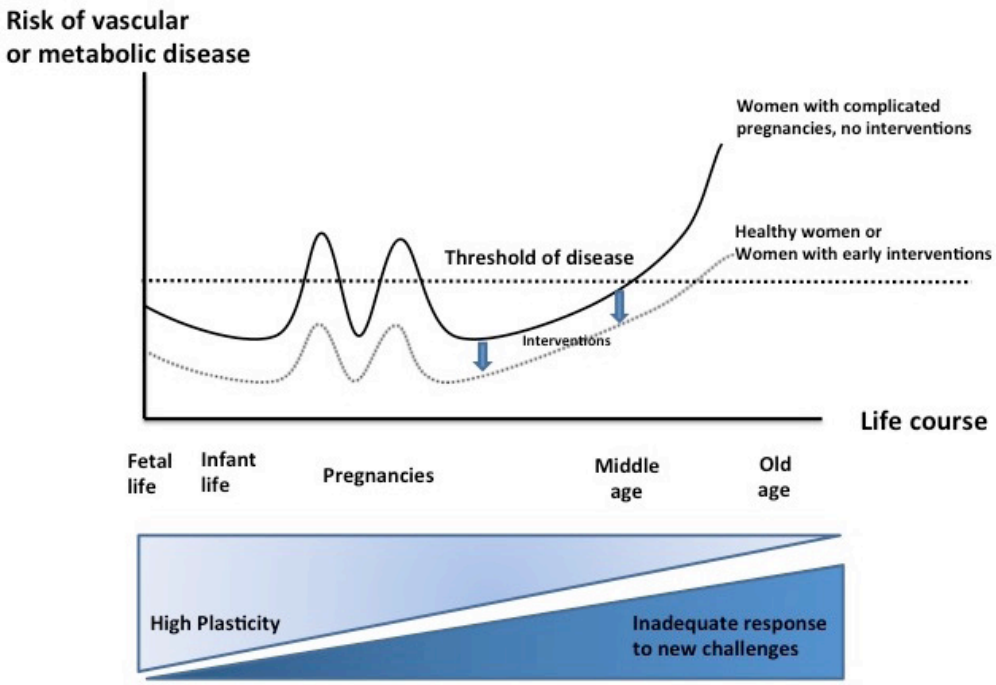

Image: Effect of developmental environment on later maternal phenotype and pregnancy complications. Figure from: Arabin B and Baschat AA (2017) Pregnancy: An Underutilized Window of Opportunity to Improve Long-term Maternal and Infant Health—An Appeal for Continuous Family Care and Interdisciplinary Communication. *Front. Pediatr.* 5:69. doi: 10.3389/fped.2017.00069

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EDITORIAL
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Editorial: Prenatal Beginnings for Better Health

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Keywords: prenatal, *in utero*, origins of health, origins of desease, perinatology

Editorial on the Research Topic

Prenatal Beginnings for Better Health

Prenatal care of pregnant women traditionally focuses on potential emergence of complications in the current pregnancy. Adverse pregnancy outcomes such as preterm labor, premature preterm rupture of membrane, gestational diabetes mellitus, and preeclampsia are known to have long-term health implications for the mother and child. Recent research developments of the specific downstream health risks of pregnancy complications hold promise for preventive measures including progesterone therapy, cerclage, acetylsalicylic acid therapy that can potentially avert the long-term health impacts. Furthermore, the drive to develop more accurate and non-invasive ways to detect pregnancies at risk of maternal and neonatal morbidities has led to investigation of biomarkers. With this trend in mind, we established this Research Topic, hosted by Obstetric and Pediatric Pharmacology, a joint division of Frontiers in Pediatrics and Frontiers in Pharmacology. Our aim is to review submissions made to this special Topic, which encompasses some of the latest discovery in biomarkers, molecular pathways of pregnancy complications and long-term health consequents of adverse pregnancy outcomes on the offspring. This Topic brings together 11 articles, with broad scope, in a novel multidisciplinary collaboration among obstetrics & gynecology, pharmacology, neurology, and pediatrics. These articles are organized around critical periods in pregnancy: pre-conception, antepartum, peripartum, and immediately postpartum.

In the pre-conception period, a woman's baseline health may foreshadow short and long-term health consequences for the offspring, as outlined by Arabin and Baschat. Women with preexisting hypertensive and metabolic risk profiles are more vulnerable to development of preeclampsia. Offspring born to mothers affected by preeclampsia, in turn, are at increased risk for hypertension, cerebrovascular accident, cognitive delay and depression, with the risk significantly increased for those affected by preterm preeclampsia. Placental stressors, inadequate delivery of nutrients *in utero* due to famine, or maternal stress due to external stressors are associated with low birth weight in the offspring, which is further linked with increased risk of cardiovascular disease, dyslipidemia, and psychiatric disorders.

On the other hand, maternal dietary supplementation has been shown to promote fetal wellbeing. One of the articles in the Topic by Mozurkewich et al. suggests possible benefit of fatty acid supplementation. In this study, women on dietary supplementation containing omega-3 fatty acid docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) had significantly high levels of metabolites of DHA and EPA in the umbilical cord blood, potentially important for reduction of preterm births and risk for infant admission to neonatal intensive care unit. While no conclusion can be drawn from this single study, it does suggest that further investigation into maternal dietary consumptions may prove beneficial for determining ways to promote fetal health.

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In the antepartum period, early *in utero* diagnosis of disease is crucial in terms of parental counseling and planning fetal interventions. Jelin et al. calls attention to skeletal dysplasias and development of molecular diagnosis, bone marrow transplant, and gene therapy *in utero.* Even skeletal diseases with significant comorbidity such as osteogenesis imperfecta may theoretically be amenable for novel treatments such as stem cell and bone marrow transplantation *in utero*. Such invasive *in utero* interventions may require simultaneous development of fetal anesthesia. Smith et al. validated remifentanil as a reasonable anesthetic agent frequently used in fetal interventions.

Shifting focus to another vital part of pregnancy, the placenta, Gumina and Su investigated the possible role of placental endothelial progenitor cells (EPCs). They suggest that EPCs, which had previously been implicated in vasculogenesis and angiogenesis, may have a role in vasculaturerelated pregnancy complications such as preeclampsia and fetal growth restriction. They highlight experiments where altered balance between EPCs of various angiogenic potential was seen in cord blood of infants affected by preeclampsia and fetal growth restrict ion. Thus, they cautiously posit EPCs may play an important role in pathogenesis of these pregnancy complications and may serve as possible targets for intervention.

Labor, the key event in the peripartum period, is intricately orchestrated by the mother and fetus. However, the precise signal exchange initiating labor is not yet fully understood. Sheller-Miller et al. hypothesize that the fetus may signal for onset of labor via exosomes, specialized intracellular signaling vesicles, in a murine model. Even less is known about pathogenesis of preterm labor. Preterm labor, delivery prior to 37 weeks' gestation is the leading cause of mortality among infants otherwise with no congenital anomalies. Infants born after preterm labor are at an increased risk of long-term intellectual and physical disabilities compared with term neonates Manuck. Manuck reviews current management of preterm labor—intramuscular progesterone for prevention, and treatment with indomethacin—and calls for further investigation into pathogenesis of preterm labor. Zhao et al. posit that ubiquitin-proteasomecollagen (CUP) pathway is implicated in molecular pathogenesis of preterm labor Johnson et al. They discovered that certain messenger RNAs associated with the CUP pathway were differently expressed in placentas and fetal membranes in women who had preterm labor or preterm premature rupture of membrane (PPROM). Their research represents one of the first steps in elucidating some of the molecular mechanisms of preterm labor and PPROM. Understanding these mechanisms may help develop targeted therapy to prevent and treat these conditions.

In the immediate postnatal period, early recognition of neonates with encephalopathy could significantly improve prognosis. Lei et al. demonstrate that neuronal nitric oxide synthase (NOS1), a marker for oxidative stress, was increased in umbilical cord blood of neonates affected by encephalopathy. They speculate that NOS1 may be a viable biomarker for early identification of neonatal encephalopathy and perinatal brain injury. An article by Graham et al. feature not only NOS1, but other biomarkers being studied that could be used as a panel to supplement existing tools to evaluate encephalopathy in neonates. Refinement of neonatal encephalopathy evaluation methods may help identify neonates who would benefit from interventions such as controlled hypothermia and cost associated with management of these neonates.

We hope that this compilation of articles highlighting the latest research in obstetrics and pediatric pharmacology will be of interest to the readers and will inspire more research in this exciting multidisciplinary approach to perinatal care.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Ubiquitin-Proteasome-Collagen (CUP) Pathway in Preterm Premature Rupture of Fetal Membranes

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Spontaneous preterm birth (sPTB) occurs before 37 gestational weeks, with preterm premature rupture of the membranes (PPROM) and spontaneous preterm labor (sPTL) as the predominant adverse outcomes. Previously, we identified altered expression of long non-coding RNAs (lncRNAs) and message RNAs (mRNAs) related to the ubiquitin proteasome system (UPS) in human placentas following pregnancy loss and PTB. We therefore hypothesized that similar mechanisms might underlie PPROM. In the current study, nine pairs of ubiquitin-proteasome-collagen (CUP) pathway–related mRNAs and associated lncRNAs were found to be differentially expressed in PPROM and sPTL. Pathway analysis showed that the functions of their protein products were inter-connected by ring finger protein. Twenty variants including five mutations were identified in CUP-related genes in sPTL samples. Copy number variations were found in COL19A1, COL28A1, COL5A1, and UBAP2 of sPTL samples. The results reinforced our previous findings and indicated the association of the CUP pathway with the development of sPTL and PPROM. This association was due not only to the genetic variation, but also to the epigenetic regulatory function of lncRNAs. Furthermore, the findings suggested that the loss of collagen content in PPROM could result from degradation and/or suppressed expression of collagens.

Keywords: sPTB, lncRNA, SNV, CNV, collagen, ubiquitin enzymes, UPS, CUP pathway

INTRODUCTION

Spontaneous preterm birth (sPTB) mainly consists of spontaneous preterm labor (sPTL) and preterm premature rupture of the membranes (PPROM). It refers to delivery that occurs before 37 gestational weeks (GWs) and is the leading cause of perinatal morbidity and mortality worldwide (Lawn et al., 2005). Etiologically, sPTB has many causes, including intra-amniotic infection, decidua senescence, and breakdown of maternal-fetal tolerance. The recognized risk factors underlying PPROM include physiologic weakening of the fetal membranes associated with apoptosis near term; dissolution of the amniochorionic matrix exacerbated by contraction-induced

shearing forces; infection and inflammation resulting from ascending genital tract colonization initiating a cytokine cascade that triggers membrane degradation; protease production and dissolution of the extracellular matrix (ECM); placental abruption with decidua thrombin expression triggering thrombin-thrombin receptor interactions and increasing choriodecidual protease production; and membrane stretching that may increase amniochorionic cytokine and protease release (Charles and Edwards, 1981; Skinner et al., 1981; Lavery et al., 1982; Taylor and Garite, 1984). The degradation of fetal membranes involved in sPTB is mediated through the activation of Toll-like receptors (TLRs) and causes an increase of matrix metalloproteinases (MMPs; Geraghty et al., 2011; Sandig and Bulfone-Paus, 2012). MMP1 and MMP8 are collagenases that have been found to degrade collagen types I–III and are upregulated in the amnion and chorion (Menon and Fortunato, 2004), which leads to collagenolysis and a decrease in the collagen content of fetal membranes (Draper et al., 1995). The increase in collagen solubility contributes to the remodeling of the ECM and further results in cervical softening and fetal membrane activation (Pollock et al., 1991). Collagen provides the major structural support for the fetal membranes, which is formed by the amnion and chorion. In addition, preterm contractions can accelerate the separation of the amnion and chorion, and then reduce membrane tensile strength, whereas cervical dilation can cause exposure of the membranes to vaginal microorganisms and reduce underlying tissue support (Strohl et al., 2010).

Genetic factors associated with PPROM have been reported. A significant association of a single nucleotide polymorphism (SNP) was found at the genes *MMP1* and *MMP8, CARD15, TLR4,* and *SERPINH1* among PPROM cases (Fujimoto et al., 2002; Wang et al., 2004, 2006). More studies have been carried out on sPTL. The gene loci of *ABCB11*, *BBS5*, *FSTL5*, *CSMD3*, *NTS*, *KLHL1*, and *NCAM2*, in addition to duplications at the loci of *OR4P4*, *OR4S2*, *OR4C6*, and *RASSF7*, have been shown to be associated with sPTL (Biggio et al., 2015).

Although non-coding RNAs (ncRNAs) are defined by the lack of a protein-coding potential, they have been found to play important roles in many biological processes (Mattick, 2009; Lipovich et al., 2010). The long non-coding RNAs (lncRNAs) are a subtype of ncRNAs with transcripts that are more than 200 nucleotides long without obvious protein-coding potential. Occasionally, lncRNAs may be translated to produce short peptides of unknown function (Fatica and Bozzoni, 2014; Ingolia et al., 2014). LncRNAs predominantly localize to the nucleus and have a lower level of expression than protein-coding regions of genes (Djebali et al., 2012). Based on the biological characteristics of transcription loci and their relationship with the associated genes, lncRNAs can be classified as exonic, intronic, or intergenic overlapping transcripts, in either sense or antisense orientation. LncRNAs may modify the expression of genes and be involved in diverse cellular processes including cell differentiation, imprinting control, and immune responses (Wilusz et al., 2009; Archer et al., 2015). The regulatory function of lncRNAs lies in their ability to alter the expression of DNA in a site-specific manner and, at the same time, bind to different proteins, bridging chromosomes, and protein complexes (Rinn and Chang, 2012; Geisler and Coller, 2013). Evidence increasingly supports the linkage of dysfunctions of lncRNAs to many human diseases, including neurodegenerative, psychiatric diseases (Faghihi and Wahlestedt, 2009), cardiovascular disease (Annilo et al., 2009), and immune dysfunction and auto-immunity (Kino et al., 2010). In our previous study, lncRNAs that are differentially expressed in human placentas delivered from PPROM and sPTL were found to be involved in more than 20 functional pathways (Luo et al., 2013). The patterns of differentially expressed lncRNAs and pathways identified from placentas of PPROM and sPTL were similar to those we observed in our study of human miscarriages (Wang et al., 2014) and of a viral-infected mouse model (Pan et al., 2015), suggesting that deregulation and dysfunction of the ubiquitin-proteasome-collagen (CUP) pathway may be one of the pathogenic mechanisms underlying the adverse outcomes of pregnancies, including PPROM.

On the basis of these findings, we hypothesized that the epigenetic regulatory role of lncRNAs in the ubiquitin proteasome system (UPS) and collagen remodeling is that they are involved in the CUP pathway in sPTB, including PPROM (Zhong et al., 2015). To test our hypothesis, we studied the lncRNAs and lncRNA-associated messenger RNAs (mRNAs) and identified gene mutations/variations associated with the CUP pathway.

MATERIALS AND METHODS

Ethics Statement

The study design was reviewed and approved by the Ethics Committee of Lianyungang Maternal and Children's Hospital, where all the specimens were collected and stored in an existing biobank, which was developed previously as a core service for the China Preterm Clinical Research Consortium. Written informed consent was obtained from the pregnant women who participated in this study. All material and data were previously coded and are anonymous to the authors of this study.

Samples

The samples used for the current study were human placentas, fetal membranes, and maternal peripheral blood. Placentas used in microarray hybridization have been described elsewhere (Luo et al., 2013). The criteria for selection of placenta samples were that they were from pregnancies with (1) no clinical signs of infection (no fever, no increase of white blood cell counts, no positive finding of amniotic fluid cultures), (2) no clinical intervention with antibiotics, steroids, or tocolytics during pregnancy, and (3) mother between 25 and 35 years of age. The placental samples were divided into two groups: preterm and full-term. The preterm group ($\leq$35 GW) was further subdivided into PPROM and sPTL. PPROM was defined as a pregnancy that had an initial clinical feature of rupture of membrane that triggered premature uterine contraction. sPTL was defined as the initial sign of labor being uterine contraction without rupture of membrane. The full-term group (between 39^{+0} and 40^{+6} GW), was divided into full-term birth (FTB) and premature rupture of membrane (PROM) at term. Ten samples of human placenta from each group (**Table 1**)—the sPTL (group A), FTB (group B),

TABLE 1 | Sample size used in the current study.

Study	Discovery	Validation	Exome sequencing
Type of sample	Placenta	Fetal membrane	Whole blood
Number of sample			
Group A (sPTL)	10	20	160
Group B (FTB)	10	20	99
Group C (PPROM)	10	20	
Group D (PROM)	10	20	
Subtotal	40	80	259

PPROM (group C), and PROM (group D)—were subjected to a discovery study with an lncRNA expression microarray (Luo et al., 2013). After the discovery study, 20 fetal membranes from each subgroup were subjected to validation with quantitative RT-PCR (qRT-PCR). The sampling process followed our in-house standard operating procedure. Briefly, immediately after delivery, the separated placentas and/or fetal membranes were rinsed with 200 ml saline twice and dried with sterilized paper towels. Placental tissues were collected with a sterilized scalpel that penetrated completely from the fetal membrane to the decidua as a cube (cm^3) of $1 \times 1 \times (2–3.5)$. A separate piece of fetal membrane (2×2 cm^2) was cut from the amniochorionic membrane (ACM) at the edge of the membrane rupture. The samples were then frozen immediately in liquid nitrogen for a minimum of 30 min before being transferred and stored in a -80°C freezer. An independent subset of 160 maternal blood samples was collected from women shortly before delivery by sPTL, and then was used for isolation of total DNAs followed by exome sequencing. An independent group of 99 women with normal FTB was subjected to sequencing analysis as the controls. These specimens had been previously banked in our existing cohort. The type and size of the samples are listed in **Table 1**. Comparisons were performed inter-group either individually (such as A vs. B) or combined (such as A+B vs. C+D).

Differential Expression Profiling of lncRNAs and mRNAs

The Arraystar Human LncRNA Array v2.0 (www.arraystar.com) was the technical platform for the discovery study. qRT-PCR was employed for validation, as reported earlier (Luo et al., 2013; Wang et al., 2014; Pan et al., 2015). In the discovery study, fold changes >2 and $p < 0.05$ were set as cut-offs and were considered significant. In the qRT-PCR study, β-actin (ACTB) was used as an internal control, and the expression values of lncRNAs and lncRNA-overlapped mRNAs were normalized to ACTB. For each RNA, the result of expression level was reported as relative expression by setting the expression value in FTB (subgroup B) at "1," and the expression value in other groups was calculated relative to this control. The data were subjected to one-way analysis of variance (one-way ANOVA) followed by an unpaired, two-tailed *t*-test. Differences were considered statistically significant at $P < 0.05$. In view of the multiple comparisons that were performed, to minimize the likelihood of a type I error, a Bonferroni correction was applied to the significance criterion (Miller, 1991). This correction is a common methodology to adjust for multiple comparisons that divides the significance criterion (usually 0.05) by the number of comparisons to derive a multiple-comparison-adjusted significance criterion. Additionally, use of FDR (False Discovery Rate) was applied to control multiple tests of correlations (Yekutieli and Benjamini, 1999).

Whole-Genome Exome Sequencing of sPTLs

Exome sequencing was performed with Hiseq 2000 (Illumina, SanDiego, CA, USA), for which the SureSelect Biotinylated Library (Agilent, Palo Alto, CA, USA) was constructed. The general workflow for calling of single nucleotide variations (SNVs), including SNPs, and of insertions/deletions (InDels) followed vendors' recommendations. Bioinformatic analysis with the Burrows-Wheeler Aligner (Li and Durbin, 2010) was used to align individual "clean data," and the genotype likelihoods were generated with SAM tools (Szklarczyk et al., 2015). Linkage disequilibrium (LD)–based multiple-sample genotype calling was performed using the LD-based Beagle (Hampson et al., 1997) for multiple-sample genotype calling. Bioinformatic analysis of co-expression and function analysis was performed with the computer programs GeneMANIA (Warde-Farley et al., 2010) and STRING (Szklarczyk et al., 2015).

RESULTS

Identification of CUP-Associated lncRNAs/mRNAs from Human Placentas

As shown in **Table 2A**, nine CUP-associated lncRNAs were identified to be differentially expressed in human placentas, with extremely high statistical significance at $P < 10^{-6}$. When the AB groups were compared to the CD groups, three lncRNAs—the ENST00000504601, CR602937, and NR_029434—were found to be upregulated, and two—the AX747492 and AK125314—were downregulated in pregnancies without rupture of fetal membranes. When sPTL (A) was compared to PPROM (C) individually, lncRNA ENST00000413033 was downregulated, but uc.173 was upregulated. LncRNA G42992 was downregulated in PPROM when compared to FTB, and ENST00000482477 was upregulated in PROM (D) vs. FTB (B).

Forty-nine CUP-associated mRNAs were differentially expressed in human placentas (**Table 2B**), mostly with considerable statistical significance at $P < 10^{-10}$. Among these mRNAs, two were the transcripts of collagen, 22 were ubiquitin enzymes, and four were proteases/proteasomes. Collagen-associated mRNAs (COL-mRNAs) were mainly upregulated in [sPTL+PPROM] vs. [FTB+PROM] and PPROM vs. sPTL, indicating that COL-mRNAs were upregulated in PPROM. Eight mRNAs of ubiquitination enzymes (UBE-mRNAs)—the UBAP1, UBAP2, USP16, USP24, UBE2L6, UBE2Q2, UBE2Z, and UBL3—were identified to be upregulated in PPROM vs. sPTL and downregulated in [sPTL+FTB] vs. [PPROM+PROM]. Seven downregulated UBE-mRNAs (UBAC2, UBE2D3, UBE2E3, UXT, USP20, USP27X, and USP50) in PPROM vs. sPTL were also

TABLE 2A | CUP-associated differentially expressed lncRNAs identified from human placentas.

	Comparison regulation	*P* value	FC	Seqname	Gene symbol	Relationship	Associated_ gene_acc	Associated_ gene_name	Associated protein_name
Collagen	A+B vs. C+D_up	4.8E−07	2.5	ENST00000504601	RP11-893F2.4	Natural antisense	NM_000088	COL1A1	Collagen alpha-1(I) chain preproprotein
	A+B vs. C+D_up	6.2E−20	2.6	CR602937		Natural antisense	NM_030582	COL18A1	Collagen alpha-1(XVIII) chain isoform 1
	A vs. C_down	8.2E−07	2.0	ENST00000413033	RP5-1106H14.1	Intronic antisense	NM_133457	COL26A1	Collagen alpha-1(XXVI) chain
Ubiquitin Enz.	A+B vs. C+D_up	1.4E−6	4.1	AX747492	lincRNA-HFE2	Intron sense-overlap	NM_006099	PIAS3	E3 SUMO-protein ligase PIAS3
	A vs. C_up	1.0E−10	3.7	uc.173-	uc.173	Natural antisense	NM_003337	UBE2B	Ubiquitin-conjugating enzyme E2 B
	C vs. B_down	4.9E−14	3.2	G42992		Natural antisense	NM_183399	RNF14	E3 Ubiquitin-protein ligase RNF14 isoform 1
Proteasome	A+B vs. C+D_up	2.8E−25	2.0	NR_029434	FLJ31306	Intronic antisense	NM_152132	PSMA3	Proteasome subunit alpha type-3 isoform 2
	A+B vs. C+D_down	2.8E−14	2.1	AK125314		Intron sense-overlap	NM_001128592	PSMG4	Proteasome assembly chaperone 4 isoform a
	D vs. B_up	1.9E−07	2.3	ENST00000482477	AC009299.5	Intronic antisense	NM_005805	PSMD14	26S proteasome non-ATPase regulatory subunit 14

A, sPTL; B, FTB; C, PPROM; D, PROM; FC, fold change; Seqname, name of lncRNA; acc, accession; Enz, enzyme.

upregulated in [sPTL+FTB] vs. [PPROM+PROM], suggesting that these eight upregulated and seven downregulated UBEs are associated with PROM in PPROM but not in sPTL. Similarly, the proteasomal protease PSMB8 was upregulated in PPROM vs. sPTL but downregulated in [sPTL+FTB] vs. [PPROM+PROM]. PRSS54 was downregulated in PPROM vs. sPTL, but PRSS33 was upregulated in [sPTL+FTB] vs. [PPROM+PROM].

Validation of Differentially Expressed CUP-lncRNAs and CUP-mRNAs

Nine pairs of lncRNAs and lncRNA-overlapped mRNAs were selected for validation with qRT-PCR. The selection was based on the following criteria: (1) the mRNAs had been found to be differentially expressed between subgroups; (2) the functional product of the mRNAs was involved in either the UPS or collagen remodeling; and (3) the differentially expressed lncRNAs were mostly antisense. The differential expression patterns (DEPs) of these RNAs are shown in **Tables 3**, **4**. In placenta samples, the greatest difference in the expression pattern of RNAs was found between the rupture-of-membrane group [PPROM + PROM] and the labor-without-membrane-rupture group (FTB + sPTL), as nearly all RNAs were transcribed at different levels with statistical significance ($P < 0.05$), except for UBE2B mRNA. When the sPTL subgroup was compared to the PROM subgroup, nine lncRNAs and seven mRNAs were found to be differentially expressed, and when the FTB to PPROM subgroups were compared, eight lncRNAs and seven mRNAs, respectively, were found to be differentially expressed among placentas. When validated with human fetal membranes (the ACMs), however, the DEP of intra-group variations was slightly different from that of placentas (**Figure 1**).

Co-expression Network and Functional Interactions among CUP-Associated Genes

CUP-associated gene loci, including *COL18A1*, *COL1A1*, *EMID2*, *PIAS3*, *PSMA3*, *PSMD14*, *PSMG4*, *RNF14*, and *UBE2B*, were subjected to analysis of their network and interactions. As shown in **Figure 2A**, all eight loci of lncRNA-mRNA pairs were present in the functional network in terms of co-expression. The whole network consists of two intensive co-expressed groups, the collagen group (COL1A1 and COL18A1) and the UPS-related group (PSMD14, PSMG4, PSMA3, UBE2B, RNF14), which were connected by PIAS3 and six other UPS-associated genes. The analysis also showed that PSMD14 and PSMA3 were both involved in the G1 DNA damage checkpoint, antigen procession, and presentation of exogenous peptide antigen via MHC class I. STRING (Szklarczyk et al., 2015) illustrated a similar result (**Figure 2B**): the proteasome-related genes PSMD14, PSMA3, and five other genes formed shared common protein homology and expression regulation, as did the collagen group, which includes COL18A1 and COL1A1. These two functional groups were then joined by RNF14, PIAS3, and UBE2B through pathways identified in published research articles. Apart from being present in proteasome subunits, PSMD14 and PSMA3 were associated with Epstein-Barr virus infection. PSMD14 consists

TABLE 2B | CUP-associated differentially expressed mRNAs identified from human placentas.

CUP	Comparison _regulation	*P* value	FC	Gene accession	Gene symbol	Unigene	Protein accession	Protein
Collagen	A+C vs. B+D_up	5.7E–18	2.76	NM_031361	COL4A3BP	Hs.270437	NP_112729	Collagen, type IV, alpha 3 (Goodpasture antigen) binding protein
	A+C vs. B+D_up	4.2E–05	3.81	NM_000494	COL17A1	Hs.117938	NP_000485	Collagen, type XVII, alpha 1
	C vs. A _up	4.3E–17	3.51	NM_031361	COL4A3BP	Hs.270437	NP_112729	Collagen, type IV, alpha 3 (Goodpasture antigen) binding protein
	C vs. A _up	9.6E–09	6.78	NM_000494	COL17A1	Hs.117938	NP_000485	Collagen, type XVII, alpha 1
Ubiquitin Enz.	A+B vs. C+D_up	1.2E–19	2.03	NM_001144072	UBAC2	Hs.508545	NP_808882	UBA domain containing 2
	A+B vs. C+D_up	1.0E–07	2.37	NM_181892	UBE2D3	Hs.518773	NP_871622	Ubiquitin-conjugating enzyme E2D 3 (homolog, yeast)
	A+B vs. C+D_up	1.8E–19	2.73	NM_006357	UBE2E3	Hs.470804	NP_872619	Ubiquitin-conjugating enzyme E2E 3 (homolog, yeast)
	A+B vs. C+D_up	7.8E–19	2.68	NM_153477	UXT	Hs.172791	NP_705582	Ubiquitously-expressed transcript
	A+B vs. C+D_up	1.7E–10	2.11	NM_001110303	USP20	Hs.5452	NP_006667	Ubiquitin specific peptidase 20
	A+B vs. C+D_up	1.8E–19	2.46	NM_001145073	USP27X	Hs.143587	NP_001138545	Ubiquitin specific peptidase 27, X-linked
	A+B vs. C+D_up	2.4E–15	2.18	NM_001098536	USP5	Hs.631661	NP_003472	Ubiquitin specific peptidase 5 (isopeptidase T)
	A+B vs. C+D_up	1.5E–18	2.14	NM_203494	USP50	Hs.677758	NP_987090	Ubiquitin specific peptidase 50
	A+B vs. C+D_down	8.3E–18	2.77	NM_016525	UBAP1	Hs.268963	NP_057609	Ubiquitin associated protein 1
	A+B vs. C+D_down	3.5E–19	2.72	NM_018449	UBAP2	Hs.493739	NP_060919	Ubiquitin associated protein 2
	A+B vs. C+D_down	1.2E–17	2.29	NM_001001992	USP16	Hs.99819	NP_006438	Ubiquitin specific peptidase 16
	A+B vs. C+D_down	3.9E–17	2.54	NM_015306	USP24	Hs.477009	NP_056121	Ubiquitin specific peptidase 24
	A+B vs. C+D_down	3.6E–15	2.01	NM_004223	UBE2L6	Hs.425777	NP_937826	Ubiquitin-conjugating enzyme E2L 6
	A+B vs. C+D_down	1.1E–19	2.39	NM_001145335	UBE2Q2	Hs.23033	NP_775740	Ubiquitin-conjugating enzyme E2Q family member 2
	A+B vs. C+D_down	5.4E–17	2.67	NM_173469	UBE2Q2	Hs.23033	NP_775740	Ubiquitin-conjugating enzyme E2Q family member 2
	A+B vs. C+D_down	1.7E–11	2.10	NM_023079	UBE2Z	Hs.514297	NP_075567	Ubiquitin-conjugating enzyme E2Z
	A+B vs. C+D_down	2.5E–17	2.47	NM_007106	UBL3	Hs.145575	NP_009037	Ubiquitin-like 3
	C vs. A _down	2.1E–10	2.07	NM_001144072	UBAC2	Hs.508545	NP_808882	UBA domain containing 2
	C vs. A _down	1.9E–06	2.51	NM_001110303	USP20	Hs.5452	NP_006667	Ubiquitin specific peptidase 20
	C vs. A _down	2.0E–09	2.27	NM_001145073	USP27X	Hs.143587	NP_001138545	Ubiquitin specific peptidase 27, X-linked
	C vs. A _down	1.2E–08	2.09	NM_203494	USP50	Hs.677758	NP_987090	Ubiquitin specific peptidase 50
	C vs. A _down	4.1E–03	2.07	NM_181892	UBE2D3	Hs.518773	NP_871622	Ubiquitin-conjugating enzyme E2D 3 (homolog, yeast)
	C vs. A _down	5.5E–09	2.70	NM_006357	UBE2E3	Hs.470804	NP_872619	Ubiquitin-conjugating enzyme E2E 3 (homolog, yeast)
	C vs. A _down	1.7E–13	2.18	NM_194259	UBE2I	Hs.302903	NP_919237	Ubiquitin-conjugating enzyme E2I (UBC9 homolog, yeast)
	C vs. A _down	5.5E–08	2.15	NM_153477	UXT	Hs.172791	NP_705582	Ubiquitously-expressed transcript
	C vs. A _up	1.3E–11	3.08	NM_016525	UBAP1	Hs.268963	NP_057609	Ubiquitin associated protein 1
	C vs. A _up	8.8E–08	2.29	NM_018449	UBAP2	Hs.493739	NP_060919	Ubiquitin associated protein 2
	C vs. A _up	2.5E–10	2.03	NM_015902	UBR5	Hs.492445	NP_056986	Ubiquitin protein ligase E3 component n-recognin 5
	C vs. A _up	2.7E–07	2.27	NM_175748	UBR7	Hs.728932	NP_786924	Ubiquitin protein ligase E3 component n-recognin 7 (putative)
	C vs. A _up	8.4E–14	2.88	NM_001001992	USP16	Hs.99819	NP_006438	Ubiquitin specific peptidase 16
	C vs. A _up	2.6E–11	2.33	NM_017414	USP18	Hs.38260	NP_059110	Ubiquitin specific peptidase 18
	C vs. A _up	2.4E–08	2.18	NM_015306	USP24	Hs.477009	NP_056121	Ubiquitin specific peptidase 24
	C vs. A _up	1.2E–08	2.03	NM_004223	UBE2L6	Hs.425777	NP_937826	Ubiquitin-conjugating enzyme E2L 6

(Continued)

TABLE 2B | Continued

CUP	Comparison _regulation	*P* value	FC	Gene accession	Gene symbol	Unigene	Protein accession	Protein
	C vs. A _up	4.8E–10	2.45	NM_001145335	UBE2Q2	Hs.23033	NP_775740	Ubiquitin-conjugating enzyme E2Q family member 2
	C vs. A _up	1.4E–11	2.47	NM_173469	UBE2Q2	Hs.23033	NP_775740	Ubiquitin-conjugating enzyme E2Q family member 2
	C vs. A _up	8.1E–08	2.31	NM_023079	UBE2Z	Hs.514297	NP_075567	Ubiquitin-conjugating enzyme E2Z
	C vs. A _up	2.5E–12	2.85	NM_007106	UBL3	Hs.145575	NP_009037	Ubiquitin-like 3
	A vs. B _up	2.4E–05	2.60	NM_199144	UBE2V1	Hs.727525	NP_954595	Ubiquitin-conjugating enzyme E2 variant 1
	A vs. B _down	6.5E–13	2.03	NM_181762	UBE2A	Hs.379466	NP_861442	Ubiquitin-conjugating enzyme E2A (RAD6 homolog)
Protease	A+B vs. C+D_up	2.4E–07	2.09	NM_152891	PRSS33	Hs.280658	NP_690851	Protease, serine, 33
	A+B vs. C+D_down	2.6E–20	2.18	NM_148919	PSMB8	Hs.180062	NP_683720	Proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)
	C vs. A_down	6.1E–06	2.20	NM_001080492	PRSS54	Hs.411239	NP_001073961	Protease, serine, 54
	C vs. A_up	1.3E–12	2.27	NM_148919	PSMB8	Hs.180062	NP_683720	Proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7)
	A vs. B_up	4.1E–05	3.23	NM_002804	PSMC3	Hs.250758	NP_002795	Proteasome (prosome, macropain) 26S subunit, ATPase, 3

A, sPTL; B, FTB; C, PPROM; D, PROM; FC, fold change; Enz, enzyme.

of JAB1/MPN/MOV34 metalloenzyme domain and was involved in the maintenance of mitochondrial structure and function (Cooper et al., 2009).

Identification of SNVs including Mutations and Copy Number Variations Associated with CUP Pathway

In the sPTL subgroup, 20 variants, including five mutations, were identified in five collagen genes, three ubiquitin ligase genes, and four ubiquitin-associated proteasome/peptidase genes (**Table 5**) identified by whole-exome sequencing (Rutishauser et al., 2001). Fourteen of these variations occurred in the intronic region of the gene, and most were deletions. For those exonic mutations, all four were missense, and one was synonymous. Copy number variations (CNVs) were found in four CUP-associated genes from women who delivered by sPTL: COL19A1, COL28A1, COL5A1, and UBAP2. All CNVs were at exons of the genes: three duplications and one deletion (**Table 6**). The lengths of these CNVs varied greatly, from 700 to more than 388,000 base pairs.

DISCUSSION

The current study is a continuing investigation of the regulatory role of lncRNAs in the molecular pathogenesis of sPTB, based on our previous studies, in which we identified differential expression of lncRNAs in sPTL and PPROM (Luo et al., 2013; Pan et al., 2015). In the current study, we analyzed the DEPs of the lncRNAs and the mRNAs that are associated with the CUP pathway that may also associate with PPROM (Zhong et al., 2015). We also identified gene mutations/variations among sPTBs, although a larger sample size will be needed to make a definitive conclusion. These findings provided evidence of the involvement of the CUP pathway in the pathogenesis of PPROM, which could be further supported by the protein interaction network, as shown in **Figure 2**. Although the algorithms of GeneMANIA (Warde-Farley et al., 2010) and STRING (Szklarczyk et al., 2015) yielded results with slight differences, a clear connection between the collagen and the UPS by an E3 ligase-like protein, PIAS3, was observed (**Figure 2B**).

Currently, the cause of preterm weakening of fetal membranes leading to PPROM remains unclear. Several studies have concluded the collagen degradation to be the major factor in remodeling of fetal membrane, as the collagen content was lowered in the ruptured membranes (Kanayama et al., 1985; Hampson et al., 1997). The strength of amnion and chorion is basically due to collagen fiber, and the process above reduces the physical strength of the fetal membranes. The major strength in the amnion was shown to be derived from collagen I (extensively in the compact layer and adjacent mesoderm) and collagen IV (a major component of the basement membrane and of the bundles connecting the mesenchymal layer and the epithelium; Bachmaier and Graf, 1999). COL18A1, COL1A1, and EMID2 were all within the collagen family, among which COL1A1 was involved in most human connective tissues, and COL18A1 and EMID2 were shown to be directly associated with the formation and remodeling of the extracellular matrix (Rebhan et al., 1993; Hoffmann et al., 2015). In this study, we have identified both gene mutations and abnormal gene expression. A missense mutation (**Table 5**) was found within the coding sequence of gene *COL23A1* from sPTL blood samples. The mutation causes the residue of the 102nd amino acid to be changed from glycine

TABLE 3 | Correlation of differentially expressed CUP-lncRNAs-mRNAs identified from human placentas.

		PPROM vs. sPTL		PPROM vs. PROM		PPROM vs. FTB		sPTL vs. FTB		sPTL vs. PROM		FTBvs. PROM		(PPROM+PROM) vs. (FTB+sPTL)		(PPROM+sPTL) vs. (FTB+PROM)	
lncRNA	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**
504601	Col1A1	Up	Up	Up*	Down*	Down*	Up*	Down*	Up*	Up*	Down*	Up	Down*	Down*	Up*	Up	Down
CR602937	Col18A1	Down*	Up*	Up*	Up*	Down*	Up*	Down*	Up	Up*	Up	Up*	Up*	Down*	Down*	Up	Up
413033	COL26A1	Up*	Up*	Up*	Up	Up*	Up	Down	Down	Down*	Down*	Down*	Down	Up*	Up*	Up	Up*
uc173	UBE2B	Down*	Up*	Up*	Up*	Down*	Up*	Down*	Down*	Up*	Down*	Up*	Up*	Down*	Down	Up	Up
G42992	RNF14	Down*	Down*	Up	Up	Down*	Down*	Down*	Down	Up*	Up*	Up*	Up*	Down*	Up*	Up*	Down
AX747492	PIAS3	Up*	Up	Up*	Up	Up*	Up	Down*	Down*	Down*	Down*	Down*	Up	Down*	Up*	Up	Up*
NR_029434	PSMA3	Down*	Up*	Up	Up	Down	Up*	Down	Down*	Up*	Down*	Up*	Up*	Down*	Down*	Up	Up
482477	PSMD14	Up*	Up*	Up	Down*	Up*	Up*	Up	Up*	Down*	Down*	Down*	Down*	Up*	Up*	Up	Up
AK125314	PSMG4	Up*	Up*	Up*	Down*	Up*	Down*	Down*	Down*	Down*	Down	Down*	Down	Up*	Up*	Up*	Down*

*Statistical significance at $P < 0.05$.

TABLE 4 | Verification of CUP-lncRNAs correlated to CUP-mRNAs with fetal amniochorionic membranes.

		PPROM vs. sPTL		PPROM vs. PROM		PPROM vs. FTB		sPTL vs. FTB		sPTL vs. PROM		FTB vs. PROM		(PPROM+PROM) vs. (FTB+sPTL)		(PPROM+sPTL) vs. (FTB+PROM)	
lncRNA	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**	**lncRNA**	**mRNA**
504601	Col1A1	Up	Down*	Up	Down	Up	Down*	Down	Up	Down	Down	Down	Up	Up*	Down*	Up	Down
CR602937	Col18A1	Up*	Down	Up*	Down	Up*	Down	Up	Down	Down	Down	Down	Up*	Up*	Down	Up*	Down
413033	COL26A1	Up*	Up	Up	Down*	Up	Down*	Down*	Down	Down*	Down	Down	Up	Up*	Down	Down	Down*
uc173	UBE2B	Up*	Up*	Up	Down	Up*	Up*	Down	Up	Down*	Down	Down*	Down*	Up*	Up*	Up	Up
G42992	RNF14	Up*	Down*	Down	Down	Up	Up*	Down*	Down*	Down	Down	Down*	Down*	Up*	Up*	Down	Down
AX747492	PIAS3	Up	Up*	Down	Up	Up*	Up*	Down	Down	Down	Down	Down*	Down	Up*	Up	Down	Up*
NR_029434	PSMA3	Down	Up	Down*	Up	Down	Up*	Down	Up	Down	Down	Up	Down	Down*	Up*	Down	Up
482477	PSMD14	Up	Up*	Up	Up	Up*	Up	Up	Down	Down	Down*	Down*	Up	Up*	Up*	Up	Up
AK125314	PSMG4	Up	Up*	Up	Up	Up	Up*	Down	Up	Down	Down	Down	Down*	Up	Up*	Up	Up*

*Statistical significance at $P < 0.05$.

TABLE 5 | Identification of CUP-associated gene mutations/variations from maternal blood specimens from sPTL.

Gene	*P*-value	Chrom	Position	Category	Ref_alle	Mut_alle	Type	AA	Significance	Description
COL23A1	0.007972	chr5	177987740	exon2	C	T	Point_M	p.G102R	Nonsynonymous	Collagen, type XXIII, alpha 1
COL4A2	0.02807	chr13	111077400	intronic	AT	A	InDel		Del_T	Collagen, type IV, alpha 2
COL4A2	0.04936	chr13	111120699	intronic	C	CT	InDel		Ins_T	Collagen, type IV, alpha 2
COL18A1	0.0086	chr21	46900356	intronic	G	C			SNV	Collagen, type XVIII, alpha 1
COL22A1	0.02772	chr8	139691995	intronic	T	TGTTA	InDel		Ins_GTTA	Collagen, type XXII, alpha 1
COL23A1	0.007972	chr5	177987780	intronic	G	A			SNV	Collagen, type XXIII, alpha 1
COL28A1	0.02708	chr7	7459630	intronic	T	TTATG	InDel		Ins_TATG	Collagen, type XXVIII, alpha 1
COL28A1	0.02755	chr7	7459616	intronic	AT	A	InDel		Del_T	Collagen, type XXVIII, alpha 1
UBE2F-SCLY	0.00987	chr2	239007755	ncRNA_ exonic	C	CATT	InDel		Ins_ATT	UBE2F-SCLY readthrough
UBR5	0.002894	chr8	103306033	exon34	T	C	Point_M	p.V1463V	Synonymous	Ubiquitin protein ligase E3 component n-recognin 5
UBR5	0.002894	chr8	103327127	intronic	A	C			SNV	Ubiquitin protein ligase E3 component n-recognin 5
UBA7	0.02729	chr3	49842881	intronic	TG	T	InDel		Del_G	Ubiquitin-like modifier activating enzyme 7
USP31	0.001936	chr16	23102027	exon7	C	A	Point_M	p.D445Y	Nonsynonymous	Ubiquitin specific peptidase
USPL1	0.005818	chr13	31233063	exon9	G	A	Point_M	p.S950N	Nonsynonymous	Ubiquitin specific peptidase like 1
USPL1	0.005818	chr13	31231806	exon9	T	C	Point_M	p.L531S	Nonsynonymous	Ubiquitin specific peptidase like 1
PSMG2	0.02867	chr18	12712817	intronic	C	CATT	InDel		Ins_ATT	Proteasome (prosome, macropain) assembly chaperone 2
PSMA4	0.03335	chr15	78836631	intronic	CTGA	C	InDel		Del_TGA	Proteasome (prosome, macropain) subunit, alpha type, 4

P val (Rubba et al., 2001), P-value of co-variants; Ref_alle, reference allele; Mut-alle, mutant allele; AA, amino acid; Chrom, chromosome; InDel, Insertion and deletion; Ins, insertion; Del, deletion; SNV, single nucleotide variation.

to arginine (Wang et al.), which could cause an interruption in the formation of the normal structure of collagen (Lee et al., 1997). It would be interesting to introduce this mutation into the mouse model to investigate whether the missense mutation may generate the sPTL phenotype. Several InDels were identified in the intronic region of collagens from the sPTL cases, indicating that these SNVs may have a genetic predisposition that might function in gene-environmental interactions, in which the environmental factor(s) may induce the epigenetic regulation that consequently may trigger the DEP and influence transcription. In fact, our data showed that mRNAs of COL1A1, COL18A1, and EMID2 (COL26A1) were all downregulated in ruptured membranes in the PPROM subgroup. Interestingly, the expression of lncRNAs 504601 and CR602937, which overlap with COL1A1 and COL18A1, respectively, has been shown to be upregulated in PPROM (**Figure 1**). Both lncRNAs 504601 and CR602937 are located at the lagging strand as the antisense, opposite from the leading strand of coding genes *COL1A1* and *COL18A1*. Like microRNAs (miRNAs; Jalali et al., 2013), these lncRNAs may also function as a suppressor to down-regulate their complementary mRNAs. Should this hypothesis be confirmed, a novel therapeutic strategy with small interfering RNA could be designed for prevention of sPTB. It would be worth to expand the sPTB cases to replicate our findings in a larger sample size among different ethnic populations globally.

Differential expression of the lncRNAs and mRNAs of ubiquitin-conjugating protein identified from expression array and qPCR suggested the involvement of the UPS in sPTL and PROM. The UPS is an ATP-dependent, non–lysosomal-proteolytic system. The whole process is shown in **Figure 3**. The protein product of three genes screened by qPCR belongs to ubiquitin ligase, functioning at different stages of ubiquitination. UBE2B, a member of the ubiquitin-conjugating enzyme family, works as an E2. RNF14 contains a RING zinc finger and can interact with E2s, acting as a ubiquitin-ligase (E3). These genes were found to be overexpressed in PPROM, suggesting that the ubiquitination process was boosted with rupture of the membrane. In the paired lncRNA-mRNA of uc173-UBE2B and G4299-RNF14, there is a clear correlation, in terms of the DEP, between lncRNAs and mRNAs. Apparently, lncRNAs were present as an activator, whereby when the lncRNA is up-regulated in PPROM, the mRNA is up-regulated accordingly. Both lncRNAs uc173 and G4299 are natural antisense. However, they may have a different epigenetic regulatory mechanism, compared to lncRNAs 504601 (COL1A1) and CR602937 (COL18A1). It is likely that the lncRNAs uc173 and G4299 might function as a scaffold to bind to transcriptional factors and facilitate the gene transcription (Engreitz et al., 2016). Previously, Faghihi et al. studied a similar phenomenon in Alzheimer's disease, describing antisense transcripts that can increase mRNA stability by making the binding sites (Faghihi et al., 2008; Faghihi and Wahlestedt, 2009).

The lncRNAs for PSMA3 and PSMD14 were both intronic antisense, but were demonstrated to have a distinct pattern.

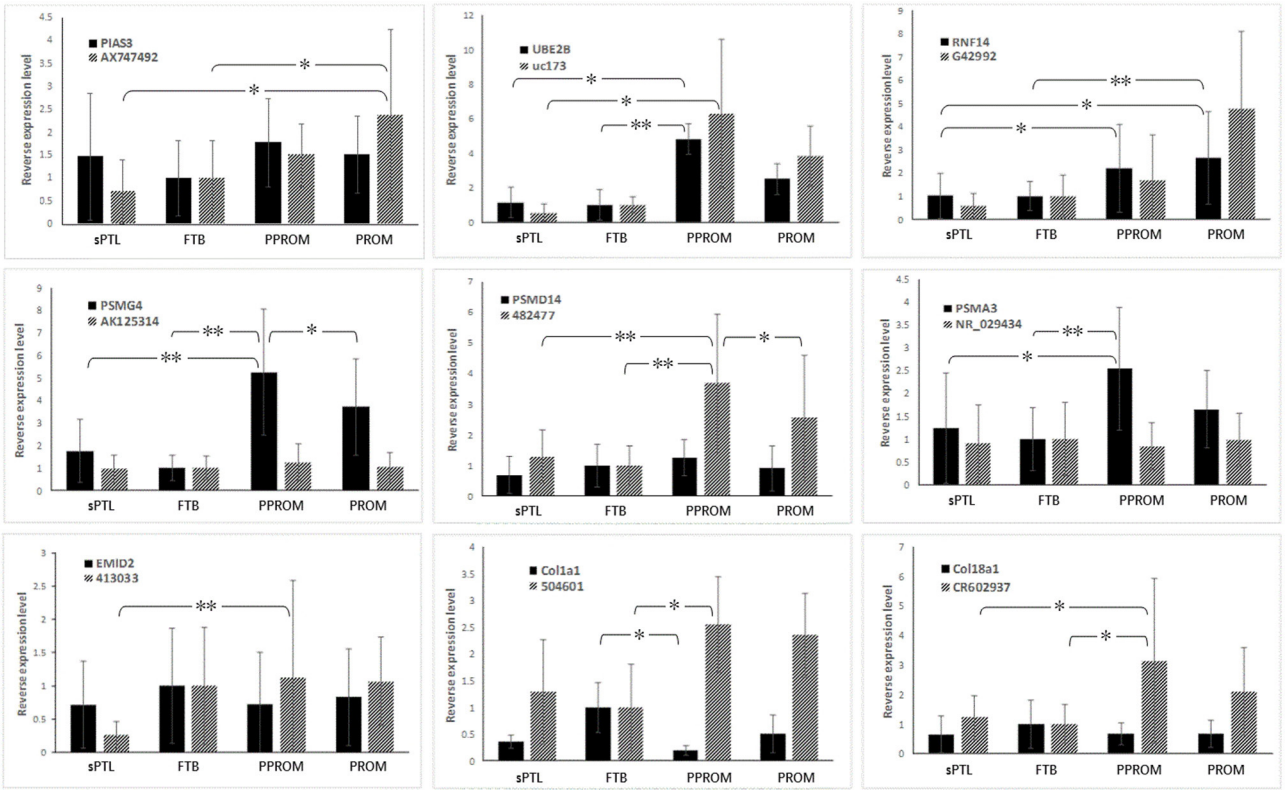

FIGURE 1 | Expression of CUP-associated lncRNAs and overlapped-mRNAs in human fetal membranes. Black bars indicate mRNA, and gray bars indicate lncRNAs. *$p < 0.05$, **$p < 0.001$.

LncRNA ENST00000482477 presented with the most varied expression pattern, and its level was shown to be greatly increased in PPROM, whereas levels of the lncRNAs AK125314 and NR_029434 remained steady among groups. Like PIAS3, both the PSMG4 mRNA and the lncRNA AK125314 that overlapped with PSMG4 were located at the same (sense) strand, and both were upregulated. Because both lncRNAs and mRNAs are transcribed as the sense strand, the possible mechanism of lncRNAs regulating mRNAs is that lncRNAs may bind to miRNAs, functioning as a sponge, which protects mRNAs from miRNA targeting and repressing. As a result, the transcription of mRNA is upregulated, and the level of mRNA is increased. In this case, the lncRNAs may act as a sink of miRNAs (Poliseno et al., 2010). The paired lncRNA of PSMA3 was intronic antisense. Hawkins and Morris (2010) reported that antisense lncRNAs can bind chromatin and chromatin-modifying proteins, facilitating epigenetic regulation. The expression pattern of PSMD14 and its paired lncRNA ENST00000482477 was similar to that of UBE2B and RNF14; the lncRNAs might regulate their mRNAs by interacting with the miRNA-binding sites.

Whether the differentially expressed lncRNAs that were identified from the human placentas derived from the sPTB are the etiological cause for, or the result of, PPROM is unknown. To better understand this, further investigation of CUP-associated lncRNA expression at the early stage of pregnancy and of the dynamic expression profile of lncRNA longitudinally during the entire pregnancy will be necessary. The lncRNAs in the maternal circulation could be assessed through quantitation of placenta-originated exosomes isolated from maternal blood. Comparing the lncRNA expression profile at early pregnancy and at term-labor may shed light on this important question.

Both pathway analyses indicated the importance of PSMA4 and PSMD14, as they appear to be the central bridge linking the collagens and ubiquitin enzymes to the proteasome proteins. PSMA4 harbors more connections with ubiquitin enzymes (**Figure 2**). Together with the expression level, we hypothesize that the suppression of collagen and the upregulation of the UPS were functionally connected and then were associated with rupture of the membranes, as a cause or a result, and within the UPS, the proteolysis process was mediated through PSMA4, rather than via PSMD14.

CNVs have been studied extensively for years, and their associations with various diseases have been proved, but the possible association between CNVs and sPTL/PPROM was studied very little in comparison. We applied maternal sera samples in the screening and identified four CNVs affecting seven genes. Results of the study by Biggio et al. of CNVs

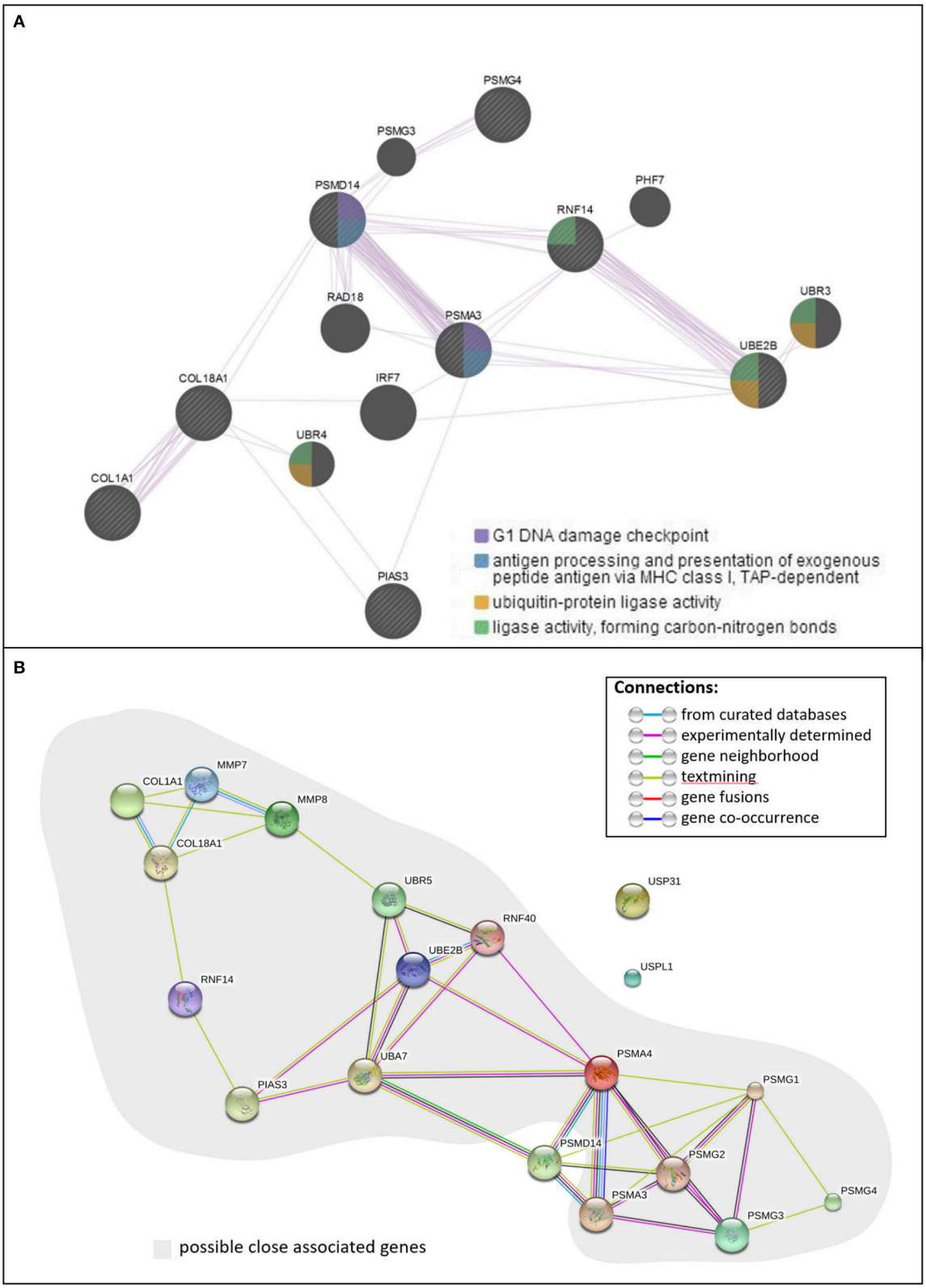

FIGURE 2 | The functional pathway analysis. (A) Function and co-expression network of CUP genes, **(B)** interactions of CUP genes.

TABLE 6 | Identification of CUP-associated CNVs from maternal blood specimens from sPTL.

Gene	Category	Chromosome	Starting position	Ending position	CNV	Allele frequency
COL19A1	exonic	Chr 6	70865930	70866694	Del	0.01
COL28A1, MIOS, RPA3	exonic	Chr 7	7528941	7917273	Dup	0.01
COL5A1, RXRA	exonic	Chr 9	137309042	137623969	Dup	0.01
UBAP2	exonic	Chr 9	33986768	34017213	Dup	0.01

Chr, chromosome; CNV, copy number variation; Del, deletion; Dup, duplication.

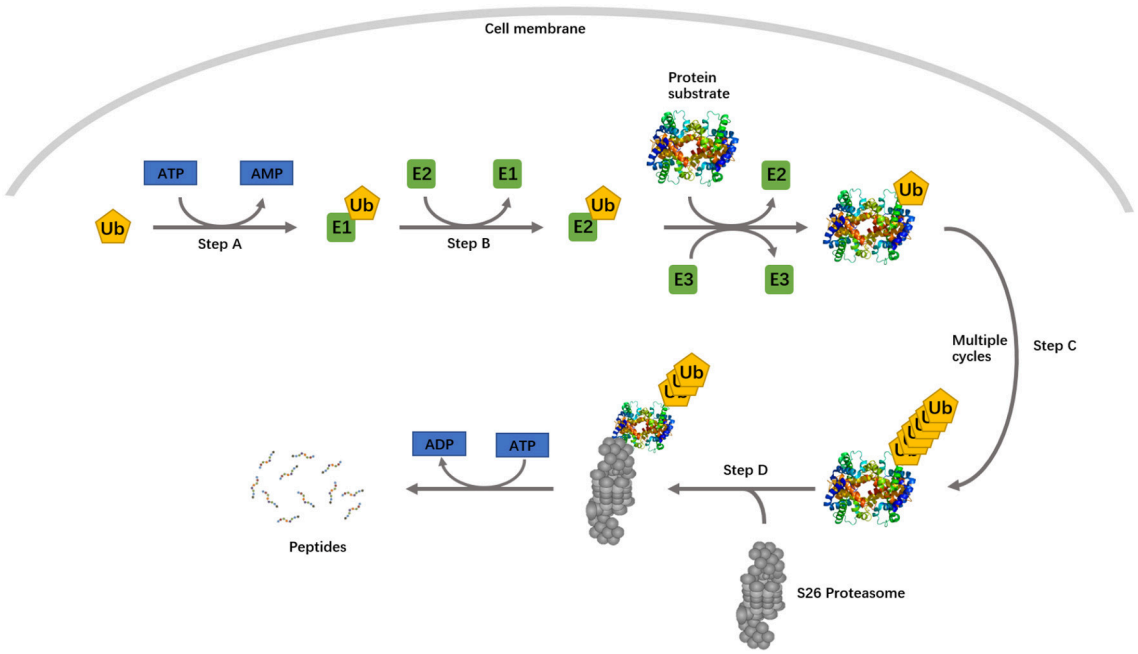

FIGURE 3 | The ubiquitin proteasome system. Step A, ubiquitin is activated by a ubiquitin-activating enzyme, E1. Step B, activated ubiquitin is transferred to a ubiquitin-conjugating enzyme, E2. In step C, ubiquitin is subsequently conjugated to target proteins in a process mediated by an E3 ubiquitin ligase. Step D, the polyubiquitylated substrate protein is degraded by the 26S proteasome. A single E1 enzyme can transfer ubiquitin to all E2s in the cells, and each of the E2s associates with a restricted set of E3s that confer substrate specificity.

in more than 1,000 American maternal and neonatal preterm birth samples and term controls showed that only neonatal, not maternal, CNVs were associated with early sPTL (Biggio et al., 2015). However, our previous work on the Chinese population indicated the contribution of maternal CNVs at the RYR1 locus to sPTL (Liu et al., 2013). Research on the Danish population reported the association of maternal CNVs in GSTT1/GSTT2 with smoking, preterm delivery, and low birth weight (Zheng et al., 2013). In the current study, the sample size may not be large enough for statistical evaluations, but the findings nevertheless suggest both race specificity for sPTL and areas for further study on the effects of these involved genes.

In conclusion, differentially expressed lncRNAs and mRNAs, polymorphisms, mutations, and CNVs identified from human placenta and fetal membrane of PPROM supported the involvement of the UPS in the development of PPROM and the possible regulatory pattern of the lncRNAs to their associated mRNAs. The results may also indicate that the loss of collagen content in PPROM was the result of not only degradation, but also of the suppressed expression, of collagen mRNAs. Furthermore, we studied the functional links of collagen to the UPS in PPROM and identified the central connector in proteasome proteins. However, the detailed mechanisms through which lncRNAs regulated their associated mRNAs in sPTL and PPROM must be further studied. On the basis of our data presented here, we propose a "two-hit" hypothesis in which genetic variations/mutations including SNVs and CNVs present as the first hit, which is genetic predisposition. Epigenetic regulation, such as lncRNAs, present as the second hit to modulate the outcome of pregnancy through the lncRNAs' epigenetic regulatory function. Our findings provide a new path for investigating the pathogenesis of sPTL and PPROM.

AUTHOR CONTRIBUTIONS

The work presented here was carried out in collaboration among all authors. NZ defined the research theme and designed the experiments. XZ, XL, JP, XD, WJ, MZha, and YY were involved in sample collection and carried out the experiments. XZ, XD, PW, WB, and NZ analyzed the data, interpreted the results, and drafted the manuscript. NZ finalized the manuscript. All authors have approved the manuscript.

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Umbilical Cord Blood NOS1 as a Potential Biomarker of Neonatal Encephalopathy

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Background: There are no definitive markers to aid in diagnosis of neonatal encephalopathy (NE). The purpose of our study was (1) to identify and evaluate the utility of neuronal nitric oxide synthase (NOS1) in umbilical cord blood as a NE biomarker and (2) to identify the source of NOS1 in umbilical cord blood.

Methods: This was a nested case–control study of neonates >35 weeks of gestation. ELISA for NOS1 in umbilical cord blood was performed. Sources of NOS1 in umbilical cord were investigated by immunohistochemistry, western blot, ELISA, and quantitative PCR. Furthermore, umbilical cords of full-term neonates were subjected to 1% hypoxia *ex vivo*.

Results: NOS1 was present in umbilical cord blood and increased in NE cases compared with controls. NOS1 was expressed in endothelial cells of the umbilical cord vein, but not in artery or blood cells. In *ex vivo* experiments, hypoxia was associated with increased levels of NOS1 in venous endothelial cells of the umbilical cord as well as in *ex vivo* culture medium.

Conclusion: This is the first study to investigate an early marker of NE. NOS1 is elevated with hypoxia, and further studies are needed to investigate it as a valuable tool for early diagnosis of neonatal brain injury.

Keywords: neonatal encephalopathy, biomarkers, umbilical veins, NOS1, diagnosis

INTRODUCTION

Neonatal encephalopathy (NE) affects 1–9 out of every 1,000 newborns (1, 2) and has a fatality rate of 9–70% (1, 3, 4); yet, it is still poorly diagnosed at birth. Clinically, NE may manifest as a subnormal level of consciousness or seizures, and is often accompanied by difficulty with initiating and maintaining respiration and depression of tone and reflexes (5). The extent of the brain injury

is later assessed by cranial ultrasound and MRI (6). Children diagnosed by clinical and neuroimaging criteria are at an increased risk of developing adverse long-term neurologic sequelae such as seizures and cognitive and motor difficulties (7).

Therapeutic hypothermia is the major medical treatment with promising long-term neurodevelopmental results, and this treatment is effective only when it is administered in the first six postnatal hours (8). Even with this treatment, approximately 40% of patients are affected with sequelae (8). A significant challenge in a timely treatment of NE is that the diagnosis and classification of the severity of the condition are often made only after the brain injury has already occurred. Based on easily accessible material, such as umbilical cord blood, early and prompt diagnosis of NE and associated prognosis would allow early intervention with hypothermia (9), erythropoietin (10), collection of umbilical cord blood for autologous stem cell transplantation (11), or stratification to other emerging therapies. Furthermore, provision of information to the injured neonates' families, who may face difficult therapeutic decisions, is a key for early intervention.

Given that NE is a rare diagnosis, studies implementing ancillary markers for the diagnosis of the condition are limited. Several biomarkers such as glial fibrillary acidic protein (GFAP), S100 calcium-binding protein B (S100B), neuron-specific enolase (NSE), adrenomedullin, and activin A were investigated, but none of them presented an opportunity for early diagnosis (12–15). A plausible candidate early marker of neurologic injury in neonates is neuronal nitric oxide synthase (NOS1). The association of the enzyme with adverse neurodevelopment is supported by animal studies (16, 17). In addition to neurons, NOS1 expression has been observed in non-neuronal tissues such as skeletal muscle, smooth muscle, and endothelial cells to control muscle contractility and local blood flow (18–20). Furthermore, studies have shown that NOS1 is expressed in cardiomyocytes in response to oxidative stress (21).

We hypothesized that NOS1 in umbilical cord blood is a plausible clinical biomarker for identification of NE due to hypoxic–ischemic events. To test our hypothesis, we pursued four aims: (1) to determine whether NOS1 is present not only in the umbilical cord but also in the umbilical cord blood of neonates; (2) to determine whether the level of NOS1 in neonates with a clinical diagnosis of NE is significantly different from that of neurologically normal neonates; (3) to identify the source of NOS1 in umbilical cord blood; and (4) to simulate hypoxic events that may lead to NE and to evaluate production of NOS1 *ex vivo*.

MATERIALS AND METHODS

Study Design

We undertook a nested case–control study within a prospective cohort of neonates, >35 weeks of gestation; cohort has been described previously (12). Cases were neonates with NE and controls were age-matched newborns, admitted to the Johns Hopkins University Hospital (JHH) Neonatal Intensive Care Unit (NICU) between April 1, 2009 and May 30, 2014 (12). The Institutional Review Board at Johns Hopkins University School of Medicine approved this study, and all participants were anonymized.

Cases (**Table 1**) were neonates clinically diagnosed with moderate to severe NE, as defined by The American Academy of Pediatrics (5), who underwent whole-body cooling as per the JHH NICU institutional protocol which follows the recommendations of Shankaran et al. (22). Controls (**Table 1**), matched with the cases by gestational age (within 1 week), were neurologically normal neonates.

For the secondary analysis, we created a subset that met stringent criteria for NE cases and controls, based on clinical exam plus two additional criteria: umbilical cord arterial pH < 7.0 and base deficit >12 (**Table 2**) (23, 24). Neurologically normal neonates had a normal exam, umbilical artery pH > 7.2, and base deficit <8.

Maternal and Neonatal Characteristics

Maternal and neonatal records were reviewed, and pertinent clinical information was extracted. Data collected included infant sex, mode of delivery, gestational age, maternal age, parity, race, and three conditions suggested being risk factors for NE (25, 26): diagnosis of preeclampsia, clinical chorioamnionitis, and IUGR. Preeclampsia was defined as proteinuria and new onset hypertension. Clinical chorioamnionitis was defined as the presence of maternal fever with at least one other finding of fetal tachycardia,

TABLE 1 | Maternal demographics and perinatal outcomes for neonatal encephalopathy (NE) cases and normal controls.

Characteristic	Neurologically normal controls (n = 37)	NE cases (n = 27)	P value
Gestational age at delivery (weeks)	39.6 ± 1.0	39.2 ± 1.4	0.70
Maternal age (years)	26.5 ± 5.7	27 ± 7.6	0.63
Nulliparous	17 (61%)	15 (56%)	0.70
White race	7 (28%)	10 (37%)	0.33
Preeclampsia[a]	3 (11%)	2 (7%)	0.18
Clinical chorioamnionitis[a]	4 (14%)	3 (11%)	0.72
Intrauterine growth restriction[a]	3 (11%)	2 (7%)	0.97
Cesarean delivery	15 (54%)	20 (74%)	0.11
Infant male sex	18 (64%)	17 (63%)	0.92

Data are presented as means and SD for the two continuous variables, and counts and percentages for the seven categorical variables.
[a]Risk factors associated with NE.

TABLE 2 | Stringent criteria for neonatal encephalopathy and neurologically normal neonates.

Encephalopathic neonates	Neurologically normal neonates
At least one clinical criteria	Neurologically normal exam
• Lethargy • Stupor or coma • Decreased or no activity • Distal flexion or complete extension • Decerebrate posture • Hypotonia or flaccidity • Abnormal primitive reflexes • Abnormal autonomic nervous system • Seizures	
Umbilical cord pH < 7.0	Umbilical cord pH > 7.2
Base deficit >12	Base deficit < 8

uterine tenderness, or purulent vaginal discharge. IUGR was defined as an estimated fetal weight less than the 10th percentile for gestational age with ultrasound performed within 3 weeks after delivery date.

Collection of Umbilical Cord Blood for Determination of NOS1

Venous umbilical cord plasma was collected from routinely discarded collected blood. Samples were stored at −80°C until utilized.

Ex Vivo Hypoxia of Umbilical Cord

Umbilical cords were obtained from healthy full-term births within 4 h. Methods previously described by Thomas et al. (27); briefly, in a sterile cell culture hood, umbilical cord was cut into 1-mm length thick slices. For normoxia, specimens were cultured in a conventional incubator preset at 37°C in a humidified atmosphere, containing 5% CO_2. Hypoxia was induced by a modular incubator chamber. To create gas hypoxia, specimens were first sealed in a humidified hypoxia incubator chamber system (Stemcell Technologies, Vancouver, BC, Canada) supplied with 1% O_2 at 37°C according to the manufacturer's instructions. After 3 h of incubation (simulating fetal hypoxic–ischemic event), umbilical cords were fixed with 4% paraformaldehyde (Affymetrix, Cleveland, OH, USA) overnight at 4°C for immunohistochemistry (IHC), or the tissue was trimmed close to the vein and artery followed by immediate storage at −80°C for western blot. Culture media was also collected and frozen at −80°C.

ELISA for NOS1

ELISA of total NOS1 was performed according to the protocol established in our laboratory for indirect "sandwich" ELISA, optimized for highest sensitivity of human NOS1 in serum, amniotic fluid, or cell lysates. Briefly, an immobilized capture antibody for human NOS1 was coated overnight in 96-well microplate (R&D, Minneapolis, MN, USA). After blocking and sample incubation, the unbound material was washed away, and a biotinylated detection antibody specific for human NOS1 (2 ng/ml, Cell Signaling Tech, Beverley, MA, USA) was used to detect the target protein utilizing a standard streptavidin-HRP format using 3,3′,5,5′-tetramethylbenzidine (TMB) as a substrate and subsequent reading at 450 nm. Biotinylation as well as prior purification of detection antibodies were done using biotinylation and protein A/G purification kits according to manufacturer's recommendations (Pierce Biotechnology, Rockford, IL, USA). Recombinant purified NOS1 was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA) for standard curve. Average for blank readings was subtracted from the averages for the duplicate readings for each sample, and total NOS1 concentrations of each sample were determined using log-linear regression. ELISA was further validated with JHH population with a separate group of samples.

Immunohistochemistry

Using a cryostat (Leica, Buffalo Grove, IL, USA), umbilical cords were sectioned into 20-μm thick slices. Sections were incubated overnight at 4°C with primary antibodies in PBS containing 0.5% Triton X-100 (Sigma, St. Louis, MO, USA) after blocking with 10% goat serum. The following primary antibodies were used: rabbit against NOS1 (1:1,000, Millipore, Billerica, MA, USA), NOS2 (1:20, Novus, Littleton, CO, USA), NOS3 (1:100, Abcam, Cambridge, MA, USA), and mouse against CD34 (1:500, Millipore, Billerica, MA, USA). CD34 was used to characterize endothelial cells in human UC. The next day, sections were rinsed with PBS, and then incubated with fluorescent secondary antibodies diluted in 1:500 for 3 h at room temperature. The following antibodies were used for immunofluorescence: goat anti-rabbit DyLight 488 (Abcam, Cambridge, MA, USA) and donkey anti-mouse Alexa Fluor 568 (Life Technologies, Grand Island, NY, USA). The sections were further stained with DAPI (Roche, Indianapolis, IN, USA) for 2 min at room temperature followed by mounting with Fluromount-G (eBioscience, San Diego, CA, USA). Images were attained using Axioplan 2 Imaging system (Carl Zeiss, Thornwood, NY, USA). To exclude a possibility that NOS1 positive cells were blood cells or of stem cell origin, double staining of NOS1/CD44 (Abcam, Cambridge, MA, USA) and NOS1/CD45 (Abcam, Cambridge, MA, USA) was also conducted.

To further confirm the distribution of NOS1 in human UC, another four NOS1 from hosts of rabbit or mouse (Cell Signaling, Beverly, MA, USA; Life Technologies, Frederick, MD, USA; Abcam, Cambridge, MA, USA; and BD Biosciences, Franklin Lakes, New Jersey, USA), one NOS2 (Abcam, Cambridge, MA, USA), and one NOS3 (Abcam, Cambridge, MA, USA) antibodies were utilized to exam the expression together with CD34 antibody. The secondary antibodies were used the same as described above.

SDS-PAGE and Western Blotting

The following were used as primary antibodies: rabbit against NOS1 (Millipore, Billerica, MA, USA), NOS2 (Novus, Littleton, CO, USA), NOS3 (Abcam, Cambridge, MA, USA) and rabbit anti β-actin (Abcam, Cambridge, MA, USA). Extracted protein (50 μg) was separated by SDS-PAGE using 4–15% gels (Bio-Rad Laboratories, Hercules, CA, USA) and then electro-transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 10% BSA (Sigma-Aldrich, St. Louis, MO, USA) in Tris-buffered saline + 0.1% Tween-20 (TBS-T, pH7.5), incubated with primary antibodies in TBS-T containing 10% BSA for 1 h at room temperature, and washed in TBS-T. Goat anti-rabbit IR Dye-800CW (Li-Cor, Lincoln, NB, USA) was used as the secondary antibody. Imaging was performed using the Li-Cor Odyssey Near Infra Red System and analyzed using Image Studio Software (Li-Cor).

Quantitative PCR (qPCR)

Vein and artery tissue were dissected from umbilical cords and frozen at −80°C until used. The tissue samples were homogenized in RLT buffer (Qiagen, Valencia, CA, USA) for 60 s using a BeadBug microtube homogenizer (Benchmark Scientific, Edison, NJ, USA) with 0.5-mm beads. Cord blood was collected into acid citrate dextrose tubes (BD Biosciences, Franklin Lakes, NJ, USA) and centrifuged at 400 × *g* for 10 min at room temperature. RNA was prepared from the umbilical cord homogenates and

cord blood leukocytes with RNEasy Mini Kit (Qiagen, Valencia, CA, USA), and cDNA was prepared using cDNA Synthesis Kit (Bio Rad, Hercules, CA, USA). qPCR was performed in triplicate in 20-μl reactions for 40 cycles, using the manufacturer's suggested protocols for temperature cycling (Bio Rad, Hercules, CA, USA). The reactions were run on a CFX384 Touch Real-Time PCR Detection System (Bio Rad, Hercules, CA, USA), using SensiFAST Probe No-ROX (Bioline, Taunton, MA, USA). Primers used were obtained from Integrated DNA Technologies (Coralville, IA, USA) for NOS1 (Fwd: AGACGCACGAAGATAGTTGAC, Rev: CCGAAGCTCCAGAACTCAC, Probe:/56-AM/TCCTTAGCC/ZEN/GTCAAAACCTCCAGAG/3IABkFQ), NOS2 (Fwd: ACTTCCACTTGCTGTACTCTG, Rev: CACCTACTTCCTG GACATCAC, Probe: CTGCTGCTCCAAAAGCTGGCC), and NOS3 (Fwd: ACGATGGTGACTTTGGCTA, Rev: TGGAGGATG TGGCTGTCT, Probe: CAGTGGAAATCAACGTGGCCGTG) or SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) for Eukaryotic 18S Ribosomal RNA (Life Technologies, Grand Island, NY, USA). Data analysis was performed with CFX Manager Software (Bio Rad).

Statistical Analysis

Data were compared using Student's *t*-test, Mann–Whitney *U* test, or Fisher exact test, when appropriate. Two-tailed Student's *t*-test or Mann–Whitney *U* test was used subsequently to determine whether data were normally distributed. Fold change of NOS1 level over mean control was calculated.

To determine the ability of NOS1 to predict NE, and the optimal cut points for NOS1, we created receiver operator characteristic (ROC) curves. Sensitivity and specificity were determined for each of these points. Statistical analyses were performed using Stata10 (StataCorp LP, College Station, TX, USA).

RESULTS

Study Population

Umbilical cord blood samples of 27 NE cases and 37 controls were used. There were no significant differences between cases and controls in: (i) maternal demographic parameters (gestational age at delivery, maternal age, nulliparous, and white race), (ii) incidence of adverse perinatal outcomes (preeclampsia, clinical chorioamnionitis, intrauterine growth restriction), or (iii) cesarean delivery or infant sex (**Table 1**). For an exploratory binding assay to detect NOS1 in umbilical cord blood of neonates, 15 NE and 24 control neonates were utilized. Eight neonates met stringent criteria for NE and were matched to ten control neonates (**Table 2**). Another 12 NE and 13 controls were utilized to validate the exploratory results by ELISA. We demonstrated the presence of NOS1 in the umbilical cord blood of all 64 neonates.

Level of NOS1 in Neonates with NE and in Neurologically Normal Neonates

In our investigational study, as determined by relative absorbance values of ELISA (**Figure 1A**), the level of NOS1 in umbilical cord blood was significantly higher in NE cases (mean = 1.392 AU, 95% CI 1.04–1.74) than in the controls (mean = 1.00 AU, 95% CI 0.77–1.23). Since these values were generated from a non-commercial ELISA (prepared in our laboratory and originally without standards/standard curve), these findings were validated by a separate experiment to determine NOS1 concentration, involving separate set of samples and standards/standard curve (NE: mean = 1.32 ng/ml; Control: mean = 0.03 ng/ml; $p < 0.05$, Student's *t*-test; **Figure 1B**).

Stringent Criteria Subset

In umbilical cord blood, the mean relative absorbance of NOS1 level was significantly higher (**Figure 2A**, $p < 0.05$, Student's *t*-test) in the neurologically at-risk neonates meeting stringent criteria (mean = 2.006 AU, 95% CI 1.425–2.587) than in the neurologically normal control neonates (mean = 1.000 AU, 95% CI 0.493–1.507). Within the group of neonates meeting stringent criteria, NOS1 was predictive of NE. The ROC curve yielded an AUC of 0.84 for NOS1 prediction of NE (**Figure 2B**).

NOS1 Expression in Endothelial Cells of Human Umbilical Vein

To localize NOS1 in the human umbilical cord, IHC for NOS1 and CD34 were performed. IHC for CD34 revealed a distribution of endothelial cells in the umbilical cord (28). NOS1 was

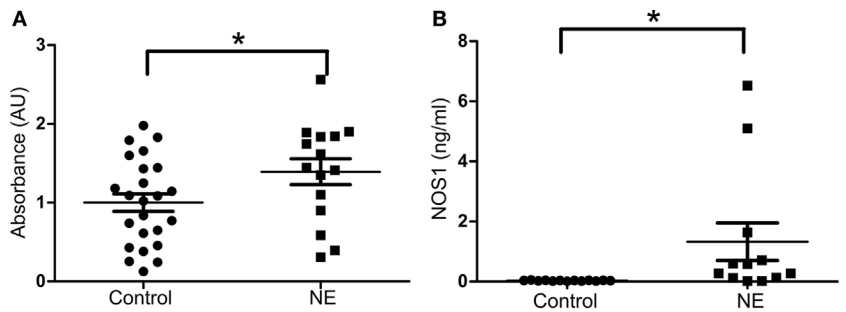

FIGURE 1 | **Expression of NOS1 in umbilical cord blood**. **(A)** Bar plot of relative absorbance in NOS1 level compared to controls based on exploratory NOS1-binding assay ($n = 15$ NE cases and 24 controls, Student's *t*-test; $^*p < 0.05$). **(B)** NOS1 concentrations were determined by ELISA using a separate set of patients ($n = 12$ NE cases and 13 controls; Student's *t*-test; $^*p < 0.05$). Data are reported as means ± SEM.

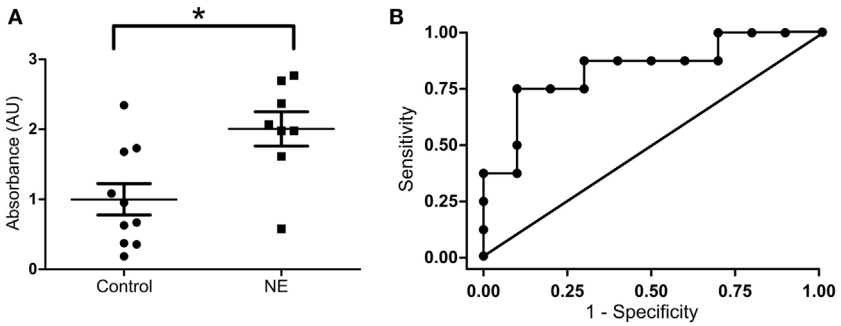

FIGURE 2 | NOS1 expression in NE patients classified by stringent criteria. **(A)** Relative absorbance of NOS1 expression compared to controls based on exploratory NOS1 binding assay ($n = 8$ NE cases and 10 controls, Student's *t*-test; $^*p < 0.05$). Bars represent mean ± SEM. **(B)** Receiver operator characteristic (ROC) curve for NOS1 expression. Area under the curve equals 0.84.

expressed mainly in the form of fine granules in the cytoplasm and, occasionally, in cell membrane. There was a co-localization of NOS1 and CD34 in the umbilical vein, as demonstrated by double-positive staining (**Figure 3A**, top panel). Neither CD44 nor CD45 cells was co-localized with NOS1 (data not shown). There was no clear NOS1 expression in umbilical artery (**Figure 3A**, bottom panel). Western blot further confirmed IHC results with significant expression of NOS1 in umbilical vein (**Figures 3B,C**, Student's *t*-test, $p < 0.05$).

Confirmatory analysis examining expression of NOS1, NOS2 (inducible NOS), and NOS3 (endothelial NOS) at mRNA and protein level in umbilical cord and leukocytes were also performed. NOS1 was only detected in umbilical cord vein (**Table 3**), while NOS2 and NOS3 expression was detected by IHC and qPCR in both umbilical cord vein and artery. Transcripts for NOS1, NOS2, and NOS3 were not detected in cord blood leukocytes (**Table 3**) using qPCR.

To further ascertain a differential expression as well as to validate the specificity of the antibodies used, another four NOS1, one NOS2, and one NOS3 antibodies were used in IHC. Similar to the above results, NOS1 expression was detected in umbilical cord vein but not in artery (Figure S1A in Supplementary Material). NOS2 (Figure S1B in Supplementary Material) and NOS3 (Figure S1C in Supplementary Material) were detected in both umbilical cord vein and artery.

Western Blot and ELISA Analyses of NOS1 Expression in *Ex Vivo* Umbilical Cord Culture

To investigate whether hypoxia induces a NOS1 response in venous endothelial cells, umbilical cord slices were cultured in a hypoxia chamber under 1% O_2. After 3 h of culture (simulating a clinical hypoxic event), the co-localization of CD34 and NOS1 increased in the hypoxic group compared with normoxic control, by IHC (**Figure 4A**) and by western blot (**Figures 4B,C**, $p < 0.05$, Student's *t*-test). We further measured NOS1 production in culture supernatant and, after 3 h of hypoxia, NOS1 increased significantly following hypoxic conditions (**Figure 4D**, Student's *t*-test, $p < 0.01$).

DISCUSSION

Our study is the first to demonstrate that NOS1, a marker of oxidative stress, is a potential early bedside clinical biomarker of NE in umbilical cord blood. In our study, the levels of NOS1 were significantly higher in the umbilical cord blood of neonates with clinical encephalopathy than in normal controls. Furthermore, our *ex vivo* experiments confirmed that NOS1 was increased with hypoxia in venous endothelial cells of umbilical cord.

There are a few biomarkers relative to neonatal brain injury. NOS1 is currently the only early biomarker that is available to aid in diagnosis of NE at delivery based on umbilical cord blood. Massaro et al. evaluated the serum biomarkers S100B and NSE in neonates treated with hypothermia and found them to be associated with clinical encephalopathy and MRI change (3), consistent with prior studies (13–15, 29). None of the biomarkers were used for diagnosis or triage of neonates to therapy. In addition, the first time point evaluated was at initiation of cooling; information was not available prior to initiation of cooling and could not assist with triage to therapy which represents a limitation of their study. Furthermore, others evaluated adrenomedullin and activin A have been evaluated as biomarkers of NE in neonatal serum but similarly have not been tested in umbilical cord blood (30). In Ennen's studies (12), GFAP in serum at 24 and 48 h after birth was predictive of brain injury on MRI in encephalopathic neonates. Similarly to other markers discussed, at birth, umbilical cord blood GFAP levels were not predictive of brain injury.

We used ELISA for NOS1, which may present as a new, fast, and easy method to perform clinical test due to high levels (nanograms) of NOS1 presented in umbilical cord blood. As the AUC of NOS1 test is perhaps superior as compared to current clinical criteria for diagnosis of NE [AUC 0.81 (31)], we speculate that implementation of this test will improve sensitivity and specificity of timely clinical diagnosis as it can be used concomitantly. Results of this test can be provided within the first several hours of life, allowing for early diagnosis and triage to therapy such as hypothermia and other novel therapeutics. One novel use for such clinical test would include early identification of which neonates

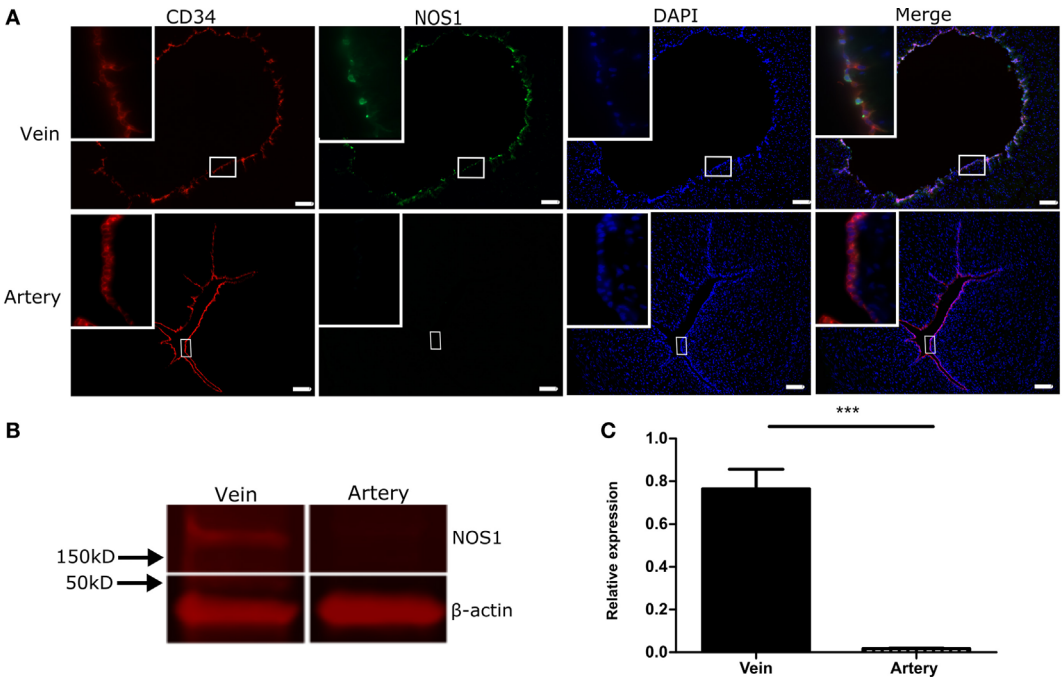

FIGURE 3 | NOS1 Expression in vessels of human umbilical cord. **(A)** Immunostaining of CD34 and NOS1 in endothelial cells of vein and artery of umbilical cord ($n = 4$ per group). **(B)** Protein expression of NOS1 in vein and artery of umbilical cord by western blot. **(C)** Densitometry analysis of western blot imaging ($n = 5$ per group; Student's t-test; $^*p < 0.05$). Scale bars = 100 μm.

TABLE 3 | Detection of NOS1, NOS2, and NOS3 in umbilical cord vein and artery.

	Immunohistochemistry (IHC)			Quantitative PCR		
	NOS1	**NOS2**	**NOS3**	**NOS1**	**NOS2**	**NOS3**
Umbilical cord artery	– (0/4)	+ (4/4)	+ (4/4)	– (0/4)	+ (4/4)	+ (3/4)
Umbilical cord vein	+ (4/4)	+ (4/4)	+ (4/4)	+ (3/4)	+ (4/4)	+ (3/4)
Cord blood leukocytes				– (0/4)	– (0/4)	– (0/4)

–, negative expression; +, positive expression. There were four samples for each group. In brackets, numerators are the numbers tested negative or positive expression by IHC or quantitative PCR.

that might benefit from storage of umbilical cord blood for later stem cell transplantation and would result in timely collection of umbilical cord blood.

Neonatal encephalopathy is a rare diagnosis and MRI diagnosis of actual brain injury in this group is an infrequent finding. The consequences of NE including death and neurodevelopmental disability can be devastating for the child and family. While one critique of our study could be a small sample size, the nested case–control study represents a valuable sample, given that NE is rare, even in our referral university center. However, our data are confirmed with simulation of hypoxia *ex vivo*, and this is an advantage of our study. Further research will concentrate on collecting information about childhood outcomes of these at-risk neonates with neurodevelopmental testing at 1–3 years of age.

Our study has several strengths. An ELISA assay quantified the level of NOS1, and this test can be used in a clinical setting to inform clinical decisions early in neonatal life. The results are reproducible, and more stringent criteria for encephalopathy yields improved prediction of NOS1 for NE. In addition to identifying neonates at risk of brain injury, a biomarker would timely predict which infants are at risk for brain injury and which are most likely to have long-term neurologic sequelae. Moreover, our study identifies a source of NOS1, and, using experimental conditions that mimic short-term hypoxic environments, we are able to observe similar findings *ex vivo*.

Perinatal hypoxia–ischemia is an important cause of NE (50–80%) (32). Nitric oxide signaling has been demonstrated to have an important role in hypoxia-induced pathological responses as all NOS isoforms critically require oxygen for activity (33). Physiologically, NOS1 depends more on oxygen and has higher rates of nitric oxide biosynthesis (34) than NOS2 and NOS3. Both NOS1 mRNA and protein expression are regulated by O_2 at transcriptional and translational level (35, 36), and therefore, it may be more sensitive to hypoxia. Corollary to that, in our study, NOS1 significantly increased in umbilical cord blood of moderate and severe NE neonates compared to control.

To investigate a plausible origin of NOS1 in umbilical cord blood, we performed a survey of all possible sources of NOS1 in umbilical cord blood and umbilical cord. We found that NOS1 was present in endothelial cells of human umbilical cord

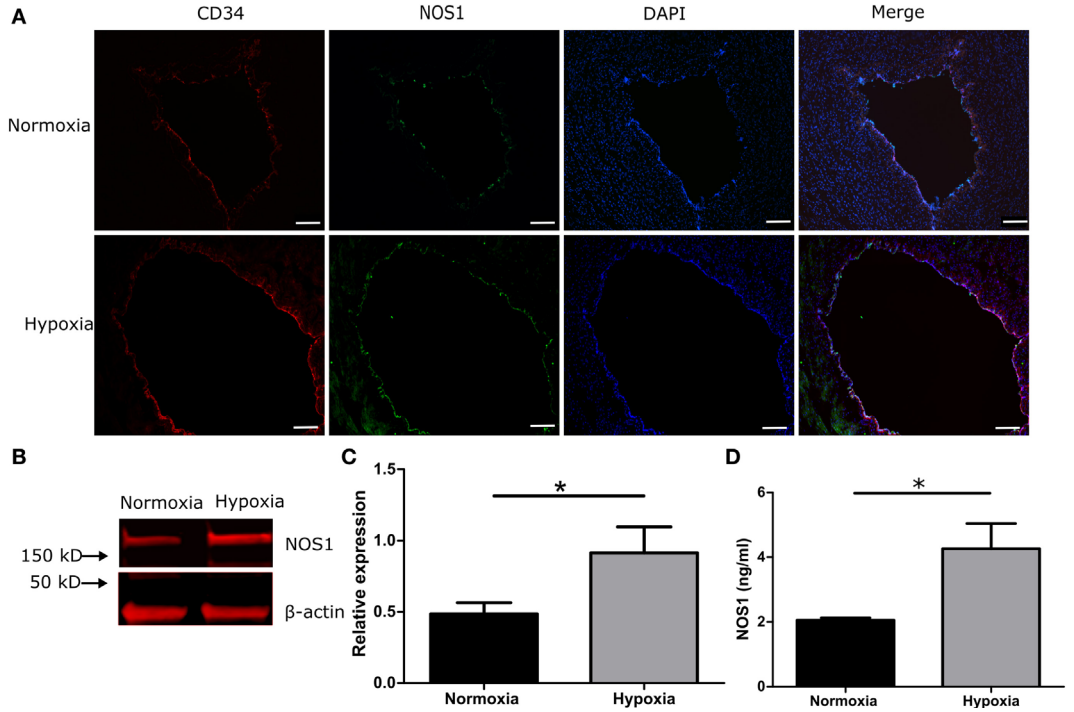

FIGURE 4 | **NOS1 expression in endothelial cells of human umbilical vein in 1% hypoxic chamber**. **(A)** Immunostaining of CD34 and NOS1 in vein ($n = 5$ per group). **(B,C)** Protein levels of NOS1 in umbilical cord by western blot ($n = 5$ per group; Student's t-test; $^{*}p < 0.05$). **(D)** NOS1 production in *ex vivo* culture supernatant by ELISA ($n = 5$ per group; Student's t-test; $^{**}p < 0.01$). Scale bars = 10 μm.

vein but not in umbilical cord artery. While our results are in keeping with a previous study (20) that showed NOS1 expression in vein of human umbilical cord by IHC, our study is the first to demonstrate a differential expression of this enzyme in umbilical vein and artery. In the adult vascular system, NOS1- and NOS3-derived nitric oxide is thought to play a major role in changes of vascular arterial tone (37, 38). In addition to regulating vascular tone, nitric oxide is an important signaling molecule in the nervous system and the immune system (39, 40). We speculate that endothelial NOS1 expression in umbilical vein may be a conserved mechanism that contributes to the fetal response to hypoxia and inflammation. Further studies to clarify the role of umbilical cord NOS1 during hypoxic and inflammatory conditions are needed.

In conclusion, we speculate that NOS1 is a novel, early biomarker for early identification of NE that can be used in determining NE together with clinical manifestations to improve diagnosis, allowing for better prognostic information and earlier interventions.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Johns Hopkins University Hospital (JHH) Neonatal Intensive Care Unit and the protocol was approved by The Institutional Review Board at Johns Hopkins University School of Medicine. All participants were anonymized.

AUTHOR CONTRIBUTIONS

JL and JR performed the experiments, analyzed data, and wrote the manuscript. SY analyzed data and wrote the manuscript. RB and SM perfumed the experiments and analyzed data. CP, EN, AZ, FN, DO, EG, and MJ designed the experiments and wrote the manuscript. IB designed and performed the experiments, analyzed data, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fped.2017.00112/full#supplementary-material.

FIGURE S1 | **Distribution of NOS1, NOS2, and NOS3 expression in umbilical cord**. **(A)** Representative image of NOS1 (green) in umbilical cord using four different antibodies. NOS1 was expressed in the venous endothelial cells (red) of human umbilical cord, but not in the artery. **(B)** NOS2 was expressed in both endothelial cells (red) of vein and artery. **(C)** NOS3 (green) was also expressed in both endothelial cells (red) of vein and artery. Scale bars = 10 μm.

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REVIEW
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Pregnancy: An Underutilized Window of Opportunity to Improve Long-term Maternal and Infant Health—An Appeal for Continuous Family Care and Interdisciplinary Communication

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Physiologic adaptations during pregnancy unmask a woman's predisposition to diseases. Complications are increasingly predicted by first-trimester algorithms, amplify a pre-existing maternal phenotype and accelerate risks for chronic diseases in the offspring up to adulthood (Barker hypothesis). Recent evidence suggests that *vice versa*, pregnancy diseases also indicate maternal and even grandparent's risks for chronic diseases (reverse Barker hypothesis). Pub-Med and Embase were reviewed for Mesh terms "fetal programming" and "pregnancy complications combined with maternal disease" until January 2017. Studies linking pregnancy complications to future cardiovascular, metabolic, and thrombotic risks for mother and offspring were reviewed. Women with a history of miscarriage, fetal growth restriction, preeclampsia, preterm delivery, obesity, excessive gestational weight gain, gestational diabetes, subfertility, and thrombophilia more frequently demonstrate with echocardiographic abnormalities, higher fasting insulin, deviating lipids or clotting factors and show defective endothelial function. Thrombophilia hints to thrombotic risks in later life. Pregnancy abnormalities correlate with future cardiovascular and metabolic complications and earlier mortality. Conversely, women with a normal pregnancy have lower rates of subsequent diseases than the general female population creating the term: "Pregnancy as a window for future health." Although the placenta works as a gatekeeper, many pregnancy complications may lead to sickness and earlier death in later life when the child becomes an adult. The epigenetic mechanisms and the mismatch between pre- and postnatal life have created the term "fetal origin of adult disease." Up to now, the impact of cardiovascular, metabolic, or thrombotic risk profiles has been investigated separately for mother and child. In this manuscript, we strive to illustrate the consequences for both, fetus and mother within a cohesive perspective and thus try to demonstrate the complex interrelationship of genetics and epigenetics for long-term health of societies and future generations. Maternal–fetal medicine specialists should have a key role in the prevention of non-communicable diseases by implementing a framework for patient consultation and interdisciplinary networks. Health-care providers and policy makers should increasingly

invest in a stratified primary prevention and follow-up to reduce the increasing number of manifest cardiovascular and metabolic diseases and to prevent waste of health-care resources.

Keywords: fetal programming, cardiovascular diseases, metabolic diseases, pregnancy as a window for future health, preventive healthcare

INTRODUCTION

The care of pregnant women is typically focused on the current pregnancy. Yet, it has been demonstrated that pregnancy complications have lifelong health implications for women and children. Barker et al. correlated low birthweight (LBW) in hunger episodes during World War II with cardiovascular disease (CVD) and type 2 diabetes (1). Several investigators have subsequently confirmed that maternal complications such as preeclampsia (PE), stress, excessive weight gain and gestational diabetes mellitus (GDM) increase chronic disease, and mortality rates in following generations (2, 3). These health risks have been attributed to perinatal programming, a mismatch between prenatally acquired attributes and critical periods in development producing health effects that are independent of a person's genetic code (Barker hypothesis, Dörners concept of functional teratology). More recently, it was recognized that adverse pregnancy outcome relating to placental syndromes is associated with maternal cardiovascular, metabolic, or thromboembolic risks and earlier mortality (**Figure 1**) (4). First-trimester screening algorithms now allow individualized prediction of fetal growth restriction (FGR), PE, spontaneous preterm birth (SPB), or GDM (5–8) by utilizing markers of pre-existing maternal "risk-profiles" that not only confer pregnancy-related risks but also lower the thresholds for disease in later life (4, 9, 10).

These observations support the hypothesis that pregnancy can interact with maternal phenotypes and modify risks for "non-communicable diseases" (NCDs) in mothers and children. This is the basis for developing interdisciplinary care paths between obstetricians, general practitioners, internists, and pediatricians that extend beyond the current pregnancy to offer population-based prevention, screening, and individualized secondary prevention. Given the increasing rate of NCDs in low-, middle-, and high-income countries such an approach requires urgent consideration by health-care providers and policy makers.

It is our aim to illustrate how pregnancy itself might serve as a screening tool for future health risks, opening important opportunities for the prevention of the most relevant diseases of our time.

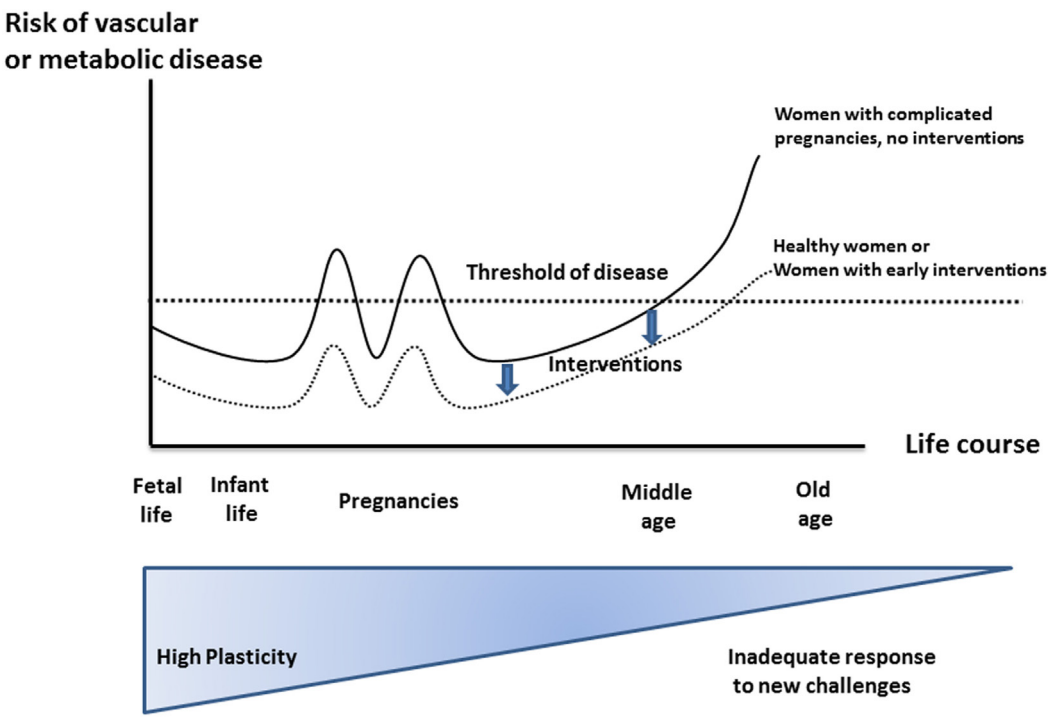

FIGURE 1 | Effect of developmental environment on later maternal phenotype and pregnancy complications. Risks for non-communicable diseases increase throughout a life course due to reduced plasticity. Increased risk factors, suspicious first-trimester screening, and pregnancy complications may be considered as the first "markers" and thus allow earlier intervention strategies compared to first symptoms of manifest chronic disease. Modified according to Sattar and Greer (4), Godfrey and Barker (11), and Godfrey et al. (12), similarly designed for Ref. (13).

METHODS

Literature searches were performed utilizing Medline, Embase, Science Direct, and Cochrane Library, proceedings from congresses on fetal origins of adult disease, the World Congress of Diabetes in Pregnancy, and the interdisciplinary workshops on the role of pregnancy complications for future maternal and child health hosted by the National Institute of Child Health and Human Development, the Society for Maternal-Fetal medicine, and the American College of Obstetricians and Gynecologists.

We selected studies relating pregnancy risk profiles to long-term cardiovascular and metabolic health of mothers and infants up to adulthood. Search terms included risk profile, miscarriage, PE, FGR, preterm birth, smoking and stress during pregnancy, subfertility, GDM, overweight, obesity and excessive pregnancy weight gain, breastfeeding, and as outcome parameters long-term maternal and infant health, CVD and CVD mortality of mothers and infants, diabetes mellitus (DM) of both mothers and infants, metabolic syndrome, thrombophilia, fetal programming, fetal origin of adult disease, growth, or body composition variable of interest such as fat mass or obesity. Full-text articles were obtained and reviewed to identify those representing the intended context; data were extracted from relevant publications. Results from prospective and retrospective human and animal studies were considered.

There is a significant overlap between cardiovascular, metabolic, and thrombotic profiles in women at risk for placental disease. Prothrombotic risks are usually managed by disease-specific interventions (i.e., anticoagulation for thrombophilia). Accordingly, for the purpose of this review, we have focused on cardiovascular and metabolic risk profiles as major precursors to NCDs to illustrate our concept.

RESULTS

Health Risks Attributable to a Cardiovascular Risk Profile

Cardiovascular diseases (heart disease, stroke) account for 31% of all deaths in the United States (US) (14). Over 2,150 Americans/day die of CVD with increasing rates for women aged 35–44 years (14, 15). Higher blood pressures in early adulthood predate increased mortality from all causes (16) and up to 77% of people have blood pressures >140/90 mmHg when they experience a first heart attack or stroke (14). At age 50, total life expectancy for normotensive compared to hypertensive men and women is 5.1, respectively, 4.9 years longer (14). In the last 15 years, the actual number of deaths attributable to high BP rose by 39% whereas the actual number of CVD deaths declined by 15% (14). However, the age-related decrease in mortality was less pronounced in women than in men (17). A mathematical model calculated that a 10% increase of treatment of early hypertension would prevent 14,000 deaths in the US/year (18). In view of these statistics, it is critical to identify those pregnancy complications that lead to persistence of high BP, CVD, and stroke and to consider preventive strategies.

Thilaganathan summarized common and unique characteristics of gestational diabetes and hypertensive disease during pregnancy raising the point that the placenta may not be the initiator of PE. Instead, pre-existing maternal hemodynamics or metabolic diseases may be the primary cause of secondary placental morphology and function (**Table 1**) (19, 20). While we separate single pregnancy complications for the purpose of this review they may all occur in one woman. In those cases, later health risks for both mother and offspring may exponentially increase.

Miscarriage

A systematic review on effects of early menopause reported that women with early-pregnancy miscarriage are more likely to develop CVD, specifically ischemic heart disease (IHD) in later life, implying lower protective effects of estrogens on serum lipids, and vessel wall anatomy as possible causes (21). Increased total serum cholesterol (22), triglycerides (TGs) (23), antithrombin III (24), factor VII, and fibrinogen (25) have also been speculated to be responsible (22, 26). Early miscarriage, either as a single or recurrent complication, carries a hazard ratio (HR) between 1.25 and 1.56 for subsequent IHD (27) (**Table 2**). This association was independent of maternal age, height, socioeconomic deprivation, chronic hypertension, and complications during the first pregnancy. The proportion of smokers was only marginally higher compared to women without miscarriage (28.4 vs. 26.8%). While the authors hypothesized that the association of IHD and miscarriage reflects common inherited thrombophilic defects, they did not find significant associations between miscarriage and family history of CVD or venous thrombotic embolism.

Fetal Growth Restriction

It has been long recognized that infant BW correlates with the mother's subsequent risk for IHD and is "aggregated within families" possibly due to genetic, physiologic, environmental, epigenetic, and socioeconomic factors (30, 43–45). Delivering an FGR infant is associated with maternal coronary artery disease, cerebrovascular disease, or cardiac insufficiency with HRs ranging from 1.35 to 3.4 for severe early-onset FGR (**Table 2**) (28). Birth weight has an inverse linear association with maternal CVD mortality and is also related to grandparental CVD and CVD mortality (29). These BW associations across generations are independent of socioeconomic, environmental, or behavioral factors, body mass index (BMI), age, or smoking status. Familial aggregation of shared determinants for risk factors associated with pregnancy complications and CVD were postulated as a potential cause for these associations (46). Other hypothesized mechanisms are based on the observation that the impact of the mother is stronger than of the father: women at high risk of CVD may be unable to mount an adequate hemodynamic response leading to FGR and/or PE. This is supported by cardiac changes seen on maternal echocardiography showing increased cardiac chamber dimensions and left ventricular hypertrophy in the index pregnancy. The likelihood of developing FGR and/or PE is elevated by many maternal demographic and medical characteristics, such as hypertension, obesity, and age that are shared risk factors for CVD (47).

TABLE 1 | Schematic similarities and differences of gestational diabetes and pregnancy hypertension, with gratitude, according to the work of Thilaganathan (19).

	Gestational diabetes mellitus (GDM)	Pregnancy hypertension
Epidemiology		
Predisposing factors	Same as for type 2 diabetes	Same as for cardiac disease
Onset of disorder	Mid to late pregnancy	Mid to late pregnancy
Effect of parity	More common in primiparity	More common in primiparity
Recurrence risk	Increased risk if previously affected pregnancy	Increased risk if previously affected pregnancy
Fetal and placental effects		
Placental histology	Some histological lesions seen more often in GDM	Some histological lesions seen more often in pregnancy hypertension
Specificity of histology	None of the placental histological lesions specific for the disorder	None of the placental histological lesions specific for the disorder
Temporal nature of lesions	Seen more frequently in early-onset and/or severe disorder	Seen more frequently in early-onset and/or severe disorder
Placental function	Increased maternal-to-fetal transplacental glucose transfer	Impaired maternal perfusion of the uteroplacental bed
Fetus	Increased fetal glucose levels lead to macrosomia	Impaired placental function leads to impaired fetal growth
Screening/diagnostic tests		
Mechanism of screening	GTT gauges pancreatic reserve	Uterine Doppler, PlGF and BP are all measures of cardiac function
Performance of screening	Better for early-onset GDM	Better for early-onset preeclampsia (PE)
Timing of screening test	Improved sensitivity the later in pregnancy it is performed	Improved sensitivity the later in pregnancy it is performed
Diagnostic test	Supra-normal glucose levels in both pregnant and non-pregnant	High BP in both pregnant population
Management		
Cure for disorder	Birth	Birth
Treatment/amelioration	Insulin—treats the biological deficit	Antihypertensive medications—treat a symptom of the disorder
Long-term maternal health	50% develop type 2 diabetes by 10 years postpartum	20% develop chronic hypertension by 10 years postpartum
Biology		
Maternal adaption	Insulin requirements increase twofold to threefold in pregnancy	Cardiac output increases by about 50% in pregnancy
Early-onset phenotypes	Present with normal or lower insulin levels compared to non-pregnancy	Present with normal or lower cardiac outputs compared to non-pregnancy
Late-onset phenotypes	Present with supra-normal (high) insulin levels compared to non-pregnancy	Present with supra-normal (high) cardiac output compared to non-pregnancy
Etiology	Inability of maternal pancreas to deal with the glucose load of pregnancy	Impaired trophoblast invasion or maternal cardiac maladaptation?

Adverse effects of FGR on cardiovascular health of the offspring were already suspected in 1977 by Forsdhal, who reported that Norwegian children, raised in poor provinces in the early twentieth century but became prosperous thereafter, suffered from excess rates of myocardial infarction as adults (48). In 1986, Barker and Osmond showed that the distribution of CVD in England was related to a person's BW (49). Maternal hunger throughout pregnancy was linked to high blood pressure in the offspring; while third-trimester deprivation led to high levels of low-density lipoprotein, cholesterol, and fibrinogen (50). The Dutch Famine Birth Cohort Study considered long-term effects of prenatal starvation among women born before, during, or after the Hunger Winter when the average supply was <1,000 calories/day (51). Intra-family sibling analysis revealed that BW was decreased when famine exposure was in the third trimester but not when it was in the first trimester. However, the expected increase in BW of the offspring with birth order was reversed after maternal exposure in the first trimester (52). The results suggested that biologic effects depend on timing of gestational exposure and are still present in subsequent generations. People conceived during the famine had doubled rates of CVD, atherogenic plasma lipid profiles and were at increased risk of schizophrenia, depression, high stress responsiveness and performed worse on tasks that correlated with accelerated aging (53) (**Table 3**).

In most pregnancy studies, the effects of famine cannot be clearly separated from those of stress especially when studied in the context of war trauma. Following the Chernobyl disaster, the perceived level of stress was a better predictor of the offspring's risk of cognitive disorders than the actual exposure to radiation (65). Conditions during and after famine periods varied between countries: in the Netherlands, World War II was followed by a period of abundance and in Russia, life conditions remained poor. The latter was not associated with adverse effects suggesting that it is beneficial when the postnatal environment matches the prenatal environment (66). There are also individual differences in susceptibility toward hunger and stress dependent on the individual genetic background (67). While starvation may play a minor role in high resourced countries, the first trimester is a vulnerable period as serious hyperemesis significantly increases the odds for cognitive and psychological diseases in the offspring (OR 3.6, $p < 0.0001$) (68).

Cardiovascular changes in FGR offspring are already present *in utero*, can persist after birth, and may produce aberrant cardiomyocyte growth in adult hearts with reversible myocardial hypertrophy (69). In humans, FGR induced cardiovascular remodeling at age 5 is ameliorated by breastfeeding but worsened by a high maternal BMI (70). Longitudinal growth analysis suggests that children with higher BP tended to be smaller during third trimester of fetal life but were normal size as infants. By contrast, children with increased aortic root diameter or left ventricular mass tend to be larger during fetal life, but of similar size during infancy (**Table 3**) (54). School age children with clustering of CVD

TABLE 2 | Impact of pregnancy complications on maternal risks for disease categories in later life (prospective, mostly retrospective cohorts, and systematic reviews).

Pregnancy disease	Sample (*n*)	Literature (Reference number/year)	Definition of health risk in later maternal life	Association of health risk HR/OR/RR (95% CI)
Cardiovascular				
>1 miscarriage	129,200	(27)/2003	Ischemic heart disease (IHD)	HR 1.52 (1.13–2.06)
2 miscarriages	129,200	(27)/2003	IHD	HR 1.25 (1.04–1.49)
≥3 miscarriages prior to first birth	129,200	(27)/2003	IHD	HR 1.56 (1.14–2.15)
Child with FGR (general)	923,586	(28)/2011	CAD, CVD, cerebrovascular disease	HR 1.39 (1.22–1.58)
Term FGR	923,586	(28)/2011	CAD, CVD, cerebrovascular disease	HR 1.38 (1.15–1.65)
Preterm FGR	923,586	(28)/2011	CAD, CVD, cerebrovascular disease	HR 3.4 (2.26–5.11)
Birthweight	783,814	(29)/2010	Maternal CVD mortality	0.74/kg (0.56–0.99)—inverse relationship
Birthweight	783,814	(29)/2010	Maternal grandfather CVD mortality	1.05 (1.01–1.09)
Birthweight (inverse relationship)	783,814	(29)/2010	Paternal Grandmother CVD mortality	0.93/kg (0.85–1.00)—inverse relationship
Low birthweight <2,500 g	119,668	(30)/2004	Cerebrovascular disease	aHR 2.51 (1.71–3.70)
Multiparity	2,533	(31)/1993	CVD	RR 1.5 (1.1–1.9)
1 birth	1,332,062	(31)/1993	CVD	1.09 (95% CI 1.03–1.15)
>5 births	1,332,062	(31)/1993	CVD	1.47 (95% CI 1.37–1.57)
>2 children	4,286	(32)/2003	Maternal CVD	OR 1.30 (1.17–1.44)
>2 children	4,252	(32)/2003	Paternal CVD	OR 1.12 (1.02–1.22)
Preeclampsia (general)	1,985	(33)/2016	Death at coronary revascularization	HR 1.61 (1.00–2.58)
Maternal placental disease	1,130,764	(34)/2012	Premature heart failure or dysrhythmia	HR 1.51 (1.26–1.80)
Maternal placental disease + FGR	1,130,764	(34)/2012	Premature heart failure or dysrhythmia	HR 2.42 (1.25–4.67)
Maternal placental syndrome	1,030,000	(35)/2005	CVD	HR 2.0 (1.7–2.2)
Maternal placental syndrome + FGR	1,030,000	(35)/2005	CVD	HR 3.1, 2.2–4.5
Maternal placental syndrome + FD	1,030,000	(35)/2005	CVD	HR 4.4, 2.4–7.9
Preterm birth 32–37 weeks	923,686	(28)/2011	CVD	HR 1.39 (1.22–1.58)
Preterm birth 28–31 weeks	923,686	(28)/2011	CVD	HR 2.57 (1.97–3.34)
Spontaneous preterm birth (SPB)	750,350	(36)/2015	Death from IHD	HR 2.26 (1.88–2.71)
SPB	750,350	(36)/2015	Total IDH	HR 1.58 (1.47–1.71)
Preterm birth 32–36 weeks	782,287	(36)/2015	Thromboembolism	aOR 1.42 (1.24–1.62)
Metabolic				
(No) Breastfeeding	23,701	(37)/2014	Increased maternal weight after 7 years	$\beta = 0.003$ (0.01, 0.003) path analysis, inverse relationship
Breastfeeding (with formula) in patients with GDM	1,010	(38)/2015	Incidence of type 2 DM after 2 years	aHR 0.64 p trend = 0.016 (formula = 1)
Breastfeeding (mostly) in patients with GDM	1,010	(38)/2015	Incidence of type 2 DM after 2 years	aHR 0.54 p trend = 0.016
Breastfeeding (exclusive) in patients with GDM	1,010	(38)/2015	Incidence of type 2 DM after 2 years	aHR 0.46 p trend = 0.016
Pregnancy weight gain > IOM limits	65,000	(39)/2011	3 years postpartum weight gain	3.06 (1.50–4.63) kg, $p < 0.001$
Pregnancy weight gain > IOL limits	65,000	(39)/2011	15 years postpartum weight gain	Mean increase of 4.72 (2.94–6.50) kg
Gestational diabetes	675,455	(40)/2009	Manifest type 2 DM	RR 7.43 (4.79–11.51)
Maternal obesity Pre-pregnancy BMI > 30 kg/m^2	46,688	(41)/2016	Hospitalization for CV events	HR 2.6 (2.0–3.4)
Premature ovarian insufficiency	190,588	(42)/2016	IHD	HR 1.69 (1.29–2.21)
Premature ovarian insufficiency	190,588	(42)/2016	Total CVD	HR 1.61 (1.22–2.12)

a, adjusted; BMI, body mass index; CAD, coronary artery disease; CVD, cardiovascular disease; DM, diabetes mellitus; FGR, fetal growth restriction; FD, fetal death; HR, hazard ratio; OR, odds ratio; RR, risk ratio (or relative risk).

risk factors had a smaller first-trimester fetal crown-rump length, lower second and third trimester estimated fetal weight but more rapid growth from 6 months onward. This suggests that even first-trimester fetal growth relates to subsequent cardiovascular risks (71). As adults, women rather than men with LBW (≤2.5 kg) have higher fasting plasma glucose, insulin, diabetes, and metabolic syndrome (**Table 3**). In both genders, height increased with BW, whereas BMI and waist circumference have a U-shaped association with BW (58).

Adequate nutrition and micronutrient density such as iron, copper, zinc, iodine, selenium, and vitamin A and D are prerequisite for fetal growth. In industrialized countries, food containing essential micronutrients is likely to be more expensive decreasing the dietary quality in low-income groups (72, 73). Even in countries without absolute food shortage (e.g., Sri Lanka) 25% of mothers and 33% of all children were malnourished and at risk of anemia, mental illness, or poor immune system (74). Exposure to unexpected contraction in the first trimester was associated with a decrease in BW which was stronger for women at home with <12 years' education and associated with increased risks for FGR (OR 1.5; 95% CI 1.1–2) (75). In recognition of the important impact of maternal nutrition and the crucial importance of preventive care, reduction of poverty and hunger during pregnancy is a defined millennium goal of the United Nations.

TABLE 3 | Impact of pregnancy complications on fetal risks for disease categories up to adulthood (selected prospective, mostly retrospective cohorts, and systematic reviews).

Pregnancy disease	Sample (*n*)	Literature (Reference number/year)	Definition of health risk in fetal life as an adult	Association of health risk HR/OR/relative risk (RR)/SD (95% CI)
Cardiovascular				
LBW and famine during gestation	975	(53)/2011	Coronary artery disease	HR 1.9 (1.0 to 3.8) adjusted for sex
LBW and first-trimester famine	726	(53)/2011	CHD	OR 3.0 (1.1 to 8.1) age and sex adjusted
LBW and famine	658	(53)/2011	Systolic BP	−4.14 mmHg/kg (−7.24 to −1.03) inverse relation
			Diastolic BP blood pressure	−2.09 mmHg/kg (−3.77 to −0.41) inverse relation
			Prevalence of hypertension	OR 0.67/kg (−0.49 to 0.93) age and sex adjusted
LBW (SGA) up to HBW (LGA)	6,239	(54)/2016	Left ventricular mass	SD score 0.05 (0.03 to 0.08) $p < 0.01$ for trend
LBW (SGA) up to HBW (LGA)	6,239	(54)/2016	Aortic root diameter	SD score 0.08 (0.05 to 0.1) $p < 0.01$ for trend
Preeclampsia (PE)	45,249	(55)/2013	High systolic BP during child and adulthood	2.39 mmHg (1.74 to 3.05)
			High diastolic BP during child and adulthood	1.35 mmHg (0.90 to 1.80)
PE	2,868	(56)/2015 Prospective cohort	Cardiovascular risk, hypertension and metabolic disease (QRISK > 75 P) at age 20	OR 2.5 (1.32 to 4.56)
Complicated HTN + birth factors	2,868	(56)/2015	Hypertension at age 20	aOR 6.25 (1.96 to 19.96)
Complicated HTN + risks at 20 years				aOR 6.74 (1.25 to 36.29)
Complicated HTN + social risks				aOR 6.63 (1.17 to 37.57)
Preterm SGA vs. term	1,756	(83)/2015	Adult hypertension	36.9 vs. 25.4%; risk factors adjusted $p = 0.006$
Neurologic				
First-trimester famine	66,321	(53)/2011	Schizophrenia ICD-6/-9	OR 2.01 (1.03 to 3.94)
First-trimester famine	737	(53)/2011	Accelerated aging, depression	$\beta = -85$ (−139 to −32), $p = 0.002$
First- and second-trimester famine	100,543	(53)/2011	Antisocial personality disorder in men ICD-6	aOR 2.5 (1.5 to 4.2)
Fetal growth restriction	1,679	(57)/2008	Hostility	(beta)SD < −0.05 (−0.14 to 0.00)
Metabolic				
First-trimester famine	2,414	(53)/2011	Increase LDL–high-density lipoprotein cholesterol ratios adult	Increase 13.9% (2.6 to 26.4)
LBW	2,546	(58)/2016	Leptin to fat mass ratio, leptin, diabetes mellitus (DM), obesity	$p < 0.05$ for all, chi-square test or ANOVA
Complicated HTN + birth factors	2,868	(56)/2015	Overweight or obesity at age 20	aOR 1.68 (1.18 to 2.39)
Complicated HTN + risks at 20 years		Prospective cohort		aOR 1.62 (1.05 to 2.52)
Complicated HTN + social risks				aOR 1.59 (1.02 to 2.48)
Early preterm	1,358	(59)/1996	High insulin at birth	OR 2.05 (1.69 to 2.42)
Early preterm	1,358	(59)/1996	High insulin in childhood	OR 1.31 (1.10 to 1.52)
Parental smoking	17,003	(60)/2014	Increased body mass index (BMI) and WC at 32 years	Increase of 0.57 kg/m^2/1.46 cm ($p \leq 0.02$)
Birth weight < 2 SD	61,311	(61)/2007	DM as an adult	OR 2.01 (1.39 to 2.91)
Birth weight ≥ 2 SD			DM as an adult	OR 2.27 (1.38 to 3.74)
Famine second trimester	702	(62)/2006	Decreased glucose tolerance at 50/58 years	Diff = 0.4 mmol/l (0.1 to 0.7), sex/BMI adjusted
LGA and GDM during pregnancy	179	(63)/2005	Metabolic syndrome at 11 years (increased insulin resistance and obesity)	OR 10.4 (1.5 to 74.4)
After ovulation induction	2,577	(64)/2014	Increased fasting glucose levels at 6 years	0.4 mmol/l (0.2 to 0.6)
After *in vitro* fertilization	2,577	(64)/2014	Increased fasting glucose levels at 6 years	0.2 mmol/l (0.0 to 0.5)

a, adjusted; BP, blood pressure; CHD, coronary heart disease; GDM, gestational diabetes mellitus; HR, hazard ratio; HTN, hypertensive disease in pregnancy; LBW, low birthweight; LGA, large for gestational age; OR, odds ratio; SGA, small for gestational age.

Parity

Older studies have demonstrated that multiparity independently increases maternal CVD risks in later life (31) (**Table 2**). A "J" shaped association between number of children and CVD was observed, lowest among those with two children and increasing with each additional child beyond two by 30–47% for women and by 12% for men (32) (**Table 2**). Specifically, in women, the number of children was inversely correlated with high-density lipoprotein (HDL) cholesterol and positively associated with TGs and diabetes. It has been concluded that lifestyle risk factors combined with child rearing result in obesity and increased rates of CVD. The 9% increase in CVD risk in women with only one child has been attributed to coexisting fertility conditions such as polycystic ovary (PCO) (76).

Hypertensive Disorders of Pregnancy (HDP), PE

Risk factors for HDP can be broadly categorized as *personal*, *cardiovascular*, *metabolic*, and *prothrombotic* (77). Approximately 80% of women with a history of PE have at least one risk factor of which the cardiovascular risk profile is most prevalent, followed by hyperhomocysteinemia, metabolic syndrome, and thrombophilia (78, 79). Both circulatory and metabolic risk profiles were associated with earlier onset PE; FGR was more likely in patients with a diastolic BP above 80 mmHg (80). All

first-trimester prediction algorithms for PE identify surrogate markers of cardiovascular and metabolic health as independent contributors (77). The most recent and largest systematic review and meta-analysis on risk factors for PE in relation to clinical risk factors included more than 25 million pregnancies (81): women with antiphospholipid antibody syndrome had the highest rate of PE (17.3%, 95% CI 6.8–31.4%). Those with prior PE had the greatest relative risk (RR) of 8.4 (7.1–9.9). Chronic hypertension ranked second with a RR of 5.1 (4.0–6.5), pre-gestational diabetes had a RR of 3.7 (3.1–4.3), pre-pregnancy BMI > 30, and ART were other risk factors suggesting that the presence of any one might suffice to designate a woman as "high risk" and support clinical prediction for the use of early prevention (81). The parallel rise in PE and maternal long-term complications supports the concept that early-pregnancy risk profiles are causally linked to maternal long-term health. Accordingly, the American Heart Association guidelines on female CVD include GDM and HDP in their risk assessment (82). Although balanced diets and active lifestyles reduce the risk for DM in women with GDM, adoption of these health behaviors is low (83).

Placental invasion, size, and function are sensitive to maternal blood flow disturbances and the placenta modulates fetal responses to the environment. The "gateway" to the fetus modifies epigenetic marks and placental gene expression leading to gender-specific diseases (84). The placental lesions of PE and severe FGR, such as "atherosis in the placental bed," are similar to atherosclerosis suggesting common genotypes, phenotypes, and earlier mortality (85). Mothers with PE have atherosclerotic disease such as angina pectoris, cardiac insufficiency, or renal disease within a mean interval of 11 years and earlier mortality is evident by 20 years after delivery in these women (86, 87).

Maternal placental syndromes combined with FGR increase the downstream risk for early hospitalization for heart failure, cardiac dysrhythmia, and CVD follows this pattern (34, 35) (**Table 2**) suggesting that dysfunction in both placental compartments amplifies the risks. Affected women should therefore have their blood pressure and weight assessed by 6 months postpartum, and a healthy lifestyle should be emphasized. Following coronary artery revascularization, middle-aged women are at higher risk for death than men. After a mean of 5 years, 41 deaths (2.2 per 100 person years) occurred in women compared to 1.1 in women without maternal placental syndrome (HR 1.96; 95% CI 1.29–2.99). The risk of death was significant in women with placental abruption (HR 2.79; 95% CI 1.31–5.96), placental infarction (HR 3.09; 95% CI 1.23–7.74), and PE (HR 1.61; 95% CI 1.00–2.58). Women with placental syndrome in two pregnancies had the highest HR of death of 4.31 (95% CI 1.71–10.89). This should be included in the informed consent process (33).

Children born to pre-eclamptic mothers are at increased risk for high BP, stroke, cognitive delay, and depression (**Table 3**) (55). As young adults, these children have a 2.5-fold increased risk of a QRISK Score above the 75th centile (95% CI 1.32–4.56, $p = 0.004$). Consideration of additional factors would allow identification of a cohort with hypertension (**Table 3**) (56). PE leads to a 40% elevated risk of later serious CVD (88); 30% of all 20-year old's with high BP had mothers with PE (95% CI 1.3–7.0; $p = 0.01$) (56). The recognition of these associations by family practitioners and pediatricians raises the possibility of tailored interventions to prevent adult hypertensive disease. The discussion on the optimal management approach to HDP during pregnancy is ongoing (89, 90).

First-trimester pregnancy-associated plasma protein-A was one of the first serum biomarkers noted to correlate with placental function and fetal growth (91). Now, more complex first-trimester screening algorithms for PE and FGR offer individual risk prediction with up to 90 and 60% sensitivity, respectively (5, 92, 93); 91% of women that are test positive at the first-trimester screen have cardiovascular and metabolic conditions amenable to therapy (8). Since many of these risk profiles pre-date before or persist after pregnancy it appears that first-trimester screening for placental disease is not only cost-effective but could have therapeutic benefits that reach far beyond pregnancy (94).

Prematurity

Prematurity and low BW are endpoints of several potentially different etiologies (95). Women who had a preterm birth more frequently develop CVD and type 2 diabetes (4, 36). There is a negative correlation with gestational age at delivery and the rates of later maternal diseases. In a recent systematic review, SPB increased maternal risks of developing or dying from IHD, stroke, and overall CVD (28, 96) (**Table 2**).

Following preterm birth, the offspring is also at higher risk for elevated BP levels in adulthood and insulin resistance in infancy (97). It is unknown if this is attributable to interactions between peripartum exposure to inflammatory cytokines, cardiovascular effects of pulmonary dysmaturity, and placental dysfunction (98–100).

Stress

Acute stress responses activate the hypothalamus–pituitary–adrenal axis (HPA) and the immune system to enable the organism for environmental threats. However, prolonged activation of the stress response may have adverse consequences. Maternal stress, anxiety, and psychological maternal disease can evoke immediate changes in blood flow to the uterus, fetal heart rate, or fetal movements (FM). However, they also induce long-term changes in fetal growth, metabolism, behavior, and cognition. Since there are no direct neural connections between mother and fetus, acute and chronic responses are likely elicited by neuroendocrine, autonomic, or vasodilatory input. Low BW by itself is associated with "hostility" in adult life, e.g., a rival cynic personality with mistrust and negative affections, which again is combined with CVD (57) (**Table 3**).

It is fascinating to imagine that the fetus may actively contribute to its own epigenesis as FM between 20 and 38 weeks transiently stimulate maternal sympathetic arousal prepare women for nurturing without becoming desensitized (101). Listening to music and singing lullabies has been shown to reduce women's experience of stress, anxiety, and depression and might simultaneously stimulate and be remembered by the fetus (102, 103).

The interplay of maternal stress on metabolic disorders in the next generation has been investigated: only 2/45 known type 2 diabetes susceptibility genes are associated with LBW, indicating that the association is mainly non-genetic. The developing

fetal brain requires some, but not overwhelming stress. FGR is associated with poor school education, smoking, drinking habits, poor social activities of mothers, and poor maternal social support (59). Even exposure to modern media "attacks" seems to reduce BW by 50 g (104). Maternal exposure to the death of a close relative is also correlated to LBW, where deregulation of the HPA was most marked during the second trimester when spiral arteries invade trophoblastic cells (105). Endocrine factors, such as β-HCG or progesterone, play gender-specific roles for growth and disease. In a multivariable regression model, increase in maternal progesterone by 1 ng/ml during the first trimester increased girls' BW by 10.2 g (95% CI 2.03–18.31); perceived worries (and smoking) predicted FGR in boys irrespective of progesterone levels (106). Fascinating reviews on maternal stress have been published (67, 107).

Smoking, Toxic Agents

Maternal smoking is one of the commonest modifiable risk factors. It has been estimated that active or passive smoking during pregnancy is responsible for at least 20% of infants with LBW (108). The effect is dose dependent (adjusted OR 2.40 for 0–9, 2.68 for 10–15, 2.88 for >15 cigarettes daily). Parental smoking also increases CVD risk in the offspring (109). After puberty, the effect of parental smoking was positively associated with BMI ($p < 0.001$) with a significant dose response for each additional 10 cigarettes. The associations of maternal smoking were stronger than for paternal smoking. At age 32, offspring of at least one smoking parent had higher BMI and waist circumference. Adjusted weight at age 17 was 63.2 kg for offspring of non-smoking parents compared to 64.6 kg of at least one smoking parent and at age 32, 68.3 and 70.5 kg, respectively (60) (**Table 3**). In many countries, pregnant mothers are exposed to wood fuel smoke. The carbon monoxide depresses placental energy-dependent processes and amino-acid transport and exhibits a reduction of infants' adjusted mean BW by −186 g (110). Variation in genes encoding enzymes modify the associations between maternal smoking and BW, but the contribution of epigenetic mechanisms, rather than genetic, underlie the long-term effects of smoke exposure as shown in aberrant placental metabolism, syncytial knot formation, or markers of placental oxidative damage (111).

Health Risks Predominantly Attributable to a Metabolic Risk Profile

Between 2011 and 2012, an estimated 14.4% of US women over 20 had high total cholesterol; overall, 65% of US women are overweight or obese, with highest rates among non-Hispanic black women (14). The International Diabetes Foundation defined metabolic syndrome as BMI > 30 kg/m^2 or WC > 80 cm (women), and at least two of the following criteria: fasting glucose > 5.6 mmol/l (100 mg/dl) or diabetes, cholesterol > 1.3 mmol/l (50 mg/dl), or medication use to low HDLs, TG levels of >1.7 mmol/l (150 mg/dl) or specific treatment and a BP > 130/85 or use of antihypertensive medication (112). It remains unclear, in how far single criteria are associated with a higher risk for stroke or CVD and the precise scientific concept of the metabolic syndrome remains controversial. The WHO has put forward specific diagnostic criteria relating to BMI, blood pressure, proteinuria, TG, and HDL (113). Each component of the metabolic syndrome increases risks for PE especially when C-reactive protein is elevated (114). *Vice versa*, women that develop PE exhibit more pronounced insulin resistance and dyslipidemia, which frequently continues after pregnancy (115–118).

The rise in the prevalence of childhood and adult obesity in low- and high-resourced countries led WHO's Director General to establish a Commission on Ending Childhood Obesity, which stressed the need for concerted and sustained action, early in the life course of mothers (and fathers) (119). The UN General Assembly proclaimed 2016 a Decade of Action on Nutrition calling upon governments to address the diet-related burden of disease (120). A series on preconception and maternal obesity in The Lancet Diabetes & Endocrinology 2016 and 2017 suggests new directions that such an initiative could take (121) and demonstrates how uncontrolled GDM is responsible for a transgenerational passage of obesity.

(No) Breastfeeding

Several studies have shown that breastfeeding is associated with decreased activation of the HPA axis, a blunted BP response to stress, and increased fat mobilization (122–124). In a prospective Danish cohort, an inverse association was observed for breastfeeding duration and weight retention up to 18 months correlating with anthropometric measures 7 years after delivery (37) (**Table 2**). In mothers, who already have symptoms of GDM, breastfeeding is protective against type 2 diabetes.

Infant feeding characteristics are associated with subsequent risk for maternal DM within 2 years (38) (**Table 2**). Potential mechanisms for these associations include prolactin-mediated preservation of pancreatic β-cells, less inflammation, and improved endothelial function. To estimate the effect of breastfeeding on maternal health, mice were randomly divided into lactated (L) and non-lactated (NL) animals (120). At 9 months, the NL group weighed significantly more compared with the L group had significantly higher systolic BP, lower ejection fraction, and higher renal artery resistive indices compared with L mice which all suggested that lactation has a direct beneficial effect. Large systematic reviews covering >9,000 abstracts and approximately 400 individual studies have demonstrated that breastfeeding reduces risks for type 2 diabetes, breast, and ovarian cancer. Early cessation or not breastfeeding was associated with a higher risk of postpartum depression. There was no relationship between a history of lactation and the risk of osteoporosis (125). Lactation duration was found to be inversely associated with common carotid intima-media thickness at 20 years; mean differences between a duration of ≥10 months compared with 0 to <1 month ranged from −0.062 mm for unadjusted models to −0.029 mm if adjusted for pre-pregnancy BMI, cardiometabolic risk factors, parity, smoking, and sociodemographics (p trend: 0.01) (38). It is not yet clear whether all observed associations between breastfeeding and maternal health are causal since breastfeeding women have a healthier lifestyle overall. Nevertheless, it was estimated that low breastfeeding rates in the US result in 4,981 excess cases of breast cancer, 53,847 cases of hypertension, and 13,946 cases of myocardial infarction. Suboptimal breastfeeding

is therefore supposed to incur a total of $17.4 billion in cost to society/year resulting from premature death (95% CI $4.38 to 24.68 billion), $733.7 million in direct costs (95% CI $612.9 to 859.7 million), and $126.1 million indirect morbidity costs (95% CI $99.00 to 153.22 million). There was a non-significant difference in additional premature deaths before 70 years (126).

Infants benefit from breastfeeding due to protection from infections and biologic signals for promoting cellular growth and differentiation. Breastfeeding reduced the severity of respiratory problems in the first 27 weeks of life (RR 0.70; 95% CI 0.55–0.88) (127) and reduces risks for acute otitis media, non-specific gastroenteritis, lower respiratory tract infections, atopic dermatitis, asthma, obesity, type 1 and 2 diabetes, childhood leukemia, sudden infant death syndrome, and necrotizing enterocolitis (125). The American Academy of Pediatrics recommends exclusive breastfeeding for approximately 6 months, followed by continued breastfeeding with complementary foods for 1 year or longer (128). The American College of Obstetrics and Gynecology asks for a multidisciplinary approach involving practitioners, family members, and child care providers to support breastfeeding mainly for underserved women (129). Breastfeeding promotion is a practical, low-cost intervention to prevent CVD, obesity, and diabetes in high-risk women, with the potential for benefits that are complementary to lifestyle interventions targeting weight loss.

Pre-Pregnancy Obesity and Excessive Weight Gain during Pregnancy

Overweight (BMI > 25 kg/m^2) and obesity (BMI > 30 kg/m^2) have become global risk factors for NCDs (130). Obesity before and during pregnancy leads to an increase of maternal mortality; more than 50% of all maternal deaths in Great Britain were overweight or obese (131). The accumulation of visceral fat tissue correlates with increasing insulin resistance and a metabolic syndrome (132). Pregnancy itself leads to obesity: approximately 75% of women are heavier 1-year postpartum than they were pre-pregnancy (133). Increased abdominal fat mass (134), which also characterizes aging (135), may affect long-term maternal health because abdominal fat mass predicts mortality better than weight or BMI (136). This is also the reason why a body fat index—a novel ultrasound index evaluating central maternal fat—seems to be more informative than BMI in terms of prediction of obstetric complications particularly for subsequent development of GDM. Also women with normal pre-pregnancy BMI need to be informed about the recommendations by the Institute of Medicine. Excess weight gain increases fat mass, especially in women with preconception obesity (137, 138). Excess pregnancy weight gain increases the risk for lifelong visceral fat retention (39, 137, 139, 140) (**Table 2**).

Mothers with excessive weight gain are less likely to breastfeed (141). In a cohort of 46,688 women, a BMI ≥ 30 kg/m^2 was associated with higher adjusted rates of cardiovascular events and related hospitalizations (142). Truncal obesity as defined by WC or waist/hip ratio has been shown to be more strongly related to certain cancer types than obesity as defined by BMI (143). Possible mechanisms that relate obesity to cancer risk include insulin resistance and chronic hyperinsulinemia, increased production of insulin-like growth factors, or high bioavailability of steroid hormones because adipose tissue-derived hormones and cytokines (adipokines), such as leptin, adiponectin, and inflammatory markers, may reflect mechanisms linked to tumor genesis.

Maternal obesity or pre-gestational DM is associated with fetal myocardial functional changes as early as the first trimester, which could explain the predisposition of offspring to CVD later in life (29, 41). Offspring of mothers with excessive weight gain and a BMI > 30 kg/m^2 are more frequently obese at age 16 compared to offspring of mothers with normal weight gain, even after adjusting for age, socioeconomic status, sex, or BW (144). As adults they demonstrate reduced life quality and life span (HR: 1.35; 95% CI 1.17–1.55) (145). These Scandinavian epidemiologic data match with animal experiments demonstrating that rats on a high-calorie diet before and during pregnancy demonstrate adipogenesis and are "programmed to early death, e.g., a shorter life" (146). Endothelial lesions, increased number of fat lobule *in utero*, as well as the U-shaped correlation of BW and obesity might play a role (3). Additionally, exposure to obesity and high-fat diet before, during, and after pregnancy promote appetite over satiety neurons in the hypothalamic arcuate nucleus leading to offspring hyperphagia and obesity (147). Finally, a high-caloric or high-fat maternal diet modulates the fetal gut microbiome and gut–brain axis causing a persistent predisposition to metabolic disease and obesity in the offspring (148, 149).

Maternal obesity is associated with sex-specific differences not only in fetal size but also in neurodevelopment reflected by gene expression signatures and the brain transcriptome. Especially male embryos of dams on the high-fat diet had a significantly lower BW than controls; dietary change in pregnancy resulted in significantly more dysregulated genes and pathways in male than in female brains (386 vs. 66, $p < 0.001$) (150).

GDM and Pre-Existing Diabetes during Pregnancy

Type 1 or type 2 diabetes may exist before pregnancy; GDM is defined as glucose intolerance first diagnosed in pregnancy and is associated with subsequent hyperinsulinemia, dyslipidemia, type 2 DM, hypertension, and CVD (151). Already in the 1950s, Pedersen et al. reported the association of GDM with DM and fetal macrosomia [c.f. (152)]. In the 1980s, Freinkel described the impact of GDM on fetal long-term health and together with Metzger on maternal glucose tolerance after birth; 30% of women with GDM have a persisting glucose tolerance and develop type 2 diabetes within 10 years (153–155). Worldwide, the incidence of GDM has increased to 7–14% (156). Weight gain above the IOM norms and GDM are associated: a case–control study of 800 women with excessive weight gain but normal glucose tolerance showed a 50% increase of GDM compared to controls with normal weight gain (118).

The hypothesis that pre-existing risk profiles play a role is supported by the fact that first-trimester prediction of GDM by history or biochemical–biophysical tests has sensitivities as high as 80% (7). The risk to develop overt DM increases with maternal age and accelerated almost 10-fold for women with GDM resulting in cumulative 15-year risk of 25% (156). A comprehensive systematic review including 205 relevant reports, 20 studies, 675,455 women, and 10,859 type 2 DM events confirmed the

increase of type 2 DM compared to normoglcyemic women (40) (**Table 2**).

Fetal programming related to GDM and DM is complex since both, low and high BW are associated with the development of a metabolic risk profile in later life. A significant association between low (OR 2.15, 95% CI 1.29–3.50) and high BW (OR 1.97, 95% CI 1.12–3.45) and later development of GDM was shown with a U-shaped relation between BW and risk of GDM (61). In families or regions where GDM was not known before an "epigenetic mismatch" between prenatal and postnatal nutrition plays an increasing role.

The hypothesis "fetal origins of disease" was proposed to explain associations between low BW and impaired glucose tolerance or CVD (157–161). The thrifty phenotype hypothesis suggests that early malnutrition induces poor development of pancreatic β-cell mass and programs the metabolic syndrome (157–162). LBW was related to high concentrations of split proinsulin, a sign of beta-cell dysfunction, linked to later high blood pressure (163, 164) and to metabolic abnormalities in combination with low physical activity and/or high-energy intake (165). The British Maternal Nutrition Study correlated prenatal micronutrient deficiency with increased insulin resistance in childhood: the offspring of mothers with combined high folate and low vitamin B12 levels were insulin resistant (166). Similarly, prenatal famine exposure was associated with impaired glucose tolerance and insulin secretion in adulthood (167, 168). Before, it was already demonstrated that a pregnancy with a LBW child indicates a risk for significantly lower rates of insulin, C-peptide, and proinsulin responses than controls. Insulin sensitivity was increased in the FGR compared to the control group (169).

Children of mothers with GDM and fetal macrosomia are at higher risk of childhood obesity and its consequences (170). Metabolic markers such as insulin resistance and high TGs are present in 21% before puberty (171) and at age 11, maternal GDM with fetal macrosomia increased the risk of metabolic syndrome 3.6-fold over controls with isolated macrosomia (63). Similarly, as adults these children are at higher risk for DM and metabolic syndrome (171, 172). A large Danish study of women with DM confirmed that as adults the offspring was at increased risk for CVD (adjusted OR 1.46; 95% CI 1.16–1.83) and for insulin-dependent DM (adjusted OR 4.7; 95% CI 3.9–5.8) compared to offspring of non-diabetic mothers. CVD was associated with FGR rather than macrosomia (OR 1.29; 95% CI 1.24–1.35) (173). Accordingly, undernutrion during pregnancy was linked to decreased glucose tolerance up to age 58 (62) (**Table 2**).

Cesarean Rates and Subfertility

Cesarean delivery and later childhood obesity are associated independently of the fact that Cesarean rates are *per se* higher in obese women and differences in the infant intestinal microbiome has been postulated as a potential explanation (174). Subfertility such as premature ovarian insufficiency or PCO is another factor that increases risk for CVD and metabolic disease (42, 76).

Children conceived through artificial reproduction techniques have higher glucose levels compared to controls (64). At the age of 5–6 years, glucose levels were increased by 0.4 mmol/l (95% CI 0.2–0.6) and 0.2 mmol/l (95% CI 0.0–0.5), respectively. Similarly, systolic and diastolic BP was elevated by 0.8 mmHg (95% CI −0.2 to 1.8) and 1.4 mmHg (95% CI 0.6–2.3). Since the duration of infertility correlates with BP in the offspring and with PE in the pregnant women, epigenetic and genetic factors have been implied (64).

Predominantly Thrombotic Risk Profile: Thrombophilia, Systemic Lupus Erythematosus (SLE), and Antiphospholipid Syndrome (APS)

Most women with a thrombotic risk profile are already aware of their diseases before pregnancy.

But pregnancy is regarded as a prothrombotic state due to its impact on coagulation. It modifies the disease or makes it visible when no symptoms were known before.

Coagulation disorders such as thrombophilia, SLE, and APS are recognized risk factors for placental dysfunction, FGR, and PE. In these conditions, aspirin or heparin therapy may decrease the rate of thrombosis and possibly of placental disease and adverse outcome (175, 176). The generalized administration of prophylactic anticoagulants is not supported until RCTs show a benefit (175). Patients with APS, SLE, and triple antiphospholipid antibodies or prior thrombosis are at risk for FGR or recurrent thrombosis and seem to have a better fetal outcome when treated with anticoagulants (177). Women with thrombophilia, SLE, and/or obstetric APLS during pregnancy have lifelong thrombosis risks requiring long-term anticoagulation and awareness during risk situations; patients with arterial events should be treated aggressively.

During pregnancy, low-dose aspirin is likely to address underlying factors that promote a prothrombotic risk profile. However, therapy of hyperhomocysteinemia may also be worthwhile as high first-trimester homocysteine levels increase the risk of PE threefold to fourfold (178, 179).

Low folate intake is an important contributor to increased homocysteine levels and is significantly more common in women who develop PE (180). Modification of homocysteine levels requires high-dose folate and an RCT evaluating a 4 mg folate daily is ongoing (181, 182). It remains to be determined if folate will benefit all women or specifically those with elevated homocysteine levels.

Children of mothers with a prothrombotic risk profile have only been investigated in small series: the neurodevelopment of 30 children born to mothers with SLE and/or APS with IgG antibeta2-glycoprotein I positive for the same antibodies at birth have been examined up to 9 years postnatally according to a Child Behavior Check List, rheumatologists, and pediatric characteristics. In all children, neurological physical exam and intelligence levels were normal. Mild behavior disorders were shown in three children possibly related to maternal disease or prematurity (183).

DISCUSSION

In 2011, the General Secretary Ban Ki-Moon declared during the first NCD-conference of WHO and the United Nations: "We

strive for an international commitment that puts NCDs high on the development agenda." Confirming a match of epidemiological data with molecular mechanisms described in human or animal research during pregnancy, opens opportunities to couple the prevention of NCDs with reduced health-care costs. This is particularly relevant for maternal-infant care (**Figure 2**). There are periods in our life cycle in which we are particularly susceptible to epigenetic influences; mediated by mechanisms that include DNA methylation, histone modification, and RNA silencing. It appears that the pathophysiology of adverse pregnancy outcome can have its origins during fertilization, gametogenesis, embryonic, fetal, and placental development and translates into downstream long-term health impacts (184, 185). We have shown that many pregnancy complications are associated with maternal and infant health risks in later life. By recognizing the sentinel circumstances during key periods in pregnancy, we are opening a unique window of opportunity for interventions to improve maternal-child health. To increase our knowledge and relevant consequences for long-term health, we have to associate findings from animal experiments and clinical measurements with transgenerational data from large populations.

New evidence is leading us to revise our understanding on the origins of placental disease and its interdependence with maternal and child health (19). It has been presumed that the increased uterine artery resistance and abnormal physiologic transformation of the placental vasculature is the initiator of placenta-based diseases such as PE and FGR. However, observations in other maternal vascular beds that are independent of early trophoblast function and the documentation of maternal risk profiles long before placental disease has developed, challenge these concepts (19, 77, 185, 186).

Even in normal pregnancy, myocardial and ventricular function decline after the second trimester (187). The findings of a drop in stroke volume index, impaired myocardial relaxation with diastolic dysfunction, and eccentric remodeling at term are suggestive of cardiovascular maladaptation to the volume overload in some apparently normal pregnancies. Following pregnancies with FGR and especially PE has shown a low cardiac

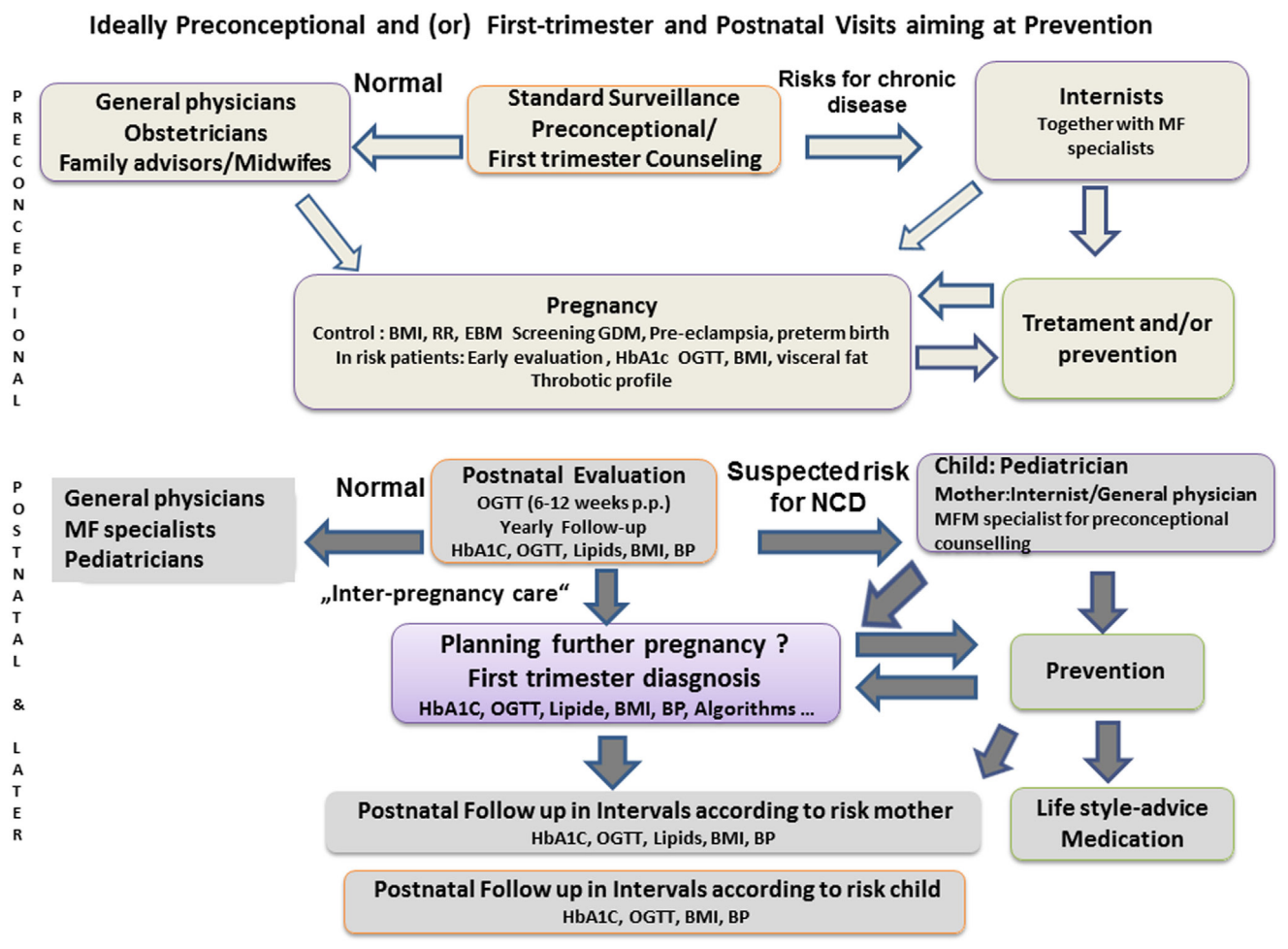

FIGURE 2 | Schematic proposal for health-care concepts to intensify interdisciplinary cooperation, pre-conceptional counseling, and postpartum consultation of women with pregnancy complications, according to Carson (112) or Bohrer and Ehrenthal (36). BMI, body mass index; BP, blood pressure; EBM, evidence-based medicine; GDM, gestational diabetes mellitus; MF, maternal fetal; NCD, non-communicable disease.

output, high resistance circulatory state, asymptomatic global diastolic dysfunction, and poor cardiac reserve (188). Postpartum follow-up has demonstrated remodeling in patients with PE, but asymptomatic left ventricular dysfunction and hypertrophy were significantly higher in preterm PE compared with term PE or matched controls. The risk of developing essential hypertension within 2 years was higher in both women with preterm PE and those with persistent left ventricular abnormal function or geometry. Specifically, these women are more likely to experience recurrent placental disease (189). Accordingly, pre-existing risk constellations seem to interact with placental development in women that develop PE or placenta-based FGR and that their risks are increased by the adverse pregnancy outcome. The synergy between individual risk constellation and their amplification by pregnancy complications is likely to be responsible for the downstream health effects as described above (190). To determine the thresholds where disease occurs requires large perinatal registers that are initiated before conception and follow women and their offspring.

The question whether later diseases in maternal life are caused by the disease itself or a pre-existing condition can also be illustrated by perinatal registers as it was performed for end-stage renal disease (ESRD). It could be concluded that familial aggregation does not explain increased ESRD risk after PE, but that PE *per se* leads to kidney damage (191).

Gestational hypertension and PE share baseline risk factors, such as a family history of DM, of myocardial infarction before 60 years and elevated TG levels while physical activity is protective (192). Extending registries across generations will amplify our ability to influence public health by even earlier preventive care to benefit mothers, fathers, and their children (193). The effects of FGR and PE on survival rates of fetuses and mothers drawn from the Norwegian registry mirror both genetic and epigenetic influences and match with the topic of this paper (**Table 4**).

Transgenerational research in rats from Nathanielsz et al. (146) not only show how obese mothers transfer obesity to their children, but also demonstrate that the effect can be limited by physical activity or a diet during pregnancy. The pictures of the second generation not only help to understand the epigenetic pathophysiology but can motivate patients with a high BMI to stick to IOM guidelines and to be physically active (**Figure 3**).

Continuous family care is relevant with a focus on selected time periods:

(a) Prenatal and early postnatal life offers a window of epigenetic plasticity when environmental factors may condition the body in ways that shape disease risk in later life (194, 195).
(b) The past as experienced by siblings, parents, grandparents, and possibly earlier generations becomes relevant for understanding our disease risks today and tomorrow (193).

In Scandinavia, registry-based perinatal epidemiology has shown the importance of sibling and generation data which allow linking of birth records across generations (193). These population-based linked materials provide research opportunities beyond cross-sectional studies where observations are often based on studies of the woman's first pregnancy. But women who stop reproducing after one pregnancy have different mortality rates than women with two or more pregnancies (196). Studying the next pregnancy conditional on outcomes of previous pregnancies is challenging and shows risk heterogeneity between women (193). Intergenerational data reveal the influence of socioeconomic and behavioral factors, and not only genetic inheritance (197).

Compared with the task of family doctors of previous times, obstetricians, and MFM specialists are predominantly focused on prenatal care and obstetric emergencies with less emphasis on the long-term outcome of women and their families. In most countries, there is not even a continuity of care between obstetric providers and other care specialties. As a result, research, development, and clinical care across this critical health-care frontier are disproportionally sparse.

As MFM specialists are in the advantageous position to screen, diagnose, and manage pregnancy-related complication in an index pregnancy they are ideally positioned to initiate care paths after pregnancy. Sentinel risk profiles need to be incorporated into care models, which will allow the initiation of personalized care paths for mothers and infants (8, 198). In the US, the diabetes prevention trial already aims to introduce

TABLE 4 | Perinatal death rates as relative risks (RRs) and later maternal death rates form cardiovascular disease in later life correlated with gestational age at birth and pregnancy disease [preeclampsia (PE) and fetal growth restriction], designed from recent data of the Norwegian data base, personal communication and with gratitude to Rolv Skjaerven, 2017.

PE during first pregnancy	Gestational weeks at delivery	Birth weight (*Z*-score)	Perinatal death (RR)	Maternal death (HR)
Yes	≥37	Large (>0)	2.0	1.8
Yes	≥37	Small (<0)	6.3	1.5
Yes	35–36	Large	4.1	**5.2**
Yes	35–36	Small	23.8	0.9
Yes	≤34	Large	29.3	**11.3**
Yes	≤34	Small	**79.4**	2.3
No	≥37	Large	1 (reference)	2 (reference)
No	≥37	Small	2.9	1.3
No	35–36	Large	4.3	2
No	35–36	Small	21.3	2.5
No	≤34	Large	51	2
No	≤34	Small	**131**	2.8

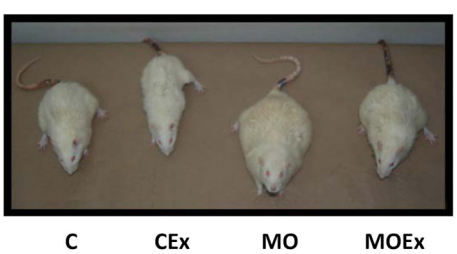

FIGURE 3 | Representative pictures of male offspring of rats at postnatal day 650. C, control diet; CEx, control diet + maternal exercise intervention; MO, maternal obesity; and MOEx, maternal obesity + maternal exercise intervention, with gratitude, according to Nathanielsz et al. (146).

a balanced diet and more active lifestyle to reduce later risks for diabetes (199). Models exist to use BP, lipids, visceral fat, BMI, and glucose tolerance at 6 and 12 months after high-risk pregnancies to define the need for inter-pregnancy care, lifestyle interventions, or therapy within specialized clinics (200–204). The fact that pregnant women are more sensitive for health-care advices should be used as a chance to intervene as early as possible (36). A potentially useful tool would be to modify the maternal "passport" as it is currently utilized in many European countries to produce a lifelong health record as required. Widely available computer technologies and app's can be designed for targeted information about risks, interventions, and evidence-based concepts and are preferred to booklets in the younger generation (205).

Meanwhile, a first guideline for follow-up of patients after previous PE has appeared, whereby optimization of modifiable cardiovascular risk factors is recommended for reproductive and pregnancy-related disorders to reduce the risk of future CVD (206). Progress will eventually become inevitable as focused patient history taking, pregnancy risk algorithms, and the existing obstetric care platform already meet WHO criteria for screening tests (207). It is our responsibility to incorporate interdisciplinary care algorithms (87, 208) (**Figure 2**).

We need to realize how epigenetic findings relate to questions of social and environmental justice and not only to individual responsibility (24). This requires recognition of the presented associations as an opportunity to modify preventive and long-term care. Such progress hinges on widespread patient and health-care provider education about the unique opportunity to identify and treat modifiable risk factors for adverse health outcomes (209, 210). Strategies to reduce long-term and intergenerational risks associated with pregnancy disorders should include access to interdisciplinary teams to substantially affect future pregnancy outcomes and chronic illness.

Policy makers need to establish preventive interventions and need to better tackle long-term risks and inequalities of perinatal care. The future MFM specialist will be less invasive, give less medicine but will interest patients in the cause and prevention of disease as Thomas Edison stated (151). We have to reduce health illiteracy, the misbalance between responsible and irresponsible resource management and thereby the burden of increasing rates of chronic diseases (211).

AUTHOR CONTRIBUTIONS

BA and AB had a substantial contribution to the conception and design of the work and its interpretation. BA had drafted the work and both BA and AB have revised it critically. Final approval and agreement to be accountable for all aspects are agreed on by BA and AB.

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OPINION
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Refining Pharmacologic Research to Prevent and Treat Spontaneous Preterm Birth

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INTRODUCTION

Preterm birth (PTB), delivery prior to 37 weeks' gestation, is the leading cause of mortality among non-anomalous neonates. Survivors carry an increased risk for lifelong intellectual, physical, and social disabilities compared with their term counterparts (Russell et al., 2007; Bodeau-Livinec et al., 2008; Vohr, 2013; Manuck et al., 2014a, 2016b; Natarajan and Shankaran, 2016). In the US alone, more than 450,000 babies are born too soon and ∼25,000 die as a result (Hamilton et al., 2015). Approximately two-thirds of all PTBs are spontaneous PTB (SPTB), and occur following preterm premature rupture of membranes, cervical insufficiency, and/or uterine contractions leading to cervical dilation. To reduce the burden of SPTB, interventions must target both prematurity prevention prior to the onset of symptoms and acute treatment once the process of acute preterm labor has begun.

Research efforts to develop treatments to improve neonatal outcomes have met some success [e.g., 17-alpha hydroxprogesterone caproate for recurrent SPTB prevention (Meis et al., 2003), antenatal corticosteroid treatment to prevent sequelae of prematurity (Roberts and Dalziel, 2006; Gyamfi-Bannerman and Thom, 2016)]. However, our ability to effectively prevent and treat SPTB remains limited. Of the available treatment options, significant inter-individual variation is appreciated. The reasons for this response variation are poorly understood and represent a critical knowledge gap contributing to thousands of SPTB every year. Refinement of patient selection for available drugs, or changing the form or dose of medication has the potential for large impact on therapeutic efficacy. Here we highlight two examples of medications currently used to reduce SPTB and discuss how cutting edge approaches may improve outcomes (**Table 1**).

PREVENTION OF RECURRENT SPTB WITH INTRAMUSCULAR PROGESTERONE

A personal history of SPTB is the strongest clinical risk factor, conferring a 2- to 4-fold risk for SPTB (Hamilton et al., 2015). In a large multi-center randomized controlled trial conducted by the NICHD Maternal-Fetal Medicine Units Network, Meis et al. studied weekly intramuscular 17-alpha hydroxyprogesterone caproate (17-OHPC) vs. placebo in women with a history of a prior SPTB. The rate of recurrent SPTB <37 weeks gestation was reduced from 55% in the placebo group to 36% in the 17P group (RR 0.66, 95% CI 0.54–0.81; Meis et al., 2003). Currently, offering 250 mg intramuscular 17-OHPC weekly from 16 to 36 weeks gestation to women with a singleton pregnancy and a history of a prior singleton SPTB <37 weeks is standard of care in the United States (Committee on Practice Bulletins-Obstetrics and The American College of Obstetricians and Gynecologists, 2012; Society for Maternal-Fetal Medicine Publications Committee, with assistance of Vincenzo Berghella, 2012). Unfortunately, 17-OHPC is only effective for some women and as many as 30–40% will experience a recurrent SPTB despite treatment. The mechanism of action of 17-OHPC has not yet been elucidated.

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TABLE 1 | Listed is a summary of example medications for prevention and treatment of spontaneous preterm birth, along with current and future approaches to optimize response to these therapeutic interventions.

Indication	Example medication	Optimizing response to therapy	
		Current	Future
Prevention of prematurity	17-OHPC	- Predict response based on clinical risk factors	- Determine optimal dose based and likelihood of response based on maternal and/or fetal genotype, methylome, transcriptome - Interrogate gene-environment interactions as etiology in variable response - Evaluation of effects of concominant drug administration on 17-OHPC levels
Treatment of acute preterm labor	Indomethacin	- None	- All of the above, plus: - Application of nanotechnology to minimize fetal exposure while maximizing tissue-specific effects

17-OHPC, 17-alpha hydroxyprogesterone caproate.

Assessing Reasons for Variable Response to 17-OHPC

Several recent studies have focused on defining which individuals are destined to have a favorable response to progesterone for the prevention of recurrent preterm birth. These investigations have identified several distinct and consistent clinical risk factors for recurrent SPTB despite 17-OHPC therapy, including black race, gonorrhea or chlamydia infection, vaginal bleeding, family history of SPTB, male fetus (Manuck et al., 2016a,c). Pharmacogenomic studies assessing recurrent SPTB among women using 17-OHPC have been pre-clinical in nature, but have also shown promising results, implicating biologically plausible pathways including the nitric oxide pathway and other genetic pathways involved with signal transduction and infection and inflammation (Manuck et al., 2014b; Manuck, 2016). Unfortunately, insufficient sample numbers, difficulties in defining adequate treatment "response" and lack of randomized studies has prevented this work from reaching direct clinical applicability (Manuck, 2016). Studies examining more acute genetic changes (e.g., epigenetic modifications) in response to the environmental stimuli of 17-OHPC are also currently limited but may offer additional avenues of research and eventual clinical application. Finally, it is possible that genotype or methylation changes affect birth outcomes only in the presence of a certain critical threshold or serum level of 17-OHPC. Many drugs taken by women for a variety of indications during pregnancy are metabolized by the cytochrome P450 enzyme system; 17-OHPC is also metabolized by the cytochrome P450 system, primarily CYP3A (Sharma et al., 2010). Interactions with co-administered CYP3A inhibitors or inducers may influence 17-OHPC drug metabolism and clinical efficacy (Sharma et al., 2008). No studies examining the effects of concomitant drug administration with 17-OHPC have been published to date, though *in vitro* data suggests these other CYP-metabolized drugs may impact 17-OHPC levels (Sharma et al., 2008).

Using State-of-the-Art Genetic Technology to Investigate Other Reasons for Variable Outcomes

Epigenetic modifications provide a mechanism by which genes interact with the environment; they affect gene expression by inducing structural changes in DNA that are maintained through cell division, respond to environmental changes including drug exposures, yet are potentially reversible and can be targets for disease therapy (Feinberg, 2007). DNA methylation at cytosine-guanine dinucleotides sites, the most commonly studied epigenetic modification in humans, guides temporal, and tissue-specific gene expression during fetal development and tissue differentiation. Even subtle environmental changes may induce epigenetic changes and have effects on phenotype (Golbabapour et al., 2011). Epigenetic biomarkers from blood have been used in a variety of complex conditions in other fields (e.g., depression, addiction/smoking). Moreover, these changes are potentially reversible and reflect dynamic interactions with the environment. For example, studies of women with breast cancer (Antoni et al., 2012; Bower et al., 2014; Stagl et al., 2015), caregivers of family members with dementia (Black et al., 2013), patients with inflammatory bowel disease (Kuo et al., 2015), and even healthy individuals (Qu et al., 2013) have shown that the body's response to chronic stress may be dynamic, mediated by glucocorticoid gene expression, and at least partly reversible **at the genomic level** when treated with cognitive behavioral therapy or relaxation techniques. Therefore, study of the methylome and transcriptome is a promising and understudied avenue of investigation in prematurity research (Fuchikami et al., 2011; Mikeska et al., 2012; Philibert et al., 2013). Methylation is tissue specific; results from blood, placental, or cervical tissue, for example typically cannot be directly compared. However, methylation studies in blood may be a reliable correlate of physiologic processes in other tissues (Smith et al., 2014). Limited studies of epigenetics in obstetrics have demonstrated identifiable differences among women delivering preterm and those with term deliveries, but more work is needed to evaluate whether these differences may be influenced (positively or negatively) by medication exposure or other known risks factors for SPTB, such as vaginal bleeding or a short cervical length.

Refining Clinical Phenotype to Improve Preterm Birth Outcome Predictions

Unfortunately, the aforementioned pharmacogenomics associations have been modest. Larger scale genome wide association studies of SPTB in general (without considering

treatment response) have also been largely disappointing. It has been hypothesized that suboptimal clinical phenotyping is a major contributing factor to the challenges of reproducibility that have characterized genetic investigations of prematurity. Many genetic studies of SPTB have limited or inconsistent phenotype information; even those that do collect detailed information tend to be based on relatively heterogeneous groups of women. We recently described a phenotype classification system. This tool uses clinical data to group women into nine distinct SPTB phenotypes (e.g., cervical insufficiency, infection/inflammation, decidual hemorrhage), and may provide further insight beyond the heterogenous PTB categorization, improving upon prior classification systems (Manuck et al., 2015).

TREATMENT OF ACUTE SPONTANEOUS PRETERM LABOR WITH INDOMETHACIN

One of the few tocolytic medications proven to prolong pregnancy is indomethacin, a non-steroidal anti-inflammatory medication that inhibits prostaglandin production by blocking the conversion of arachidonic acid to prostaglandin. Indomethacin is metabolized by CYP2C9 and carboxy esterase (Agúndez et al., 2009). Cells in the amnion, chorion, decidua, and myometrium exhibit enhanced prostaglandin production in response to cytokines interleukin-1β, interleukin-6, and tumor necrosis factor-α, and may help initiate the parturition cascade (Romero et al., 1989a,b; Mitchell et al., 1990). It is therefore hypothesized that indomethacin has a direct effect in the inhibition of the inflammatory response. Indomethacin has been shown to be more effective than placebo in prolonging pregnancies with threatened preterm labor by >48 h, and use for women with acute preterm labor may permit administration of a course of antenatal corticosteroids. Despite this, indomethacin has not been proven to improve short- or long- term neonatal outcomes (Niebyl et al., 1980; Panter et al., 1999; Klauser et al., 2014).

Though somewhat effective, indomethacin is not without potential risk to the fetus, and these risks have limited its use. Indomethacin freely crosses the placenta and enters fetal circulation. Premature closure of the physiologically patent ductus arteriosus is a recognized side effect of indomethacin and is the leading reason why tocolysis with indomethacin is generally limited to short courses of therapy (48–72 h; Moise, 1993). Some studies suggest that neonates exposed to antenatal indomethacin may have an increased risk of other adverse effects including periventricular leukomalacia and necrotizing enterocolitis (Major et al., 1994; Amin et al., 2007), although results have been somewhat inconsistent between studies.(Dudley and Hardie, 1985; Macones and Robinson, 1997; Loe et al., 2005) Despite these safety concerns, indomethacin is one of the first line recommended tocolytics by the American Congress of Obstetricans and Gynecologists, and short courses of indomethacin are widely used in the US for acute tocolysis due to its relative effectiveness compared to other agents (American College of Obstetricans and Gynecologists' Committee on Practice Bulletins-Obstetrics, 2016).

Assessing Reasons for Variable Response to Indomethacin

Determination of individuals most likely to remain pregnant 48 h after indomethacin therapy is a challenge, as placebo-controlled tocolytic studies are limited and retrospective studies are fraught with confounding. The diagnosis of "preterm labor" remains imperfect, and studies have traditionally used varying definitions to determine participant eligibility. Many women initially diagnosed with acute preterm labor will eventually deliver at term. We are unaware of any reported studies of indomethacin pharmacogenetics in the setting of pregnancy, though investigation of maternal and/or fetal genome, epigenome, methylome may shed additional light on variation in response in a similar fashion to pharmacogenomics investigations of 17-OHPC as outlined above.

Application of Nanomedicine to Target Indomethacin Delivery to Gestational Tissues

If women who are most likely to benefit from indomethacin treatment for acute preterm labor can be identified through pharmacogenomic or other investigations, the issue of potential toxicity to the fetus must be resolved in order for this therapy to be administered optimally. Recently, nanomedicine has been investigated as one potential way to overcome this limitation. Nanomedicine provides a mechanism to vector drugs (e.g., through liposomes) preferentially to diseased or target tissues in the body, while limiting exposure (and thus toxicity) to healthy tissues (and in the case of pregnancy, the fetus). Specifically engineered liposomes, encapsulated with indomethacin and with a surface oxytocin receptor antagonist, have been designed for this purpose, to deliver indomethacin directly to the myometrium while limiting fetal exposure. In initial murine studies, these liposomes successfully delivered indomethacin to the uterus and inhibited prostaglandin production—thus maintaining its pharmacologic effects—while reducing fetal exposure by 7.6-fold.(Refuerzo et al., 2015) In subsequent murine models, these specifically engineered liposomes prolonged pregnancy by 31% and reduced the rate of PTB by 15% (Refuerzo et al., 2016). The application of nanotechnology has great promise as a solution to the fetal toxicity appreciated with indomethacin, and refinement of these techniques will expand researcher's abilities to investigate other therapeutics for SPTB prevention and treatment with lower concern for fetal exposure.

SUMMARY

Preterm birth is a devastating obstetric complication leading to fetal mortality and morbidity. Despite extensive research, there remains much to learn regarding the pathophysiological mechanisms associated with SPTB. We understand even less about the role that epigenetic regulation and subsequent altered gene expression play as an etiologic factor for this condition. Progestogens and tocolytic drugs can reduce neonatal morbidity and mortality by preventing or halting SPTB through poorly defined mechanisms. Additional individual

patient characteristics, including maternal and fetal genotype and gene-environment interactions likely influence response. Other factors, beyond the scope of this article—such as cytochrome P450 enzyme activity, maternal body mass index, and other considerations that may impact volume of drug distribution may also impact response to medications for SPTB. Studies of all SPTB therapeutics should collect and incorporate rigorous clinical phenotype information and biologic sample data whenever possible, in order for further refine and integrate clinical phenotype, genotype, and response to preventative and therapeutic medications.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Endothelial Progenitor Cells of the Human Placenta and Fetoplacental Circulation: A Potential Link to Fetal, Neonatal, and Long-term Health

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The fetoplacental circulation plays a key role in both short- and long-term outcomes, and aberrant flow indices as manifested by abnormal fetal Doppler velocimetry within this compartment have been associated with significant adverse consequences. These include fetal growth restriction, which often coexists with preeclampsia, and long-lasting medical issues as a result of both the underlying pathology and prematurity such as bronchopulmonary dysplasia, chronic lung disease, and neurodevelopmental delay. Furthermore, it is also clear that exposure to an abnormal *in utero* environment increases risk for long-term, adulthood issues such as cardiovascular disease. Endothelial progenitor cells (EPCs) have been implicated in vasculogenesis and angiogenesis, and they have been isolated from both human placenta and umbilical cord blood. This review outlines the extensive nomenclature of EPCs, summarizes existing literature surrounding human placental and umbilical cord blood EPCs, explores their potential role in pregnancy complications and adverse perinatal outcome, and highlights key areas where future investigations are needed.

Keywords: endothelial progenitor cells, umbilical cord blood, placenta, endothelial colony-forming cells, circulating progenitor cells

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INTRODUCTION

Of the three main components that shape placental function—the maternal uteroplacental circulation, placental trophoblast, and fetoplacental blood flow—it is the fetoplacental circulation that has been clinically demonstrated to be most highly related to adverse perintal outcome. For instance, pregnancies complicated by fetal growth restriction (FGR) with abnormal fetal Doppler velocimetry (e.g., umbilical arteries, middle cerebral artery with concern for "brain-sparing," and ductus venosus) are at significantly elevated risks for stillbirth and neonatal death (1, 2). Survivors are at higher risk for chronic medical problems and neurodevelopmental delay (1, 2). Furthermore, even if a growth-restricted fetus emerges from the perinatal and early childhood periods without adverse consequences, multiple lines of evidence suggest that they remain at increased risk for long-term, adulthood issues such as cardiovascular disease (3–5).

From a structural perspective, scanning electron microscopy, stereological analysis, and mathematical modeling suggest that FGR placentas demonstrate impaired placental vascular angiogenesis (6–8). Although this is just one condition in which the fetoplacental vasculature is impaired, it highlights the importance of this compartment in pregnancy and long-term outcome.

Recently, there has been substantial focus on endothelial progenitor cells (EPCs) and their role in vasculogenesis, angiogenesis, and even re-endothelialization of injured vessels. This field continues to evolve in many areas including nomenclature, methods of isolation and culture, and mechanistic roles during pathogenesis. EPCs have been isolated from the human placenta and umbilical cord blood, and in comparison to those derived from adult peripheral blood mononuclear cells (PBMNCs), demonstrate unique features such as enhanced proliferative and clonogenic potential. This suggests that placental and/or cord blood EPCs might play a role in development of the fetoplacental vasculature, and thus, may be potential targets for treatment modalities aimed at improving pregnancy, fetal, neonatal, and long-term outcomes.

EPC HISTORY

The initial discovery of a population of putative EPCs from adult peripheral blood in the late 1990s (9) changed two widely accepted paradigms. First, it challenged the existing notion that vasculogenesis occurs only during fetal development. Second, it disputed the concept that angiogenesis in adults is only able to arise through extension of mature vascular endothelium. Since this initial discovery, much has been uncovered about the function and classification of EPCs. In this review, we discuss the current nomenclature, history of various sub-populations, EPCs isolated from both umbilical cord blood and placenta, and the association of EPCs with adverse pregnancy outcomes.

Isolation of an EPC population was first performed by Asahara and colleagues (9). However, the identification method used to isolate these cells did not include a unique identifier specific to EPCs. As such, many groups have since worked to further characterize and develop a method to unequivocally identify EPCs. Unfortunately, a universal, indisputable approach to ascertaining a progenitor population has yet to be found. Progress, however, has been made to further characterize EPCs and EPC sub-types, and along the way, new nomenclature and identification techniques have been introduced. Differing names and techniques can be confusing and makes it difficult to decipher if previous reports are applicable to current investigation. To best understand the current state of the field, it is helpful to have an appreciation for where the field began.

The initial identification of EPCs utilized Ficoll centrifugation of peripheral blood to obtain a mononuclear cell population, and within this population, either $CD34^+$ or $Flk\text{-}1^+$ (also known as vascular endothelial growth factor receptor 2 or kinase insert domain receptor) cells were isolated with magnetic beads coated with the respective antibodies. These two antigens were individually targeted because both are expressed by hematopoietic stem cells prior to differentiation. Enriched cells were plated under various conditions. Attached $CD34^+$ cells after 7 days of culture appeared spindle-like and expressed endothelial-specific markers including *Ulex europaeus* agglutinin-1, factor VIII, CD31, endothelial nitric oxide synthase, and DiI-labeled acetylated LDL. They also demonstrated an endothelial cell-like phenotype, with the ability to produce nitric oxide in response to acetylcholine and vascular endothelial growth factor. *In vivo*, these cells were incorporated into foci of neovascularization in a rabbit model of unilateral hindlimb ischemia. In total, these investigators concluded that PBMNCs isolated with anti-CD34 or anti-Flk-1 were able to differentiate into endothelial cells, and this method of isolation and identification became the standard for assessing EPCs.

EPC ISOLATION *VIA* CELL CULTURE

Different cell culture methods of isolating EPCs utilize principles from the culture conditions as mentioned above, with each technique resulting in the isolation of different cell types. The method isolating endothelial colony-forming cells (ECFCs) is widely accepted as the closest representation of an EPC *in vitro* population. Isolation of ECFCs includes Ficoll centrifugation of PBMNCs, plating on collagen-1 coated plates, culturing in complete EGM-2 media (cEGM-2; Lonza) with 20% FBS, and expansion of colonies that appear typically between 14 and 21 days after isolation (**Figure 1**). This method produces colonies with cobblestone morphology indicative of an endothelial cell type. Functionally, these cells are able to migrate and form capillary-like structures, proliferate, and repopulate from a single cell (**Figure 2**) (10). These cells can sustain multiple passages in culture, but most experts recommend use of low passage number cells (P2–5) for experimental purposes. This cell type is also known as late outgrowth EPCs, blood outgrowth endothelial cells (10, 11), and can be defined as low or high proliferative potential (LPP and HPP, respectively) (12).

While this method is accepted, it has limitations. Currently, there are no studies linking ECFCs, an *in vitro* population, to physiologic cell populations. Another issue with this method is the quantity of blood required to produce colonies. Estes et al. has recommended a minimum of 16 mL of peripheral blood for the isolation of ECFCs in healthy adults (13). When isolating from umbilical venous cord blood, which has a higher percentage of ECFCs than adult peripheral blood (12), a minimum of 5 mL is required, although 10–20 mL is recommended. However, in conditions where the pregnancy is affected by certain pathologies, especially those resulting in preterm delivery, collecting an appropriate amount of cord blood can be difficult. Obtaining an adequate amount can also become problematic when trying to isolate ECFCs from infants and children.

Other cell culture isolation methods have been reported in the literature including colony-forming unit-endothelial cells (CFU-ECs) (14) and CFU-Hill (15) and early outgrowth EPC. A description of the nomenclature, isolation method, and limitations are outlined in **Table 1**. All of these names refer to similar cells, in that they have a spindle-like morphology, do not incorporate into vessels *in vivo*, and are likely of myeloid or lymphoid progenitor background (10, 16, 17).

EPC ISOLATION *VIA* FLOW CYTOMETRY

Another commonly used method for identifying EPCs is flow cytometry, and similar to cell culture methods of isolation, there are many differing sets of antigens used. The most recent

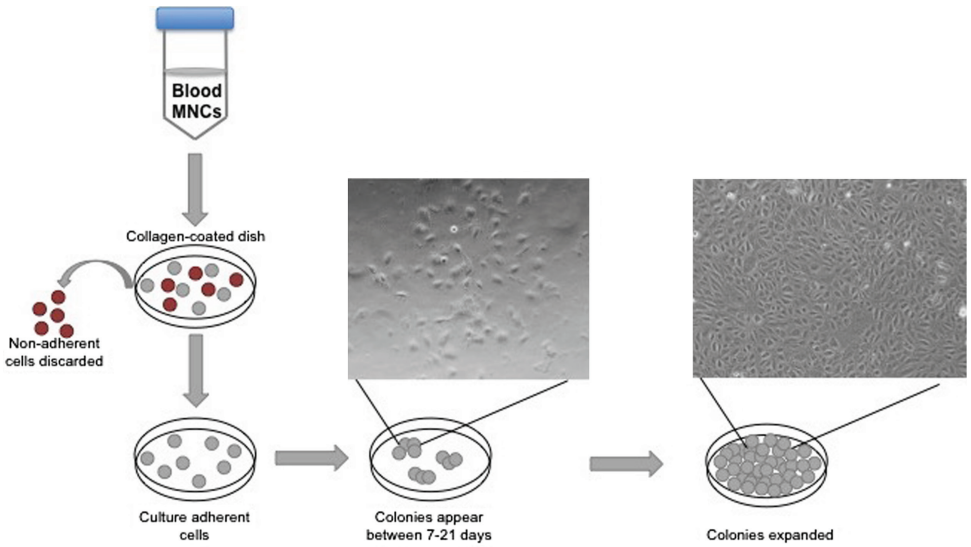

FIGURE 1 | **Isolation of endothelial colony-forming cell schematic**. The diagram depicts peripheral blood mononuclear cell separation *via* a Ficoll gradient, plating on collagen, and the appearance of colonies with cobblestone morphology.

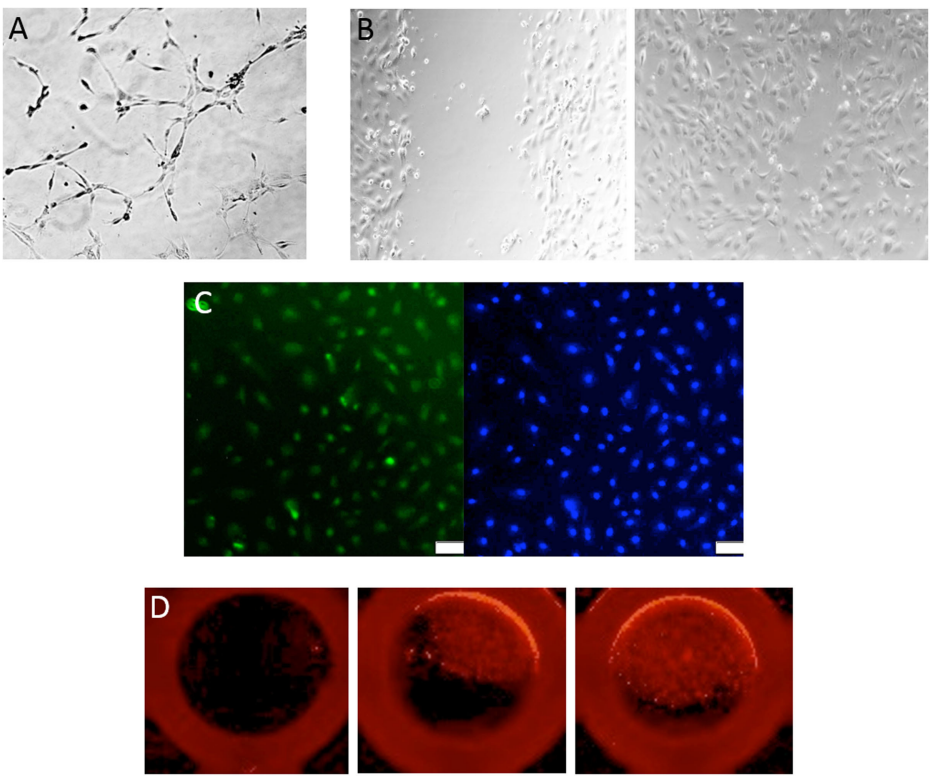

FIGURE 2 | **Assessment of endothelial colony-forming cell (ECFC) function**. **(A)** Representative image of a tube formation assay, where ECFCs are capable of forming capillary-like structures with the formation of branches and closed loops. **(B)** A classic wound migration demonstrates that ECFCs are able to migrate and close the wound. **(C)** ECFC proliferation is shown with BrdU staining in green (DAPI in blue). **(D)** Single-cell assay shows that ECFCs are capable of repopulation from a single cell.

TABLE 1 | The most commonly used nomenclature, isolation method, and associated identification markers of endothelial progenitor cells (EPCs) *in vitro*.

Cell culture models				
Name	**Other names**	**Isolation method**	**Identifying markers**	**Reference**
Endothelial colony-forming cells	Blood outgrowth endothelial cells Late outgrowth EPCs	Peripheral blood mononuclear cells (PBMNCs) are isolated from peripheral blood with a Ficoll gradient. Cells are plated on collagen-1, grown in cEGM-2, and colonies appear between 14 and 21 days in culture	Expression of CD31; CD141; CD105; CD146; CD144; vWF; Flk-1; CD34; CD133; CD117; eNOS Negative staining for CD45, CD14 Single-cell assay: able to repopulate from a single cell Able to form capillary structures *in vitro* Cobblestone morphology	(12, 18)
Colony-forming unit-endothelial cells	Early outgrowth EPCs, CFU-Hill EPCs	PBMNCs are isolated from peripheral blood similar to above, plated on fibronectin, grown in M199 medium, and colonies appear between 5 and 7 days in culture	Expression of CD34, vWF, CD144, Flk-1, UEA-1, Tie-2 Dil-Ac-LDL uptake Negative staining for VCAM-1 Spindle-like morphology	(10, 14, 19)

vWF, von Willebrand factor; Flk-1, vascular endothelial growth factor receptor-2; eNOS, endothelial nitric oxide synthase; UEA-1, Ulex europaeus agglutinin-1; VCAM-1, vascular cell adhesion molecule-1; Tie-2, TEK receptor tyrosine kinase; Dil-Ac-LDL, Dil-conjugated acetylated low-density lipoprotein.

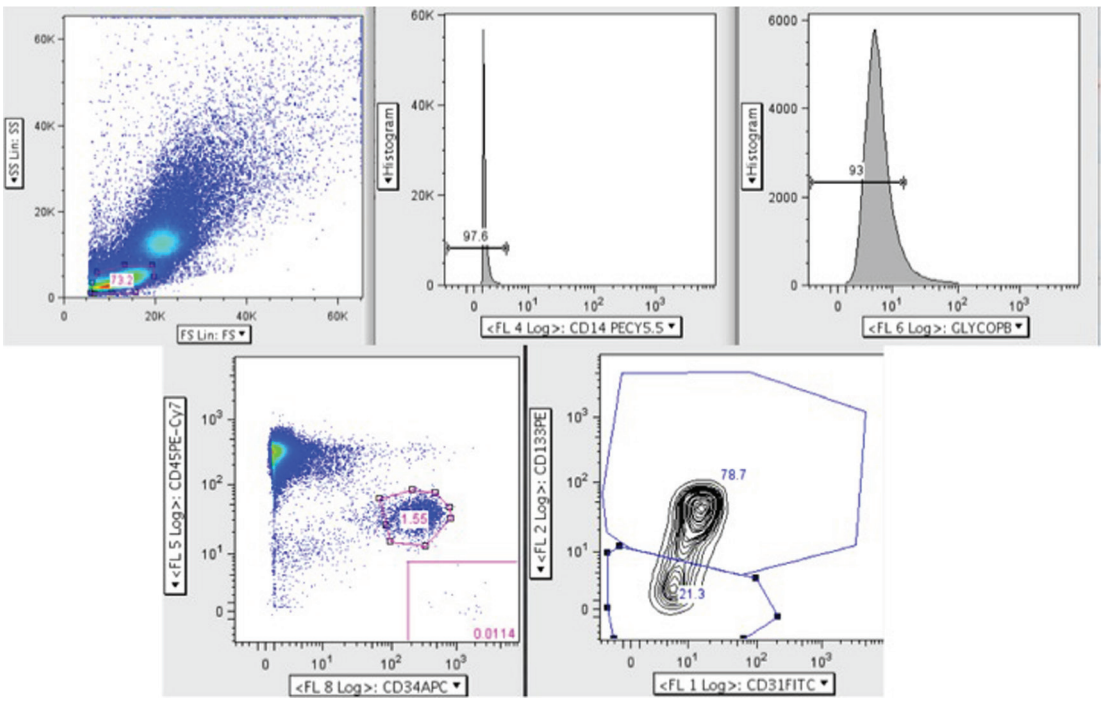

FIGURE 3 | Circulating progenitor cell (CPC) flow cytometry gating strategy adapted from Gumina et al. (23). Here, peripheral blood mononuclear cells were analyzed by polychromatic flow cytometry. Live mononuclear cells were selected and then gated for $CD14^-$ and glycophorin A^- cells to exclude erythrocytes and macrophages. Next, the $CD45^{dim}$ and $CD34^+$ population was selected from which the pro-angiogenic ($CD45^{dim}$ $CD34^+$ $CD31^+$ $AC133^+$) and non-angiogenic ($CD45^{dim}$ $CD34^+$ $CD31^+$ $AC133^-$) CPCs were identified.

version identifies circulating progenitor cells (CPCs) and allows for sub-group analysis of pro-angiogenic CPCs ($CD45^{dim}$ $CD34^+CD31^+AC133^+$) and non-angiogenic CPCs ($CD45^{dim}$ $CD34^+CD31^+AC133^-$) (**Figure 3**) (13, 20). The current limitations of this method include at least 1×10^6 cells for staining. This is not an issue when performed alone, but when paired with cell culture isolation, cell quantity may be inadequate. Additionally, as there are steps required for multiple antigen staining, these cells need to be fixed, and therefore it is not possible to culture these cells for ECFC comparison. It is important to note that ECFCs exist in the flow cytometry literature as well but are distinct from CPC populations. Specifically, ECFCs are a rare population thought to be identifiable with the CPC staining profile but instead of $CD45^{dim}$, they are $CD45^-$ (12, 21). Older methods using a combination of $CD34^+$Flk-1^+CD133^+ are likely incorporating angiogenic macrophages (22), which confound much of the older literature. **Table 2** provides a description of the nomenclature and staining protocols in the literature.

TABLE 2 | The most commonly used nomenclature and associated staining protocol for flow cytometry identification of endothelial progenitor cells (EPCs).

Flow cytometry		
Name	**Staining protocol**	**Reference**
EPC/circulating endothelial precursor	$CD45^-$ and/or $CD34^+AC133^+KDR^+$	(9, 24)
Circulating endothelial cell	$CD31^{bright}CD34^+CD45^-CD133^-$	(25)
Pro-angiogenic circulating progenitor cell (CPC)	$CD45^{dim}CD34^+CD31^+AC133^+$	(13, 20)
Non-angiogenic CPC	$CD45^{dim}CD34^+CD31^+AC133^-$	(13, 20)
Endothelial colony-forming cell	$CD45^-CD34^+CD31^+AC133^-CD146^+$	(13, 21)

PLACENTAL AND UMBILICAL CORD BLOOD EPCs

There has been considerable interest in alternative sources for EPCs beyond adult peripheral blood or bone marrow isolation. Various groups have demonstrated the presence of EPCs within both the human placenta and umbilical cord blood. Investigation of EPCs within the placenta has been limited, while literature surrounding cord blood EPCs has been much more robust. Nevertheless, a few different laboratories have described isolation of ECFCs from the human placenta, although some differences exist such as surface molecule expression (26–28). For example, Sölder et al. isolated $CD45^-CD34^+CD133^+Flk\text{-}1^+$ fetal endothelial cells from the placenta and showed that they were able to form tubes on a Matrigel assay (28). By contrast, $CD45^-CD34^-CD31^+Flk\text{-}1^+CD144^+$ cells were isolated by Rapp and colleagues, and these cells were able to form chimeric blood vessels in an *in vivo* vasculogenesis bioassay (26).

Unlike placentally derived EPCs, umbilical cord blood EPCs have been more extensively investigated. Earlier studies isolated EPCs that were characterized *via* early methodologies first described by Asahara and colleagues (9), which again, was the first description of a putative EPC population in adult peripheral blood (29, 30). Thus, these early cord blood EPCs are more consistent with CFU-ECs and not ECFCs. More recent studies of cord blood ECFCs demonstrate expression of various endothelial-derived surface markers (**Table 1**), with these cells exhibiting significant clonogenic and proliferative potential (12, 31). Importantly, cord blood ECFCs are enriched and display enhanced clonogenic and proliferative potential in comparison to adult peripheral blood (12). However, adult peripheral and cord blood ECFCs do not show any difference in tube formation capability or induction of vascular cell adhesion molecule-1 with inflammatory stimuli (12). When compared to placental ECFCs, though, cord blood ECFCs form significant fewer blood vessels in an *in vivo* vasculogenesis assay (26).

UMBILICAL CORD BLOOD EPCs AND DISEASE

In spite of the current limitations in identifying EPCs, there are numerous studies that have indicated reduced colony number and/or dysfunction of EPCs isolated from the umbilical cord blood of pregnancies complicated by preeclampsia, FGR, and gestational diabetes mellitus (GDM). In this review, we focus solely on studies that analyze either ECFCs in cell culture or CPCs derived from flow cytometry in order to avoid further confusion with other isolation methods that likely produce cell types independent of EPCs. It is important to note that other literature exists analyzing other cell populations described in **Tables 1** and **2**.

With regard to preeclampsia, different groups of investigators have demonstrated both reductions in circulating number and abnormal function of ECFCs isolated from the venous cord blood of babies born to preeclamptic mothers (23, 32, 33). Specifically, Gumina et al. showed a decrease in both pro- and non-angiogenic subsets of CPCs identifiable by flow cytometry in pregnancies complicated by preeclampsia in comparison to normotensive controls. All three reports also indicate fewer ECFC colony numbers in their respective preeclamptic populations. From a functional perspective, two groups reported that ECFCs from cord blood of preeclamptic pregnancies demonstrated diminished growth and migration (23, 32), while other laboratories found no difference in ECFC tube formation (23, 33). However, von Versen-Höynck et al. demonstrated a deficiency in preeclamptic ECFC tube formation, with partial improvement with vitamin D3 treatment (32).

Similar findings have been demonstrated in pregnancies complicated by FGR. Cord blood from FGR-complicated pregnancies showed fewer CPCs and ECFCs in comparison to controls, although this was seen only in arterial cord blood (34). ECFCs from the FGR offspring also showed diminished proliferation and migration. Furthermore, FGR ECFCs implanted into mice prepared for an *in vivo* vasculogenesis bioassay resulted in a sixfold increase in *de novo* capillary formation in comparison to controls (34). Taken together, the abnormalities seen in cord blood ECFCs in preeclampsia and FGR may be one mechanism that contributes to placental dysfunction and long-term elevated risks for cardiovascular disease in these offspring.

There is conflicting data regarding CPCs and ECFCs from the cord blood of GDM pregnancies. For example, one group of investigators found a decrease in CPCs and the CPC:non-CPC ratio in cord blood from GDM pregnancies in comparison to controls, but there was no difference in ECFCs, suggesting that endothelial function is intact at birth (35). By contrast, others have shown a decrease in ECFC colonies, proliferation, migration, and tube formation in cord blood of pregnancies complicated by GDM (36). From a mechanistic perspective, fetal ECFCs exposed to *in vitro* hyperglycemia demonstrated impaired migration and diminished tube formation in comparison to those exposed to normoglycemic conditions (36). ECFCs from GDM pregnancies, however, were also found to be resistant to hyperglycemia-induced senescence (36, 37). In total, this suggests that although cord blood EPCs in GDM pregnancies may have undergone a phenotypic alteration that renders them tolerant to a hyperglycemic environment, they still demonstrate functional abnormalities that may contribute to the increased risks of cardiovascular disease in offspring of diabetic women.

CURRENT LIMITATIONS AND FUTURE AREAS OF INVESTIGATION

There are several limitations within the field that may amplify discrepancies between findings in different studies. First, a comprehensive characterization of ECFCs in relation to normal physiology of the fetus and neonate is lacking. Additionally, knowledge of gestational age norms is also essentially non-existent, and this further hampers the field of investigation regarding EPCs and other pathogenic conditions that relate more directly to impaired placental vascularization, including FGR and stillbirth. Second, there are methodological issues that have yet to be standardized. For example, when ECFC number is assessed, it refers to the number of colonies that appear. ECFC colonies typically arise between 14 and 21 days *in vitro*, although colonies can still develop beyond this time frame (33). Thus, discrepancies in the literature may be a result of when the colonies are counted, and this may be one reason why studies differ in their interpretation of how ECFCs are specifically impacted by each condition. Third, controversy also exists when assessing ECFC function. Functionality is most commonly evaluated by measures of proliferation, migration, and ability to form capillary-like structures. As discussed above, all of these cellular processes can be assayed with various techniques and each technique can have slight differences that result in differing findings. Additionally, few studies incorporate *in vivo* models such as ischemic injury animal models in which a Matrigel plug embedded with patient-derived ECFCs is injected into the area of ischemia and ECFC incorporation into newly formed vessels is later analyzed. This model (38, 39) would yield a better understanding of ECFC function in a physiological setting.

In addition to methodological issues, it is also possible that different study populations are being investigated. For example, preeclampsia can present across a wide gestational age range and with varying degrees of severity. However, it has been shown that ECFCs are enriched at different gestational ages within umbilical cord blood, with gestational age likely to affect findings (31). Furthermore, the effect of the severity of the condition itself on ECFCs has also not yet been explored. Because number and function of ECFCs has been associated with adverse neonatal outcomes such as moderate or severe bronchopulmonary dysplasia, which itself has also been linked to severity of preeclampsia and FGR, it is not inconceivable that the status of the disease may affect ECFCs (18). As another example, ECFCs are increased in infants affected by chorioamnionitis, further suggesting that *in utero* environment may play a role on umbilical cord blood and placental EPCs (18).

Finally, in addition to continued cord blood EPC research, further investigation is also needed with regard to placental EPCs. The few existing studies utilize different isolation methods, demonstrate slight differences in immunophenotype, and perhaps most compellingly, suggest that there might be enhanced colony formation and functional characteristics in comparison to umbilical cord blood EPCs. As the field continues to advance, umbilical cord blood and placental EPCs are areas ripe with opportunity to better understand mechanisms underlying pregnancy-related diseases and adverse perinatal outcome. Continued investigation may yield preventative treatments or interventions for these pregnancy and perinatal complications in the future. Yet, this field has the potential to provide treatment targets beyond perinatal and neonatal outcomes by further elucidating mechanisms of fetal programming effects that contribute to increased risks for disease later in life.

AUTHOR CONTRIBUTIONS

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Antibiotic Therapy for Chorioamnionitis to Reduce the Global Burden of Associated Disease

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Chorioamnionitis is associated with significant maternal and neonatal morbidity and mortality throughout the world. In developed countries, great progress has been made to minimize the impact of chorioamnionitis, through timely diagnosis and appropriate treatment. In the global setting, where many women deliver outside the healthcare facilities, this diagnosis is frequently overlooked and not properly treated. In addition to its impact on maternal health, a significant proportion of neonatal morbidity and mortality can be prevented by both recognition and access to readily available treatment. With the increasing focus on saving the most vulnerable members of society, we echo the need for providing parturient women with suspected chorioamnionitis universal access to appropriate therapy. We describe known effective antibiotic therapies for chorioamnionitis and provide an overview of additional potential antimicrobial treatments that might be effectively implemented in areas with limited access to care.

Keywords: chorioamnionitis, neonatal sepsis, intrapartum antibiotics, global maternal health, intraamniotic infection

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Chorioamnionitis significantly contributes to maternal and neonatal poor outcomes. It represents Intrauterine Infection and/or Inflammation (Triple-I) (Higgins et al., 2016). In the short-term, chorioamnionitis can be associated with maternal sepsis, multi-organ dysfunction, stillbirth and death. Among surviving newborns, chorioamnionitis is associated with asphyxia, and early onset neonatal sepsis (EONS) well as long-term neurologic sequelae including cerebral palsy (Johnson et al., 2014). Antibiotics have been the mainstay of intrapartum therapy, and proven to reduce associated maternal and newborn morbidity. Chorioamnionitis can be clinically diagnosed by the presence of maternal fever, prurulent discharge, and tachycardia in the mother or her fetus (Higgins et al., 2016). Additional diagnostic methods include placental or amniotic fluid evaluation, but have more limited clinical applicability. Timely diagnosis and appropriate treatment with empiric antibiotic therapy and fetal delivery improves maternal and neonatal outcomes. Unfortunately, throughout the world, access to antibiotics and treatment for chorioamnionitis is limited (Laxminarayan et al., 2016). A variety of antibiotics have been described for their use in similar infections. We present a summary overview of current standard of care antimicrobial therapy as well as additional antibiotics that can be considered to facilitate treatment in resource-limited settings.

MATERNAL AND NEONATAL MORBIDITY ASSOCIATED WITH CHORIOAMNIONITIS

Chorioamnionitis, or Triple-I, is associated with significant maternal morbidity including the need for cesarean delivery, uterine atony, postpartum hemorrhage, as well as post-partum wound infections (Johnson et al., 2014). Approximately, 1 in 30 deliveries is complicated by the presence of chorioamnionitis, however the prevalence increases with decreasing gestational age at the time of delivery and has been associated with an estimated 25% of preterm deliveries (Ericson and Laughon, 2015). In the absence of treatment, up to 1 in 5 newborns exposed to chorioamnionitis develop early onset neonatal sepsis (Chan et al., 2015). Definitive treatment is achieved with delivery of the uterine contents, while temporization with antibiotic therapy may permit trial of labor and vaginal delivery rather than immediate surgical cesarean delivery. Antibiotic therapy can improve fetal signs of infection such as fetal tachycardia and minimize some of the associated maternal morbidities (Johnson et al., 2014). Therefore, while antibiotic therapy cannot eradicate the infection, it is an important pillar of management to prevent exacerbation of disease prior to delivery (Higgins et al., 2016).

Fetal sequelae from chorioamnionitis span from acute short-term disease to life-long morbidities (Johnson et al., 2014). The Fetal Immune Response Syndrome (FIRS) contributes to the significant morbidity of the condition, suggesting that even with antibiotic treatment of the offending organism, in some cases the inflammatory response may still cause significant morbidity, and is a major cause of stillbirth or neonatal death (Gibbs, 2002; Kallapur et al., 2014). Early onset neonatal sepsis can result in multi-organ dysfunction including life-threatening symptoms of respiratory distress and hemodynamic instability, and can be associated with chronic lung disease and neurologic injury among survivors. These complications are exacerbated among preterm infants and likely account for a significant proportion of global neonatal mortality, with urgent need for research to better delineate its impact are urgently needed (Lozano et al., 2012). It is important to note that routine administration of antibiotics in some cases of spontaneous preterm birth (e.g., preterm premature rupture of membranes) has the potential to mitigate neonatal sequelae (Cousens et al., 2010). Chorioamniontis in the global setting is a potential cause of stillbirth (Gibbs, 2002). Therefore, proper diagnosis of chorioamnionitis, and prompt administration of antibiotics to the laboring mother can temporize management until delivery, or in some cases during referral and triage to a tertiary care center and has the potential to avert death and long-term morbidity in a significant number of women and children (Gülmezoglu et al., 2016).

The burden of chorioamnionitis is most profound in Low and Middle Income Countries (LMIC) (Chan et al., 2016). Worldwide, an estimated 40 million births occur at home, mostly in LMIC and usually in the absence of skilled birth attendants and without access to proper preventative care as well as diagnostics and proper treatment for possible infection (Liu et al., 2016). A combination of poor conditions and poor hygiene contribute to neonatal mortality, with evidence that clean delivery practice has the potential to almost halve the risk of neonatal mortality (Seward et al., 2012). Neonatal infections, prematurity, and intrapartum related events account for the vast majority of the neonatal deaths worldwide. In the presence of overt chorioamnionitis, timely administration of antibiotics has the potential to minimize the risk of overt maternal or fetal sepsis (Chan et al., 2015) and has been associated with a 40% reduction in the neonatal infectious sequelae (Tita and Andrews, 2010).

EXISTING GUIDELINES FOR TREATMENT OF SUSPECTED CHORIOAMNIONITIS

General guidance for treatment for chorioamnionitis includes antibiotic coverage of the causative pathogens (Higgins et al., 2016). While widely subject to demographic and geographic variability, common organisms associated with chorioamnionitis include Ureaplasma (47%), Mycoplasma (30%), Gardnerella vaginalis (25%), bacteriodes (30%), gram negative rods including Escherichia Coli (8%), and Group B Streptococcus (15%) (Tita and Andrews, 2010). GBS specifically has wide variability in prevalence among global populations (Le Doare and Heath, 2013). The common feature of these organisms is that they represent genital microbes that can ascend and cause a strong inflammatory response.

Based on our knowledge of causative pathogens, a variety of antibiotic regimens are used to cover the presumed bacterial etiology of chorioamnionitis (Greenberg et al., 2012). However, current consideration for antimicrobial treatment for chorioamnionitis includes a combination of an aminopenicillin and gentamicin, with clindamycin or metronidazole generally added when cesarean delivery is performed (Tita and Andrews, 2010; Higgins et al., 2016). Of interest, neither antibiotic provides coverage against mycoplasma, which is a common organism associated with chorioamnionitis (Tita and Andrews, 2010). There are to date limited randomized controlled trials to evaluate superiority of antibiotic regimens to treat amniotic infections during ongoing labor to demonstrate effectiveness (Chapman et al., 2014). This suggests an absence of evidence basis for recommended type of treatment for clinical chorioamnionitis in a limited resourced setting. Therefore, current section of antibiotic regimen is driven not by absolute science, but limitations with existing research to demonstrate treatment superiority or non-inferiority.

Treatment of chorioamnionitis is challenged by accurate diagnosis, using clinical judgment in the assessment of signs of maternal fever, maternal and/or fetal tachycardia, and purulent cervical discharge (Johnson et al., 2014). Isolated maternal fever may arise from other causes, and is not necessarily an indication for diagnosis and treatment of chorioamnionitis (Higgins et al., 2016). Diagnosis can also be made on histologic placental evaluation after delivery, as well as microbial testing of amniotic fluid, each with significant limitations regarding clinical utility, particularly in a low resource setting (Johnson et al., 2014). Additional, there appears to be imperfect

TABLE 1 | A selected list of antibiotics and routes of administration, as included in the WHO guide of essential medications (WHO, 2016), with pregnancy category, half-life, and indication of placental passage efficacy.

Antibiotic class	Antibiotic	Pregnancy category	Route	Half-life (hours)	Placental transfer
Penicillins	Benzyl PCN	B	Injection	0.5	Incomplete
	Benzathine PCN G	B	Injection	30–50	Incomplete
	PCN V	B	Oral	0.5	Incomplete
	Procaine PCN G	B	Injection	20–40	Incomplete
Aminopenicillins	Ampicillin	B	Injection	1	Complete
	Amoxicillin	B	Oral	1.3	Complete
Penicillins: (Pellicinase Resistant)	Cloxacillin	B	Oral, Injection	0.5	Incomplete
Cephalosporins	Cefazolin	B	Injection	2	Complete
	Cephalexin	B	Oral	1	Complete
Cephalosporins:	Ceftriaxone	B	Injection	8	Complete
3rd Generation	Cefotaxime	B	Injection	1	Complete
	Ceftazidime	B	Injection	2	Complete
Vancomycin	Vancomycin*	C	Injection	4–6	Incomplete
B-Lacatmase Inhibitors	Amoxicillin/Clavulanate	B	Oral	1.0	Complete
Carbapenams	Imipenam+Cilastin	B	Injection	1–2	Incomplete
Aminoglycosides	Gentamicin	C	Injection	2–4	Incomplete
Macrolides	Erythromycin	B	Oral, Injection	1–1.5	incomplete
	Azithromycin	B	Oral	12	Incomplete
	Clarithromycin	C	Oral	5–7	Complete
Chloramphenicol	Chloramphenicol	C	Oral, Injection	1.2	Complete
Lincosamide	Clindamycin*	B	Oral, Injection	2–3	Complete
Fluoroquinolones	Ciprofloxacin	C	Oral, IV	3.7	Incomplete
Nitroimidazole	Metronidazole		Oral, injection, suppository	9	Complete
Nitroheterocylic	Nitrofurantoin	B	Oral	0.33	Incomplete
	Spectinomycin	B	Injection	2	Incomplete
Anti-Folate Agents	Trimethoprim/Sulfa	C	Oral, Injection	12	Incomplete
	Trimethoprim	C	Oral	12	Incomplete
Tetracycline	Doxycycline	D	Oral	12–16	Complete

Adapted in part from Grayson et al. (2010), Roberts et al. (2008), and WHO (2016).
PCN, Penicillin.
**Indicates WHO complementary medication, to be considered for specific clinical circumstances.*

correlation between these diagnostic methods (Chan et al., 2016). Although chorioamnionitis is clearly associated with preterm labor and delivery, the evidence does not support the routine administration of antibiotics to women in preterm labor with intact membranes in the absence of overt signs of infection, with caution that such prophylaxis may exacerbate poor outcomes (Subramaniam et al., 2012). Antibiotics for preterm premature rupture of membranes are indicated and effective in reducing the risk of a number of early morbidities, including respiratory distress syndrome and infection, without having a significant impact on mortality (Cousens et al., 2010). The burden of neonatal disease due to Group B streptococcal infection has seen dramatic improvement with standardized treatment following universal screening in pregnancy (Koenig and Keenan, 2009). It remains universally sensitive to penicillin, with alternative antibiotic regimens considered only in cases of allergy. Therefore, intrapartum antibiotics to prevent EONS are effective and have reduced substantially the incidence of EONS in countries where they are implemented (Dutta et al., 2010).

ADDITIONAL ANTIBIOTICS THAT CAN BE CONSIDERED FOR TREATMENT OF SUSPECTED CHORIOAMNIONITIS

A number of antibiotics might be considered for use in pregnancy to treat amniotic infection, appreciating those listed in the WHO Guide of Essential Medications (**Table 1**) (WHO, 2016). Considering fetal organ immaturity and/or ongoing organ development, initial evaluation must consider potential fetal organ toxicity or teratogenic effects, even when given just prior to delivery. It is important to note, that while pregnancy category may not strictly contradict administration, other considerations might preclude their use as with fluoroquinolones (ACOG, 2016). Secondary considerations would include route of administration. This would be particularly relevant for developing regimens that can be implemented in LMIC. Therefore, many of these agents would be reasonable potential candidates in LMIC. As challenges remain in recommendation of superior treatment regimens for chorioamnionitis when compared to others, consideration of

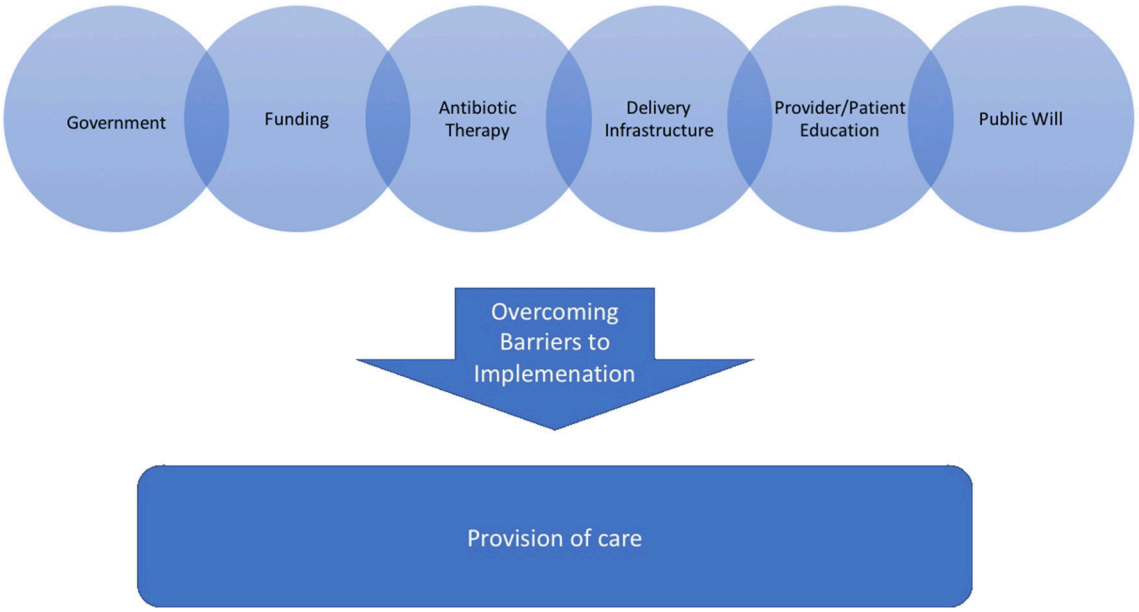

FIGURE 1 | A pictorial description of the factors that are needed for implementation of a policy for antibiotic therapy for chorioamnoninits into a society where it is otherwise not accessible.

a variety of agents might be considered to provide effective treatment for chorioamnionitis in a low resource setting. Consideration of Trimethoprim/Sulfa for use is considered in light of potential association with kernicterus in neonates (Thyagarajan and Deshpande, 2014).

The WHO list of essential medications provides guidance on a number of antibiotics that are most safe, efficacious, and cost-effective for priority conditions (WHO, 2016). Potential candidates for antibiotic therapy included on this list are further described with their half-life as well as relevant pharmacokinetic data on placental cross over (**Table 1**). Although treatment for chorioamnionitis before delivery tends to leave a short interval prior to delivery, duration of medication in case of needed repeat dosing may be considered. Given that chorioamnionitis represents an intra-amniotic infection, consideration of placental transmission of the medication would seem relevant. For each medication, individual considerations should be taken into account among other factors. Such factors include the compound stability, ease of administration, and medication cost, all of which are considered in the creation of the WHO list of essential medications. These factors may largely depend on geographic or local factors specific to communities. Additionally, medications that require weight-based considerations for dosing, such as aminoglycosides, macrolides, or vancomycin, might be challenging to implement. B-lactam antibiotics warrant consideration for increased dosing for particularly obese patients, which might be considered where obesity is a particular issue (Pevzner et al., 2011). Cultural acceptability of a regimen is important prior to implementation.

Accepted therapeutic regimens including ampicillin and gentamicin have similar antibiotic coverage compared to extended B-Lactamase agents (e.g., ampicillin/sulbactam). While multiple agents permit extended coverage, single agent regimens would seem to at least provide a modicum of convenience if not pragmatic integration in lower resource areas. Oral agents would have to consider their onset to action and bio-availability relative to IM or IV dosing, as time-conscious treatment of chorioamnionitis remains important. IV dosing will have pragmatic limitations in most communities. Cost will also have significant implications for universal provision, which would contribute to considering one agent over another.

STEPS TO IMPLEMENTATION OF UNIVERSAL TREATMENT FOR CHORIOAMNIONITIS

Globally, there remains an unmet need of intrapartum treatment of chorioamnionitis. Many steps will be made before this need can be met. Antibiotic therapy will need to be affordable, transportable, and readily administered. In addition, regimens will need to demonstrate non-inferiority to standardized regimens. Universal birth attendance is an unmet goal in many LMIC. As efforts bend to millennium development goals help improve the safety of childbirth, attention will move to significant causes of morbidity such as chorioamnionitis. Just as with uterotonics for postpartum hemorrhage and magnesium for seizure prophylaxis, antibiotic therapy for intrapartum infection will be necessary to reduce the morbidity of childbirth.

Attempts to create implementable regimens in various regions will rely on multiple factors for implementation (**Figure 1**). Evaluation of simplified regimens will be necessary to

demonstrate effective policies but also to encourage widespread implementation of effective treatment modalities (Zaidi et al., 2013). Effective implementation of antibiotic therapy for chorioamnionitis will require continued success with Sustainable Development goals aiming to promote well-being for mothers and children. Public Health efforts to minimize the maternal and neonatal morbidity associated with chorioamnionitis will continue. As attention is directed toward areas where treatment is not currently available, those pregnancies in low resource settings will benefit. As much focus as will need to be put on birth attendance and appropriate delivery hygiene, provision of appropriate antibiotic therapy for chorioamnionitis will remain important.

CONCLUSIONS

Various antibiotics and classes of antibiotics may be considered for effective treatment of chorioamnionitis. Ethical demonstration of benefit is problematic, given that a number of agents have long been considered effective therapy (Higgins et al., 2016). We outline a number of different antibiotic types that might be considered when developing treatment algorithms for chorioamnionitis, particular in LMIC were current treatment is far less than what a population might need to minimize maternal and neonatal complications. Effective treatment of chorioamnionitis will require more than just antibiotic therapy; it will require a birth companion competent to diagnose and treat the condition, an infrastructure to provide such antibiotics to the appropriate providers. As global efforts to increase the safety of birth advance, there is an urgent need for antibiotic therapy to appropriately manage chorioamnionitis in order to optimize maternal and neonatal outcomes.

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CJ, RA, and AF: Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; and drafting the work or revising it critically for important intellectual content; Final approval of the version to be published; Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Skeletal Dysplasias: Growing Therapy for Growing Bones

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Skeletal dysplasias represent a large and diverse group of rare conditions affecting collagen and bone. They can be clinically classified based on radiographic and physical features, and many can be further defined at a molecular level (Bonafe et al., 2015). Early diagnosis is critical to proper medical management including pharmacologic treatment when available. Patients with severe skeletal dysplasias often have small chests with respiratory insufficiency or airway obstruction and require immediate intubation after birth. Thereafter a variety of orthopedic, neurosurgical, pulmonary, otolaryngology interventions may be needed. In terms of definitive treatment for skeletal dysplasias, there are few pharmacotherapeutic options available for the majority of these conditions. We sought to describe therapies that are currently available or under investigation for skeletal dysplasias.

Keywords: hypophosphatasia, mucopolysaccharidosis, osteogenesis imperfecta, achondroplasia, *Asfotase alfa*, enzyme replacement therapy

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INTRODUCTION

A skeletal dysplasia is diagnosed in 1 of 5,000 births each year (Orioli et al., 1986). Severity is highly variable and can range from mild short stature to perinatal lethality. Among patients with less severe forms that allow survival are many who will require significant care for the duration of their lives. Despite research in the area of skeletal dysplasias that has elucidated a great deal about the genetic pathophysiology of these conditions, there remain limited therapeutic options.

The ability to accurately diagnose a skeletal dysplasia *in utero* has improved in recent years due to advances in prenatal ultrasound and molecular diagnosis. There are, however, limitations to ultrasound; certain skeletal dysplasias may not be seen until the third trimester (e.g., some forms of osteogenesis imperfecta), while others may manifest as late as infancy or early childhood (e.g., pseudoachondroplasia). In general, the earlier the dysplasia manifests, the more significant are its effects in terms of prenatal and postnatal morbidity and mortality. When sonographic findings are present, parents benefit from prenatal counseling and anticipatory guidance. Understanding the inheritance pattern is also important to direct recurrence risk estimates for future pregnancies, particularly when both parents are affected with a skeletal dysplasia. Inheritance depends on the specific dysplasia and may include autosomal dominant, autosomal recessive and X-linked inheritance, or a combination of these when both parents are affected.

Medical management of patients with skeletal dysplasias is dictated by the underlying pathogenesis along with the type and severity of physical manifestations in the realm of orthopedics, neurosurgery, otolaryngology, and pulmonary services. Pharmacologic treatment for these conditions is disease-specific (**Table 1**), and 4 conditions/groups will be discussed.

TABLE 1 | Therapeutic options for skeletal dysplasias.

	Clinical therapy	Investigational/other therapy
Osteogenesis Imperfecta	Bisphosphonates, growth hormone, Teriparatide	*In utero* stem cell transplantation, Raloxifene, Denosumab, Sclerastin, Anti-TGFβ
Hypophosphatasia	*Asfotase alfa*	Human parathyroid hormone*, bone marrow transplant*, gene therapy*
Mucopolysaccharidosis (MPS)		Stem cell transplantation, chaperone therapy, gene therapy
MPS Type I	Laronidase	Bone marrow transplant
MPS Type II	Idursulfase	
MPS Type IVa	*Elosulfase alfa*	
MPS Type VI	*Galsulfase*	
Achondroplasia		C-type natriuretic peptides, meclozine, human parathyroid hormone, human growth hormone

**No longer employed, or under investigation.*

OSTEOGENESIS IMPERFECTA

Osteogenesis imperfecta (OI), also known as "brittle bone disease," is characterized by short stature, bone deformation, bone fragility, and osteoporosis (Forlino et al., 2011). There are over a dozen types of OI with a prevalence of 1–2 per 20,000 children (Yap and Savarirayan, 2016). Pathogenic mutations in OI have been uncovered in the following genes: *COL1A1, COL1A2, SERPINF1, CRTAP, LEPRE1, PPIB, SERPINHI,* or *FKBP10*. The majority of cases are due to an autosomal dominant mutation in *COL1A1* and *COL1A2* resulting in types I–IV, classified based on severity (Yap and Savarirayan, 2016). These mutations affect formation of type I collagen, which is the most prominent connective tissue in bone and skin.

Type I patients have a quantitative deficiency of collagen and often have blue sclerae and fractures without deformity. Patients with Types II–IV have defective forms of collagen caused by improper formation of the triple helix resulting in continual remodeling with a dominant negative effect. Type II OI is perinatally lethal, due to severe intrauterine fractures and bone deformities (Harrington et al., 2014). Type III is the most severe nonlethal type of OI, presenting with normal mental capacity (Krakow, 2015) and progressive deformities resulting in loss of ambulatory abilities (Krakow, 2015). Type IV OI is milder than type III, but still severe, commonly characterized by vertebral fractures and short stature (Krakow, 2015). Types II and III are generally detectable *in utero* (Krakow et al., 2008; Krakow, 2015), whereas the milder types of OI, types I, IV, and V, are usually diagnosed after birth (Harrington et al., 2014). Patients with type II and type III OI likely have a small chest cavity, and may require mechanical ventilation if demise is not inevitable secondary to severe fractures (Harrington et al., 2014).

In addition to the autosomal dominant mutations in COL1A1 and COL1A2, 5–10% of cases are due to a mutation in another gene. Type V OI, due to an autosomal dominant mutation in IFTITM5, is recognized by hypercallus formation and calcification of the intraosseous membrane (Liu et al., 2016). An autosomal recessive mutation in SERPINF1 is responsible for type VI (Harrington et al., 2014). Additional types of OI are autosomal recessive and are not well-recognized because they are very rare. Examples include the following types and (genes): type VII (CRTAP), type VIII (LEPRE1), type IX (PPIB), type X (SERPINH1), and Type XI (FKBP10; Harrington et al., 2014; Krakow, 2015).

Of the skeletal dysplasias, osteogenesis Imperfecta currently has the most pharmacologic treatment options. Overall the goal is to improve bone density and decrease fractures, and there are several drug families currently employed to do this (Lee et al., 2016). Medications include bisphosphonates, growth hormones, denosumab, and teriparatide. Additional therapies are under investigation; Raloxifine improves outcomes in murine models, and mesenchymal bone marrow transplant has promise when initiated *in utero*.

Bisphosphonates

Bisphosphonates are one of the major pharmacotherapeutic agents clinically prescribed for OI (Dwan et al., 2014; Harrington et al., 2014). Bisphosphonates suppress bone remodeling and inhibit calcification by inactivating osteoclasts (Dwan et al., 2014; Harrington et al., 2014), which, in turn, decreases areal bone mineral density (aBMD), primarily in the spine, hip, and femur and decreases the incidence of fractures (Ward et al., 2010). There is variation in fracture incidence, aBMD, and bone pain among these agents (alendronate, pamidronate, etc.; Dwan et al., 2014) and in their route of administration (intravenous vs. oral). Adverse side effects to long-term use of bisphosphonates have been found, including cumulative micro damage, cartilage calcification (Sinder et al., 2013; Vasanwala et al., 2016), and osteonecrosis of the jaw in elderly patients (Dwan et al., 2014; Harrington et al., 2014). Bisphosphonates have been found to be less effective after 2–4 years of treatment, so intermittent treatment may be more beneficial in some cases.

Growth Hormone

Osteogenesis imperfecta is not typically associated with growth hormone deficiency, but growth hormone treatment can be beneficial by increasing aBMD and growth velocity in children with OI (Antoniazzi et al., 2010). In a randomized study, recombinant growth hormone (rGH) was combined with bisphosphonate therapy (Antoniazzi et al., 2010). Although, the rate of fractures did not differ, lumbar spine and wrist bone mineral density increased. There was no apparent decrease in

fracture rate overall (Harrington et al., 2014), yet the combination of rGH and bisphosphonates has not been shown to increase fracture rate either (Antoniazzi et al., 2010).

Teriparatide

Teriparatide is a bone stimulating recombinant form of parathyroid hormone used in anabolic therapy to treat osteoporosis (Vahle et al., 2002; Orwoll et al., 2014). When given in concert with bisphosphonate therapy, teriparatide has been shown to increase aBMD (Orwoll et al., 2014) and accelerate the healing of fractures (Rozen et al., 2007) in adults with Type I OI. In more severe forms of OI (types III and IV), teriparatide treatment showed no increase in aBMD compared to control groups (Orwoll et al., 2014).

Future OI Therapies

Development of OI treatments is ongoing. Studies have demonstrated an increase in bone healing by callus formation when teriparatide is combined with BMP-7, a recombinant protein in bones (Morgan et al., 2008). *Raloxifene* has been shown to decrease the rate of bone fractures in mice (Berman et al., 2016) and may prove to be useful to decrease fractures in future human trials of OI. *Denosumab* is an antibody currently used to prevent fractures in post-menopausal women with osteoporosis (Cummings et al., 2009; Shaker et al., 2015) and is being evaluated to treat OI. *Sclerostin antibody* has been shown in studies to increase bone formation, therefore improving bone mass (Sinder et al., 2013; Shaker et al., 2015), which is essential to OI preventative measures. *Anti-TGF*β *therapy* may also prove to be beneficial in decreasing osteoblast signaling and bone resorption (Shaker et al., 2015) in future OI treatment.

There are presently no clinically available *in utero* therapies for any of the skeletal dysplasias, however *bone marrow* and *mesenchymal stem cell transplantation* are currently under study for *in utero* treatment of OI (Mehrotra et al., 2010; Harrington et al., 2014). Case series demonstrate safety, with transient improvements in bone growth and decreases in fractures (Chan and Götherström, 2014). A larger clinical trial is underway (Chitty et al., 2016).

HYPOPHOSPHATASIA

Hypophosphatasia (HPP) is a rare metabolic disorder resulting from a loss-of-function mutation in the ALPL gene with corresponding deficiency of tissue-nonspecific alkaline phosphatase (TNSALP) (Millán and Plotkin, 2012; Whyte et al., 2015; Yap and Savarirayan, 2016). There are 6 recognized clinical forms of HPP with varied severity, all correlated to insufficient mineralization of bone and teeth as well as osteomalacia in adults (Yap and Savarirayan, 2016). Although, mild forms of HPP are found in adolescents and adults, HPP that manifests in the fetus is almost always associated with infantile and perinatal lethality (Millán and Plotkin, 2012; Yap and Savarirayan, 2016) due to abnormal skeletal development and respiratory complications resulting from a small chest with pulmonary hypoplasia (Nishioka et al., 2006).

Current Treatments for HPP

Asfotase alfa is a human recombinant TSNALP currently used to safely treat HPP by reestablishing TSNALP levels for proper degradation of inorganic pyrophosphate and consequential regulated bone mineralization (Nishioka et al., 2006; Whyte et al., 2016; Yap and Savarirayan, 2016). One study of this subcutaneous form of enzyme replacement therapy has demonstrated increased strength and agility as a result of improved bone mineralization (Whyte et al., 2016). An immense increase in perinatal and postnatal survival rates has been observed in patients treated with *asfotase alfa* (Whyte et al., 2016). In an open label study, infants with a previously perinatal lethal condition who were treated with *asfotase alfa* survived to have average stature with mainly defects in tooth enamel (Whyte et al., 2012).

Potential Treatments for HPP

Prior to the availability of *asfotase alfa*, several treatments were under investigation for HPP. These included *bone marrow transplants* (Millán and Plotkin, 2012), *parathyroid hormone (PTH)* treatment (Millán and Plotkin, 2012), and *fetal gene therapy*. Although, fetal gene therapy appeared to improve postnatal development in murine models (Sugano et al., 2011), its potential benefits are no longer under investigation due to the dramatic clinical benefits of enzyme replacement therapy with *asfotase alfa*.

MUCOPOLYSACCHARIDOSIS

Mucopolysaccharidoses (MPSs) are a type of lysosomal storage disease, a rare group of disorders that results in symptoms secondary to a defect in lysosomal function leading to abnormal storage of glycosaminoglycans (GAGs) in the bones, heart, brain, liver, or spleen (Muenzer, 2014; Regier and Tanpaiboon, 2016). The enzyme deficiency is specific to the type of MPS. MPSs are progressive, so most cases become lethal as the patient ages (Walkley, 2009) as GAG builds up and causes multiple organ failures (Muenzer, 2011). GAGs are also involved in complex secondary signaling pathways which can create permanent cellular damage (Muenzer, 2014), so early diagnosis and treatment are essential to patient longevity (Clarke, 2011). Early diagnosis, however, is often difficult in patients with normal cognitive abilities (Muenzer, 2011; Lachman et al., 2014; Regier and Tanpaiboon, 2016). There are seven types of MPS, each of which is caused by an autosomal recessive disorder, except for MPS II, which is X-linked recessive and generally occurs only in males (Valayannopoulos and Wijbug, 2011; Muenzer, 2014). Current treatments for the mucopolysaccharidoses are focused mostly on enzyme replacement therapy (ERT) and hematopoietic stem cell transplantation (HSCT; Clarke, 2011).

Enzyme replacement therapy (ERT) involves intravenous administration of the deficient enzyme specific to each type of MPS (Valayannopoulos and Wijbug, 2011; Haneef and Doss, 2016). ERT is currently used to treat MPS I, MPS II, and MPS VI (Muenzer, 2014; Haneef and Doss, 2016). Its use in the treatment of MPS IV type A (Morquio) is under *observational* study now that it has been FDA approved (Haneef and Doss, 2016; Regier

and Tanpaiboon, 2016). Although, repeated administration ERT may improve some of the symptoms of MPS including respiratory function and mobility, it cannot reverse existing skeletal disease (Muenzer, 2014).

MPS I is caused by deficiency of α-L-iduronidase (IDUA; Wang et al., 2009; Valayannopoulos and Wijbug, 2011; Ou et al., 2016), a critical enzyme in the GAG degradation pathway of heparin sulfate and dermatan sulfate (Kakkis et al., 2001; Muenzer, 2011; Regier and Tanpaiboon, 2016). MPS I is categorized into 3 groups based on its presentation and severity: Hurler's syndrome (severe; lethal by age 10); Hurler-Scheie syndrome (moderate; lethality by age 20); and Scheie's syndrome (mild; possible normal life span; Kakkis et al., 2001). Larodinase is used in ERT for MPS I, and has been shown to safely reverse some MPS symptoms, not including those relating to skeletal disease (Wraith et al., 2004; Muenzer, 2014).

MPS II is the only form of MPS that is inherited as an X-linked recessive disorder. It results from a deficiency of iduronate-2-sulphatase (Muenzer, 2011) which, similar to MPS I, leads to insufficient degradation of dermatin sulfate and heparin sulfate (Muenzer, 2011; Haneef and Doss, 2016; Motas et al., 2016). Idursulfase administration has resulted in reduced non-central nervous system symptoms of MPS II (Haneef and Doss, 2016).

MPS *IVA* and *VI* are difficult to distinguish because of their varied presentation (Lachman et al., 2014), however treatment is dependent on the diagnosis. MPS IVA results from deficiency of N-acetylgalactosamine-6-sulfatase (GALNS) corresponding to the buildup of keratan sulfate (KS) and chondroitin-6-sulfate (C6S) (Regier and Tanpaiboon, 2016). Clinical treatment for MPS IVA with *elsosulfase alfa* is underway (Regier and Tanpaiboon, 2016). *Galsulfase* is currently the only treatment for *MPS VI* and has been successful in improving pulmonary function and mobility (Motas et al., 2016).

Hematopoietic stem cell transplantation (HSCT) and *bone marrow transplantation* are used to introduce unaffected donor cells into the body and correct the lysosomal disorder by the accompanied addition of the enzyme that was previously lacking (Haneef and Doss, 2016). HCST is recommended in treating Hurler syndrome (MPS I) with ERT pre-treatment for the best results (Clarke, 2011; Muenzer, 2014). HSCT has been less successful in patients with MPS II or severe forms of MPS III (Muenzer, 2014; Motas et al., 2016). Bone marrow transplant was effective in reversing CNS symptoms (Walkley et al., 1994) in MPS I, MPS II, MPS VI, and MPS VII but is associated with a high complication rate (Haneef and Doss, 2016).

Future Therapies for MPS

Chaperone therapy could potentially treat MPS by assisting in the proper folding of proteins to create functional enzymes that break down GAG (Kakkis et al., 2001). *Gene therapy* is promising in finding a cure for skeletal dysplasias if the mutated gene can be correctly identified and modified to produce functional proteins, but development of this technique will take continued research (Kakkis et al., 2001).

ACHONDROPLASIA

Achondroplasia (ACH) is an autosomal dominant disorder generally resulting from a specific gain-of-function mutation (G380R) in the fibroblast growth factor receptor 3 (*FGFR3*) (Wang et al., 2013; Krakow, 2015; Yap and Savarirayan, 2016). As the most common type of nonlethal skeletal dysplasia, occurring in 1–2 of every 20,000 live births (Savarirayan and Rimoin, 2002; Yasoda et al., 2009; Klag and Horton, 2016), ACH is the cause for most cases of dwarfism (Klag and Horton, 2016; Yap and Savarirayan, 2016). The common clinical presentation of ACH includes short stature, relative macrocephaly with frontal bossing (Matsushita et al., 2014), and rhizomelic limb shortening (Faruqi et al., 2014; Krakow, 2015). There are usually no cognitive impairments associated with ACH unless symptoms result due to a complication of another manifestation (Gordon, 2000). Severe symptoms may result due to spinal stenosis at the foramen magnum.

Abnormal long-bone development typically is not obvious until after the first half of the second trimester thus, early prenatal diagnosis of ACH by ultrasound is not common (Krakow et al., 2008; Krakow, 2015). When achondroplasia is suspected, prenatal molecular diagnosis can be employed to evaluate for the specific G380R mutation, however sequencing of *FGFR3* may be preferred if hypochodroplasia is on the list of differential diagnoses. Affected parents may also desire prenatal molecular diagnosis or pre-implantation genetic diagnosis to evaluate for a known maternal or paternal mutation (Wang et al., 2013).

Pharmacologic treatments for ACH are currently in stage 2 clinical trials (Wendt et al., 2015; Klag and Horton, 2016). Treatments are aimed at regulating the function of FGFR3 in growth plate formation (Matsushita et al., 2014). FGFR3 negatively regulates bone growth (Faruqi et al., 2014). Its elevated function leads to irregular endochondral ossification (Wendt et al., 2015; Yap and Savarirayan, 2016) and underdeveloped linear bone growth (Liu et al., 2016) resulting from interrupted differentiation of chondrocytes (Yasoda et al., 2009; Wendt et al., 2015; Klag and Horton, 2016).

C-type natriuretic peptides (CNP) increased linear bone growth in murine models with ACH by antagonizing FGFR3 signals (Wang et al., 2013; Faruqi et al., 2014; Klag and Horton, 2016). Most CNPs are active in the body for less than 3 min (Klag and Horton, 2016) before they are removed by the natriuretic clearance receptor (NPR C) and neutral endopeptidase (NEP) (Wendt et al., 2015). Constant intravenous infusions of these short-lived CNPs would be required to observe any improvement of endochondral ossification in patients (Yasoda et al., 2009; Wendt et al., 2015). *BMN 111* (vosoritide) is a type of CNP that is resistant to digestion by NEP, and therefore has a longer circulation period in the body (Klag and Horton, 2016). Recent studies have shown an increased annual linear bone growth in cynomolgus monkeys (Wendt et al., 2015) and in children with ACH after daily subcutaneous administration of BMN 111 (Klag and Horton, 2016). BMN 111 is the most promising of CNP treatments and the only treatment for ACH that has made it to clinical trials (Klag and Horton, 2016).

Possible Future Treatments of Achondroplasia

Meclozine is an oral antihistamine that has been shown to block negative signaling of FGRF3 in chondrocytes (Matsushita et al., 2014; Klag and Horton, 2016) and increase linear bone growth in mice, both with ACH and wild-type (Matsushita et al., 2014). Furthermore, intermittent injection of *human parathyroid hormone [PTH]* in mice was accompanied by increased chondrogenesis and recovered bone growth (Xie et al., 2012). *Human growth hormone (hGH)* can increase short-term bone growth velocity in children, but is an ineffective therapy in adults (Savarirayan and Rimoin, 2002; Yasoda et al., 2009; Matsushita et al., 2014). A short-term study found increased chondrogenesis by *statin* administration, specifically rosuvastatin (Yamashita et al., 2014), but long-term analysis has not been done.

CONCLUSION

The currently available clinical therapies for patients with skeletal dysplasia are predominantly palliative in nature, however enzyme replacement therapy is now available for certain skeletal conditions. The ability to perform enzyme replacement therapy requires knowledge of the underlying molecular diagnosis as well as the pathogenic pathway by which the mutation affects bone growth and/or development. In addition to enzyme replacement therapy, bone marrow transplant is a less specific form of therapy that is clinically beneficial for several skeletal dysplasias. It is also being trialed *in utero* for osteogenesis imperfecta. A lack of suitable biomarkers accounts for the deficiency of therapeutic treatments (Briggs et al., 2015). Identifying these biomarkers will enable more useful treatment methods, but there is also associated difficulty with targeting the specific mutations, even when their identity is known (Tomatsu et al., 2013). Advances in molecular technology enable a more rapidly confirmed diagnosis as well as an earlier diagnosis in childhood and even *in utero*. This is key to allow for useful therapies specific to the underlying diagnosis. Our diagnostic capabilities will allow for a more personalized approach to treatment and targeted gene therapy as a foreseeable approach in the future.

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Exploring the Pharmacokinetic Profile of Remifentanil in Mid-Trimester Gestations Undergoing Fetal Intervention Procedures

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Background: Indications for surgery during pregnancy have increased. Specifically fetal interventions have increased from conditions that were considered lethal like twin-twin transfusion syndrome and severe fetal anemia to non-lethal conditions like myelomeningocele. The optimal anesthetic agent for *in utero* surgery is yet to be determined. Success of the procedure is often dictated by the efficacy of the anesthetic to immobilize the fetus without over-sedating mom. Remifentanil is used as preferred agent due to its short half-life however pharmacokinetics in pregnancy is unknown.

Objective: To determine the pharmacokinetic parameters of remifentanil in a mid-trimester pregnant patient population undergoing fetal intervention.

Study Design: A validated liquid chromatography assay with ultraviolet absorbance was employed to estimate maternal serum remifentanil levels. Blood samples were obtained at baseline and at selected time points: 5, 15, 30, 45, 60 min after the beginning of the remifentanil infusion and at 15, 30, and 60 min post end of infusion.

Results: Ten pregnant patients were enrolled in the study however only eight patients had sampling obtained at all time points. The mean gestational age was 22.2 (±2.7) weeks, maternal age was 27.8 (±5.1) years and body mass index was 29.6 (±6.3). After receiving a continuous infusion of remifentanil, mean total dose was 975.3 μg, C_{min} was 2.0 ng/mL and C_{max} was 8.4 ng/mL. A two-compartment model best described the plasma remifentanil data. Mean pharmacokinetic parameters were: volume of distribution (Vd_c) = 124.6 L (16.2–530.8 L), maternal remifentanil total clearance (Cl_t) = 170.7 L/h (17.7–486.9 L/h), and half-life ($t_{1/2}$) = 0.6 h (0.2–0.9 h). The maternal remifentanil area under the curve (AUC) ranged from 2.7 to 21.7 μg/L*h. The mean alpha-acidic glycoprotein was 124.8 mg/dL (81.3–149.8).

Conclusion: The pharmacokinetic profile of remifentanil in pregnant women is similar to previously reported general population profiles. This data did provide potential rationale for the clinical observations why when remifentanil is dosed based on non-pregnant guidelines, it did not uniformly provide adequate fetal immobilization as per anecdotal perception of operating fetal surgeons. These findings are important for the development of further clinical studies to optimize dosing for surgery during pregnancy including the estimation of placental transfer and total fetal exposure.

Keywords: fetal anesthesia, pregnancy pharmacokinetics, remifentanil, twin-twin transfusion syndrome

INTRODUCTION

Despite the increasing popularity of specific anesthetic agents used for fetal intervention procedures such as remifentanil, very little information about their pharmacological and toxicological profiles in the maternal-fetal population exist (Dershwitz et al., 1995). Limited information exists about the long-term effects of fetal exposure to anesthetics, and yet, based on limited animal and human studies, a great public concern has been raised about the potential long-term neurotoxic effects of anesthetic exposure in early life (Loepke and Soriano, 2008). Fetal life is a period of active brain development, with a hypothesized greater susceptibility to environmental and pharmacologic insults (Palanisamy, 2012). The indications for fetal surgery have increased in recent years, with an expected increase in the number of procedures to be performed in the future (Deprest et al., 2010). Given the significant maternal and fetal morbidity inherent to fetal surgery, these procedures are reserved for conditions that are either lethal or associated with significant long-term disability, such as twin-to-twin transfusion syndrome, congenital diaphragmatic hernia and myelomeningocele. Additionally, fetal exposure to anesthetic agents can occur at any time during pregnancy for a maternal complication requiring operative intervention such as acute appendicitis.

Remifentanil is a category C potent ultra-short acting synthetic opioid drug. It is FDA approved for sedation and analgesia in both adults and children. It is usually combined with other medications for induction and maintenance of general anesthesia and is typically administered intravenously as a bolus or as a continuous infusion. Remifentanil is metabolized via rapid hydrolysis by non-specific plasma and tissue esterases. The terminal half-life is 10–21 min (Westmoreland et al., 1993).

When compared with other anesthetic agents such as diazepam for fetal intervention procedures, remifentanil produces maternal sedation and analgesia as well as fetal immobilization in a more rapid and pronounced manner. This has the potential to decrease the total surgical time and subsequently the length of fetal exposure to anesthetic agents (Van De Velde et al., 2005). Evidence also shows that remifentanil may have neuro-protective and other cell protective properties (Fodale et al., 2008; Jeong et al., 2012). However, the optimal dose needed to provide adequate fetal sedation is not clearly studied. Anecdotal experience has observed that when dosing is based on non-pregnant studies, it unfortunately does not uniformly provide adequate fetal immobilization. This led to the current study to determine the pharmacokinetic profile of this drug in pregnancy. The primary objective of this study was to define the pharmacokinetic (PK) parameters of remifentanil in a pregnant patient population in mid-gestation. A secondary objective of the study was to evaluate the alpha acidic glycoprotein (AAG) levels on remifentanil pharmacokinetics. AAG is a serum "stress plasma protein" with a short 7 to 10 day half-life that readily binds to medications. Remifentanil is reported to have greater than 80% plasma protein binding (PPB), with over 75% specifically to AAG, however the impact of gestational stress, such as twin transfusion syndrome, on AAG levels has not been evaluated. This basic knowledge of the maternal pharmacokinetics of remifentanil and better understanding of the impact of gestational stress on AAG levels will open the doors for further toxicological and fetal (PK/PD) research.

MATERIALS AND METHODS

Patient and Study Design

Patient enrollment was approved by the institutional review board (IRB) of the University of Texas Health Science Center at Houston and Children's Memorial Hermann Hospital, Houston, TX. The population selected included patients undergoing laser photocoagulation of placental anastomoses for twin-to-twin transfusion syndrome (TTTS). Patients underwent standardized ultrasound evaluation and counseling in order to determine if they were candidates for fetal intervention for TTTS. Only after they had been deemed appropriate surgical candidates by the faculty of The Fetal Center were they offered enrollment and consented to participate in the study.

Remifentanil Infusion

The remifentanil infusion was initiated at 0.05–0.08 $mcg \cdot kg^{-1} \cdot min^{-1}$ just prior to surgical procedure upon the transfer of the patient to the operating room table. The infusion was initiated with a higher dose followed by continuous intravenous infusion adjusting the rate based upon the patient and the fetus's sedation level and physiological response. The initial infusion rate and any changes made by the anesthesiologist or surgeon in order to attain optimal operative conditions were documented on the data collection sheet.

An additional intravenous catheter was placed in the contralateral arm then the one used for the remifentanil infusion for pharmacokinetic (PK) sampling. Plasma concentrations of

remifentanil were obtained at baseline and at selected time points: 5, 15, 30, 45, 60 min after the beginning of the remifentanil infusion and at 15, 30, and 60 min post end of infusion (**Figure 1**). Due to limitations set by the IRB for the number of samples that can be obtained from a pregnant patient, the above time points were chosen.

An initial baseline sample of 12 mL was drawn; 6 mL used for PK and the remaining 6 mL was used for determining the AAG levels. All the other samples were 6 mL. The intravenous catheter was flushed with normal saline after each blood sample collection to help prevent clotting thus, the initial 2 mL of blood was discarded prior to each PK sample being drawn. The samples were placed on ice, transported to the analytical core laboratory and processed within 15 min of collection. The collection tube was centrifuged under refrigeration (4°C) 1000 × G for 10 min to separate the cellular elements from the plasma. Maternal plasma samples were then stored at −80°C until the time of analysis.

Remifentanil Sample Analysis

Remifentanil concentrations were quantified by validated high pressure liquid chromatography (HPLC) assay according to parameters described in the CDER Guidance for Industry Bioanalytical Assay Method Validation (Guidance for Industry: Bioanalytical Method Assay Validation, 2001). Remifentanil was isolated from maternal plasma samples by solid phase extraction after the addition of 10 μL of fentanyl 10 μg/mL as the internal standard. Liquid chromatographic separation was achieved by isocratic mobile phase: the mobile phase consisted of a composition of 60% of the following mixture: acetonitrile: methanol: 50 mM potassium phosphate buffer, pH3: water (180:120:48:652), and 40% methanol adjusted pH 7.2 with flow rate 1 mL/min with a elution on a Phenomenex PhenoSphere C1, 150 × 4.6 mm, 5 μm particle size packing analytical column (Phenomenex, Torrance, CA). The remifentanil peak eluted at 9.20 ± 0.2 min and fentanyl retention time was 11.50 ± 0.2 min identified by photodiode array detector at a wavelength of 210 nm. The remifentanil assay was found to be linear over 2–100 ng/mL with a correlation coefficient (r) of 0.997. The coefficient of variation for the remifentanil assay ranged from 0.16 to 14.9%. The alpha1-acidic glycoprotein (AAG) was determined using the Quantikine ELISA technique (R&D systems, Inc. Minneapolis, MN, USA).

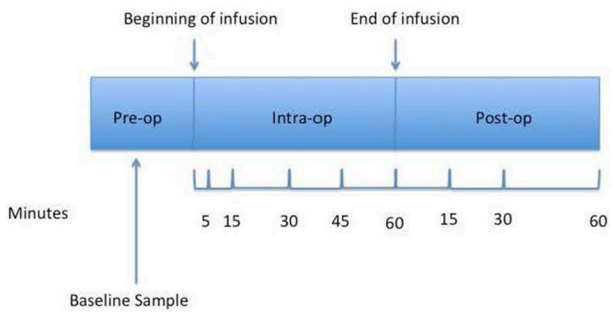

FIGURE 1 | Sample draw timeline. The figure shows all time points at which blood samples were drawn.

Pharmacokinetic Analysis

The plasma concentration time data was fit using ADAPT II Software Version 4.0. (BMRS, University of Southern California, Los Angeles, CA, USA) to determine optimal PK model to describe the data (D'argenio and Schumitzky, 1979). Model discrimination was based on the Aikaike inspection criteria (AIC), Schwartz, weighted sum of squares, sum of squares and linear correlation (R^2) as well as visual inspection of the data fit. Systemic exposure (AUC) was calculated: AUC ($\mu g \cdot L^{-1} \cdot h^{-1}$) = [total dose (μg)/total clearance (L/h)].

RESULTS

A total of 10 pregnant patients were enrolled in the study however only eight patients had complete sampling; these eight patients comprised the study population. The mean gestational age was 22.2 (±2.7) weeks. The mean maternal age was 27.8 (±5.1) years and BMI was 29.6 (±6.3). Five of the patients were Hispanic and 3 were Caucasian (**Table 1**). The mean C_{min} = 2.0 ng/mL and the mean C_{max} = 8.4 ng/mL after receiving continuous infusion of remifentanil with a mean total dose of 975.3 mcg. The mean remifentanil concentration per minute was 12.6 ± 7.8 ng/ml (**Table 2**).

A two-compartment model best described the plasma remifentanil data. Mean PK parameters were: volume of distribution (Vd_c) = 124.6 L (16.2–530.8 L), maternal remifentanil total clearance (Cl_t) was 170.7 L/h (17.7–486.9 L/h), and half-life ($t_{1/2}$) was 0.6 h (0.2–0.9 h). The maternal remifentanil AUC ranged from 2.7 to 21.7 $\mu g \cdot L^{-1} \cdot h^{-1}$ (**Table 2**). The mean AAG was 124.8 mg/dL (81.3–149.8 mg/dL) in these pregnant patients.

DISCUSSION

The pharmacokinetics of remifentanil in normal healthy non-pregnant adults has been shown to be similar in both genders (Egan, 1995; Minto et al., 1997; Scott and Perry, 2005). The data from this study suggested that the PK profile in pregnant women is similar to other healthy non-pregnant women with a few notable differences. The mean volume of distribution in this study was 124.6 L (±170.2), which is higher than normal healthy individuals (25–40 L) (Westmoreland et al., 1993). This was approximately a 3.3-fold increase in the volume of distribution, which is not unexpected in pregnancy. There is a 40–50% increase in the maternal blood volume in pregnancy which starts early in the first trimester and peaks at around 32 weeks gestation (Hytten and Paintin, 1963). Thus, this increased maternal circulating volume contributes to the greater volume of distribution noted in the current study in pregnant patients. This might be an attributing factor to the clinical observation of delay in fetal immobilization that had prompted this study. Previous studies have shown a significant amount of transfer of remifentanil across the placenta (Welzing et al., 2011; Heesen et al., 2013). However, there is also rapid metabolism and

TABLE 1 | Demographics and infusion data.

Patient	Age	Gestational age (weeks)	Race	Gravity and parity at the time of evaluation	TTTS stage	BMI	Starting infusion ($mcg \cdot kg^{-1} \cdot h^{-1}$)	Starting infusion dose (mcg/h)	Duration of infusion (min)
1	28	21	White	G2P1	III	31	0.3	1480	85
2	30	26	White	G2P1	I	24	0.08	396	82
3	21	25.7	White	G2P1	IV	43	0.12	792	73
4	24	21.4	Hispanic	G4P3	I	21	0.2	672	81
5	32	18.9	White	G2P0	III	30	0.5	2280	84
6	33	21.4	Hispanic	G6P4	II	30	0.05	241	62
7	33	19.4	White	G3P1	II	29	0.1	366	67
8	21	23.6	Hispanic	G2P1	II	29	0.1	438	70

Demographics and infusion data for each patient in the study.

TABLE 2 | Pharmacokinetic results.

	Remifentanil C_{min} (ng/mL)	Remifentanil C_{max} (ng/mL)	Mean concentration during infusion (ng/mL)	Maternal total clearance Cl_t (L/h)	Volume of Distribution Vd_C (L)	Mean Half-life (h)	AUC ($\mu g \cdot L^{-1} \cdot h^{-1}$)	AAG level (mg/dL)
Mean	2.0	8.4	4.8	170.7	124.6	0.6	10.6	124.8
Standard deviation	1.3	5.0	2.7	185.2	170.2	0.2	6.7	24.2
CV%	67.4	60.2	57.8	108.5	136.7	43.4	63.7	19.4

Pharmacokinetic results with mean, standard deviation, and coefficient of variation (CV%).

redistribution of remifentanil Kan et al. (1998). have shown an umbilical vein: maternal serum (UV:MA) ratio of 0.88 ± 0.78 and an umbilical artery: umbilical vein ratio of 0.29 ± 0.07. This was similarly demonstrated in a randomized trial by Ngan Kee et al. (2006) where the UV:MA ratio was 0.73 ± 0.17. During the current PK study there was a concurrent pharmacokinetic study in pregnant women undergoing intrauterine transfusions that also received sedation with remifentanil that observed a similar transfer fraction (UV:MA) of 0.64 ± 0.52. These studies would indicate that transplacental transfer/distribution of remifentanil into the fetal compartment may also contribute to the greater volume of distribution noted in the current study.

The maternal remifentanil clearance in our study was 170.7 L/h (±185.2), which is lower than the clearance previously reported in non-pregnant women (250–300 L/h) (Westmoreland et al., 1993). The half-life in our study was 0.6 h (±0.2) as compared to non-pregnant women where it ranges from 0.2 to 0.4 h (Westmoreland et al., 1993). We therefore found a 1.8 fold decrease in the remifentanil clearance, which would explain the increase in the half-life by about 1.5-fold. Typically in pregnancy the renal blood flow and the glomerular filtration rate (GFR) are increased by approximately 50% (Dunlop, 1981). This increase in the GFR results in an increase in the elimination of medications that are cleared by renal excretion and a corresponding shorter half-lives (Pacheco and Hankins, 2013). However, remifentanil is predominantly metabolized by non-specific circulating esterases to an acid metabolite GI-90291 (Westmoreland et al., 1993). This metabolite is excreted through the kidney. The clearance of remifentanil is therefore not affected directly by the maternal renal function.

The decrease in clearance could be related to the increase in the AAG levels noted in the current study as compared to the non-pregnant population. The mean level of 125 mg/dL observed in these patients was nearly two-fold higher than the mean level of 62.6 mg/dL previously reported by Chu et al. (1981) Approximately 70% of remifentanil is bound to plasma proteins; one third to albumin but most of it, greater than two-thirds, is bound to AAG decreasing its free-fraction available for drug activity. Certain plasma proteins like albumin are decreased in pregnancy, while others like sex hormone binding globulins are increased (Pacheco and Hankins, 2013). These changes in the protein concentrations are important in determining the drug response (Wood and Wood, 1981). AAG is a physiological stress protein, hence in conditions associated with more physiological stress, higher levels of AAG will be observed. For example, Chu et al. (1981) did not find any difference in the AAG levels in uncomplicated pregnancies when compared to non-pregnant women (62.6 ± 18.8 mg/dL). However, they did find that pregnancies complicated by acute or chronic inflammation resulted in an increase in AAG levels (Chu et al., 1981). The current study found that the AAG was elevated (124.8 mg/dL ± 24.2). Although the study patients did not have any significant medical history, their pregnancies were complicated by the need for fetal surgery. The effects of fetal surgery on maternal stress have not been studied, however any surgery would appear to cause significant psychological stress. Coussons-Read et al. (2007) demonstrated an increase in the pro-inflammatory cytokines

and acute phase reactants in cases of increased prenatal stress. The increased AAG concentration results in an increase in the amount of protein-bound of remifentanil, decreasing the free fraction of the active remifentanil. Hence the elevated AAG levels may also have contributed to the clinical observations of delay fetal immobilization when pregnant women are dosed based on non-pregnant dosing guidelines. In addition, subsequently a two-fold decrease in its clearance that was observed in this study. As a result of the decreased clearance and longer elimination half-life, a more prolonged interval may be necessary to reach a steady state in the pregnant woman. The delay in reaching steady state is another factor have contributed to the clinical observations of delay fetal immobilization when pregnant women are dosed based on non-pregnant dosing guidelines.

This study is limited by its small sample size. While enrolled 10 pregnant patients and were able to obtain adequate samples from 8 women. This is a prospective study of pregnant women in their second trimester undergoing a fetal procedure and we were able to obtain pharmacokinetic studies in these women, which have enhanced our understanding of remifentanil effects in pregnant women.

In conclusion, the PK profile of remifentanil in pregnant and non-pregnant women was similar but with differences in volume of distribution, clearance, half-life and AAG levels. While limited by a small sample size, this data did provide some potential rationale for the clinical observations why when remifentanil is dosed based on non-pregnant guidelines, it does not uniformly provide adequate fetal immobilization hence higher remifentanil doses may be required to achieve adequate fetal immobilization. Clinically these higher remifentanil doses may not have a significant impact on patient outcomes given the benefits of remifentanil in obtaining adequate fetal analgesia and its rapid clearance from the fetal circulation. These findings are important for the development of further clinical studies to optimize dosing for surgery during pregnancy including the estimation of placental transfer and potential total fetal exposure.

AUTHOR CONTRIBUTIONS

JS: Co-Investigator, Study conception, design, sample analysis, pharmacokinetic analysis, manuscript preparation. RD: Patient consent, data analysis, manuscript preparation. PA: Co-Principal Investigator, study conception, design, patient consent, data analysis, manuscript preparation. AG: Co-Investigator, study design, Sample acquisition, data collection, patient monitoring, manuscript preparation. RJ: Sample acquisition, data collection, patient monitoring. NB and EG: Patient navigator, sample acquisition, data monitoring. KM: Principal Investigator, study conception, design, patient enrollment, patient consent, data analysis, manuscript preparation.

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Feto-Maternal Trafficking of Exosomes in Murine Pregnancy Models

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Timing and initiation of labor are well-orchestrated by signals communicated between the fetal and maternal compartments; however, how these signals are communicated is not completely understood. Fetal exosomes, intercellular signaling vesicles, may play a key role in the process. The objective of this study was to evaluate exosome trafficking *in vivo* from fetal to maternal compartments. Pregnant CD-1 mice were intra-amniotically injected on gestational day 16 and 17 with exosomes isolated from primary human amnion epithelial cells fluorescently labeled with the lipophilic dye 1,1-dioctadecyl-3,3,3,3-tetramethylindotricarbocyanine iodide (DiR). All our analyses were performed on samples collected on Day 18. After 24 h, mice were imaged using Bruker MS FX PRO *In vivo* Imager and tissues were collected. *In vivo* imaging of mouse showed fluorescence in the uterus, on the exosome-injected side whereas the uterine tissues from the uninjected side and saline and dye alone injected animals remained negative. Histological analysis of placenta showed exosome migration from the fetal to the maternal side of the placenta. Fluorescence released from exosomes was seen in maternal blood samples as well as in maternal uterus and kidneys. This study demonstrates that exosomal cargo can be carried through systemic route from the fetal to the maternal side of the uterine tissues during pregnancy, supporting the idea that fetal signals can be delivered via exosomes.

Keywords: parturition, signaling, microvesicles, CD-1 mice, oxidative stress

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INTRODUCTION

Human parturition is widely accepted as an inflammatory process initiated by environmental, endocrine, and physiological factors, although the precise mechanisms involved are still unclear (Gonzalez et al., 2011; Reinl and England, 2015). Normal term parturition consists of well-orchestrated events involving both fetal and maternal compartments (Blackburn, 2014; Gao et al., 2015). On the maternal side, activation of the decidua, myometrial functional progesterone withdrawal, and cervical ripening are all considered mechanistic signals associated with parturition (Shynlova et al., 2013; Romero et al., 2014). Both endocrine and paracrine fetal biochemical signals released from matured organs, such as increased cortisol production by fetal adrenals or surfactant

protein-A from fetal lungs, can induce parturition (Mendelson, 2009; Gao et al., 2015; Reinl and England, 2015; Menon et al., 2016b). Our laboratory has investigated a new fetal signaling mechanism initiated by fetal membrane senescence in response to inflammation and oxidative stress that builds up in the amniotic cavity at term (Menon, 2014; Menon et al., 2014, 2016b; Behnia et al., 2015). This leads to senescence-associated sterile inflammation through the release of inflammatory cytokines, chemokines, matrix degrading enzymes and growth factors, termed senescence-associated secretory phenotype (SASP; Menon et al., 2013, 2016a; Menon, 2014; Behnia et al., 2015). Senescent cells also secrete damage associated molecular patterns (DAMPs), which are well known inflammatory mediators released from dying cells communicating cellular damage (Srikrishna and Freeze, 2009; Garg et al., 2014).

Although the senescent signal action is predominantly localized, these signals of cellular stress may get carried to maternal tissues, signaling fetal maturity and prompting delivery of the fetus (Natasha et al., 2014; Zhang et al., 2014). Distant senescent signaling is likely facilitated through intercellular signaling vesicles called exosomes (Sheller et al., 2016). Exosomes are 30–100 nm endosome-derived vesicles with specific characteristics that separate them from other larger particles such as microvesicles and apoptotic bodies (György et al., 2011; Akers et al., 2013). First described as modulators of the immune response to cancer cells, exosomes have also been found to contribute to angiogenesis and metastasis (Bobrie et al., 2011; Rodríguez et al., 2014). The current research involving exosome signaling in tumorigenesis via immune cell modulation has increased interest in their role in inflammatory disorders, such as asthma, arthritis and inflammatory bowel disease (Eldh et al., 2010; Corrado et al., 2013; Rodríguez et al., 2014). Since inflammation is an underlying theme in the initiation and progression of labor (Shynlova et al., 2013; Gomez-Lopez et al., 2014; Menon, 2014; Srikhajon et al., 2014; Reinl and England, 2015), it is likely that exosomes play an important role in cell signaling during term labor.

Exosome size facilitates transport between cells and tissues, while their contents, which reflect the functional state of the cell of their origin, may regulate the phenotype of the target cell (Raimondo et al., 2011; Kobayashi et al., 2014; Mulcahy et al., 2014). Ongoing studies in our laboratory have shown that myometrial cells treated with exosomes from amnion epithelial cells (AECs) cultured under oxidative stress conditions induce a contractile phenotype through the activation of NFκB and gene transcription activation of contraction associated proteins COX-2 and Connexin 43.

Although studies show exosomes can induce functional changes in myometrial cells, we do not know if the fetal membrane-derived exosomes can reach the maternal tissues to induce labor. The objective of this study was to determine the biodistribution of exosomes *in vivo* in pregnant animal models. By injecting fluorescently labeled amnion cell-derived exosomes into the amniotic fluid of pregnant CD-1 mice, we observed the migration of exosomes from the fetal to the maternal tissues.

MATERIALS AND METHODS

Patient Inclusion Criteria

No subjects were recruited or consented for this study since we used discarded placenta from normal term, not-in-labor cesarean sections that were de-identified before they were received by lab staff, as described previously (Sheller et al., 2016). Placental samples obtained for this study were from the John Sealy Hospital at The University of Texas Medical Branch (UTMB) at Galveston, TX, USA. The collection of placenta was approved by the institutional review board at The University of Texas Medical Branch at Galveston in compliance with all applicable Federal regulations governing the protection of human subjects (#11-251 April 2013). This protocol allowed us to collect discarded placental specimens after normal term cesarean deliveries or vaginal deliveries as an exempt protocol that does not require subject's consent.

Isolation and Culture of Human Amnion Epithelial Cells (AECs)

All reagents and media were warmed to 37°C prior to use. The amniotic membrane was processed within 15 min after delivery as described previously (Lim et al., 2013; Menon et al., 2013; Sheller et al., 2016). Primary AECs ($n = 4$) were cultured in T75 flasks containing complete media consisting of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 media (DMEM/F12; Mediatech Inc., Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 10% Penicillin/Streptomycin (Mediatech Inc.) and 100 μg/mL epidermal growth factor (EGF; Sigma-Aldrich) at 37°C, 5% CO2, and 95% air humidity to 60–65% confluence.

Exosome Isolation

Culture media was removed and cells were serum starved for 1 h in DMEM/F12 with 5% pen/strep prior to treatment with exosome-depleted media (DMEM/F12, 5% pen/strep and 10% exosome-depleted FBS) for 48 h. FBS (Sigma-Aldrich) was depleted of exosomes by ultracentrifugation at 100,000 × *g* for 18 h then filter-sterilized with 0.22 μm filter (Millipore, MA, USA) (Soo et al., 2012; Kobayashi et al., 2014). Culture media were collected and stored at −80°C until exosome isolation. Media was thawed overnight then isolated using differential ultracentrifugation as described previously, (Sheller et al., 2016) with the following modifications. After the 2 h 100,000 × *g* centrifugation, the supernatant was removed and the exosome pellet was resuspended in PBS. The sample was then split: half was centrifuged for 1 h at 100,000 × *g* while the other half was labeled with DiR. The final pellets were resuspended in cold PBS and stored at −80°C.

Labeling of Exosomes with DiR

To fluorescently label exosomes for *in vivo* imaging, we resuspended the pellet centrifuged at 100,000 × *g* for 2 h in 7.0 mL 7.5 μM DiR (Life Technologies, Carlsbad, CA, USA) in PBS. After mixing, the exosomes were incubated in the DiR/PBS solution for 15 min at room temperature in the dark and then

ultracentrifuged at 100,000 × g for 1 h. The final pellet was resuspended in 50 μL PBS and stored at −80°C.

Exosome Characterization Using Transmission Electron Microscopy (TEM) and Western Blot

To show that exosomes isolated from primary AECs exhibit classic exosome shape and morphology, Transmission Electron Microscopy (TEM) studies were performed as described previously (Sheller et al., 2016), with the following modification: exosomes were fixed in 5% buffered formalin; then, 5 μL of exosome suspension were dropped onto the grid and left to dry at room temperature for 10 min. To show exosome and amnion cell markers, we performed a Western blot as described previously (Sheller et al., 2016).

Animals

All animal procedures were approved by the Animal Care and Use Committee of Johns Hopkins University. Timed-pregnant CD-1 mice, outbred mice reflecting diverse genetic backgrounds in humans, were purchased from Charles River Laboratories (Houston, TX, USA) and received on gestational day 9 (E9). Animals had access to food and water *ad libitum* freely during housing and the experimental period. To determine the biodistribution of exosomes *in vivo*, we anesthetized pregnant CD-1 mice on E16 ($n = 9$) and E17 ($n = 9$) with continuous isoflurane in oxygen and performed intrauterine injections of DiR-labeled exosomes or phosphate-buffered saline solution (PBS).

Mice were subjected to mini-laparotomy, as illustrated in **Figure 1**. Using a Hamilton syringe, saline ($n = 3$ per gestational day) or DiR-labeled exosomes in PBS ($n = 6$ per gestational day) were injected intra-amniotically into each gestational sac on the right side of the cervix (maximum of five injections). The left side of the uterus was not injected and served as an internal control. Surgical incisions were closed, and the dams recovered in individual cages.

After 24 h, animals were imaged under anesthesia on both the dorsal (after hair removal) and abdominal sides using the Bruker In Vivo MS FX PRO Imager (Bruker, Billerica, MA, USA). Upon completion of live imaging, the animals were sacrificed by carbon dioxide inhalation according to the IACUC and American Veterinary Medical Association guidelines. The fetus contained within the uterus was collected in 4% paraformaldehyde (Sigma-Aldrich) and analyzed by histology for the presence of exosomes. Uteruses were also removed from saline- and exosome-injected mice and imaged using IVIS 200 (PerkinElmer, Inc., Waltham, MA, USA). Any image modifications (brightness, contrast, and smoothing) were applied to the entire image using Image J (open source). Maternal plasma was collected for exosome isolation.

Embryos were removed and fixed in 4% PFA at 4°C overnight. The next day, specimens were washed with PBS extensively and immersed in 30% sucrose until saturation, followed by cryosection at a 20-μm thickness. All photographs were taken with Zeiss AxioPlan 2 Microscope System (Jena, Germany). Routine hematoxylin and eosin (H&E) histochemical staining were performed on the neighbor sections.

Maternal Plasma Exosome Isolation to Localize Trafficking of Exosomes

To determine whether exosomes injected into the amniotic cavity can reach the maternal side via the systemic route, we analyzed serum samples for fluorescently labeled exosomes in maternal serum. Due to the low volume of serum collected, samples were pooled prior to isolation. Exosomes were isolated from maternal plasma, as described above, and the final pellet was resuspended

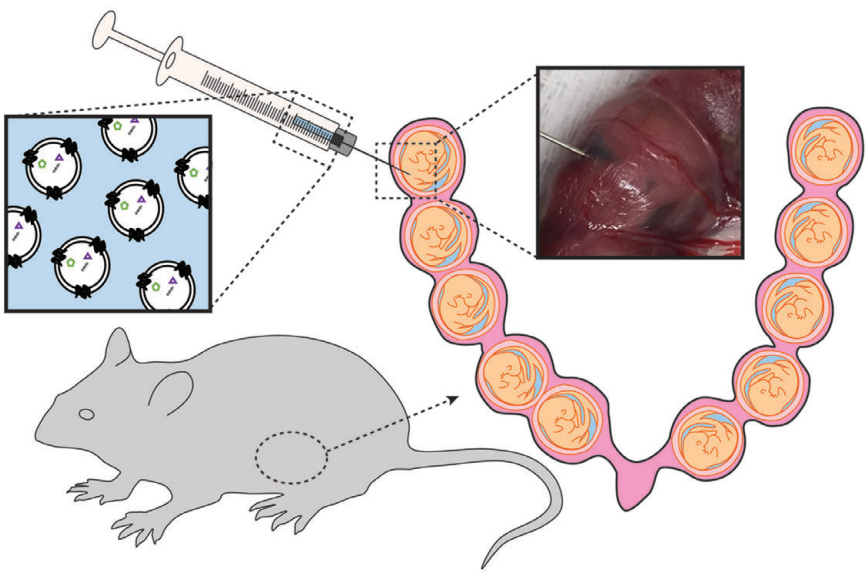

FIGURE 1 | Illustration of exosomes injected into the amniotic cavity of pregnant mice.

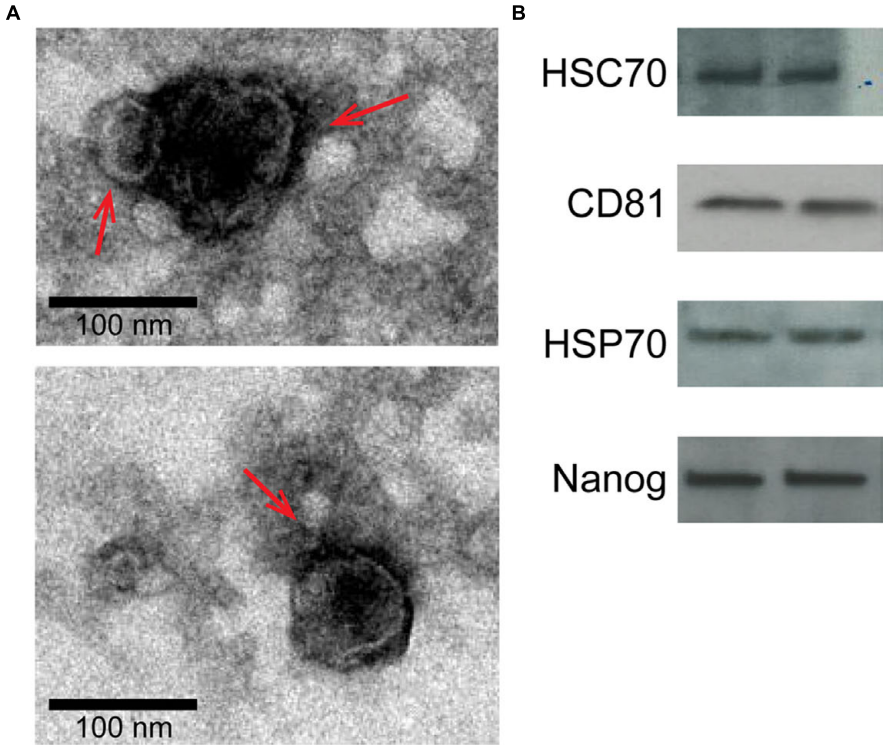

FIGURE 2 | Characterization of two representative exosome samples isolated from primary amnion cells. (A) Electron microscopy showing cup-shaped vesicles that have a size distribution of 30–100 nm (arrow indicates exosomes; scale bar represents 100 nm). **(B)** Western blot analysis showing the presence of exosome markers HSC70, CD81 and HSP70, as well as embryonic stem cell marker, Nanog, indicating amnion epithelial cell origin.

in 100 μL 1:1 glycerol/ethanol solution. To determine if the isolated exosomes contained fluorescence, we pipetted 50 μL of each sample and control in duplicate into a black 96-well plate (Corning) and imaged them on the Biotek Synergy H4 Hybrid (Biotek, Winooski, VT, USA). Wavelength was set for excitation at 745 nm and emission at 779 nm. A serial dilution of DiR in 1:1 glycerol/ethanol solution was used as the positive control, while the glycerol/ethanol solution was used as a negative control. Values were determined using relative fluorescence units (RFU). Upper and lower limits were established by DiR serial dilution (positive control, RFU $>$ 50) and the glycerol/ethanol solution (negative control, RFU $<$ 50). Statistical analysis was not performed as we analyzed pooled sample sets ($n = 2$ in each group) and therefore we report a fold change between the two groups.

RESULTS

Amnion Epithelial Cell-Derived Exosome Characterization

Isolated exosomes were characterized using TEM and Western blot for exosome and amnion markers as described previously (Sheller et al., 2016). TEM analysis revealed vesicles with classic exosome size and morphology (**Figure 2A**), consistent with previously published reports for exosomes (Redman and Sargent, 2008; Kshirsagar et al., 2012; Salomon et al., 2014; Mitchell et al., 2015). Western blot analysis was performed to determine exosome-enriched markers HSC70, CD81, and HSP70, as well as embryonic stem cell marker Nanog (**Figure 2B**).

Exosome Trafficking in Pregnant Mice

To determine the trafficking of exosomes injected into the amniotic cavity, we imaged animals 24 h after injection. As a negative control, saline was injected into the amniotic cavity and imaged after 24 h. Fluorescent signals could not be seen in saline-injected mice when imaged (data not shown).

Fluorescently labeled exosomes were injected into mice and imaged after 24 h. Regardless of the gestational day, exosome-injected mouse images showed fluorescence on the dorsal side (**Figure 3A**), although the signal remained on the injected side. When uteruses were removed and imaged (**Figure 3B**), fluorescent signals were seen only on the injected sides and not on the uninjected sides, confirming the dye stays contained within the exosomes and does not leak from the membrane of the exosomes. On gestational day 18, images of the maternal kidneys from saline- and exosome-injected mice were also taken (**Figure 3C**). Fluorescent signals were not seen in the saline-injected kidneys, but fluorescence was seen in the kidneys of the exosome-injected mice.

Histologic analyses of placental and uterine tissues were performed to observe exosome trafficking in the reproductive

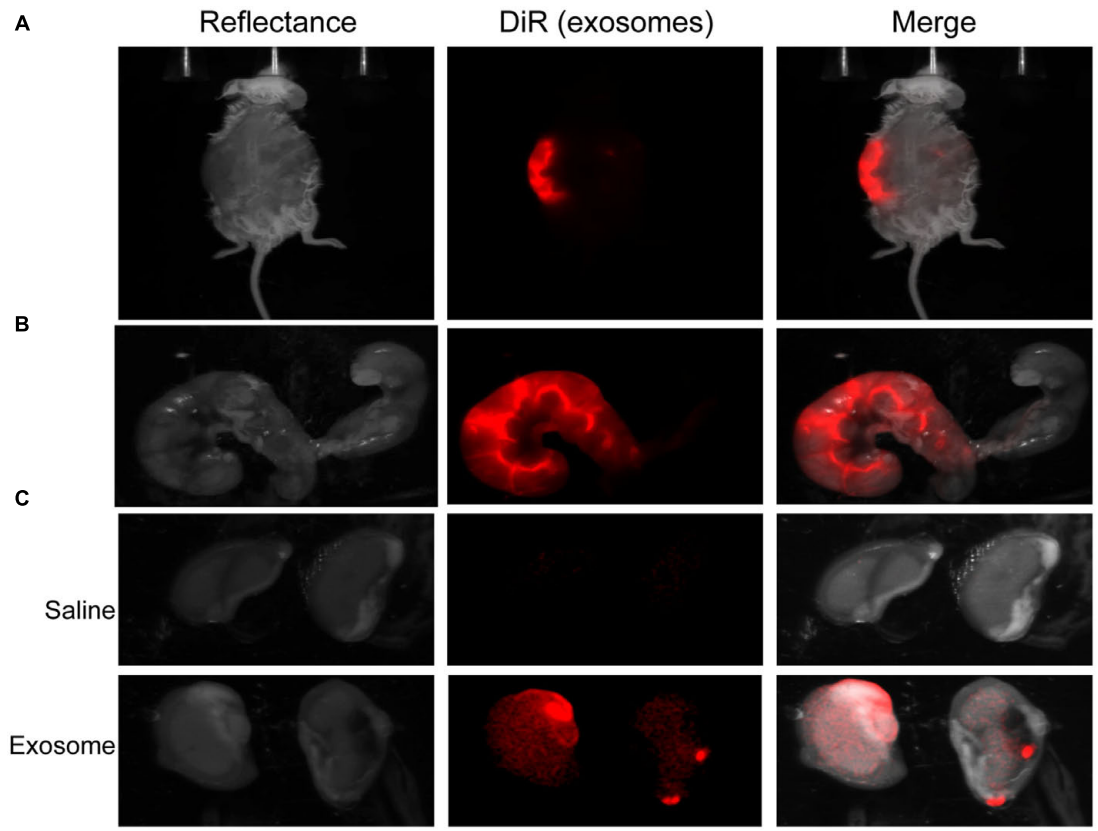

FIGURE 3 | ***In vivo*** **imaging of pregnant mouse 24 h post injection.** Exosomes stained with DiR (red) were injected into five different embryonic sacs on one side of the uterus. **(A)** Dorsal image after removal of hair using Nair. **(B)** Uterus was removed post sacrifice. Red fluorescence indicated embryonic localization of DiR labeled exosomes. Uninjected side (right) lacks fluorescence. **(C)** Kidneys from saline-injected mice (top) do not have fluorescent signal while kidneys from exosome injected mice (bottom) have fluorescent signal. Merge is an overlay of reflectance and DiR.

tissues. On E17, the collected exosome- and saline-injected mouse placentas showed no signals (**Figure 4**); however, on E18, signals emerging from exosomes can be seen on the maternal side of the placentas, whereas signals were not seen on the fetal side or in the saline-injected placentas (**Figure 5**). *In vivo* imaging of the uterine tissue showed saline-injected uterine tissue did not have fluorescent signals, whereas uterine tissues from the exosome-injected animals showed fluorescent signals (**Figure 6**). Fluorescent signals were validated using the Biotek Synergy H4 Hybrid, in which the exosome-injected uterine tissues on day 18 had sixfold higher RFU than the saline-injected tissues (data not shown).

Exosomes Traffic to the Maternal Serum

To validate the observations above and trafficking of exosomes through a systemic route, we analyzed exosomes isolated from maternal serum for fluorescence. Exosome injection solution was also analyzed for fluorescence to ensure exosomes were successfully labeled. Serum from saline-injected mice on E17 and E18, as well as from mice injected with exosomes on E17, had an RFU below 50, similar to the negative control (glycerol/ethanol solution). The serum from the exosome-injected mice on day 18 had an RFU above 50, indicating the exosomes from the amniotic fluid can traffic through the maternal serum later in pregnancy and may be dependent on timing. We report a 2.3-fold higher RFU in the serum of animals injected with DiR labeled exosomes compared to saline controls.

In summary, we demonstrated that exosomes may diffuse to the placental side where they can reach maternal tissues through a systemic route.

DISCUSSION

It is generally considered that the timing and initiation of labor are well orchestrated by communications between the fetus and the mother (Smith et al., 2002; Challis et al., 2005). However, how these signals are communicated between the fetal and maternal compartments is poorly understood. As intercellular signaling vesicles that can travel long distances through tissues and fluids (Raposo and Stoorvogel, 2013; Mincheva-Nilsson and Baranov, 2014; Bátiz et al., 2015; De Toro et al., 2015), exosomes may be carriers of these signals.

This study was performed to observe exosome trafficking *in vivo*. After labeling exosomes with the near-infrared dye DiR, we determined that exosomes injected intra-amniotically

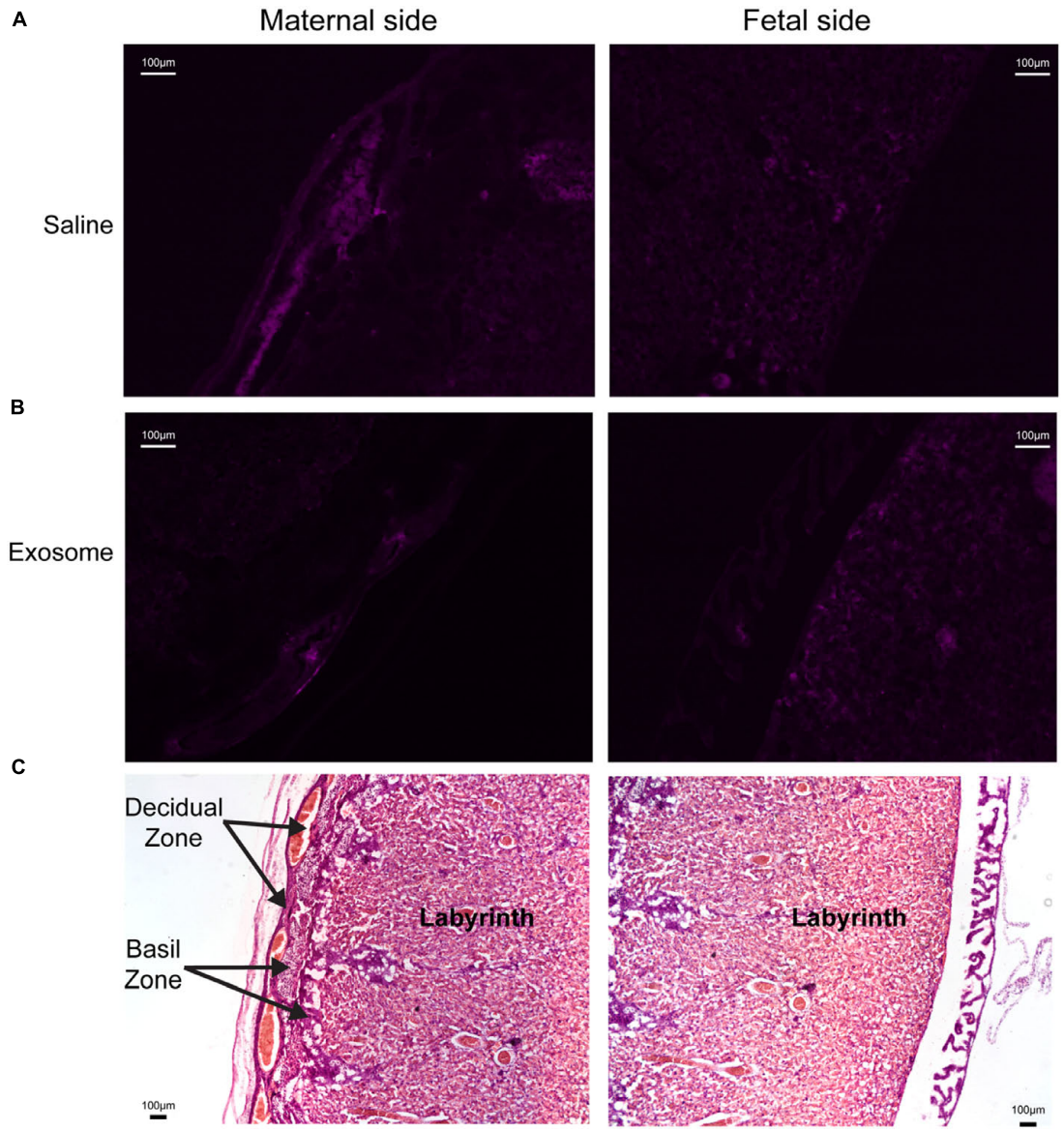

FIGURE 4 | Maternal and fetal sides of the placenta collected from injected mice on gestational day 17. (A,B) Lack of fluorescence in saline and exosome injected mouse placenta on either maternal or fetal sides. **(C)** H&E staining of maternal and fetal sides of the placenta (scale bars represent 100 μm). Arrows indicate regions of the placenta.

into pregnant mice can be imaged and monitored for their migration. DiR-labeled exosomes injected on E17 were observed in the maternal plasma and kidneys and on the maternal side of the placenta and uterus on E18, indicating migration from the amniotic cavity to the maternal side. Our study shows that exosomes, which potentially carry signals for the initiation of parturition, can traffic from the amniotic fluid into the placenta and systemically spread through circulation.

Exosomes are characterized by their contents, which reflect the physiological status of the origin cell and can regulate the phenotype of the target cell (Kambe et al., 2014; Bátiz et al., 2015; Sheller et al., 2016; Willms et al., 2016). At term, oxidative stress and inflammation build up in the amniotic cavity, causing cellular senescence of the fetal membranes and subsequent release of signals of cellular damage (Menon, 2014; Behnia et al., 2015, 2016; Polettini et al., 2015; Menon et al., 2016b). Senescent signal action is primarily localized, although we have shown signals of cellular damage are also packaged into exosomes from AECs treated with the oxidative stress inducer cigarette smoke extract (Sheller et al., 2016). It is likely that exosomes carrying signals of cellular damage can reach maternal tissues and contribute to parturition.

Though our study answers basic questions about exosome trafficking *in vivo*, it does not include activation of inflammatory

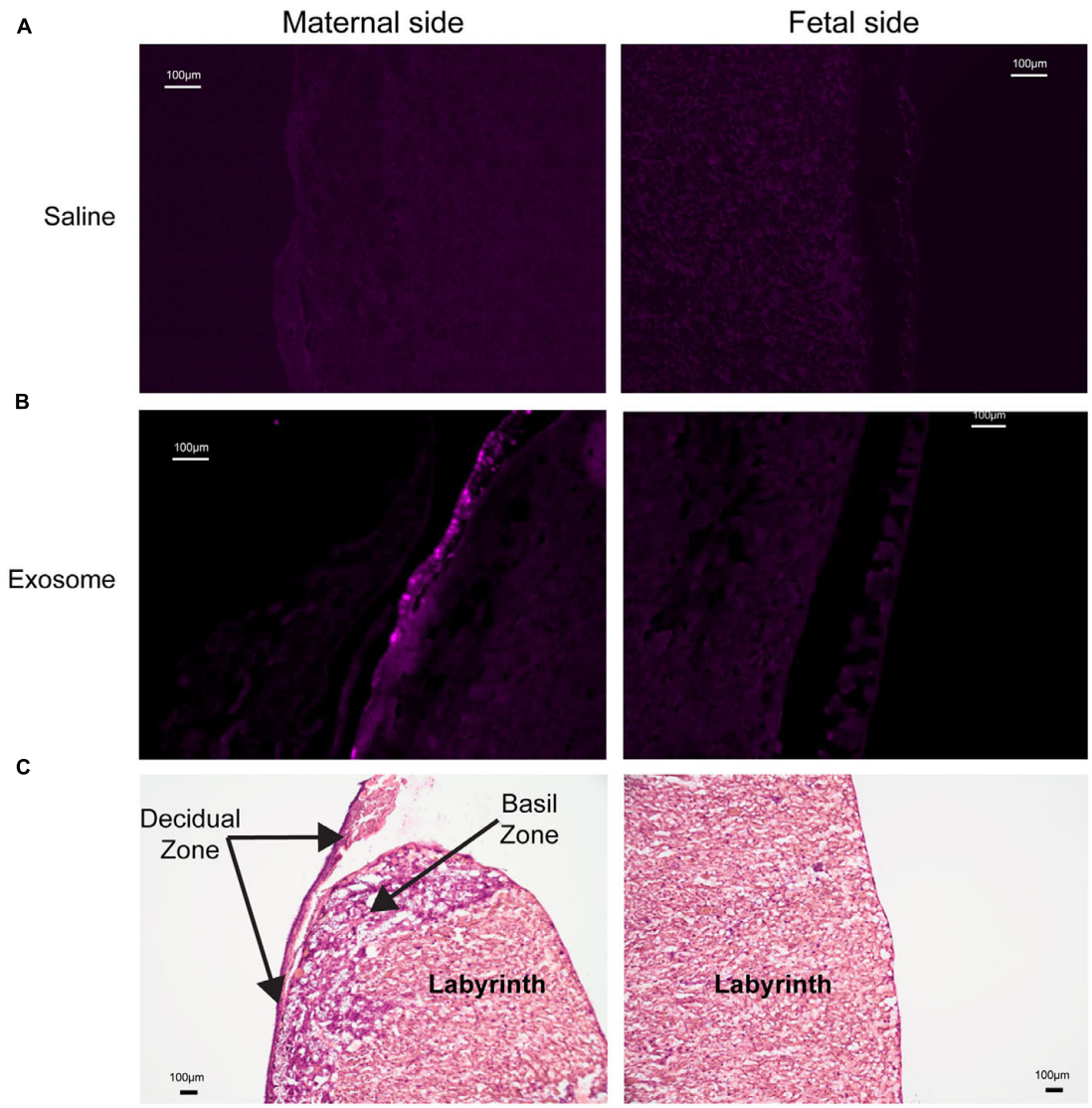

FIGURE 5 | Maternal and fetal sides of the placenta collected from injected mice on gestational day 18. (A) Saline injected mouse placenta showed no fluorescence on maternal or fetal sides. **(B)** Exosome injected mouse placenta showed fluorescence on maternal side but lacks signal on the fetal side of the placenta. **(C)** H&E staining of maternal and fetal sides of the placenta (scale bars represent 100 μm).

pathways involved in the initiation and progression of labor. We only allowed for 24 h prior to imaging and tissue collection, which may not be sufficient time for exosomes to migrate to the target tissues or cause functional changes. Determination of initiation of parturition at term or preterm based on signals carried by exosomes was beyond the scope of this study. Exosomes were stored at −80°C until the day of injection. Although the stability of amnion epithelial cell-derived exosomes during freeze-thaw cycles has been not been evaluated for this study, there have been published reports demonstrating exosome stability in various biological fluids from pregnancy (Sarker et al., 2014). The number of exosomes injected was random, which may have an effect on the trafficking and eventual response to exosome signaling. Our ongoing studies using a specific number of exosomes will determine the effect of the quantity required to cause a functional change and specific pregnancy outcomes like preterm or term parturition. Future studies using live imaging will also determine the timing required for exosome migration between feto-maternal compartments. We will also understand differences between exosomes from amnion cells grown under standard conditions and oxidative stress conditions, including activation of inflammatory pathways related to parturition and preterm birth rates.

In summary, we have demonstrated that exosomes injected into the amniotic cavity of pregnant mice can traffic to the maternal tissues. Specifically, exosomes injected on gestational day 17 migrated to the maternal side of the placenta, the

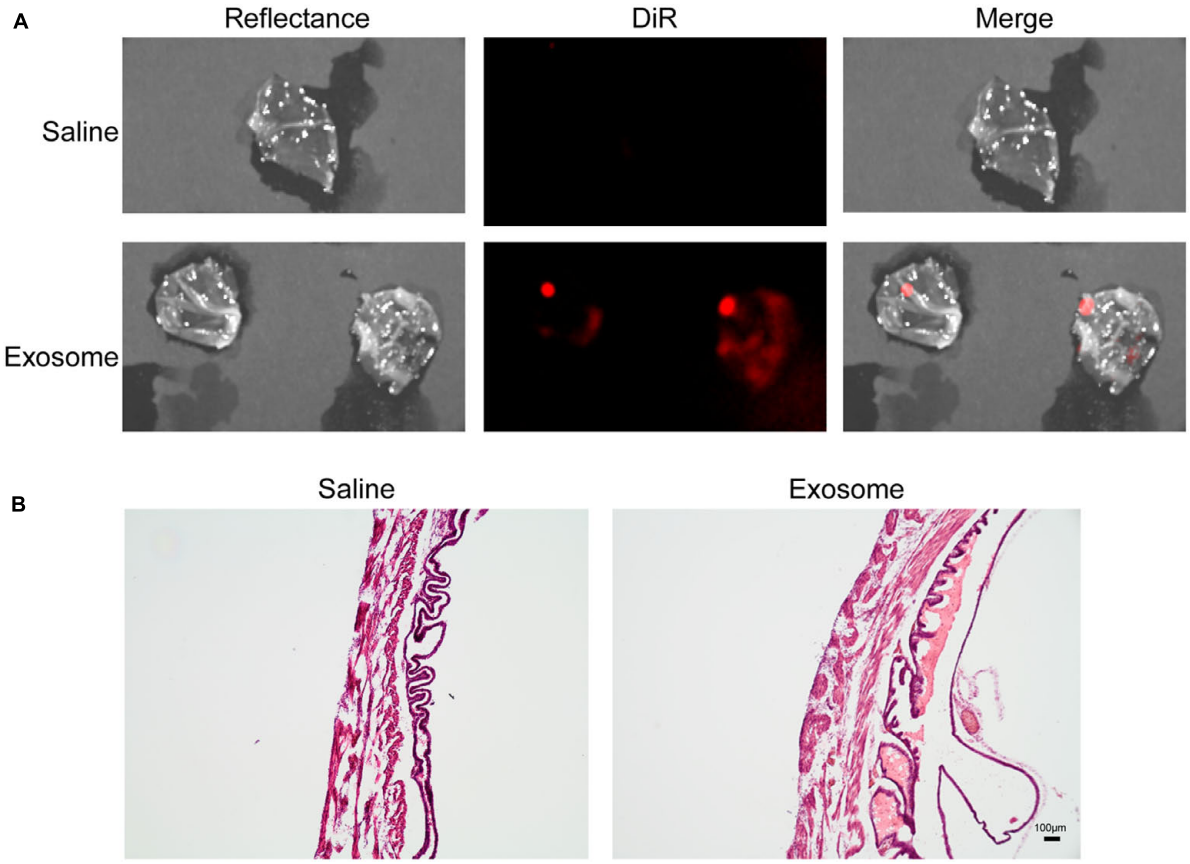

FIGURE 6 | *In vivo* imaging of uterine tissues collected from gestational day 18. (A) Saline-injected (top) mouse uterus showed no fluorescent signal, while exosome-injected (bottom) mouse uterus showed fluorescence, indicating exosomes traffic to the maternal tissues. **(B)** H&E staining of uterus tissue from saline (left) and exosome (right) injected mice (scale bars represent 100 m). Merge is an overlay of reflectance and DiR.

maternal serum, and the maternal kidneys. So we propose that exosomes can traverse through various tissue layers and or it can reach various tissues through a systemic route. Although not tested, the diffusion of exosomes through various layers could be a concentration dependent process and the quantity very well may determine the fetal signal strength. Oxidative stress is expected to increase exosome numbers (Salomon et al., 2013) and at term, oxidative stress induced increased production of exosomes from fetal tissues may increase exosome quantity and thus signal strength. This study demonstrated that fetal signals can be carried as exosomal cargo through either diffusion between tissues or through systemic route from the fetal to the maternal side during pregnancy. This supports the postulate that fetal signals that can contribute to the initiation of human parturition can be delivered via exosomes.

AUTHOR CONTRIBUTIONS

RM and IB conceived and designed the experiments. SS-M and JL performed the experiments. RM, SS-M, and JL analyzed the data. RM, IB, GS, and CS contributed reagents/materials/analysis tools. RM, SS-M, JL, and IB wrote the paper.

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Pathway Markers for Pro-resolving Lipid Mediators in Maternal and Umbilical Cord Blood: A Secondary Analysis of the Mothers, Omega-3, and Mental Health Study

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The omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are precursors to immune regulatory and specialized pro-resolving mediators (SPM) of inflammation termed resolvins, maresins, and protectins. Evidence for lipid mediator formation *in vivo* can be gained through evaluation of their 5-lipoxygenase (LOX) and 15-LOX metabolic pathway precursors and downstream metabolites. We performed a secondary blood sample analysis from 60 participants in the Mothers, Omega-3, and Mental Health study to determine whether SPM and SPM precursors are augmented by dietary EPA- and DHA-rich fish oil supplementation compared to soy oil placebo. We also aimed to study whether SPM and their precursors differ in early and late pregnancy or between maternal and umbilical cord blood. We found that compared to placebo supplementation, EPA- and DHA-rich fish oil supplementation increased SPM precursor 17-hydroxy docosahexaenoic acid (17-HDHA) concentrations in maternal and umbilical cord blood ($P = 0.02$). We found that the D-series resolvin pathway marker 17-HDHA increased significantly between enrollment and late pregnancy ($P = 0.049$). Levels of both 14-HDHA, a maresin pathway marker, and 17-HDHA were significantly greater in umbilical cord blood than in maternal blood ($P < 0.001$, both).

Keywords: resolvins, EPA, DHA

INTRODUCTION

A variety of putative health benefits have been attributed to omega-3 fatty acids, including prevention of cardiovascular disease, reduction of chronic inflammation, reduction in depressive symptoms, and prevention of insulin resistance (Delarue et al., 2006; Gonzalez-Periz et al., 2006; Hassan and Gronert, 2009). In pregnancy, omega-3 fatty acid supplementation has been shown to reduce early preterm births (<32–24 weeks; Makrides et al., 2006, 2010; Szajewska et al., 2006; Horvath et al., 2007) as well as to decrease risk for infant admission to a neonatal intensive care unit (Makrides et al., 2010).

Omega-3 fatty acids were originally thought to exert these beneficial effects by competitively inhibiting formation of pro-inflammatory arachidonic acid-derived eicosanoids (Gonzalez-Periz et al., 2006). However, more recently, omega-3 fatty acids have been shown to be the precursors to novel classes of specialized pro-resolving mediators (SPM) that are formed by lipoxygenase (LOX) or cyclooxygenase enzymes analogous to the formation of the arachidonic acid-derived SPM class of lipoxins. These ω-3 polyunsaturated fatty acid (PUFA)-derived mediators are classified as E-series resolvins [eicosapentaenoic acid (EPA) metabolites], D-series resolvins [docosahexaenoic acid (DHA) metabolites], maresins (DHA metabolites), and protectins (DHA metabolites) (Fredman and Serhan, 2011; Serhan, 2014). *In vitro* and *in vivo* experimental evidence strongly suggests that increased resolvin, maresin, and protectin formation after dietary supplementation may explain many of the observed health benefits of dietary ω-3 PUFA supplementation (Gronert, 2008; Grenon et al., 2015; Murphy, 2015; Serhan et al., 2015).

In humans, many of the prominent DHA-derived resolvins are primarily synthesized through the interaction of 15-LOX with 5-LOX. A key intermediate is the 15-LOX product 17-hydroperoxy-DHA; its metabolite 17-hydroxy docosahexaenoic acid (17-HDHA) is pathway marker for the D-series resolvins. 17-Hydroperoxy-DHA is also the rate-limiting intermediate for the formation of protectin D1, which is 15-LOX product and that does not require 5-LOX (Serhan and Petasis, 2011). The primary hematologic cell types that generate 17S-HDHA are eosinophils (high expression) (Miyata et al., 2013) monocytes (low expression and high expression in macrophages) and polymorphonuclear leukocytes (low expression but inducible during resolution) (Serhan, 2014). By contrast, DHA-derived maresins are formed via the 12-LOX intermediate 14-hydroperoxy-DHA and further conversion by 5-LOX. 14-HDHA is the pathway marker for maresin formation and 12-LOX activity, an enzyme that is highly expressed in platelets and at lower levels in macrophages (Barden et al., 2014). 4-HDHA is another 5-LOX metabolite, that in animal models has been shown to mediate the antiangiogenic effect of omega-3 fatty acids (Sapieha et al., 2011) (**Figure 1**). By contrast, the EPA-derived resolvins are primarily synthesized through the cytochrome P450 intermediate 18S-hydroxyeicosapentaenoic acid (18S-HEPE), that is further converted by 5-LOX (Barden et al., 2014).

In healthy, non-pregnant volunteers, endogenous formation of maresins, resolvins, and protectins may be amplified by dietary omega-3 fatty acid supplementation (Fredman and Serhan, 2011; Gomolka et al., 2011). Due to the short half-life and rapid elimination of the potent resolvins, protectins, and maresins, direct quantification and detection of SPM in archived tissue samples may be challenging (Psychogios et al., 2011; Skarke et al., 2015). However, their specific metabolic intermediates and pathway markers have established activation of SPM pathways and their formation *in vivo* both in humans and animal models of inflammatory diseases. In particular 18-HEPE, 17-HDHA and 14-HDHA are established markers for the activation of the E-series resolvin, D-series resolvin and maresin biosynthetic pathways, respectively (Weylandt et al., 2012; Barden et al., 2014).

Endogenous synthesis of specialized pro-resolving lipid mediators and their pathway markers in maternal and fetal serum has not been previously studied. The proposed study represents a secondary analysis of stored maternal and fetal (cord blood) serum samples from subjects who participated in a double blind randomized controlled trial comparing EPA-rich fish oil (1060 mg EPA plus 274 mg DHA) and DHA-rich fish oil (900 mg DHA plus 180 mg EPA) supplementation with soy oil placebo for prevention of perinatal depression among women at risk (Mozurkewich et al., 2013). We performed this ancillary study in order to compare maternal and neonatal activity of resolvin, protectin, and maresin pathway markers (RPM). We also aimed to study whether RPM differed in maternal and umbilical cord blood from subjects who were supplemented with EPA-rich fish oil, DHA-rich fish oil, versus placebo. We hypothesized that pathway markers of DHA-derived resolvins and protectins would be enhanced in umbilical cord blood compared to maternal blood, due to the preferential transplacental transport of DHA in late pregnancy. We further hypothesized that dietary supplementation with EPA- and DHA-rich fish oils would amplify resolvin, protectin, and/or maresin pathway activity compared with placebo supplementation.

Ethics

The parent trial was registered at Clinicaltrials.gov at NCT00711971. This parent study and this secondary analysis were approved by the University of Michigan Institutional Review Board at HUM00004684. This secondary analysis was deemed exempt by the University of New Mexico Health Sciences Center Human Research Protection Organization where the data analyses were carried out.

MATERIALS AND METHODS

This study was a secondary analysis using LC/MS/MS (liquid chromatography/mass spectroscopy/mass spectroscopy) of stored plasma samples that were collected as part of a prospective, blinded randomized controlled trial of fish oil supplementation for prevention of depressive symptoms among women at risk (Mozurkewich et al., 2013). The parent trial was carried out at two medical centers in southeastern Michigan, the University of Michigan Health System and St. Joseph Mercy Health System. One hundred twenty-six subjects, of whom 118 completed the trial, enrolled in the study between October 2008 and May 2011. Potential subjects were excluded if they were eating more than two fish meals weekly, or if they were taking omega-3 fatty acid supplements, including prenatal vitamins with DHA (Mozurkewich et al., 2013). They were randomly assigned to receive EPA rich fish oil (1060 mg EPA plus 274 mg DHA), DHA rich fish oil (900 mg DHA plus 180 mg EPA), or soy oil placebo, and were followed longitudinally through pregnancy from their enrollment at 12–20 weeks' gestation, through 6 weeks postpartum. Maternal blood was drawn at enrollment (visit 1) and again at 34–36 weeks gestation (visit 3). Umbilical cord blood was obtained after the delivery of the infants born to mothers enrolled in the study (visit 4). Serum aliquots prepared from

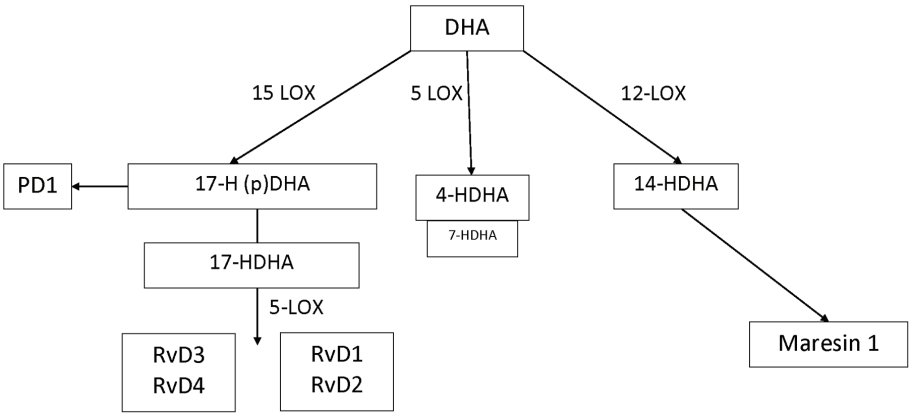

FIGURE 1 | DHA metabolome.

each blood collection and were stored at −70°C (Mozurkewich et al., 2013). All samples were processed and stored within 12 h. Results of the blood sample analyses for fatty acids have been previously described (Mozurkewich et al., 2013).

This exploratory study involved a subgroup analysis of serum samples collected from 60 mother–infant pairs of study participants. Due to cost considerations, it was not possible to perform analyses for the whole study cohort. Samples for analysis were selected based on availability of complete sample sets and adherence to the protocol as ascertained by capsule counts.

Stored samples were analyzed by LC/MS/MS based lipidomic analysis to quantify levels of ω-3 PUFA-derived SPM. In brief, 400 pg of class specific deuterated internal standards prostaglandin E2 (PGE2-d4), lipoxin A4 (LXA4-d5), leukotriene B4 (LTB4-d4), 15(S)-hydroxyeicosatetraenoic acid [15(S)-HETE-d8], eicosatetraenoic acid (arachidonic acid-d8) and docosahexaenoic acid (DHA-d5) were added to each sample prior to processing and extraction to calculate the recovery of specific classes of oxygenated fatty acids. Lipid antacids were extracted by solid phase with SampliQ ODS-C18 cartridges (Agilent Technologies). Eicosanoids and docosanoids were identified and quantified by LC/MS/MS-based lipidomics based on our published methods (Hassan and Gronert, 2009). Extracted samples were analyzed by a triple-quadrupole linear ion trap LC/MS/MS system (MDS SCIEX 3200 QTRAP) equipped with a Kinetex C18 mini-bore column. The mobile phase was a gradient of A [water/acetonitrile/acetic acid (72:28:0.01, v:v:v)] and B [isopropanol/acetonitrile (60:40, v:v)] with a 450 μl/min flow rate. MS/MS analyses was performed in negative ion mode and prominent fatty acid metabolites quantified by multiple reaction monitoring (MRM mode) using established and specific transitions as previously described (González-Périz et al., 2009; Hassan and Gronert, 2009; Sapieha et al., 2011; von Moltke et al., 2012; Kalish et al., 2013; Pruss et al., 2013). Calibration curves (1–1000 pg) and specific LC retention times for each compound were established with synthetic standards (Cayman Chemical, Ann Arbor, MI, USA). Structures were confirmed for selected autacoids by MS/MS analyses using enhanced product ion mode with appropriate selection of the parent ion in quadrupole 1. EPA, DHA, and arachidonic acid metabolomes were constructed. For inclusion, levels of ω-3 PUFA-derived SPM needed a signal-to-noise ratio of at least 3:1. When the signal-to-noise ratio did not meet this inclusion criteria, we used the lower limits of detection, which were 1 pg/ml for 4-HDHA, 5 pg/ml for 14-HDHA, and 2 pg/ml for 17-HDHA.

Statistics

For comparison of the baseline characteristics between the three treatment groups, we used analysis of variance (ANOVA). Comparisons for categorical variables were done using Fisher's exact tests. For other outcome measures computed descriptive statistics reported as mean and standard error of the mean for continuous data and frequency (%) for binary and categorical data. For values below the limits of detection, we imputed the lower limit of detection for the assay used. Data with right-skewed distributions were logarithmically transformed. We analyzed log-transformed resolvin pathway marker concentrations across visits 1, 3, and 4 (with visit 4 representing neonatal cord blood), with repeated measures (RM) ANOVA with "visit" as the repeated factor and the three "groups" (EPA, DHA, and placebo) as the grouping factor. Where there was no difference between individual treatment groups versus placebo, the treatment groups (EPA and DHA) were combined for subsequent RM ANOVA exploratory analysis. In instances in which significance was found by RM ANOVA, further exploratory analyses were carried out in the following manner. *Post hoc* tests of change at visit 3 (post-supplementation) were done by paired *t*-test. *Post hoc* comparisons between groups were done using unpaired *t*-tests. Pearson's correlation coefficient was computed to describe the relationship between log-transformed serum DHA levels at each study visit and log-transformed 4-HDHA, 14-HDHA, and 17-HDHA. For the purposes of the correlation analyses, the values below the lower limit of detection were removed. An additional correlation analysis was carried out to evaluate the relationship between maternal DHA at 34–36 weeks and cord blood 17-HDHA. A *P*-value of 0.05 was considered statistically significant. We conducted an exploratory analysis of the effect of labor on 17-HDHA formation by

comparing log transformed 17-HDHA concentrations in cord blood collected after cesarean section without labor versus cord blood from birth after labor using unpaired *t*-tests.

RESULTS

The baseline demographic characteristics of the study subjects in the subset chosen for this analysis did not differ significantly between the treatment groups. Capsule compliance was similar between the three groups with subjects in the EPA group having an overall compliance of 69%, subjects in the DHA group with 71% compliance, and subjects in the placebo group with 76% compliance. This difference was not statistically significant ($P = 0.60$, generalized linear models). There was no difference in mean age at screening, gravidity, parity, or history of prior preterm delivery among the treatment groups (ANOVA, all $P \geq 0.50$) There was no difference in ethnicity or race (Fisher's exact test, both $P > 0.35$; **Table 1**).

TABLE 1 | Demographic table.

	EPA	DHA	Placebo	Significance
N	19	21	20	
Age at screening	29.0 ± 5.5	30.4 ± 4.8	30.9 ± 6.1	NS
Gravidity	2.37 ± 1.38	2.33 ± 1.15	2.20 ± 1.91	NS
Parity, term	0.68 ± 0.95	0.90 ± 1.04	0.60 ± 0.88	NS
Preterm	0.105 ± 0.315	0.095 ± 0.301	0.200 ± 0.523	NS
Av. compliance	0.69 ± 0.25	0.71 ± 0.20	0.76 ± 0.22	NS
White	16	17	16	NS

Av = average; NS = not significant.

D-series resolvins, E-series resolvin, protectins, and maresins were not consistently detected; peaks were below acceptable signal-to-noise ratio for the 3200 QTRAP MRM-based identification and quantification. The E-series resolvin pathway marker, 18-HEPE, was below detectable limits in 96% of the samples. Thus no further analysis of the EPA metabolome was carried out.

There was no significant difference in 4-HDHA levels between the groups at study entry or at any subsequent study visit. Log transformed 4-HDHA levels did not significantly differ across the study visits and were not significantly different in the maternal blood versus umbilical cord blood (**Figure 2**). Log transformed 4-HDHA concentrations were significantly correlated with DHA levels (Pearson's correlation coefficient $4 = 0.51$, $P < 0.001$; **Figure 3**).

Log transformed 14-HDHA did not differ significantly between early and late pregnancy. 14-HDHA levels were significantly increased in umbilical cord blood compared to maternal blood at both maternal blood draw time points ($P < 0.001$). There was no significant treatment effect of EPA- and DHA-rich fish oil supplementation compared to placebo on 14-HDHA levels, in either maternal blood or umbilical cord blood (**Figure 4**). However, in an analysis of combined blood samples from all study visits, 14-HDHA levels were significantly correlated with DHA levels (Pearson's correlation coefficient 0.47, $P < 0.001$; **Figure 5**).

For the total cohort, 17-HDHA levels significantly increased in maternal blood between enrollment and 34–36 weeks ($P = 0.049$). Concentrations of 17-HDHA in umbilical cord blood were significantly greater than in maternal blood both at study entry and at 34–36 weeks gestation ($P < 0.001$; **Figure 6**). In the total sample set, 17-HDHA levels were significantly correlated with DHA levels (Pearson's correlation coefficient 0.34, $P < 0.02$; **Figure 7**).

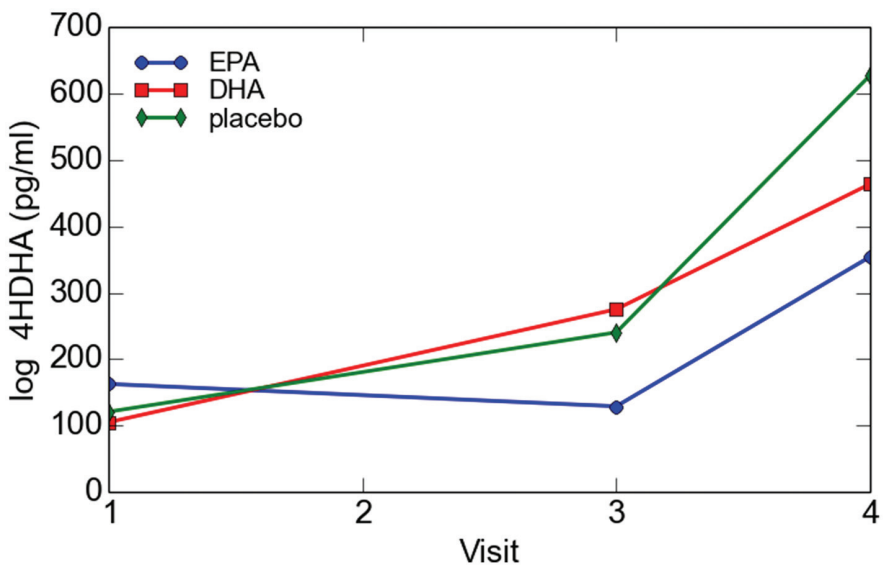

FIGURE 2 | Maternal and cord blood log 4-HDHA concentration.

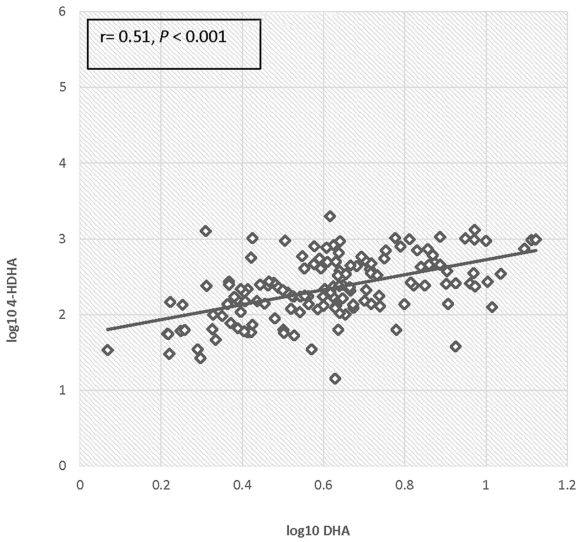

FIGURE 3 | Correlation of log DHA fraction with log 4-HDHA concentration.

When the EPA- and DHA-rich fish oil groups were individually compared with placebo using repeated measures modeling, there was a trend toward higher 17-HDHA activity that did not reach significance ($P = 0.07$). By contrast, when the two supplementation groups were combined for the purpose of analysis, 17-HDHA activity was significantly higher in the combined EPA- and DHA-rich fish oil group than in the placebo supplemented group ($P = 0.02$).

However, when the EPA- and DHA-rich oil groups were together compared with placebo at the post-supplementation time points using the *t*-test, there were no significant differences between the treatment groups and placebo at either of the post-supplementation time points. Although there was a trend toward treatment effect for supplementation at visit 3 ($P = 0.11$, *t*-test) this effect was not statistically significant. Likewise, although concentrations of 17-HDHA in umbilical cord blood were higher in the treatment groups as compared to placebo, this trend was not significant ($P = 0.33$). Of interest, cord blood levels of 17-HDHA were 5-fold higher after exposure to labor than in the instance of elective cesarean section, but this difference did not reach statistical significance.

DISCUSSION

The main finding of our study is that the maresin pathway precursor 14-HDHA and the resolvin pathway marker 17-HDHA were significantly enhanced in cord serum compared to maternal serum in early and late pregnancy. This finding is consistent with the observation of augmentation of DHA percentage in umbilical cord serum compared to maternal serum that we observed in the parent study and that has also been reported in other studies (Mozurkewich et al., 2013; Baack et al., 2015). Our study also suggested a treatment effect for EPA- and DHA-rich fish oil supplementation for the resolvin pathway precursor 17-HDHA, but prospective randomized studies are needed to determine whether resolvins, protectins, and maresins, themselves, are augmented by supplementation (Colas et al., 2014).

Previous studies examining the presence of SPM in adults have looked at subsets with specific diseases, including asthma, Alzheimer's disease, multiple sclerosis, chronic kidney disease, peripheral artery disease, and rheumatoid arthritis (Serhan, 2014; Grenon et al., 2015; Mas et al., 2016) as well as in unselected adults (Psychogios et al., 2011). In the reproductive sphere,

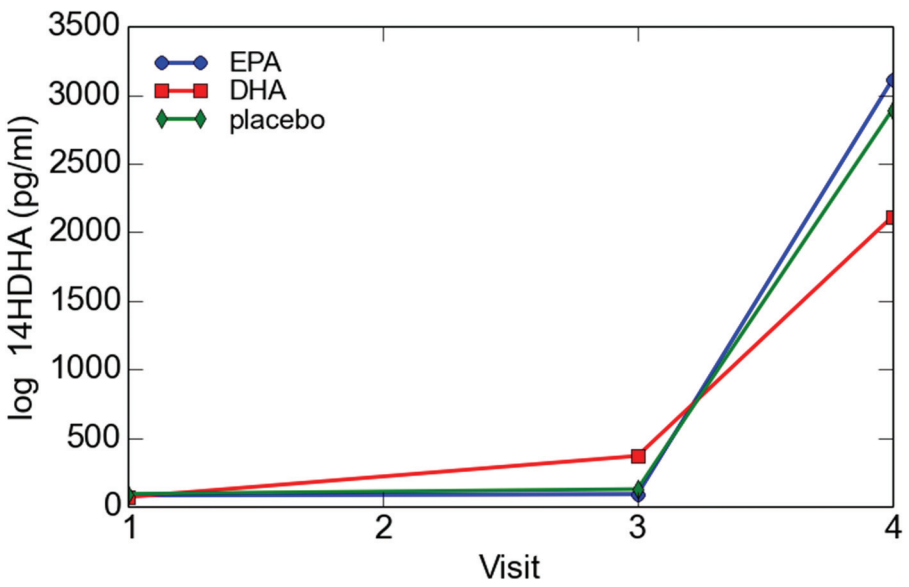

FIGURE 4 | Maternal and cord blood log 14-HDHA concentrations.

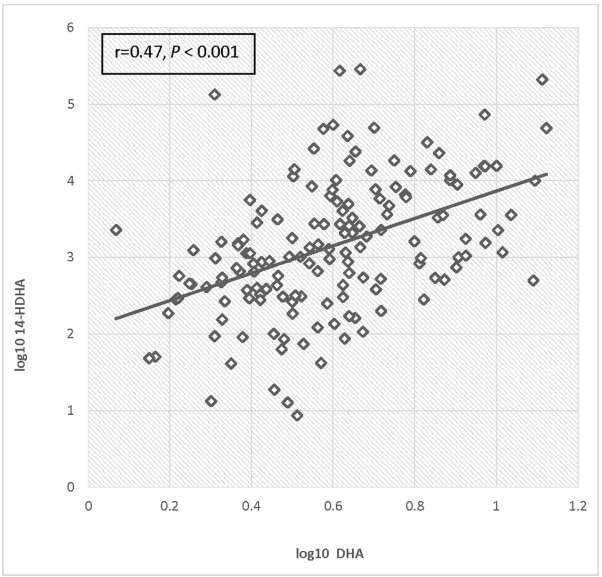

FIGURE 5 | Correlation of log DHA fraction with log 14-HDHA concentrations.

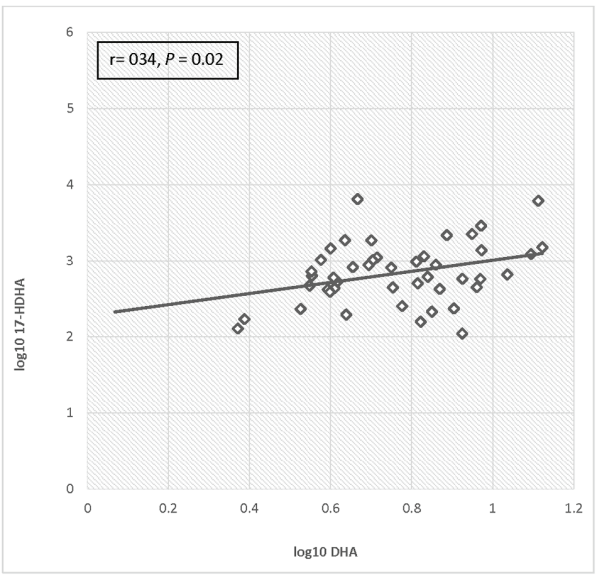

FIGURE 7 | Correlation of log DHA fraction with log 17-HDHA concentrations.

maternal omega-3 fatty acid supplementation with 3.7 g DHA plus EPA was shown to increase resolvin and protectin precursors in human placental tissues; likewise, a high n-3 PUFA diet has been shown to increase SPM precursors in rat placentas (Jones et al., 2013; Keelan et al., 2015). This study differs from these prior evaluations in that it assessed resolvin, protectin, and maresin pathway activity in human umbilical cord blood in relation to maternal dietary supplementation with DHA and EPA.

Our study was limited by the small sample size as well as cost constraints that precluded analysis of the entire trial cohort. Similarly, the sample subset analyzed was not randomly chosen, but was selected based on maternal compliance with supplementation and by availability of complete sample sets. In terms of quantifying ω-3 PUFA-derived SPM our analysis was limited to quantifying DHA-derived resolvin, maresin, and protectin pathway markers, which likely was due to using an LC/MS/MS system with 1–10 pg limits of quantification. The DHA-derived resolvin pathway precursors we evaluated were below the limits of detection in some of our samples, and the precursors of the E-series resolvins were detected in only a few

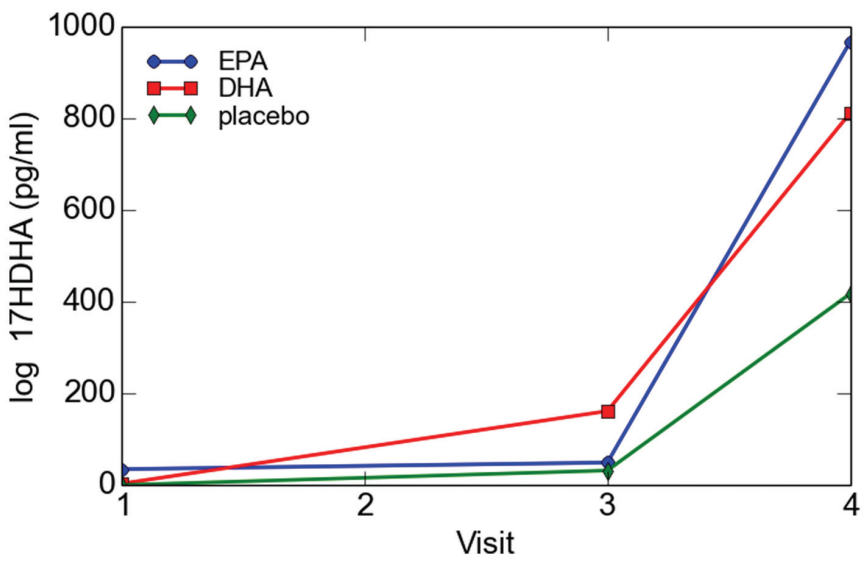

FIGURE 6 | Maternal and cord blood log 17-HDHA concentrations.

samples. In addition, although several investigations in animal models suggest that SPM may act to inhibit preterm delivery and insulin resistance (González-Périz et al., 2009; Hellmann et al., 2011; Yamashita et al., 2013), our study was not designed to determine whether increased SPM levels after supplementation would confer any clinical benefit.

Another potential weakness of this study is the possibility that autoxidation may have led to higher levels of some pathway markers rather than enzymatic conversion of DHA. Our protocol for the timing of specimen processing and storage was also chosen to minimize autoxidation artifacts. The patients and treatment-specific lipidomic profiles and differing levels of the monohydroxy metabolites 4-HDHA, 14-HDHA, and 17-HDHA in each sample also suggest that autoxidation did not significantly contribute to the overall lipidomic profiles.

It is important to note that resolvins, protectins, and maresins are produced in self-limited inflammatory leukocyte-rich exudates and are temporally regulated during the resolution phase of acute inflammation (Serhan, 2014). This may explain the increase in cord blood levels of 17-HDHA after exposure to labor, which requires activation of inflammatory pathways, compared to elective cesarean section (Gonzalez et al., 2011). Lipid mediators have also been found to be 10–100 times higher in serum compared to plasma (Colas et al., 2014). Finally, higher levels of SPMs were recently reported in human milk when compared to those observed in peripheral blood samples of healthy individuals (Weiss et al., 2013).

Thus, future research examining SPM pathway activity in serum during phases of acute inflammation, such as normal and abnormal labor would be of great interest. We have previously reported that DHA-rich fish oil supplementation increased DHA levels in maternal and umbilical cord blood, compared to placebo (Mozurkewich et al., 2013). While we did not demonstrate any effect of fish oil dietary supplementation on SPM themselves, the trend toward treatment effect of supplementation on resolvin pathway markers in our small sample should lead to larger studies evaluating the effect of supplementation on SPM and any associations with clinical outcomes. Further, given the reported high concentration of SPMs in human milk, it would be beneficial to collect the serum of breastfed neonates of mothers supplemented with omega-3 fatty acids for examination of newborn host defense and development. Continued research on specialized SPM profiling is also needed because human resolution phenotypes have only recently been developed (Morris et al., 2010). Now that studies have proven the presence of SPMs in adult and fetal tissue, as well as their potential for modulation, the stage has been set for further identification of SPM function and relationship to nutrition and disease. Specifically, further research is needed to elucidate the potential benefits for prenatal augmentation of RPM in the fetal compartment.

AUTHOR CONTRIBUTIONS

EM designed and carried out the parent study and this secondary analysis and wrote the paper. CC, DB, and VR helped to carry out the study and wrote the paper. MG carried out the blood sample analyses described in this analysis. KG designed this secondary analysis, carried out blood sample analyses, and wrote the paper. ZD designed and carried out the parent study and wrote the paper. CQ carried out the statistical analyses and wrote the paper.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer AF and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review.

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REVIEW
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Blood Biomarkers for Evaluation of Perinatal Encephalopathy

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Recent research in identification of brain injury after trauma shows many possible blood biomarkers that may help identify the fetus and neonate with encephalopathy. Traumatic brain injury shares many common features with perinatal hypoxic-ischemic encephalopathy. Trauma has a hypoxic component, and one of the 1st physiologic consequences of moderate-severe traumatic brain injury is apnea. Trauma and hypoxia-ischemia initiate an excitotoxic cascade and free radical injury followed by the inflammatory cascade, producing injury in neurons, glial cells and white matter. Increased excitatory amino acids, lipid peroxidation products, and alteration in microRNAs and inflammatory markers are common to both traumatic brain injury and perinatal encephalopathy. The blood-brain barrier is disrupted in both leading to egress of substances normally only found in the central nervous system. Brain exosomes may represent ideal biomarker containers, as RNA and protein transported within the vesicles are protected from enzymatic degradation. Evaluation of fetal or neonatal brain derived exosomes that cross the blood-brain barrier and circulate peripherally has been referred to as the "liquid brain biopsy." A multiplex of serum biomarkers could improve upon the current imprecise methods of identifying fetal and neonatal brain injury such as fetal heart rate abnormalities, meconium, cord gases at delivery, and Apgar scores. Quantitative biomarker measurements of perinatal brain injury and recovery could lead to operative delivery only in the presence of significant fetal risk, triage to appropriate therapy after birth and measure the effectiveness of treatment.

Keywords: biomarkers, neonatal encephalopathy, hypoxic-ischemic encephalopathy, neuronal injury, Glial injury

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INTRODUCTION

Worldwide it is estimated that 1.15 million babies develop hypoxic-ischemic encephalopathy (HIE) every year (Lee et al., 2013). Up to 60% of infants with HIE will die or have severe disabilities by the age of 2 including mental retardation, epilepsy, and cerebral palsy (Pierrat et al., 2005). The costs related to HIE exceed $11 billion annually in the U.S. (Lawn et al., 2011). HIE is defined by a constellation of symptoms in the neonate, and no definitive diagnostic test is available (American College of Obstetricians Gynecologists, 2014). One of the greatest challenges in perinatal medicine is assessing the fetus during labor and the neonate shortly after birth for evidence of brain injury. The presence of meconium, non-reassuring fetal heart rate tracing, Apgar scores, umbilical artery

blood gases, and physical exam, are tools currently used to identify brain injury in the fetus and neonate but they all both separately and collectively lack precision. Amplitude integrated EEG can detect early changes associated with brain injury and has been used to determine prognosis and predict long term outcomes, (Merchant and Azzopardi, 2015) however, interference from hypothermic environments can reduce the prediction of HIE prognosis, and amplitude integrated EEG cannot determine the time of injury (Thoresen et al., 2010).

One of the major challenges that needs to be solved is the early discrimination of mild-moderate injury from severe injury. The availability of therapies such as whole-body hypothermia, which must be instituted within 6 h of birth, make the rapid identification of a baby with neurologic injury critically important. How to objectively and quantitatively identify the fetus and neonate with brain injury may be solved by borrowing an approach from traumatic brain injury research. Extensive effort has been applied in the field of traumatic brain injury to identify acute blood biomarkers as diagnostics to identify these patients, discriminate severity, monitor treatment efficacy and as prognostics for recovery and long-term disabilities (Diaz-Arrastia et al., 2014).

Blood biomarkers are utilized as a diagnostic tool to identify patients with a possible disease or abnormal condition, e.g., elevated glucose levels for the diagnosis of diabetes mellitus and elevated levels of cardiac troponin for diagnosis of acute myocardial infarction (Atkinson et al., 2001). Biomarkers can be used to predict the stage of a disease and its severity, such as measuring the concentration of prostate-specific antigen in the blood to detect the level of tumor growth and metastasis (Mouhieddine et al., 2015). Following brain injury a destructive cascade of biological events continues over hours and days that may worsen the patient's condition following a primary insult and a secondary reperfusion phase. The focus of biomarker development in traumatic brain injury has been on the identification of moderate to severe injury; (Mondello et al., 2012a) however, the greatest potential impact of blood biomarkers to change clinical practice in perinatology is for mild injury since diagnostic and prognostic challenges presented for mild injuries are more difficult to identify and monitor (Diaz-Arrastia et al., 2014). There is no current standard therapy for mild injury and increasingly late outcomes suggest that mild injury results in identifiable pathology.

Similar to traumatic brain injury, it is unlikely that a single biomarker will reflect the full picture of the injured brain for a multifaceted complex disease such as HIE (Diaz-Arrastia et al., 2014). Combining biomarkers from multiple cellular pathways has shown superior sensitivity and specificity in the identification of traumatic brain injury (Diaz-Arrastia et al., 2014). Simultaneous measurements of neuronal and glial biomarkers may complement each other to identify distinct injury mechanisms and determine the timing of injury. A study of severe traumatic brain injury found that glial biomarker elevations are primarily a reflection of focal mass lesions, and that diffuse injuries primarily result in neuronal biomarker elevations (Mondello et al., 2012a). Since patients with diffuse injuries may require different therapies from those with focal lesions, such a combination of biomarkers may enable us to select patients for targeted therapies (Saatman et al., 2008; Diaz-Arrastia et al., 2014). In perinatal medicine this might allow differentiation of large major vessel stroke from more diffuse hypoxic-ischemic injuries. The ideal biomarker panel may include multiple biomarkers produced by different brain cell types, and currently available multiplex immunoassay platforms make it possible to measure up to 10 biomarkers with a high degree of sensitivity from small volumes of plasma (Diaz-Arrastia et al., 2014). Investigators have proposed developing a point of care handheld device that can quickly and accurately measure brain injury biomarkers similar to the handheld dextrometer of diabetic patients that can measure a glucose level within seconds (Bressan et al., 2014).

Optimally, the level of the brain injury biomarkers should correlate with the size, location and severity of the lesion, clinical outcome and response to treatment (Mouhieddine et al., 2015). Ideally, serum biomarkers should provide information on the pathophysiology of injury, improve stratification of patients by injury severity, assist in the monitoring of secondary insults and injury progression, monitor response to treatment and predict functional outcome (Papa et al., 2008). Circulating brain injury biomarker levels in neonatal HIE could indicate brain injury and reflect the extent of damage, solving a clinical dilemma in the discrimination of mild vs. moderate-severe injury (Lv et al., 2015).

Brain injury biomarker protein discovery can be either hypothesis driven or discovery driven. In the discovery driven method samples from normal and brain injured patients are compared with mass spectrometry to identify differences in circulating brain proteins. Unlike hypothesis driven research that identifies potential biomarkers before testing them in cases and controls, discovery driven research collects a huge amount of information first then extracts questions and answers from the data (Guingab-Cagmat et al., 2013). As currently there is no gold standard for diagnosing mild traumatic brain injury, not even by conventional assessment through neuroimaging techniques, (Niogi and Mukherjee, 2010) these approaches have been applied to identify brain specific markers of injury. During brain injury, neural proteins or their breakdown products are released into the extracellular environment reaching the cerebrospinal fluid (CSF) in relatively high concentration, and the blood stream via the compromised blood-brain barrier (Guingab-Cagmat et al., 2013; **Figure 1**). In premature infants in particular, the blood brain barrier is particularly fragile, potentially increasing the likelihood that brain proteins may reach the circulation after injury (Dammann and Leviton, 1997). Clearance and half-life of the biomarkers contribute to the final concentration that can be measured in blood (Guingab-Cagmat et al., 2013). Review of recent research in traumatic brain injury suggests significant potential for crossover of traumatic brain injury biomarkers to the brain injured fetus and neonate. The following biomarkers and classes of biomarkers may be used as tools to diagnose neonatal brain injury, follow its treatment efficacy and provide prognostic information.

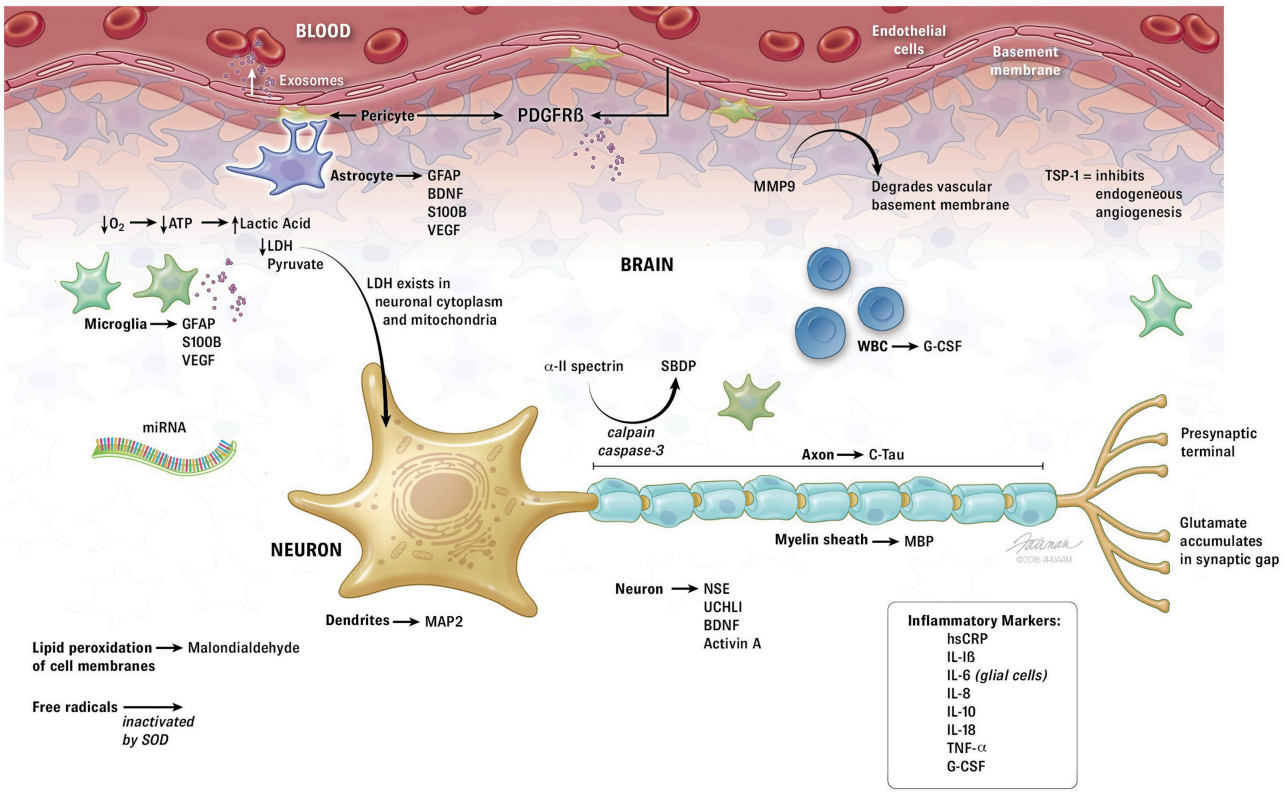

FIGURE 1 | Summary of biomarkers related to perinatal hypoxic-ischemic encephalopathy. ATP, adenosine triphosphate; BDNF, brain derived neurotrophic factor; C-Tau, cleaved-Tau; G-CSF, granulocyte colony stimulating factor; GFAP, glial fibrillary acidic protein; hsCRP, high sensitivity C-reactive protein; IL, interleukin; LDH, lactate dehydrogenase; MAP, microtubule-associated protein; MBP, myelin basic protein; miRNA, micro ribonucleic acid; MMP, matrix metalloproteinase; NSE, neuron specific enolase; PDGFR, platelet derived growth factor receptor; SBDP, spectrin breakdown products; SOD, superoxide dismutase; TNF, tumor necrosis factor; TSP, thrombospondin; UCH, ubiquitin carboxy-terminal hydrolase; VEGF, vascular endothelial growth factor.

GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP)

Of the numerous candidate biomarkers for traumatic brain injury GFAP holds the most promise (Guingab-Cagmat et al., 2013). One of the main strengths of GFAP as a brain injury biomarker is that it is only found within the central nervous system (CNS; Galea et al., 1995). GFAP is a cytoskeletal intermediate filament protein that forms networks that support astroglial cells and is found only in the astroglial cytoskeleton (Guingab-Cagmat et al., 2013). Astrocytes are important to brain injury because their foot processes comprise part of the blood brain barrier, and with disruption after injury astrocyte damage results in early release of GFAP (Vos et al., 2004; Guingab-Cagmat et al., 2013). GFAP levels peak at 1–2 days following severe brain trauma, and are normal in patients with other trauma that does not include traumatic brain injury, an indication of GFAP's brain specificity (Schiff et al., 2012). In a study of 81 patients with traumatic brain injury, serum GFAP levels in the 1st 24 h post-injury were significantly higher in patients with a focal mass lesion compared to patients with diffuse injury (Mondello et al., 2011). GFAP may be an appropriate and sensitive candidate for the diagnosis of focal brain injury such as contusion or intracerebral hemorrhage (Yokobori et al., 2013). GFAP may also be important in the differential diagnosis of various types of stroke, which is clinically relevant as immediate treatment of stroke depends on whether the stroke is ischemic or hemorrhagic (Guingab-Cagmat et al., 2013). In neonates, serum GFAP levels have been shown to be significantly elevated at the time of birth and during the first week of life in term and near-term infants with HIE that have abnormal brain MRI scans at 1 week of life (Ennen et al., 2011; Massaro et al., 2013; Chalak et al., 2014) and in premature neonates that develop periventricular white matter injury (Stewart et al., 2013). In addition, GFAP may provide insights into the pathobiology of therapeutic hypothermia as significant elevations in GFAP occur after rewarming from therapeutic cooling for HIE in the neonates that later have an abnormal MRI, suggesting that the neonates with severe injury manifest reperfusion injury post-therapeutic cooling (Schiff et al., 2012). This may offer an opportunity to discriminate mild from moderate to severe HIE and triage high risk neonates to evolving adjunctive therapies.

NEURON-SPECIFIC ENOLASE (NSE)

NSE is 1 of 5 isozymes of the glycolytic enzyme enolase found in central and peripheral neurons and red blood cells and has

been shown to be elevated following cell injury (Skogseid et al., 1992). NSE is not normally secreted into extracellular fluids by intact neurons, but when axons are damaged NSE is upregulated in an attempt to maintain homeostasis (Yokobori et al., 2013). It is highly expressed in neuronal cytoplasm and has been shown to have the sensitivity and specificity to detect neuronal cell death (Selakovic et al., 2005). Given its location and abundance NSE should possess relatively high specificity and sensitivity for axonal injury, and accordingly NSE seems to have excellent potential as a therapeutic monitor in the neurological intensive care unit and for the determination of long-term prognosis (Gradisek et al., 2012). A limitation of NSE is its lack of brain specificity, being abundant in red blood cells which leads to the occurrence of false positive results in the setting of hemolysis (Papa et al., 2008). Increased cerebrospinal fluid (CSF) and serum levels of NSE have been reported after traumatic brain injury, and NSE concentrations were associated with severity of injury, CT scan abnormalities and outcome (Selakovic et al., 2005; Guingab-Cagmat et al., 2013). NSE has been shown to provide quantitative measures of brain damage and to improve the diagnosis and prediction of ischemic stroke, intracerebral hemorrhage, seizures, comatose patients after cardiopulmonary resuscitation for cardiac arrest, and traumatic brain injury (Isgro et al., 2015). There is a positive correlation between NSE levels and infarct volume after acute ischemic stroke, and serum levels of NSE in the first few days of ischemic stroke may serve as a useful marker to predict stroke severity and early functional outcome (Isgro et al., 2015).

Release patterns of NSE and S100B were investigated in 66 traumatic brain injury patients, and in those with cortical contusions NSE was highest on the 1st measurement on day 1 after injury, decreasing from this peak over 4 days, while S100B slowly increased over the 4 day period (Herrmann et al., 2000). Serum concentrations of NSE and S100B were significantly correlated with contusion volume, but the 1st sample taken was the most valid indicator of traumatic brain injury severity (Herrmann et al., 2000). Similarly in 152 pediatric traumatic brain injury patients higher NSE concentrations were associated with worse outcome, and initial and peak NSE concentrations correlated with Glasgow Outcome Scale score at all time points investigated, particularly in children $\leq$ 4 years of age (Berger et al., 2007).

S100B

S100B is the principal low affinity calcium-binding protein in astrocytes and is considered a marker of astrocyte injury and death (Papa et al., 2008). Although S100B is glial specific and expressed primarily by astrocytes and Schwann cells, it is also found in several non-nervous system cells such as adipocytes, chondrocytes, skin, glioblastoma, and melanoma cells (Zimmer et al., 1995). S100B has a serum half-life of only 2 h, (Guingab-Cagmat et al., 2013) but is stable in blood and not affected by hemolysis (Lv et al., 2015). Since S100B is produced outside the CNS, general trauma without brain injury can increase its levels (Rothoerl and Woertgen, 2001). S100B spikes up after hemorrhagic shock and correlates with shock severity which decreases it's value as a single biomarker for traumatic brain injury (Guingab-Cagmat et al., 2013). It may be possible to use S100B as a biomarker of brain injury if measured immediately after injury; however, most mild brain injury patients are not evaluated at the time the injury occurs (Guingab-Cagmat et al., 2013).

S100B levels in blood at 24 h post-traumatic brain injury provides an early and sensitive biomarker for the prediction of brain damage which may be useful in defining early risk patterns (Egea-Guerrero et al., 2013). Elevated serum levels of S100B correlate with MRI abnormality and neuropsychological examination after mild traumatic brain injury (Ingebrigtsen et al., 1999). In 265 patients with traumatic brain injury S100B levels added substantial information regarding patient outcome in excess of that provided by age, Glasgow Coma Scale, pupil reaction and CT scan, and elevated S100B levels were best correlated to CT-visible intracranial pathology (Thelin et al., 2013). It has been estimated that adding the measurement of S100B to clinical decision tools for mild traumatic brain injury patients could potentially reduce the number of CT scans by 30% (Biberthaler et al., 2006). Furthermore, S100B below a threshold level may also safely eliminate the need to obtain a CT scan in patients with mild traumatic brain injury (Unden et al., 2013). A significant correlation has been found between the volume of contusion visible on CT scan and serum S100B concentration (Raabe et al., 1998).

Cord blood concentration of S100B $>$ 2.20 μg/L has a sensitivity of 87% and specificity of 88% in predicting moderate to severe HIE (Qian et al., 2009). Serum concentrations of S100B 2 h after birth of 8.5 μg/L predict the occurrence of severe neonatal HIE (Nagdyman et al., 2001). Salivary S100B levels may also be able to predict neonatal neurologic abnormalities related to asphyxia (Gazzolo et al., 2015). In patients with severe traumatic brain injury S100B appears to be a more accurate biomarker for predicting intracranial hypertension than NSE, particularly in the early post-injury phase (Yokobori et al., 2013). In neonates with HIE treated with hypothermia or normothermia, both NSE and S100B levels were highly elevated following asphyxia, however, serum S100B levels were lower in the hypothermia group and strongly correlated with the neurodevelopmental outcome (Roka et al., 2012).

UBIQUITIN CARBOXY-TERMINAL HYDROLASE L1 PROTEIN (UCHL1)

UCHL1, also known as neuronal-specific protein gene product 9.5, is a cysteine protease that is predominately expressed in neurons, but is also expressed in small amounts in neuroendocrine cells (Guingab-Cagmat et al., 2013). This small (25 kDa) enzyme comprises about 2% of the total soluble protein in the brain and hydrolyzes the C-terminal bond of ubiquitin or unfolded polypeptides (Setsuie and Wada, 2007). Because of its high and specific expression in brain tissue, increased levels of UCHL1 have been suggested as a marker of CNS injury such as acute cerebral ischemic disease and early stage severe traumatic

brain injury (Lv et al., 2015). Serum UCHL1 is a useful biomarker of severe traumatic brain injury both in the acute phase and in the 1st week after injury (Mondello et al., 2012b). In 251 patients with suspected mild to moderate traumatic brain injury UCHL1 outperformed GFAP and S100B in reducing CT scan use without sacrificing sensitivity (Welch et al., 2016). Of the 3 biomarkers, UCHL1 had the best test performance to differentiate between subjects with normal vs. abnormal CT scans (Welch et al., 2016). When obtained within 6 h of injury UCHL1 in combination with GFAP was very sensitive for a positive head CT, and was thought to provide the objective evidence clinicians desire when trying to reduce the use of CT scans in patients with mild traumatic brain injury (Welch et al., 2016). Serum UCHL1 levels were higher in patients with diffuse injury, in contrast to GFAP levels which were higher in patients with focal mass lesions (Mondello et al., 2011). High levels of UCHL1 have been found in the umbilical cord blood of neonates with HIE associated cortical injury and subsequent movement and cognitive disorders (Massaro et al., 2013; Douglas-Escobar et al., 2014).

CLEAVED TAU (C-TAU)

Tau is an intracellular, microtubule associated protein that is highly enriched in axons which stably assembles axonal microtubule bundles and participates in anterograde axoplasmic transport (Yokobori et al., 2013). Tau is preferentially localized in the axon, and Tau lesions are related to axonal disruption (Higuchi et al., 2002). Under normal conditions axonal Tau is below the level of detection, however, after axonal injury activated calpain depolymerizes microtubules in both perikarya and neurites to form filamentous Tau inclusions which are a pathologic hallmark of axonal injury (Yokobori et al., 2013). A cleaved form of Tau has been investigated as a potential biomarker of CNS injury (Papa et al., 2008). C-Tau levels in the CSF are significantly elevated after traumatic brain injury, and these levels correlate with clinical outcome (Zemlan et al., 2002). Although levels of C-Tau were also elevated in plasma from patients with severe traumatic brain injury, there was no correlation between plasma levels and clinical outcome (Chatfield et al., 2002). Serum Tau protein concentrations are significantly increased in bilirubin encephalopathy of newborns, and there is significant correlation between Tau levels and the severity of brain injury (Okumus et al., 2008).

MICROTUBULE-ASSOCIATED PROTEIN 2 (MAP2)

MAP2 is primarily expressed in the nervous system and is one of the most abundant proteins in the brain (Yokobori et al., 2013). MAP2 is generally dendrite-specific and potentially a good candidate biomarker for dendritic injury (Kobeissy et al., 2008). A study of 16 patients with severe traumatic brain injury found that serum MAP2 concentrations correlated with neurologic outcome at 6 months after injury (Mondello et al., 2012c). Perinatal asphyxia has been shown to affect the distribution of MAP2 in the brainstem of children (Covenas et al., 2014).

MYELIN BASIC PROTEIN (MBP)

MBP is the major component of the myelin sheath, and has a crucial role in the maintenance of myelin structure and function (Barbarese et al., 1988). It is one of the most abundant proteins in white matter, representing 30% of the protein content of myelin (Yokobori et al., 2013). Under normal conditions only a small amount is released into the blood stream, but in white matter brain injury the concentration of MBP in blood and CSF increases rapidly reflecting the severity of myelin damage which allows MBP to be used as a specific biomarker of white matter lesions or nerve fiber demyelination (Lv et al., 2015). MBP diminishes significantly in the contused rat cortex as early as 2 h after traumatic brain injury reaching its lowest level at 48 h (Ottens et al., 2008). Serum and CSF MBP has been studied as a biomarker for traumatic brain injury and for determination of outcome (Yokobori et al., 2013). Levels of serum MBP in neonates with moderate to severe HIE was significantly higher than those with mild HIE and no injury (Lv et al., 2015).

SPECTRIN BREAKDOWN PRODUCTS (SBDP)

Alpha-II-spectrin is the main structural component of the cortical membrane cytoskeleton and is particularly abundant in axons and presynaptic terminals (Riederer et al., 1986). Alpha-II-spectrin is a major substrate for both calpain and caspase-3 cysteine proteases, and is cleaved into breakdown products that may serve as biomarkers of brain injury (Guingab-Cagmat et al., 2013). A signature of caspase-3 and calpain activation is cleavage of several common proteins such as cytoskeletal alpha-II-spectrin (Ringger et al., 2004). Since calpain and caspase-3 are major executioners of necrotic and apoptotic cell death during ischemia and trauma, SBDP may provide crucial information not only on the severity of brain injury, but also on the underlying pathophysiological mechanisms associated with cell death (Guingab-Cagmat et al., 2013). Calpain and caspase-3 mediated SBDP levels in CSF are significantly increased in patients after traumatic brain injury (Pineda et al., 2007). Average SBDP values measured early after injury correlate with severity of injury, CT scan findings and outcome at 6 months post-injury (Pineda et al., 2007). This is consistent with activation of a continuum of cell death mechanisms over different time courses following severe traumatic brain injury, and shows that SBDP are potentially useful biomarkers of severe traumatic brain injury and may provide information about the timing of injury (Papa et al., 2008).

BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

BDNF is a neurotrophin secreted by CNS neurons and astrocytes that is involved in neuronal survival and synaptic plasticity. BDNF promotes the growth, differentiation, regeneration and repair of neurons (Lv et al., 2015). Proinflammtory cytokines enhance neurotrophic signaling via expression of BDNF (Werner

and Stevens, 2015). Decreased levels of serum BDNF have been associated with higher clinical severity of traumatic brain injury and with decreased 6-month functional outcome scores (Korley et al., 2016). After traumatic brain injury serum BDNF is acutely decreased correlating with injury severity, and therapies that increase brain BDNF expression, such as environmental enrichment, show promise for cognitive recovery (Failla et al., 2016). In 113 traumatic brain injury patients with serum BDNF levels measured 0–6 days (acute) and 6–12 months (chronic) post-injury, serum BDNF levels were reduced after traumatic brain injury at all time points (Failla et al., 2016). Acute serum BDNF may be a viable predictive biomarker for mood and cognitive complications within the 1st year after traumatic brain injury, and BDNF may provide a treatment window in the acute phase that affects long term recovery (Failla et al., 2016). Chronic serum BDNF may be reflective of injury severity and serve as a potential biomarker for tracking treatment response and effectiveness in real-time (Failla et al., 2016). If serum BDNF levels are persistently elevated in neonates with HIE this suggests severe brain injury and a poor prognosis (Imam et al., 2009).

ACTIVIN A

Activin A is a trophic factor that regulates neuron proliferation and is a member of the transforming growth factor β superfamily (Lv et al., 2015). Activin A has been shown to protect from hypoxic-ischemic damage in cell culture and animal models (Mukerji et al., 2007). Hydrogen peroxide treatment increased activin mRNA twofold in surviving cortical neurons, and inhibition of activin with neutralizing antibodies caused neuronal death (Mukerji et al., 2007). After transient focal cerebral ischemia in adult mice, activin mRNA increased at 1 and 4 h ipsilateral to the infarct but returned to control values at 24 h after reperfusion (Mukerji et al., 2007). Activin was also increased after 2 h of 11% hypoxia (Mukerji et al., 2007). Activin mRNA increased at 1 h, but not 4 or 24 h after hypoxia, similar to the time course of erythropoietin and vascular endothelial growth factor induction (Mukerji et al., 2007). This shows that activin is an early-regulated gene response to transient ischemia and hypoxia (Mukerji et al., 2007). Because activin responds to oxidative challenge protecting neurons, it may have a role as a potential therapy in stroke injury (Mukerji et al., 2007). In full term neonates with moderate to severe HIE, activin A is significantly elevated in CSF, and it may be a reliable early indicator for the identification of HIE (Imam et al., 2009).

MATRIX METALLOPROTEINASE-9 (MMP-9)

MMP-9 is involved in the breakdown of the blood-brain barrier by degrading brain vasculature basement membrane components (Lv et al., 2015). Under the effect of inflammatory mediators and oxygen free radicals MMP-9 is activated, and the basement membrane of the blood-brain barrier is damaged, increasing permeability and causing secondary vascular source cerebral edema, which is part of the pathophysiology of HIE (Lv et al., 2015). Serum MMP-9 is significantly elevated in neonates with HIE, and its elevation is related to the time of onset (Liu et al., 2009). A sustained increase of serum MMP-9 concentrations in neonates with HIE indicates worsening blood-brain barrier damage leading to brain damage and edema (Lv et al., 2015).

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF)

VEGF is an angiogenic factor secreted by astrocytes and microglia that is overexpressed in hypoxia-ischemia and protects neurons and glial cells by promoting the proliferation and angiogenesis of vascular endothelial cells (Lv et al., 2015). Brain VEGF mRNA expression increases and reaches a sustained peak at 12 h of life for a duration of 14 days or longer in animal models of hypoxia-ischemia (Liang and Wang, 2005). Plasma concentrations of VEGF increase with increased severity of neonatal HIE (Lv et al., 2015).

PLATELET DERIVED GROWTH FACTOR RECEPTOR β (PDGFRβ)

The integrity of the blood-brain barrier is essential for proper neuronal functioning, and pericytes are crucial for maintaining blood-brain barrier integrity. Brain capillary pericyte dysfunction results in blood-brain barrier breakdown and contributes to neurological injury (Sagare et al., 2015). PDGFRβ is expressed in the brain by vascular mural cells, brain capillary pericytes and arterial vascular smooth muscle cells, and is a marker for blood-brain barrier disruption (Sagare et al., 2015). In cultures of human brain pericytes exposed to hypoxia PDGFRβ is a biomarker of pericyte injury (Sagare et al., 2015). Elevated PDGFRβ in biofluids in patients with neurodegenerative disorders likely reflects ongoing pericyte injury and supports its potential to be developed and validated as a biomarker of brain pericyte injury and blood-brain barrier dysfunction (Sagare et al., 2015). Inflammation, induced either by trauma or hypoxia, can induce blood-brain barrier disruption by altering tight junction function leading to paracellular leakage and affecting vesicular processes leading to transcytotic leakage of potential biomarkers (Banks et al., 2015). To date PDGFRβ has not been studied in neonatal HIE.

THROMBOSPONDIN-1 (TSP-1)

Angiogenesis is a fundamental endogenous process for brain development and repair. TSP-1 is the 1st identified endogenous angiogenesis inhibitor, and its expression is upregulated after intracerebral hemorrhage (Dong et al., 2015). TSP-1 may be released into the CSF from damaged brain tissue with recirculation into the peripheral blood (Dong et al., 2015). In 110 patients with intracranial hemorrhage compared to age and gender matched healthy controls increased plasma TSP-1 concentrations following intracranial hemorrhage were independently associated with injury severity and short and long term clinical outcomes (Dong et al., 2015). TSP-1 may be a useful complementary tool to acutely assess the

severity of injury and predict poor clinical outcomes following acute intracranial hemorrhage which may make it an ideal biomarker to determine prognosis in premature neonates with intraventricular hemorrhage. TSP-1 has not been studied in neonates with HIE.

INFLAMMATION RELATED MARKERS

Because neuroinflammation is such a prominent feature of traumatic brain injury, cytokines have been investigated as potential biomarkers. Cytokines play both harmful and curative roles in traumatic brain injury, readily cross the blood-brain barrier and may relay important information on the severity and prognosis of injury (Banks et al., 2016). High sensitivity C-reactive protein is a sensitive marker of inflammation and tissue injury whose concentration increases rapidly in brain tissue following hypoxia-ischemia. In 74 neonates with HIE high sensitivity C-reactive protein, interleukin-6 and tumor necrosis factor-α (TNF-α) were significantly increased, and high levels correlated with a poor prognosis (Shang et al., 2014). High sensitivity C-reactive protein reaches a peak at day 3 of life then begins to decrease; if the serum concentration fails to decrease the prognosis is poor (Tian and Yan, 2012).

Interleukin-1β (IL-1β) promotes brain damage through the release of free radicals, stimulating inflammatory reactions, and enhancing the toxicity of excitatory amino acids (Lv et al., 2015). In neonates with HIE elevated serum IL-1β levels are associated with neurological abnormalities at 6–12 months (Liu and Feng, 2010). In 16 deceased full term asphyxiated infants there was increased expression of IL-1β in the hippocampus in those with seizures, and all cases with seizures displayed alteration in the blood-brain barrier as assessed by immunohistochemistry for albumin (Schiering et al., 2014). The authors speculated that seizure development may lead to secondary brain damage, and that IL-1β may aid in the development of therapeutic targets for neonatal seizures (Schiering et al., 2014).

IL-6 is produced by glial cells and has a protective effect on the CNS by inhibiting the synthesis of TNF-α and IL-1 and promoting nerve growth factor secretion. However, high concentrations of IL-6 can induce inflammation and increase vascular permeability leading to cerebral edema (Lv et al., 2015). When the cord blood of 50 neonates with HIE was compared to 113 controls, IL-6 levels were significantly elevated, aiding the diagnosis of brain injury, and were related to prognosis (Wu et al., 2012).

IL-8 is a neutrophil chemotaxis factor that recruits neutrophils to injured areas, and through enhanced IL-1β and TNF-α neurotoxicity increases brain injury (Lv et al., 2015). IL-8 increases in the acute phase of injury in neonates with HIE, and the more severe the injury the higher the IL-8 levels (Lv et al., 2015). In a study of 13 neonates with HIE and epilepsy, most inflammatory factors in the serum were decreased after 8–72 h, however, serum IL-8 levels remained high indicating that IL-8 might be an early biomarker for the diagnosis of neonatal HIE with epilepsy (Youn et al., 2012).

IL-10 plays a protective role in brain tissue by inhibiting the secretion of IL-1β, IL-8, and TNF-α, inhibiting the production of chemokines, decreasing leukocyte aggregation, and reducing inflammatory responses in the brain (Lv et al., 2015). IL-10 levels are significantly elevated in the acute phase of injury in neonates with HIE (Wang et al., 2003).

IL-18 is an anti-inflammatory factor that stimulates the expression of IL-1β and IL-8, and can both protect brain tissue and aggravate brain damage (Felderhoff-Mueser et al., 2005). Serum levels of IL-18 are elevated in neonates with HIE, and levels correlate with the severity of brain damage (Guo et al., 2014).

TUMOR NECROSIS FACTOR ALPHA (TNF-α)

Systemic inflammation leads to increased CNS inflammation and injury through direct transport of inflammatory agents or inflammatory cells across the blood-brain barrier, and it has been proposed that infection-induced upregulation of TNF-α can produce or worsen brain injury (Leviton, 1993). This hypothesis is supported by various studies showing that pro-inflammatory cytokines such as TNF-α produced by the placenta can cross the blood-brain barrier (Jin et al., 2015).

Intra-amniotic infections lead to preterm delivery which increases the risk of neurologic morbidity in preterm neonates. Elevated amniotic fluid levels of the inflammatory cytokines TNF-α, IL-1β, and IL-6 are linked with white matter injury in preterm neonates, (Yoon et al., 1997) and elevated cord blood levels of these cytokines correlate with neonatal cerebral lesions on MRI (Duggan et al., 2001). In preterm infants, an elevated inflammatory response during the perinatal period correlates with long term morbidities including cerebral palsy, necrotizing enterocolitis, bronchopulmonary dysplasia, and chronic lung disease (Jin et al., 2015). Elevated inflammatory cytokines measured in neonatal blood correlate with periventricular white matter injury, ventriculomegaly and severe germinal matrix hemorrhage assessed by ultrasound (Nelson et al., 2003). At this point extensive literature strongly supports the bioplausibility of all of these inflammatory cytokines as biomarkers of neonatal HIE.

GRANULOCYTE COLONY STIMULATING FACTOR (G-CSF)

G-CSF mobilizes stem cells and is currently used to promote the production of neutrophils in chemotherapy patients that develop neutropenia. G-CSF has neuroprotective properties after peripheral administration and is protective against carbon monoxide toxicity and ischemia (Banks et al., 2016). Serum levels of G-CSF correlate with severity of stroke (Yu et al., 2012) and are diagnostic for gliomas (Yildiz et al., 2011). G-CSF was the only cytokine of 23 measured whose serum levels were elevated

after traumatic brain injury in a murine model (Dohi et al., 2014). High levels of G-CSF are associated with better functional outcome and reduced lesion volume in intracranial hemorrhage (Sobrino et al., 2009). G-CSF is elevated 2 h after controlled cortical injury, but not at later time points in mice, which may make it useful in determining the timing of injury (Dohi et al., 2014). Plasma G-CSF levels correlate with neuroinflammation in the same mouse models (Dohi et al., 2014) and in human studies where they correlate with time since injury and total severity of injury (Banks et al., 2016). In a neonatal rat HIE model treatment with G-CSF has been shown to attenuate long term brain damage (Fathali et al., 2010).

OXIDATIVE STRESS RELATED MARKERS

Free radicals cause lipid peroxidation of cell membranes, and the antioxidant enzyme superoxide dismutase and the lipid peroxidation product malondialdehyde reflect the extent of oxidative damage to cells (Lv et al., 2015). Excess free radicals consume a large amount of superoxide dismutase and produce a large amount of malondialdehyde, which may allow these compounds to be used for the early prediction of neonatal HIE, but they are not brain specific (Qin et al., 2005).

METABOLISM RELATED MARKERS

Since perinatal hypoxia-ischemia leads to brain injury through an increase in anaerobic metabolism, clinical testing of metabolites may help identify the neonate with HIE and follow their recovery (Lv et al., 2015). Hypoxia leads to an increase in anaerobic glycolysis, decreased ATP production, and accumulation of lactic acid. Lactate dehydrogenase exists in neuronal cytoplasm and mitochondria, and its role is to catalyze the oxidation of lactate to pyruvate. At the onset of neonatal HIE lactate dehydrogenase activity and lactate production are increased, and a combination of lactate dehydrogenase and NSE may be used to identify brain injury (Lv et al., 2015). Detection of lactate dehydrogenase with other metabolites may have an important role in neonatal HIE diagnosis and prognostic evaluation (Lv et al., 2015).

Extracellular glutamate primarily mediates excitotoxicity and is involved in the pathophysiological processes of brain ischemia (Lv et al., 2015). During HIE ATP synthesis decreases, glutamate transport is inhibited and glutamate accumulates in the neuronal synaptic cleft subsequently leading to neuronal death (Lv et al., 2015). Because glutamate is brain specific, it can be a sensitive biomarker for brain injury, and if monitored early may aid in the early diagnosis of HIE in the neonate and could be a marker of therapeutic efficacy as ancillary therapies are developed for HIE (Lv et al., 2015).

MICRORNA (miRNA)

MicroRNA (miRNA) are being investigated as promising serum biomarkers for neurotrauma. miRNA may be stable in the circulation which supports their potential use as disease biomarkers (Egea et al., 2012). miRNAs are endogenously expressed ∼22 nucleotide long noncoding RNAs that control a wide spectrum of cellular function, and bind to target regions of certain genes to control their expression by either repression or activation of mRNA translation/transcription (Chen et al., 2015). miRNAs play important roles in developmental and functional aspects of the CNS and in many neurological diseases, which potentially make them important candidates for brain injury diagnosis (Chen et al., 2015). Emerging data suggest that exosomal miRNA may provide potential biomarkers in acute ischemic stroke (Chen et al., 2015). Studies of endothelial cell cultures and *in vivo* rat focal ischemia models have shown significant reductions in serum miR-126 detected at 3 h after permanent ischemia but not transient ischemia, which suggests that changes in serum miR-126 may be able to distinguish severe permanent ischemia from milder injury after transient ischemia (Chen et al., 2015). miR-126 was selected as the initial candidate biomarker of neurovascular damage in stroke because it is specifically and highly expressed in endothelial cells and is known to be involved in the regulation of vascular integrity, endothelial function, and angiogenesis (Chen et al., 2015). miRNAs have been shown to be altered in plasma before the first spontaneous seizure and have been proposed as putative biomarkers of epileptogenesis (Roncon et al., 2015). Recently miRNAs have been found to be involved in the pathophysiology of HIE, including the regulation of excitatory amino acid toxicity, oxidative stress, inflammatory reactions and apoptosis (Lv et al., 2015). Plasma miRNA profiles compared between severe traumatic brain injury patients and healthy volunteers found that decreases in the levels of miR-16 and miR-92a and increased levels of miR-765 were good markers of severe traumatic brain injury at 25–48 h after injury (Redell et al., 2010). Specific miRNAs seem to be good candidate biomarkers for distinguishing focal and diffuse brain injury or for accurate determination of raised intracranial pressure (Yokobori et al., 2013). Animal models of neonatal HIE have shown specific miRNA changes following injury using microarray (Weiss et al., 2012). Expression of miR-21 is found in astrocytes. A study of 49 cases of neonatal HIE showed that serum miR-21 was significantly increased and may be a marker for the early diagnosis of neonatal HIE (Chen and Yang, 2012).

EXOSOMES

A potentially transformative finding in traumatic brain injury research has been the identification of exosomes, which are nano-sized extracellular vesicles that have key roles in cell signaling and undergo membrane fusion so that they readily cross the blood-brain barrier. Exosomes contain proteins, miRNA and other nucleic acids, and are believed to play a major role in disposal of cellular waste (Werner and Stevens, 2015). Exosomes may serve as vehicles for targeted delivery of repair-inducing molecules and possibly as novel biomarkers (Werner and Stevens, 2015). They have been implicated in an array of signaling processes involving astrocytes, oligodendrocytes, microglia, neurons, and neural stem cells (Rajendran et al., 2014). Increased levels of exosomes have been found after traumatic brain injury in humans (Patz

et al., 2013). Injured neurons release the microtubule-associated protein Tau which is then carried by exosomes (Rajendran et al., 2014). Exosomes may represent ideal biomarker containers, as RNA and protein transported within the vesicles are protected from enzymatic degradation, and there is considerable interest in developing assays to evaluate blood-borne brain-derived exosomes, often referred to as the "liquid brain biopsy" (Werner and Stevens, 2015).

CONCLUSION

Blood biomarkers discovered in traumatic brain injury could significantly improve the management of neonates with HIE, particularly those with mild and moderate injury, by providing more accurate early diagnosis and prognosis, and for monitoring therapies in the acute care setting (Papa et al., 2008). Biomarkers could help determine severity and mechanism of injury and quantitatively measure injury progression which would provide major opportunities for clinical research (Papa et al., 2008).

Serum based combined multi-marker analysis reflecting glial and neuronal cell damage should be considered for understanding more precise pathophysiological mechanisms of brain injury. Glial injury can be assessed by GFAP in the blood and axonal injury by C-Tau and spectrin protein breakdown products (Guingab-Cagmat et al., 2013). The serum levels of glial and neuronal biomarkers (S100B, GFAP, UCHL1) at the time of admission after brain injury have been shown to correlate with clinical outcome and are sensitive and specific in determining the severity of injury (Lee et al., 2015). With UCHL1 levels increasing in diffuse injury and GFAP levels increasing in focal injury, a glial:neuronal ratio has been proposed as a novel indicator to differentiate focal and diffuse injury, and has been found to be more accurate when measured at < 12 h after injury (Mondello et al., 2012a). Several potential epilepsy biomarkers have been proposed in recent years including blood biomarkers of inflammation, blood-brain barrier damage and brain injury. Given the complexity of epilepsy it is unlikely that a single biomarker is sufficient for predicting epileptogenesis, but a combinatorial approach may be able to identify appropriate biomarkers at different stages of the evolution of the disease (Loscher et al., 2013).

The pathology of perinatal HIE is very heterogeneous and one "magic" biomarker may not be the solution, but a panel of biomarkers may prove to be most useful in distinguishing the different pathologic-anatomic processes that comprise the injury (Papa et al., 2008). The brain consists of many elements, and depending on the mechanism and severity of injury, various damage patterns may be reflected by different combinations of biomarkers (Lee et al., 2015). Biomarkers will probably supplement existing tools, such as the Glascow Coma Scale and neuroimaging, for the initial classification of brain injury in the near future (Papa et al., 2008). As the hypoxic injury pattern on MRI is not diagnostic for 7–14 days, a blood biomarker that could fill the clinical gap for predicting current and worsening neurological status or long-term disability and would have great clinical utility. With the combinations of different pathophysiology related to each biomarker, a multi-biomarker analysis would seem to be the most effective way to assess brain injury and would likely increase diagnostic accuracy (Yokobori et al., 2013).

Blood biomarkers offer an objective and quantitative way to identify and follow a neonate with brain injury. They could be used to triage neonates to the current standard of 72 h of hypothermia as well as investigational therapies such as erythropoietin, xenon gas, melatonin and various forms of stem cell treatment. Blood biomarkers could possibly provide information about the extent of injury in the acute phase before ultrasound or MRI can identify abnormalities and determine the timing of injury as well. Blood biomarkers measured noninvasively using near-infrared spectroscopy (Torricelli et al., 2014) or optoacoustic techniques (Petrov et al., 2012a,b) may also allow quantitative monitoring of the fetal brain for injury during the intrapartum period through a dilated cervix with ruptured membranes via a probe attached to the fetal head similar to the currently used fetal scalp clip for measuring heart rate. Rather than imprecise measures of fetal and neonatal brain injury such as fetal heart rate abnormalities, meconium, cord gas at delivery, and Apgar scores, a multiplex combining glial and neuronal biomarkers could provide objective evidence of the extent and pattern of injury which would lead to improved identification of the brain injured fetus or neonate, operative delivery only in the presence of a significant risk of HIE, triage to appropriate therapy after birth and serve as an objective measure of the effectiveness of treatment for the brain injured baby.

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All authors listed, have made substantial, direct, and intellectual contribution to the work, and approved it for publication.

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