ADVANCES IN THE PREVENTION AND TREATMENT OF INFLAMMATION-ASSOCIATED PRETERM BIRTH

EDITED BY: Jeffrey A. Keelan and John P. Newnham

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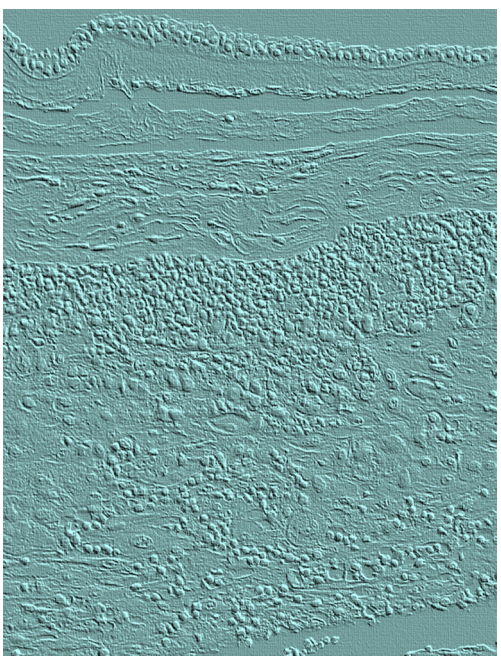
ADVANCES IN THE PREVENTION AND TREATMENT OF INFLAMMATION-ASSOCIATED PRETERM BIRTH

Topic Editors:
Jeffrey A. Keelan, The University of Western Australia, Australia
John P. Newnham, The University of Western Australia, Australia



Histological image of the fetal membranes from a pregnancy with acute chorioamnionitis (image graphically enhanced).

After decades of intensive research and over 10,000 publications, preterm birth remains a major global obstetric healthcare problem. Each year, early birth is responsible for the deaths of more than one million infants worldwide and is a major cause of life-long disability. Preterm birth places an enormous financial burden on our healthcare systems, resulting in long-term adverse health outcomes and lost productivity for many people.

Preterm birth is a syndrome, associated with several different aetiologies; hence, potential treatment strategies need to be matched to pathophysiology in order to be effective. There is now unequivocal evidence that inflammation is causally involved in a majority of spontaneous preterm deliveries. However, the triggers of inflammation, and the strategies by which it can be safely and effectively prevented and treated, remain the subject of ongoing investigation and debate. While intraamniotic infection is an important cause of inflammation-associated preterm birth, particularly in very preterm deliveries, 'sterile' inflammation is actually a more common finding associated with preterm birth.

It is likely that the nature, localisation, timing and extent of the inflammatory insult all determine the obstetric outcome and degree of risk to the fetus. These factors will also influence

the success of approaches that might be employed to achieve better pregnancy outcomes. Despite our increased understanding of the causes and significance of intrauterine inflammation, we have yet to translate this knowledge into effective therapeutic strategies for preventing prematurity and mitigating its consequences for the neonate.

In this Research Topic we review recent progress in treating and preventing inflammation-associated preterm birth, approaching the topic from both the causal and therapeutic perspectives. With global attention increasingly focussed on the need to translate knowledge discovery into clinical translation, we hope this EBook will provide a stimulating and timely discussion that will focus research and lead to improved healthcare outcomes for women and children.

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EDITORIAL
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Editorial: Advances in the Prevention and Treatment of Inflammation-Associated Preterm Birth

Jeffrey A. Keelan and John P. Newnham*

School of Women's and Infants' Health, The University of Western Australia, Perth, WA, Australia

Keywords: preterm birth, inflammation, infection, fetus, oxidative stress, anti-inflammatory agents, antimicrobials, probiotics

The Editorial on the Research Topic

Advances in the Prevention and Treatment of Inflammation-Associated Preterm Birth

Despite the widely appreciated fact that preterm birth (PTB) is a syndrome and the potential consequence of many different pathways (1), there is now unequivocal evidence that inflammation lies at the core of the majority of the pathophysiological causes of PTB. Inflammation within the amniotic cavity – characterized by chorioamnionitis and/or elevated levels of amniotic fluid cytokines and chemokines – is the major driver of preterm labor less than 34 weeks gestation and an important contributor to later preterm deliveries (2–4). However, the causes of the inflammation and the strategies by which it can be safely and effectively prevented and treated are less clear and the subject of ongoing investigation and debate. While ascending bacterial infection is a particularly important and well-studied cause of inflammation-associated PTB in very preterm deliveries, other "sterile" causes are dominant at later gestational ages. The nature, localization, timing, and extent of the inflammatory insult likely determine the obstetric outcome and degree of risk to the fetus. These factors also dictate possible pharmacotherapeutic approaches that might be employed to achieve better pregnancy outcomes – namely, minimal neonatal morbidity and optimal long-term health and development of the child.

In this research topic, we have invited contributions from a range of internationally recognized clinicians and scientists relating to inflammation-associated PTB from both the causal and therapeutic perspectives. To place the discussions in the broader clinical context, Newnham et al. describe the problem of PTB and major intervention strategies that are being applied in clinics around the world to prevent PTB. They outline the evidence supporting efficacy and safety, and the likely impact of clinical implementation on PTB rates. They contend that we are now in a position to translate research into clinical care, although such interventions will require integration and coordination of multidisciplinary teams, tailored to different communities and resource settings. Next, Kemp discusses the immunological factors involved in pathogenesis of fetal inflammatory response syndrome (FIRS), with special emphasis on the role of pattern recognition receptors, defensins, and complement activation. He reviews, in detail, the evidence for a fetal contribution to the overall intrauterine inflammatory response to microbial infection and its significance with respect to neonatal sequelae.

Following on, Payne and Bayatibojakhi outline the roles played by different microorganisms (bacteria, fungi, yeasts, and viruses) in the process of infection-driven PTB and the contribution of microbiome studies to our understanding of this topic. They reiterate the important point that intra-amniotic infection is frequently a polymicrobial disease and discuss technical aspects related to the generation and interpretation of microbiome data – in particular the "disconnect" between

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Pietro Ghezzi, Brighton and Sussex Medical School, UK

****Correspondence:***
Jeffrey A. Keelan
jeff.keelan@uwa.edu.au

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DNA-based identification and presence of viable microorganisms. The recent study by Prince et al. (5) further contributes to this topic, providing novel links between the microbiome and the metabolome in preterm deliveries. Continuing with the microbial-inflammation theme, Ireland and Keelan move the focus to the maternal response to infection and the serological response to *Ureaplasma*, in particular. While this intracellular microorganism is known to be commonly associated with inflammation-driven PTB, the relatively low incidence of infection-associated PTB in the presence of high vaginal *Ureaplasma*-colonization rates (~50% in pregnancy) remains unexplained. This review explores the role of the maternal host response to colonization in determining risk and pregnancy outcomes. Next, Menon overviews the role of oxidative stress as a driver of PTB and preterm prelabor rupture of membranes (PPROM). He examines the evidence supporting the contribution of reactive oxygen species (ROS) to the pathogenesis of PPROM and PTB and the pregnancy conditions that may give rise to ROS generation and downstream adverse effects: MAPK activation, telomere reduction, DNA damage, senescence, and apoptosis. He suggests that the release of secreted senescence biomarkers may play a key role in the triggering of PTB and PPROM and, as such, are targets for new interventional strategies. A recent publication from his group reinforces the theme of this review (6).

From inflammatory pathophysiology we move to therapy. Ng et al. commence by exploring the potential of novel anti-inflammatory drugs to block inflammatory signaling and prevent the release of cytokines and other mediators that trigger labor and delivery and cause inflammation-associated fetal morbidity. They discuss the efficacy of a range of compounds and then discuss the barriers to clinical translation and the challenges of adopting such anti-inflammatory strategies in different pregnancy scenarios. Next, Yang et al. examine the potential application of administration of probiotics to prevent PTB. They initially discuss the evidence supporting an association between altered vaginal microbiome and adverse pregnancy outcome, looking at the relationship between the presence of different *Lactobacillus* species and PTB rates, in different populations. They, then, review the evidence from their own studies (and others) on the properties and applications of probiotics, such as *Lactobacillus rhamnosus* GR-1, for preventing PTB. They conclude that the strategy holds promise, but large scale clinical trials are required with appropriate power to demonstrate the benefits and risks. A recent trial of oral probiotics (*L. rhamnosus* GR-1 plus *L. reuteri* RC-14) supports the potential effectiveness of the approach in terms of normalizing vaginal microbiota (7).

Lamont, then, reviews the evidence around the use of antibiotics for the prevention of PTB, looking at the results of clinical trials and meta-analyses and pointing to errors in their design and conclusions. He stresses the importance of applying the right therapy at the right time to the right cohort of patients and points to the success of studies using clarithromycin early in pregnancy to treat women with abnormal vaginal microbiota. Finally, Keelan et al. discuss the potential applications of a new macrolide antibiotic, solithromycin, in the prevention and treatment of pregnancy infections and PTB. Drawing on their own studies in human placentas and the pregnant sheep model, combined with other data on antimicrobial efficacy, they discuss the potency of solithromycin against the organisms that typically infect the amniotic cavity. This review also highlights solithromycin's unique ability to cross the placenta and treat the infection at its source following oral maternal administration.

AUTHOR CONTRIBUTIONS

Both authors are editors of the research topic and contributed to the writing and editing of the editorial.

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Strategies to prevent preterm birth

John P. Newnham*, Jan E. Dickinson, Roger J. Hart, Craig E. Pennell, Catherine A. Arrese and Jeffrey A. Keelan

School of Women's and Infants' Health, The University of Western Australia, Perth, WA, Australia

Edited by:
Anna Rubartelli, IRCCS AOU San Martino IST, Italy

Reviewed by:
Pablo Pelegrin, Fundación para la Formación e Investigación Sanitarias de la Región de Murcia – Hospital Universitario Virgen de la Arrixaca, Spain
Patrizia Rovere Querini, Ospedale San Raffaele and Vita-Salute University, Italy

****Correspondence:***
John P. Newnham, School of Women's and Infants' Health, The University of Western Australia, 35 Stirling Highway – M550, Crawley, Perth, WA 6009, Australia
e-mail: john.newnham@uwa.edu.au

After several decades of research, we now have evidence that at least six interventions are suitable for immediate use in contemporary clinical practice within high-resource settings and can be expected to safely reduce the rate of preterm birth. These interventions involve strategies to prevent non-medically indicated late preterm birth; use of maternal progesterone supplementation; surgical closure of the cervix with cerclage; prevention of exposure of pregnant women to cigarette smoke; judicious use of fertility treatments; and dedicated preterm birth prevention clinics. Quantification of the extent of success is difficult to predict and will be dependent on other clinical, cultural, societal, and economic factors operating in each environment. Further success can be anticipated in the coming years as other research discoveries are translated into clinical practice, including new approaches to treating intra-uterine infection, improvements in maternal nutrition, and lifestyle modifications to ameliorate maternal stress. The widespread use of human papillomavirus vaccination in girls and young women will decrease the need for surgical interventions on the cervix and can be expected to further reduce the risk of early birth. Together, this array of clinical interventions, each based on a substantial body of evidence, is likely to reduce rates of preterm birth and prevent death and disability in large numbers of children. The process begins with an acceptance that early birth is not an inevitable and natural feature of human reproduction. Preventative strategies are now available and need to be applied. The best outcomes may come from developing integrated strategies designed specifically for each health-care environment.

Keywords: preterm birth, prevention, progesterone, smoking, pregnancy

INTRODUCTION

Each year, 15 million babies are born preterm (1). Many may look forward to a normal life, but others may die or live a life of disability. Worldwide the rate of preterm birth is 11.1% but varies with geography and race, ranging from 15% or more in some parts of Africa to 5–6% in several European nations (2) and possibly lower in some parts of East Asia (2).

In most countries, the rate of preterm birth has risen in recent decades and worldwide now represents the largest cause of neonatal death (3) and the second largest direct cause of death in children up to 5 years of age (2). Discovering how to lower the rate of this major complication of pregnancy needs to be one of the highest priorities in contemporary health care.

Prevention of early birth, however, presents several great challenges. The condition merely describes an event that occurs before its due time, and is not a diagnosis in itself. There are many pathways leading to preterm birth and the prevention of each requires different types of scientific inquiry and clinical strategies, which together encompass a wide array of measurement systems and clinical interventions across many health-care disciplines (4).

With such a great challenge, what evidence do we have that prevention of preterm birth is possible and feasible?

LESSONS FROM POPULATIONS IN TRANSITION

Changing rates of preterm birth in populations in transition provide some evidence that environmental and lifestyle factors may be involved, and these may be amenable to intervention.

In China, the rate of preterm birth is not known with certainty as there is no national obstetric data reporting system, but the estimated rate is thought to be relatively low by international standards. Hospital-based reports suggest rates ranging from 3 to 6% (5–8). A geographic-based study employing ultrasound confirmation of gestational age in early pregnancy in Jiangsu Province indicated rates of 2.6 and 2.9% in urban and rural regions, respectively (9). These rates appeared to rise as Chinese women lived in increasingly Westernized environments, with preterm birth rates of 4.4% in China-born women in Western Australia; 5.6% in non-resident Chinese women living in Hong Kong; and 7.6% for Hong Kong women with residency status. The factors underpinning these differing rates suggest that environment and lifestyle may be involved in modifying preterm birth rates and that factors operating outside traditional China may somehow have increased the rate by several percentage points. These figures provide an indirect clue as to the potential magnitude of the effect of preventative strategies, at least in women of Chinese origin living in Western environments.

Mexican women after migration to USA also experience an increase in risk of preterm birth. Long-term immigrants who had lived in USA for more than 5 years were shown to have a 1.9-fold greater risk of delivering preterm, and a 1.5 greater risk of giving birth to a low-birth weight infant when compared with more recent arrivals (10). The factors involved in the increasing preterm birth risks in these population groups are uncertain, but long term Mexican immigrant women have been shown to have

higher parity, more pregnancy complications, fewer planned pregnancies, and to smoke. An almost doubling in preterm birth rate as these lifestyle factors are adopted by immigrant women suggests that appropriate interventions may reduce the rate of early birth by nearly half.

OPPORTUNITIES TO PREVENT PRETERM BIRTHS

"Green shoots" are now appearing in the field of preterm birth prevention. At least in high-resource settings, some strategies are suitable for immediate use in clinical practice, have a high likelihood of success, and in many regions have already been adopted in part or in whole. Other strategies have potential and feasibility, but evidence for their effectiveness is still uncertain. Finally, there are initiatives in place aimed at other clinical end-points but which may incidentally prevent some cases of early birth.

The levels of evidence employed in this review and that enable description of potential effectiveness in high-resource settings are shown in **Table 1**, and the potential strategies that are feasible and suitable for implementation are shown with their level of evidence in **Table 2**.

Table 1 | Levels of evidence for intervention studies as used by the Australian National Health and Medical Research Council (NHMRC) and employed in this review.

Level	Intervention
I	Systematic review of level II studies
II	Randomized controlled trial
III-1	Pseudo-randomized controlled trial (i.e., alternate allocation or some other method)
III-2	Comparative study with concurrent controls Non-randomized experimental trial Cohort study Case-control study Interrupted time series with control group
III-3	Comparative study without concurrent controls Historical control study Two or more single-arm study Interrupted time series without a parallel control group
IV	Case series with either post-test or pre-test/post-test outcomes

https://www.nhmrc.gov.au/_files_nhmrc/file/guidelines/developers/nhmrc_levels_grades_evidence_120423.pdf

Table 2 | Strategies to prevent preterm birth feasible for implementation and likely to be successful in high-resource settings.

Strategy	Possible reduction in PTB	Level of evidence
Prevent non-medically indicated late preterm/early term birth	55% (11)	III-3
Progesterone supplementation	45% (12)	I
Cervical cerclage	20% (13)	III-1
Tobacco control		
Prevent smoking in pregnancy	20% (14)	III-2
Smoke-free legislation	10% (15)	III-3
Judicious use of fertility treatments	63% (16)	I
Dedicated preterm birth prevention clinics	13% (17)	III-2

*Levels of evidence as defined in **Table 1**.*

STRATEGIES SUITABLE FOR IMMEDIATE USE

There are six strategies currently available with various levels of evidence of effectiveness that are suitable for translation into clinical practice in high-resource settings and have a high chance of successfully preventing a proportion of preterm births.

PREVENTING NON-MEDICALLY INDICATED LATE PRETERM BIRTH

The most feasible approach to rapidly lowering the overall rate of preterm birth is to address non-medically indicated late preterm birth. Late preterm birth is defined as birth between 34 weeks 0 days and 36 weeks 6 days and these infants account for 70% of all preterm births (18). In USA, the rate of preterm birth increased by one-third over the last 25 years and this increase has resulted almost entirely from a rise in late preterm births (19). In the period 1990–2006, the late preterm birth rate for singleton births increased 20% from 6.7 to 8.1%. Similar increases in late preterm birth have been described in other countries during this time period, including South America (20), France (21), and Australia (22, 23).

Late preterm birth is a potential danger to the child. Infants born in the late preterm period are physiologically and metabolically immature. Their brain mass is approximately 70% of that of a term infant and the ongoing process of myelination is reduced accordingly (24). In the neonatal period, late preterm infants are at increased risks of death, admission to neonatal intensive care, respiratory distress and need for mechanical ventilation (25), apnea, temperature instability, hypoglycemia, hyperbilirubinemia, poor feeding, separation from their mother, and re-admission after discharge (24). In childhood, late preterm infants are at increased risk of death (18), cerebral palsy (26), speech disorders (27), growth delay and stunting (28), developmental delay (29), behavioral problems including attention deficit disorder (24, 30, 31) and learning difficulties (31, 32). The financial costs are considerable, not just for the health-care system in the short term, but for the individual, the family, and the society in terms of life-long productivity.

Recently, the effect of birth in the early term period has received increasing attention (33). Early term birth has been defined as birth between 37 weeks 0 days and 38 weeks 6 days gestation (24) and accounts for approximately 17% of all births. Rates of early term birth have risen in recent years in many regions. Neonatal and infant morbidities are increased in the early term period, when compared with births at 39 weeks gestation or more, but the magnitude of excess risk is less than in the late preterm birth age group. As an example, a recent Canadian study observed that the adjusted relative risk for admission to neonatal intensive care was

6.14 (95% CI 5.63, 7.03) for late preterm birth and 1.54 (95% CI 1.41, 1.68) for early term birth (34). For neonatal respiratory morbidity, the adjusted relative risks were 6.16 (95% CI 5.39, 7.03) and 1.46 (95% CI 1.29, 1.65), respectively. In a comparison of outcomes after planned Cesarean section in 19 centers in USA, it was observed that when compared with births at 39 completed weeks, rates of admission to a neonatal intensive-care unit (NICU), need for mechanical ventilation, and treatment for sepsis or hypoglycemia were increased 1.8- to 4.2-fold for births at 37 weeks and 1.3–2.1 for births at 38 weeks (35).

A significant proportion of all late preterm and early term births result from obstetric or medical complications of pregnancy, while others may be precipitated out of concern for maternal or fetal well being. As a result, assessment of outcomes after birth is confounded by the relative contributions of prematurity and any abnormalities in the pregnancy that contributed to the early gestational age at birth. The role of such biological determinants has been investigated and found to be amplified at earlier gestational ages, but quantification of the true relative contributions of gestational age and biological determinants remain challenging and will require further investigation (34).

Strategies addressing the increase in late preterm and early term births will be enhanced by an understanding of the demographic characteristics of those most at-risk. In a review of the factors contributing to the rise in preterm birth rates at 36 and 37 weeks gestation in USA between 1992 and 2002, non-Hispanic white births were found to be the greatest contributor (36). Rates of early preterm birth in Hispanic and black births remained relatively constant. The factors underpinning the rise in non-Hispanic white early births is likely to be socioeconomic and include access to health care and possibly maternal request for intervention.

If then the major contributor to rising preterm birth rates in recent years has been late preterm birth, is it possible to intervene and can it be done with safety? The clinical reasoning behind many cases of early intervention is to prevent stillbirth. In a 10-year population-based study in New South Wales, Australia encompassing more than 100 hospitals and approximately one-third of all births in Australia, the number of planned interventions before the estimated due date was found to have increased to 26% of all singleton births >32 weeks gestation (22). Stillbirth rates were unchanged over this time period, indicating that at least at a population level, the increased intervention did not improve infant survival.

Researchers in Denmark have reported the outcomes of the first randomized controlled trial of elective Cesarean section planned for 38 weeks, versus 39 weeks, gestation (37). An original sample size of 1010 participants was increased to 1270 when a high rate of non-compliance was observed. There were small reductions in rates of admission to NICU in the delayed delivery group, but the differences were not statistically significant. Interpretation of these findings is confounded by uncertainty as to whether the study was under-powered and by the unexpected high rate of earlier delivery in the planned later birth group (23).

The effects of introducing policies to lower the rate of elective delivery before 39 weeks gestation have been reported as a retrospective cohort of prospectively collected data (11). The study involved 27 facilities across 14 states in USA. After an education campaign and declaration of an intent to reduce non-medically indicated birth <39 weeks gestation on the basis of patient safety, medical staff were allowed to choose one of three approaches: (1) a "hard stop" approach in which elective delivery <39 weeks would be refused by hospital staff and the policy would be enforced; (2) a "soft stop" approach in which compliance would be left to individual clinicians but that any departure from policy would be referred to a local peer review committee for "evaluation and potential action"; and (3) an "education only" approach.

During the study period, the rate of elective delivery between 37 and 39 weeks gestation fell significantly from 9.5% of all births in 2007 to 4.3% of births in 2009. The rate of elective early term delivery was significantly reduced in both the "hard stop" and "soft stop" groups, and the reduction in the "hard stop" group was double that in the "soft stop" group. There was a small decline in the "education only" group, but the difference did not achieve statistical significance. Interestingly, there was considerable variation between facilities in their rates of elective delivery <39 weeks before the intervention commenced. Each facility had self-selected their choice of the three strategies. The large reduction in rates of early birth in some facilities, which began with high levels of intervention indicates that any such intervention may be most effectively targeted at those with the highest rate of early intervention. Overall, in the study facilities during the observation period, the rate of term newborn intensive care admission fell 15% from 8.9 to 7.5% (CI 0.79, 0.92), while stillbirths rates were unchanged.

The findings of this intervention cohort study provide strong evidence that the rate of late preterm and early term birth may be lowered and result in a 15% reduction in admissions to neonatal intensive care. Such an approach does not appear to increase the rate of stillbirth. Education alone did not significantly improve outcomes, but the process of education was aimed solely at the health-care providers, and it is yet to be seen if a combined approach of providing education to both the women themselves and the health-care personnel may result in a more favorable outcome.

The price for obstetricians and their hospitals of delaying birth, however, is an increase in the number of births out-of-hours. In a secondary analysis of the Danish randomized controlled trial of planned Cesarean birth at 38 or 39 weeks gestation (38), planned delivery at 39 weeks compared with 38 weeks resulted in a 60% increase in unscheduled Cesarean sections and a 70% increase in deliveries outside regular working hours. Further research is urgently required to determine the most effective strategies by which late preterm and early term births may be minimized without deleterious impacts on the health-care system and while maintaining patient safety. Future strategies may be most effective if they recruit to the cause not just the health-care professionals but also the pregnant women and their families.

PROGESTERONE SUPPLEMENTATION

For several decades, there has been interest in the potential use of progesterone supplementation to prevent preterm birth but a series of recent studies has now provided strong evidence for their usefulness.

Ironically, the mechanism by which progesterone may delay birth remains uncertain. In non-primate placental mammals, the

uterine quiescence of pregnancy is maintained by high circulating levels of progesterone and falling levels herald the onset of labor (39). In human beings, there is no such decline in circulating levels before labor (40). Two possible mechanisms of action are proposed. First, progesterone has an anti-inflammatory action that may counteract the inflammatory process that is involved in initiation of labor (41). Second is a possible functional withdrawal of progesterone through changes in progesterone receptors and their transcriptional activity at a tissue level (41–43).

Progesterone has been administered in several formulations. For preterm birth prevention, natural progesterone is used (44). Synthetic progesterones, such as medroxyprogesterone acetate are not used as they have significant androgenic activity. Natural progesterones can be given vaginally, orally, or by injection. Vaginal progesterone has the advantage of being locally available and has few side effects although some women complain of vaginal irritation. The half-life is 13 h (45) and daily treatment is required. Various doses are employed ranging from 90 to 400 mg but there is no evidence that any one dose is superior to another. An alternative agent is 17 α-hydroxy-progesterone (17P) caproate, which is also a natural progesterone conjugate but with a longer half-life of 7 days (44). 17P is administered intramuscularly and is given once each week. Both these progesterone formulations are considered to be safe in pregnancy.

In women with a past history of preterm birth, progesterone has been shown in meta-analysis of RCTs to significantly reduce the risk of preterm birth <34 weeks (RR 0.31 95% CI 0.14–0.69), preterm birth <37 weeks (RR 0.55, 95% CI 0.42–0.74), perinatal death (RR 0.50, 95% CI 0.33–0.75), need for assisted ventilation (RR 0.40, 95% CI 0.18–0.90), necrotizing enterocolitis (RR 0.30, 95% CI 0.10–0.89), and admission to neonatal intensive care (RR 0.24, 95% CI 0.14–0.40) (46).When given to women with a past history of preterm birth, there is no evidence for a difference in effectiveness between daily natural vaginal progesterone and weekly intramuscular 17P injections. As a result of these findings, the American College of Obstetricians and Gynecologists recommends that "progesterone supplementation for the prevention of recurrent preterm birth should be offered to women with a singleton pregnancy and a prior spontaneous preterm birth due to spontaneous labor or premature rupture of membranes" (47).

There is also strong evidence that progesterone treatment may prevent preterm birth in women shown to have a short cervix on ultrasound imaging in mid-pregnancy. Meta-analysis of individual patient data of five trials has shown that vaginal progesterone given to pregnant women in the mid-trimester with a short cervix (≤25 mm) is associated with a significant reduction in the rate of preterm birth <28 weeks (RR 0.50, 95% CI 0.30–0.81), <33 weeks (RR 0.58, 95% CI 0.42–0.80) and <35 weeks (RR 0.69, 95% CI 0.55–0.88), in addition to significant reductions in risk of newborn complications including respiratory distress syndrome, need for mechanical ventilation, admission to neonatal intensive care, and composite morbidity and mortality (48).

If progesterone is so effective in preventing preterm birth in women with a short cervix in mid-pregnancy, should all pregnant women be screened for cervical length at this time? The question is of great importance and remains controversial. In one of the major and most important trials in this field, Hassan and colleagues allocated at random asymptomatic women with a singleton pregnancy and short cervix (10–20 mm) between 19 weeks 0 days and 23 weeks 6 days to receive either vaginal progesterone gel or placebo daily (12). Preterm birth and the major complications of prematurity were halved by the treatment, consistent with the findings from other studies. The numbers of women, however, required to achieve this reduction were large. A total of 32,091 pregnant women were screened to identify 733 with a cervix length between 10 and 20 mm. 268 women declined to participate or were excluded, leaving 236 randomized to the treatment group and 229 to the placebo. The primary outcome of birth <33 weeks was observed in 21 cases in the treatment group (8.9%) and 36 cases in the placebo group (16.1%), preventing the early birth of 15 cases out of 36 eligible. Therefore, if we were to assume that introduction into clinical practice were to involve administration of vaginal progesterone to all women with a cervix between 10 and 20 mm in mid-pregnancy, and replacing the use of placebo with active treatment and avoiding the refusal to participate of the approximately one-third of eligible women observed in the research study, then screening 32 thousand asymptomatic pregnancies would identify 733 suitable women (2.3%) resulting in prevention of birth <33 weeks in 47 cases (14.7 per 10,000).

There is no doubt that any clinical strategy that would prevent the preterm birth of so many infants would be of considerable benefit to our patients and their families. The cost-effectiveness, however, would be dependent on the health-care environment and availability of appropriate resources and funds. In a decision analysis model comparing no routine cervical length screening with a single routine ultrasound trans-vaginal cervical length measurement at 18–24 weeks gestation, with the women with a short cervix then offered vaginal progesterone treatment, the policy appeared to be cost effective (49). In US dollars in the year 2010, for every 100,000 women screened, $12 million could be saved and 424 quality-adjusted life-years gained.

At this time, the American College of Obstetricians and Gynecologists has not recommended routine cervical length screening for all pregnancies, probably as a result of the large numbers requiring to be screened, and a perceived need for further studies to be conducted across a variety of health-care settings (47, 50). Practice guidelines are required in each clinical environment that enable the effectiveness of this protocol to be adopted within the resources and expertise that can be harnessed for the challenge. As our use of progesterone expands, we also need to remain mindful that we do not yet have data describing the complete safety of their use for later child and adult life.

Progesterone has also been evaluated as a potential treatment in other conditions that may lead to preterm birth. Studies conducted to-date have shown that progesterone treatment is not effective in preventing preterm birth in multiple pregnancies, preterm labor, or preterm pre-labor rupture of membranes (46, 47).

CERVICAL CERCLAGE

Cervical cerclage is the surgical placement of a suture or tape around the cervix in an attempt to prevent dilatation and subsequent preterm birth. The procedure was first described by

Shirodkar in 1955 and involved dissection of the bladder superiorly to enable placement of the suture as close to the internal os as possible (51). A simplified procedure was described by McDonald 2 years later in which bladder dissection is not performed, minimizing the intervention but possibly leaving the suture lower in the cervix (52).

The decision to insert a cervical cerclage in mid-pregnancy is based on one of the three scenarios. First, a history of preterm births, classically recurrent and painless second trimester losses. Second, shortening of the cervix on ultrasound imaging. Third, short or dilated cervix on physical examination (13).

The mechanistic basis by which cerclage is effective is simplistically described as physical closure of the cervix, but the concept of cervical "incompetence" remains as much an enigma today as it was when described by Shirodkar in 1955 (51). As a result, the benefits of the procedure need to be carefully balanced against the potential risks and alternative therapies. The effects of cerclage on the cervico-vaginal microbiota may be clinically important, although has not yet been investigated.

Cerclage compared with no treatment

Meta-analysis of the RCTs that have compared cervical cerclage against no treatment has shown a significant reduction in preterm births of 20% (average RR 0.80, 95% CI 0.69–0.95) and with a reduction in perinatal deaths although this difference did not quite reach statistical significance (RR 0.78; 95% CI 0.61–1.00) (13). However, cerclage was associated with higher rates of fever, vaginal discharge, and vaginal bleeding, together with a significant increase in delivery by Cesarean section (RR 1.19, 95% CI 1.01–1.40).

Cerclage compared with progesterone

Only one trial has attempted to directly compare ultrasound-indicated cervical cerclage with a progesterone (53). The progesterone was 17P given intramuscularly. The trial was halted prematurely, and the sample size was too small to make meaningful conclusions (13).

No trial has compared vaginal progesterone versus cerclage for ultrasound-detected cervical shortening in mid-pregnancy (13). An indirect comparison using adjusted indirect meta-analysis of trials was reported by Conde-Agudelo et al. (54). The analysis included trials of singleton pregnancies with a history of previous preterm birth and in which ultrasound imaging had demonstrated a short cervix in mid-pregnancy. Both vaginal progesterone and cerclage were found to be effective in preventing preterm birth and improving perinatal outcomes. Neither treatment, however, was superior to the other.

At this time, the evidence guiding clinical practice in making a decision to insert a cervical cerclage versus administration of progesterone is incomplete. Decisions need to be based on informed consent and include patient and clinician preference, as well as the local availability of surgical resources and expertise.

Cerclage compared with pessary

Cervical pessaries have been proposed as an alternative method of preventing preterm birth (55). A range of designs has been proposed and some success has been described. At this time, however, the role of pessaries and the most effective clinical protocols for their use remain under investigation.

PREVENT CIGARETTE SMOKING

Tobacco smoking in pregnancy causes preterm birth in addition to a dose-dependent reduction in birthweight (14, 56, 57). The exact mechanism by which preterm birth is triggered is uncertain and probably relates to the vasoconstrictive effects of nicotine, the increase in circulating levels of carbon monoxide, or other as yet unknown effects from the 4000 chemically active components in tobacco smoke. The woman herself does not need to be the smoker for there to be an increased risk of preterm birth. Second-hand smoke is associated with an increased risk of early birth, as well as stillbirth, low birthweight, and respiratory disorders in childhood (58).

The nicotine in cigarette smoke is addictive and produces the positive feelings inherent in addictive behaviors. Strategies aiming to prevent smoking in pregnancy, however, are complicated by the many factors that contribute to the decision-making processes in women who elect to smoke while pregnant. In high-income countries, rates of smoking in pregnancy have declined in recent years but the reduction has not been in all groups (59, 60). Smoking in pregnancy in high-income countries is now a marker of social disadvantage and remains common in many indigenous groups (61). There are also cultural factors that contribute, resulting in complex interplays between socio-economic disadvantage, social isolation, cultural background, migration, and mental health (62–64). Hence, anti-smoking campaigns that are effective in one demographic group may alienate others and be either ineffective or even be at-risk of producing an opposite effect.

Nevertheless, the risk of preterm birth attributable to smoking has been estimated as more than 25% and reducing smoking rates in pregnant women must be of highest priority (65). Psychosocial interventions appear to be moderately effective. Pooled data from 14 studies describing a variety of psychosocial interventions have shown a significant reduction in smoking rates (RR 0.82, 95% CI 0.70–0.96) (64). The number need to treat to prevent one case of preterm birth was 71.

Pharmacological interventions may be of less value. Nicotine replacement therapy is the only pharmacotherapy for smoking cessation in pregnancy that has been adequately tested. A review of the six published trials was unable to confirm benefit (66). Further studies involving different demographic groups and alternative agents are required.

In contrast, smoke-free legislation appears to be of great benefit (15). A review of 11 studies involving local or national bans and including more than 2.5 million births showed that smoke-free legislation was associated with a significant 10% reduction in preterm births (95% CI −18.8 to −2.0). The data in this analysis were observational rather than randomized, but the likelihood of causality was increased by the dose-dependent nature of the effect with comprehensive smoking laws appearing to produce the greatest benefit. It is likely that the major action on pregnancy outcomes from the legislative changes resulted from reductions in second-hand smoke effects. It is of interest that the reduction in preterm birth rates was not associated with a similar effect on rates of low

birthweight. Maternal smoking in pregnancy is known to produce a dose-dependent reduction in birthweight, and it is possible that second-hand smoke exposure may act to trigger preterm birth acting through a more rapid and different pathway to chronic exposure to cigarette smoke where there is a most definite and consistent effect on fetal growth.

JUDICIOUS USE OF FERTILITY TREATMENTS

The advent of fertility assistance has contributed to a significant increase in the rate of preterm birth. Central to this contribution has been an increase in the incidence of multiple pregnancies. In USA, the incidence of multiple births has doubled from 1.8% of all births in 1972 to 3.5% in 2011 (67), and this rise can be attributed to an increase in the use of medically assisted reproduction. It has been estimated that in the US in 2011, 36% of twin births and 77% of triplet and higher order multiple births were due to medically assisted conception (67).

Data on *in vitro* fertilization (IVF) cycles in many countries are relatively easy to analyze; however, the data capture on less invasive treatments, such as ovulation induction and intra-uterine insemination cycles (often combined with ovarian stimulation) are less readily available. These treatments often involve ovarian stimulation and clinicians and patients may elect to proceed to fertility treatment when several follicles are potentially available for ovulation. In such circumstances, multiple pregnancies may result. In 2000, the incidence of twin gestations resulting from non-IVF fertility treatment was estimated to be 20% (68).

The risk of preterm birth that may result from fertility treatment can best be addressed by education of the attending healthcare practitioners. The rate of multiple gestations resulting from IVF treatment can be reduced to relatively low levels. In Australia and New Zealand, single embryo transfer has been embraced widely resulting in a multiple pregnancy rate following IVF of only 6.9% (69). Transferring a single embryo minimizes the risk of multiple pregnancy but requires an environment of high competence and patient education as the overall pregnancy rate is less (16). In recent years, the percentage of embryo transfers that were single in Australia and New Zealand was 69%, compared to 40% in the UK (www.HFEA.gov.uk). Meanwhile, in USA, the percentage of single embryo transfers was 21%, resulting in IVF being responsible for one in five multiple births in that country in 2011 (67).

Currently, one in 25 children born in Australia has resulted from IVF procedures (70). In Denmark, the percentage is almost 5% (70). As the use of IVF technology spreads progressively across the world, measures are required to ensure that responsible ovulation induction treatment and a single embryo transfer approach in IVF treatment are embraced to minimize the risk of multiple pregnancies and risk of preterm birth.

Multiple pregnancies are not the only pathway by which fertility treatment can lead to preterm birth. It is well established that there are other obstetric and perinatal complications that may befall a mother and her infant as a result of IVF treatment (71, 72). At first, it was thought that additional perinatal risks resulted purely from complications of multiple pregnancies but data from several countries in which single embryo transfer is common have shown additional risks even in the presence of a single fetus. The causes of these additional risks are unclear. One contributing factor may be the underlying cause of the subfertility. Evidence for an effect of subfertility itself has come from observations that women with a history of subfertility who conceive spontaneously have a significantly worse perinatal prognosis than those with normal fertility (73–75), and women who require intra-uterine insemination have a significantly worse perinatal outcome than women who spontaneously conceive (74, 76). Ironically, some of the worst perinatal outcomes exist for women who conceive a singleton pregnancy as a result of IVF treatment, with an approximate doubling of the risk of stillbirth, growth restriction, preterm delivery, and neonatal nursery admission for their baby (71, 72, 77, 78). Hence, a woman with subfertility has an increased perinatal risk due to her subfertility. Further, the perinatal mortality of a single fetus conceived after a double embryo transfer procedure is significantly greater than a singleton conceived from a single embryo transfer (79).

Despite the trend toward single embryo transfer, the incidence of monozygotic twinning is believed to remain increased by IVF treatment by an additional 1–5% (80). The risk of monozygotic twinning is particularly increased by the procedures of assisted hatching (81) and blastocyst transfer in comparison to early embryo transfer (82). As a result, these procedures increase the risk of preterm birth.

Finally, children born as a result of assisted reproductive technology have an excess risk of birth defects when compared to spontaneously conceived children, further increasing the chance of obstetric intervention and preterm birth (83).

DEDICATED PRETERM BIRTH PREVENTION CLINICS

In recent years, many health regions and hospitals have developed dedicated preterm birth prevention clinics. These clinics and their associated services have employed a wide variety of criteria outlining who should attend and the protocols for management. The first large-scale attempt to determine the effectiveness of such a program was the West Los Angeles Preterm Birth Prevention Project in which eight prenatal county clinics in California were allocated at random to be experimental or control clinics (84). The intervention was based on providing additional education to the women and offering more clinic attendances. In the experimental group, there was a 19% reduction (9.1–7.4%) in the preterm birth rate when compared with that of the control clinics. This difference in rates was statistically significant when the number of patient risk factors was taken into account. In pregnancies of black women, the preterm birth rate was 15% in the experimental clinics and 22% in the control clinics. Secondary interventions of bed rest, social work assistance, and oral synthetic progesterone medication were of no additional benefit.

More recently, most dedicated preterm birth prevention clinics have focused on newer diagnostics and therapeutic interventions including assessment of vaginal microbiology, fibronectin testing, ultrasound detection of shortened cervix, antibiotic use, progesterone therapy, cervical cerclage, and Arabin cervical pessaries. A survey of 23 dedicated preterm birth prevention clinics in UK in 2012/13 revealed considerable heterogeneity in protocols and practices suggesting a need for effective networking and coordination of such services (85). Also, there was considerable variation

in the criteria for referral and attendance, reflecting the fact that risk scoring systems have generally been unhelpful in predicting preterm birth (86). Most clinics attempt to target women with a history of preterm birth or recurrent mid-pregnancy loss, previous preterm pre-labor rupture of membranes, or previous loop excision or cone biopsy of the cervix (85).

Using a retrospective cohort design, investigators from Utah, USA reported a significant reduction in recurrent preterm birth (48.6 versus 63.4%) in women who attended a dedicated clinic and with lower rates of composite major neonatal morbidity (5.7 versus 16.3%) (87). The intervention was consultative and consisted of three standardized clinic attendances with routine prescription of 17-alpha hydroxyprogesterone caproate, as well as sonographic measurement of cervical length. Using a similar study design, investigators from Ohio, USA reported on the outcomes from their preterm birth prevention clinic after adoption of an accelerated appointment process and prophylactic treatment with progesterone (88). After adjustment for major confounders, these changes to their practice resulted in a significant 25% decrease in spontaneous preterm birth.

Despite the relatively large number of preterm birth prevention clinics now operating in various parts of the world, a review of their effectiveness could identify only three trials that qualified for inclusion in the analysis, and with only one study providing outcome data on most end-points (17). When data from the three trials were pooled, there were fewer preterm births in the treatment group compared with controls, but the difference between groups was not statistically significant (RR 0.87, 95% CI 0.69–1.08). The authors concluded that adequate randomized controlled trials of preterm birth prevention clinics may never be performed as such clinics have become an accepted part of antenatal care in many countries.

In addition to the enhanced provision of expert care and application of effective interventions for at-risk women, an important function of dedicated preterm birth prevention clinics may also be to alleviate maternal anxiety. Stress has been thought for many years to be a possible cause of some cases of early birth, and research is needed on the potential benefits or otherwise of attendance at such clinics (89).

STRATEGIES WITH PROMISE BUT REQUIRING MORE RESEARCH

TREATMENT OF INTRA-UTERINE INFECTION

Intra-uterine infection and inflammation play a well-recognized role in the etiology of spontaneous preterm labor, particularly in deliveries less than 32 weeks gestation (90) or those complicated by preterm pre-labor rupture of membranes (91). The primary reservoir for such infection is the vagina. Vaginal microorganisms are hypothesized to breach the cervical barrier, colonize the fetal membranes, and eventually the amniotic cavity (91, 92). The vigorous inflammatory response ultimately leads to preterm birth.

The microorganisms most commonly isolated from the amniotic fluid are very small bacteria of the Class Mollicutes, namely *Ureaplasma* and *Mycoplasma* species (93). Numerous other bacteria have also been identified in infected amniotic fluid samples including *Streptococcus*, *Fusobacterium*, and *Enterobateriaceae*. The frequent presence of these organisms does not necessarily denote causation, but there is evidence from several sources to support a role for some of these organisms in the causal pathway to preterm labor. In experimental sheep, intra-amniotic injection of *Ureaplasma* spp. elicits a robust intra-uterine inflammatory response and enhanced lung maturation (94–96). Intra-amniotic injection with *Ureaplasma* spp. in chronically catheterized Rhesus macaques drives intra-uterine cytokine and prostaglandin production, chorioamnionitis and preterm labor, replicating the disease pathogenesis and ontogeny observed in human pregnancy (97).

The relationships between vaginal microbiota and ascending infection resulting in preterm birth remain uncertain. For several decades, investigators have explored a role for bacterial vaginosis. Bacterial vaginosis is a common genital condition among women of reproductive age characterized by a disturbance in normal vaginal microbiota with a loss of H_2O_2-producing *Lactobacillus* spp., an increase in vaginal pH, and an increase in Gram-variable coccobacilli, anaerobic organisms, and genital mycoplasmas (98). There are well-established associations between bacterial vaginosis and preterm birth (99), but the extent of a causative role is not certain. What is known with certainty, however, is that bacterial vaginosis varies dramatically with race and that studies need to be specific for different population groups (98, 100).

Antibiotic treatment of bacterial vaginosis is generally ineffective in preventing preterm birth (101–103). It should be noted, however, that antibiotics commonly used to treat bacterial vaginosis are ineffective against *Ureaplasma* and *Mycoplasma* spp., which are the organisms most frequently associated with preterm birth. These organisms are best treated with macrolide antibiotics, the most frequently used of which are erythromycin and azithromycin. In addition, the transplacental passage of these drugs is poor and unlikely to reach levels sufficient to eradicate infection (104–107). Newer macrolide antibiotics, such as solithromycin, with greater efficacy and better transplacental passage, offer promise and may prove in time to be more effective (108, 109).

There is some evidence, however, that treatment earlier in pregnancy may be more effective in preventing preterm birth. Lamont and colleagues have shown that administration of clindamycin to women with abnormal vaginal flora before 22 weeks gestation may reduce the rate of subsequent preterm birth (110). This possibility is the topic of a separate review in this series where it will be discussed at length.

In summary, while a role for vaginal infection in the causal pathway to many cases of early preterm birth seems clear, at this time translation of that knowledge into an effective treatment strategy has yet to be widely adopted. The field is the subject of active investigation and progress can be anticipated in the near future.

NUTRITIONAL INTERVENTIONS

There has been considerable research on the interactions between nutrition and risk of preterm birth, but the many environments and demographic groups included in these studies complicates interpretation. A low pre-pregnancy body mass index (BMI) has been associated with an increased risk of preterm birth, while obesity has been shown to be protective (111). Obesity, however,

predisposes pregnant women to diabetes and pre-eclampsia often leading to iatrogenic early birth.

It has been known for many years that there are associations between preterm birth and low serum levels of many micronutrients. Proving a causative role and using supplementation to reduce preterm birth rates has, however, so far remained elusive.

In a large cohort study, a strong association was observed between use of pre-conception folate supplementation for one year or more and reduction in risk of preterm birth before 32 weeks gestation, but not at later gestational ages (112). However, the randomized controlled trials designed to investigate the effects of peri-conception folate supplementation on rates of neural tube defects did not reveal any reductions in rates of miscarriage or low birthweight (113). It is entirely possible that observational studies of folate use may describe a population of women at lower risk of preterm birth for other reasons. Further research is required before peri-conception folate supplementation can be considered to be an effective strategy to prevent preterm birth.

Research is underway investigating the possibility that maternal intake of omega-3 long-chain polyunsaturated fatty acids may prevent preterm birth and improve birth weight. The impetus for this hypothesis was the observation that women living in the Faroe Islands who have a high consumption of fish oil also have pregnancies of longer gestational ages and infants of high birth weight (114). A recent randomized controlled trial using the $n-3$ (omega-3) long-chain polyunsaturated fatty acid docosahexaenoic acid (DHA) in the last half of pregnancy resulted in fewer preterm births before 34 weeks gestation, longer gestations and shorter hospital stay for preterm infants (115). Such results are encouraging but further research is required before supplementing the maternal diet with omega-3 fatty acids to prevent preterm birth can be recommended (116).

AMELIORATION OF MATERNAL STRESS

It has been shown that women with high levels of psychological or social stress are at increased risk of preterm birth (117–119). Randomized controlled trials of interventions aiming to relieve stress or provide comforting reassurance have not been successful in preventing early birth suggesting that multiple other confounding factors are contributing to the relationship between stress and preterm birth (120).

TREATMENT OF PERIODONTAL DISEASE

It has been known for many years that periodontal disease is associated with preterm birth (121). Inflamed and infected periodontal tissues could stimulate preterm labor either by translocation of periodontopathic organisms, or by stimulation and release of inflammatory mediators and prostaglandins into the maternal circulation (122). Disappointingly, randomized controlled trials of treating periodontal disease during pregnancy have failed to lower the rate of preterm birth (123–125). It seems likely that the alterations in maternal immune responses that cause periodontal disease also predispose women to preterm birth, but that treating periodontal disease during pregnancy will neither cause nor prevent this major complication of pregnancy. Nevertheless, further research is required to investigate if there is any benefit in reducing rates of preterm birth by treatment of periodontal disease before conception as the randomized controlled trials conducted so far have initiated treatment in mid-pregnancy.

PREVENTION OF SURGICAL TREATMENT FOR CERVICAL INTRA-EPITHELIAL NEOPLASIA

It is well established that surgical treatments of cervical intra-epithelial neoplasia (CIN) predispose women to preterm birth in subsequent pregnancies, including early preterm birth (126). Such treatments aim to prevent cancer of the cervix and the possible risks for future pregnancies have always been judged against the need to prevent life-threatening cancer. It has now been shown that the vast majority of cases of CIN are caused by human papillomavirus (HPV) infection (127). The discovery and introduction of a vaccine to prevent HPV infection can now be expected to dramatically reduce the prevalence of pre-invasive abnormalities of the cervix and hence decrease the need for surgical treatments that may predispose women to subsequent preterm births (128). In addition to saving lives of young women who are vaccinated, it is likely that time will show that discovery of the vaccine to prevent HPV infection will have serendipitously improved outcomes for the next generation by also preventing early birth. Population-based vaccination of young women to prevent HPV vaccination needs to be given high priority.

CONCLUSION

In recent decades, advances in newborn care have resulted in improved outcomes for large numbers of children who have been born too early but this progress has not been matched by similar advances in our ability to prevent preterm birth. Times have changed. We now have increasing evidence that a variety of interventions have potential to significantly and safely prevent a meaningful proportion of preterm births. Translation of recent discoveries into clinical practice will have different requirements for high and low-resource settings, and for different population groups. For each setting, the best chance of success will come from an integrated implementation strategy that harnesses both the health-care personnel and the pregnant women for whom they provide care. The interventions are in many cases multi-disciplinary and require the participation of personnel from multiple fields including those who make local and national policies. This process begins with awareness across the medical and general communities that preterm birth is one of modern health care's greatest challenges, but that prevention in many cases is now possible.

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Preterm birth, intrauterine infection, and fetal inflammation

Matthew W. Kemp*

School of Women's and Infants' Health, The University of Western Australia, Perth, WA, Australia

Edited by:
Claudia Monaco, Catholic University Rome & Imperial College, UK

Reviewed by:
Marian Kacerovsky, University Hospital in Prague, Czech Republic
Ana María Franchi, The National Scientific and Technical Research Council (CONICET), Argentina

***Correspondence:**
Matthew W. Kemp, School of Women's and Infants' Health, The University of Western Australia, M550, 35 Stirling Highway, Crawley, Perth WA 6009, Australia
e-mail: matthew.kemp@uwa.edu.au

Preterm birth (PTB) (delivery before 37 weeks' gestation) is a leading cause of neonatal death and disease in industrialized and developing countries alike. Infection (most notably in high-risk deliveries occurring before 28 weeks' gestation) is hypothesized to initiate an intrauterine inflammatory response that plays a key role in the premature initiation of labor as well as a host of the pathologies associated with prematurity. As such, a better understanding of intrauterine inflammation in pregnancy is critical to our understanding of preterm labor and fetal injury, as well as on-going efforts to prevent PTB. Focusing on the fetal innate immune system responses to intrauterine infection, the present paper will review clinical and experimental studies to discuss the capacity for a fetal contribution to the intrauterine inflammation associated with PTB. Evidence from experimental studies to suggest that the fetus has the capacity to elicit a pro-inflammatory response to intrauterine infection is highlighted, with reference to the contribution of the lung, skin, and gastrointestinal tract. The paper will conclude that pathological intrauterine inflammation is a complex process that is modified by multiple factors including time, type of agonist, host genetics, and tissue.

Keywords: preterm birth, fetus, inflammation, infection, injury

INTRODUCTION

Preterm birth (PTB) is presently defined as delivery before 37 weeks' completed gestation from the last known menstrual period (1); the lower limit of PTB varies between countries but is generally set at 20 or 22 weeks' completed gestation (2). PTB is often further sub-classified into late (32 or 34–36 completed weeks' gestation), early (<32 completed weeks' gestation), and very early (<28 completed weeks' gestation) delivery (2, 3). These definitions are now viewed as somewhat arbitrary in nature and susceptible to classification bias because of the methodologies used to assess gestational age (self-reported last menstrual period vs. ultrasound measurement) (2, 4, 5). These measures also fail to account for a host of pregnancy characteristics (e.g., infection, maternal health status, and fetal distress) that are likely key to both the treatment of the preterm infant and our attempts to better understand and prevent PTB. As such, a number of investigators have recommended the adoption of an information-rich, phenotypic PTB classification system that considers births occurring after 16 and before 39 weeks of completed gestation (5).

Preterm birth is a significant global health issue. Rates of PTB vary with ethnicity, geography, and a range of lifestyle factors (6); in 2003, the rate of PTB for Asian, Caucasian, and African American women in the United States was 10.5, 11.5, and 17.8%, respectively (7). Complications of prematurity account for 29% of global neonatal deaths (approximately 1 million) each year and 3.1% of total disability adjusted life years in the global burden of disease (8, 9). Underscoring the scope of the PTB problem, recent data suggest that just a 5% relative reduction (from 9.59 to 9.07%) in the rate of PTB across 39 countries with a very high-human development index would yield some 58,000 fewer preterm deliveries and a saving of US$3 billion (10).

From a pathophysiological perspective, PTB is a highly complex and incompletely understood syndrome (11, 12). Evidence exists to implicate a host of factors including uteroplacental ischemia, cervical disease, decidual hemorrhage, stress, infection, and inflammation in the initiation of prematurity (12). Inflammation (with or without hypoxia) is also strongly implicated in a host of fetal morbidities. It is hypothesized that these, and perhaps other factors, acting either independently or in concert, trigger a suite of changes in gestational tissues that shift the uterus from a state of quiescence to one of activity in the initiation of labor. Gotsch and colleagues have described the tripartite uterine components of a (p. 6) "common pathway of parturition" as including an increase in myometrial contractility coupled with ripening of the cervix and activation of the fetal membranes and decidua (3, 12).

The immune system is hypothesized to play a key role in regulating the above processes, and a compelling body of evidence exists to suggest that both term and preterm labor are characterized by significant pro-inflammatory changes in gestational tissues (12–14). One point of difference is that the inflammation identified in term labor is commonly thought to be lower in magnitude than that identified in PTB (3, 12). Labor-associated inflammatory changes are characterized by immunocyte infiltration and significant increases in the expression of interleukin (IL)-1β, IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, and tumor necrosis factor (TNF)-α expression in the fetal membranes, cervix, amniotic fluid, and placenta. Expression of pro-inflammatory mediators is, in turn, hypothesized to result in (i) increases in prostaglandins, which promote uterine contractility; (ii) degradation of the chorioamnion extracellular matrix; and (iii) ripening of the cervix by matrix metalloproteinases and reduced expression of tissue metalloproteinase inhibitors (14).

A complex regulatory network involving Th1 (IL-12 polarized $CD4^+$ T-cells characterized by interferon-γ expression), Th2 (IL-4 polarized $CD4^+$ T-cells characterized by IL-4, IL-10, and IL-13 expression), Treg ($CD4^+CD25^+$ T-cells expressing the transcription factor FOXP3), Th17 ($CD4^+$ T-cells that secrete IL-17a and IL-17f), and uterine natural killer cells is hypothesized to be of critical importance in the establishment and prolongation of pregnancy (13–15). This network, in turn, is susceptible to regulation by the pro- and anti-inflammatory actions of a host of endocrine factors including progesterone, estrogen, human chorionic gonadotrophin, and luteinizing hormone (14, 15).

There is also good evidence to suggest that pro-inflammatory signaling following activation of the innate immune system, in response to injury or infection, plays an important role in the premature initiation of labor (11). Infection is most commonly identified in the earliest of preterm deliveries, those occurring before 28 weeks of gestational age. Goldenberg et al. suggest that the 25–40% of preterm deliveries ascribed to intrauterine infection may be a minimum estimate due to potential culture and sample selection bias (11). Similarly, histologic chorioamnionitis, a hallmark of intrauterine infection, has been reported in nearly 70% of preterm deliveries between 20 and 24 weeks' gestational age, but in only 16% of cases delivered at 34 weeks (16). Recent data indicate that intrauterine infection is frequently polymicrobial in nature. Although bacterial nucleic acids have been identified in chorioamnion from both term and very preterm deliveries, samples from very preterm deliveries have been found to contain the largest diversity and distribution of bacterial species (17). The microorganisms most commonly identified in cases of PTB include the *Ureaplasma* spp. (in particular, *Ureaplasma parvum*), *Mycoplasma hominis*, and the *Fusobacterium* spp. There is also increasing evidence for the importance of *Candida* spp. in intraamniotic infection (18–20) and much remains to be understood in relation to the potential for viral infection to play a role in the pathological processes that underpin PTB.

We have previously speculated that the development of efficacious therapies for infection-associated PTB is likely predicated on our ability to resolve both intrauterine infection and the concomitant intrauterine inflammation that results (21). However, we also suggest that any attempts to regulate intrauterine inflammation must be undertaken with caution; regulated expression of cytokines plays an important role in the development of a number of fetal organs, including the skin, lung, and brain. To this end, identifying the origins and nature of the pathological inflammation that accompanies intrauterine infection is an important step in our efforts to deliver targeting anti-inflammatory therapies.

There is a wealth of data available to describe the pro-inflammatory responses of placental, uterine, cervical, and chorioamnion tissues to intrauterine infection. The contributions of somatic fetal tissues to both intrauterine inflammation and the fetal inflammatory response syndrome (FIRS; defined on the basis of a cord blood plasma IL-6 concentration >11 pg/mL) associated with intrauterine infection are, in contrast, much less well characterized.

The remainder of this paper will focus on data provided by basic science and clinical studies to (i) describe the capacity of fetal tissues, in particular, the skin, lung, gastrointestinal tract to mount a pro-inflammatory reaction in response to stimulation of the innate immune system by microbial agonist; and (ii) comment on some of the evidence, to date, that suggests an important role for these fetal tissues in driving the intrauterine inflammatory response implicated in PTB and fetal injury.

CAPACITY OF FETAL TISSUES TO MOUNT A RESPONSE TO INFECTION AND INJURY VIA THE INNATE IMMUNE SYSTEM

The innate immune system is a critical element of host defense against microbial invasion. Broadly speaking, the innate immune system comprises humoral (endogenous antibodies and complement) and cellular (recognition of conserved pathogen motifs by natural killer cells, monocytes, macrophages, and non-immune cells including endothelial cells and neuronal cells, stimulating phagocytosis and or the release of pro-inflammatory cytokines, chemokines, and defensins) elements (22). Together, these act in concert to recognize and resolve infection in addition to serving as a means of coupling innate immune responses to the induction and amplification of a subsequent adaptive immune system response. A detailed treatment of the innate immune system is beyond the scope of this work and a number of excellent review articles have recently dealt with elements of the innate immune system including complement (23), toll-like receptors (TLRs) (24), Nod-like receptors (25), and defensins (26). Rather, this work will take three key elements of the innate immune system, namely, pattern-recognition receptors, complement, and defensins, and explore the evidence for their expression in developing fetal tissues.

PATTERN-RECOGNITION RECEPTORS

Pattern-recognition receptors (PRRs) are innate immune system receptors that recognize structurally stable microbial elements (27). In addition, PRRs can recognize molecules that are released in response to tissue injury or stress (24, 27). Their immunological role is considered to be of great importance as their conserved ability to recognize pathogens and/or injury allows for an initial defensive reaction, which simultaneously recruits elements of the adaptive immune system to respond to a potential infectious threat. PRR ligand binding results in the activation of multiple signaling factors (including NF-κB and interferon-regulatory factors), yielding cytokine and chemokine expression, with downstream effects including enhanced phagocytosis, pyrexia, increased rates of hematopoiesis, elevated type-I interferon expression, and pyroptosis (27).

Toll-like receptors are a large family of transmembrane proteins, and perhaps the best characterized class of PRRs. TLRs 1–6, 10, and 11 are found in the plasma membrane and TLRs 3, 7, and 9 in endosomal membranes. While most TLRs have quite defined ligand targets (e.g., TLR 5 recognizes flagellin, TLR 7 recognizes single stranded RNA), TLR 2 and TLR 4 both recognize a wide range of ligands including high-mobility group box protein 1 (HMGB1), peptidoglycan, mucins, hemagglutinin, and porins for TLR 2; and lipopolysaccharide (LPS), vesicular stomatitis virus glycoprotein G, mannan, heat shock protein (HSP) 60, HSP 70, and HMGB1 for TLR 4 (24).

Studies in human fetal autopsy samples have detected the presence of TLRs from an early gestational age. Immunohistochemical studies have demonstrated TLR 3 expression in neuronal and glial

cells in preterm brains from approximately 23 weeks' gestational age (28). Petrikin and colleagues used a PCR array platform validated by hydrolysis probe qPCR to analyze the temporal expression of TLRs in the human fetal lung at 60-, 90-, and 130-day gestational age. TLRs 1–8 and 10 were identified in lung samples from 60-day gestational age fetuses, indicating that TLR signaling is likely functional from very early in fetal development. Interestingly, with the exception of TLR 4, the expression of all TLR transcripts increased significantly (between 1.43- and 9.20-fold) from 60 to 130 days of gestation (29). Flow cytometry studies have demonstrated that TLR4 is expressed on the surface of $CD14^+$ monocytes harvested from preterm infants delivered at <30 weeks' gestational age, although at levels significantly lower than that seen in preterm infants born later than 30 weeks' gestation. A similar pattern was identified for TLR4 mRNA transcripts in $CD14^+$ monocytes (30). Increasing expression of TLR 2 in the lung with gestational age was identified in studies of fetal ovine TLR expression between 108 and 145-day gestational age (31). Again using qPCR, Sow et al. demonstrated the presence of mRNA transcripts for TLRs 4, 5, 7, and 8 at 115-day gestational age in the fetal ovine lung (32).

Toll-like receptor expression has also been characterized in the fetal ovine skin and spleen across the second trimester of pregnancy; work by Nalubamba and co-workers suggests that TLRs 1–10 are expressed in the spleen at levels approaching or often exceeding that seen in the adult from 60 days of gestation (33). TLR expression in the fetal skin was also found to be extensive, although transcripts for TLRs 9 and 10 were not identified between 60 and 90 days of gestation (33). A recent study by Iram and colleagues reported that skin samples taken from embryonic (9–11 weeks' gestation) to fetal (12–13 weeks' gestation) autopsies expressed the same range of TLRs as adult skin. Their data also suggested that, mRNA transcripts for TLRs 1–5 were more highly expressed in these early gestation skin samples than in adult skin samples (34).

In summary, these studies provide strong evidence to suggest that in the developing fetus: (i) TLRs are expressed from very early in gestational development; (ii) TLR expression is widespread in both AF-exposed (lung, skin) and internal (brain, spleen, immunocytes) fetal tissues; and (iii) the ontological pattern of TLR expression likely varies between tissues, which, in turn, potentially impacts the ability of individual tissues to mount a response to tissue invasion and injury by microorganisms.

In the cytosol, retinoic acid-inducible gene 1 (RIG-1)-like helicases, including RIG-1 and melanoma differentiation-associated gene 5 (MDA5), detect double stranded viral RNA and play an important role in the regulation of interferon expression in response to viral infection (35, 36). Work in embryonic chickens indicates that MDA5 is expressed in both the fetal spleen and lung from at least embryonic day 10 (35). In studies utilizing primary fetal buffalo fibroblast cells, Poly I:C treatment resulted in significant increases in RIG-1, MDA-5, and interferon-β mRNA expression, suggesting that the presence of a functional innate immune response to simulated viral infection (36). RIG-1, MDA5, and interferon-β1 mRNA transcripts have also been shown to be up-regulated in primary human cord blood mast cells in response to stimulation with antibody enhanced dengue virus infection (37). NOD-like receptors are a third class of PRRs; NOD-1 and NOD-2 recognize specific peptidoglycan derivatives (meso-diamino-pimelic acid and muramyl dipeptide, respectively) to initiate pro-inflammatory NF-κB and MAP kinase signaling (38). Several other NOD-like receptors are implicated in multiple immunomodulatory roles including inflammasome function and type-I interferon production (27). A recent analysis of NOD-like receptor Nlrp6 inflammasome elements by Kempster and colleagues demonstrated that pycard, caspase-1, and IL-18 (an inflammasome substrate) mRNA transcripts are detectable in the fetal ovine jejunum at 100-day gestational age. In the same study, Nlrp6, pycard, and caspase-2 transcripts were detected in the fetal rat intestine and lung at both embryonic day 16 and 20 (39).

Considered together, these data from animal and human studies suggest that functional TLR, RIG-1-like helicase, and Nod-like receptor PRR systems are widely expressed from early in pregnancy (**Table 1**). As such, it appears reasonable to conclude that these innate immune system effectors are capable of playing a role in the fetal response to intrauterine infection.

COMPLEMENT

The complement system is a central component of the humoral innate immune system (40). To date, three pathways of

Table 1 | Summary of pattern-recognition receptor expression in human and animal fetal tissues.

PRR class/type	Fetal studies			Reference
	Component	Organism and tissue	Fetal gestational age (days)	
Toll-like receptors	TLR3	Human neuronal and glial cells	161	(28)
	TLRs 1–8 and 10	Human lung	60	(29)
	TLR2	Ovine lung	108	(31)
	TLRs 4, 5, 7, and 8	Ovine lung	115	(32)
	TLRs 1–10	Ovine spleen	60	(33)
	TLRs 1–5	Human skin	84	(34)
RIG-1-like receptors	MDA5	Avian spleen and lung	10	(35)
NOD-like receptors	Pycard, caspase-1, IL-18	Ovine jejunum	100	(39)
	Nlrp6, Pycard, caspase-2	Rodent intestine and lung	16	(39)

complement activation have been identified (i) the classical pathway, which is activated in response to antigen–antibody complex formation; (ii) the constitutively active, antibody–antigen complex independent alternative pathway; and (iii) a more recently identified lectin pathway, which is activated following the formation of complexes between mannin binding lectin and surface expressed microbial mannose (41). Cleavage of C3 into C3a and C3b is a key step in complement activation common to all three activation pathways. C3b is a multifunctional complement mediator; it binds foreign cells, identifying them for phagocytosis. In addition, C3b interacts with C5 to initiate a cleavage cascade that results in the formation of a lytic membrane attack complex that damages the membrane integrity of foreign cells (41). Complement proteins also have a range of innate immunomodulatory functions (42); for example, C5a and C3a act as chemoattractants via interactions with CD88 and C5L2 receptors, recruiting immunocytes (including lymphocytes and neutrophils) to sites of microbial invasion or localized inflammation (40).

Although much remains to be understood in relation to the ontogeny of fetal complement expression, there are a number of clinical studies that demonstrate elements of the complement system are expressed from early in gestation (40, 43). At term, serum complement factors' concentrations have been reported to range from 36 to 79% of normal adult levels (44). As summarized by Grumach et al., complement pathway components are detectable as early as 5 weeks' gestation. The authors note that (p. 267), "one may assume that all the components may be detected by week 18–20 of pregnancy" (43). CH_{50} (a sheep erythrocyte-based lysis assay to determine the functional activity of the classical and terminal complement pathways) studies with preterm infants suggest that functional complement activity at 27–31 weeks of gestation is approximately one-third of the normal adult level (40, 45). Woolach and colleagues reported an AP_{50} (a rabbit erythrocyte-based lysis assay that employs a calcium chelator to inactivate the classical and lectin pathways to isolate alternative pathway functional activity) value of approximately 50% normal adult levels for preterm infants born between 28 and 33 weeks' gestation (44). A similar pattern is apparent in fetal sheep. Analysis of data from modified CH_{50} assays using serum from fetuses delivered at 125-day (term is between 145 and 150 days) revealed a pattern of lytic activity similar to that of maternal sheep serum. In contrast, maximum lysis using serum from earlier gestational ages (85 and 95 days) was approximately twofold lower (46).

Together, these data suggest that the fetus is equipped with a functional complement system from early in gestation, although it is active at lower levels than in adults.

DEFENSINS

Defensins are a class of antimicrobial proteins that are released from a variety of cell types in response to the detection of tissue injury or microbial pathogens. They are found at highest concentrations in cells that play a role in host defense including leukocytes (where they are stored in granules) and Paneth cells in the small intestine. Defensins are also produced by a range of epithelial cells, either constitutively or following stimulation by microbial agonist (47). Human beings have six α-defensins (HαD) and at least 28 β-defensins (HβD), although only HβD 1–6 and 23 have been well characterized (26). HαD 1–4 are commonly termed human neutrophil peptide (HNP) 1–4 due to their constitutive synthesis by neutrophil precursors in bone marrow (47). HαD 5 and 6 are commonly termed human defensins (HD) 5 and 6. They are expressed by intestinal cells in addition to a number of types of epithelial cells. In mammals, epithelial cells are the primary sources of HβDs (26).

As elegantly reviewed by Yang and colleagues, defensins play a multitude roles in host defense. Both HαDs and HβDs exhibit differential microbiocidal activity by the formation of pore-like structures in microbial cell membranes, resulting in membrane disruption and depolarization (47). HβD 1 and HβD 2, for example, are effective at killing *Escherichia coli* and *Pseudomonas aeruginosa* but lack HβD 3's ability to kill *Staphylococcus aureus* (26). Defensins have also been shown to have anti-viral activity, can neutralize microbial toxins, and act as both chemoattractants and activators of phagocytic activity. Importantly, they have also been shown to mobilize dendritic cells and T-lymphocytes, bridging the innate and adaptive immune responses to combat infection (26). Given their importance to host microbial defense, a number of investigators have undertaken studies in human beings and animals in an attempt to clarify the ontological development of fetal defensin expression across gestation.

A mounting body of evidence exists to suggest that defensins are expressed early in gestation across a wide range of organisms. Isoforms of the β-defensin-like gene have been identified in an expressed sequence tag library derived from the early developmental stages of the olive flounder, *Paralichthys olivaceus* (48). Working with fertilized chick eggs over a 12-day time course, Meade and co-workers demonstrated the expression of 13 avian β-defensins at 3-day post-laying. Interestingly, differing patterns of both temporal and spatial (head vs. abdomen) expression were evident in the magnitude of avian β-defensin expression over the 12-day time course, suggesting that a process of developmental regulation with embryonic development (49). Data from a more recent histological study using immunolabelling suggest that peridermal granules in the developing embryonic chick skin contain avian β-defensin 9, indicating that these organelles play a role in both host defense and epidermal barrier formation (50). In sheep fetuses, initial work by Huttner et al. demonstrated the presence of ovine βD1 and βD2 mRNA transcripts in jejunum, ileum, caecum, and colon of 130-day gestational age fetuses (51). These findings were later replicated, in part, by work undertaken in late gestation (120–140-day gestational age) sheep fetuses, with Meyerholz and colleagues reporting the identification of ovine βD2 expression in both the small and large intestine (52).

Data from human fetal studies also suggest that a pattern of spatial and temporal regulation for defensins. Using semi-quantitiative PCR, Starner et al. demonstrated that HβD1, HβD2, but not HβD3 were expressed in the fetal lung at 42 weeks' gestational age; all three HβDs were undetectable at 18 and 22 weeks of gestation. Interestingly, the human cathelicidin, LL-37, was found to be expressed at all three gestational time points, with the highest apparent expression at 18 weeks' gestation (53). A distinct pattern of defensin expression is also apparent in the fetal skin. Immunohistochemical studies have shown that HβD2 and HβD3 staining is absent from the fetal skin at 10–15 weeks of gestational

age, and that HβD3 is expressed in the *stratum corneum* at 18–24 weeks' gestation (54). *In vitro* studies with primary human fetal keratinocytes suggest that HβD1 and HβD2 mRNA is often more highly expressed at 22 weeks' gestation than in samples from neonatal and adult skin (55). Defensin proteins have also been identified in human amniotic fluid and *vernix caesosa* samples from term pregnancies without chorioamnionitis and in the amniotic fluid of women with preterm labor (56, 57).

FETUS, INFECTION, AND INTRAUTERINE INFLAMMATION

Fetal inflammation is strongly associated with impending preterm labor, preterm prelabor rupture of membranes (PPROM) and is also an independent risk factor for subsequent neonatal morbidity (58). Elevated levels of cord blood IL-6 at birth have, for example, been demonstrated to predict the development of bronchopulmonary dysplasia (59). In a now classic study, Gomez and colleagues proposed that a fetal cord blood plasma IL-6 concentration in excess of 11 pg/mL was indicative of a FIRS, a condition that the authors describe as being (p. 201) "frequently associated with microbial invasion of the amniotic cavity" (58).

Intrauterine infection is associated with chorioamnionitis and funisitis, which are, in turn, inversely related to gestational age at delivery (16, 60). To further investigate the relationship between intrauterine infection and fetal inflammation, Yoon and colleagues examined putative relationships between umbilical cord blood IL-6 concentration, funisitis, amniotic-fluid microbial culture, and neonatal sepsis in a cohort of 315 preterm (20–35 weeks' gestation) infants. The presence of oligohydramnios, which is associated with a range of neonatal morbidities, has also been associated with elevated cord blood IL-6 concentrations in women with PPROM (61). Preterm infants with funisitis were found to have elevated rates of chorioamnionitis and positive amniotic-fluid cultures, a lower gestational age at delivery, and higher cord blood IL-6 concentrations than those without funisitis. These data suggest that funisitis is also associated with a FIRS (60). Fetal plasma IL-6 levels have also been shown to be significantly associated with inflammatory lesions in the chorioamnion, leading to the conclusion that funisitis and chorioamnionitis are histological markers of FIRS (62).

Interestingly, subsequent work by Lee et al. demonstrated that, in an analysis of patients with intrauterine inflammation (defined on the basis of an elevated matrix metalloproteinase 8 concentration in the amniotic fluid), cord blood plasma C-reactive protein was lower in the absence of proven amniotic-fluid infection, compared to cases where infection was confirmed by culture. These data suggest that although FIRS is possible in the absence of intraamniotic infection, the strongest fetal inflammatory response is associated with a culturable amniotic infection (63). Recent studies suggest that rather than having a simple binary relationship, it is important to take into account the magnitude of amniotic-fluid inflammation when predicting pregnancy outcomes (64). On the basis of these data, it is also tempting to speculate that a similar pattern may be applicable with regards to cord blood plasma IL-6 levels and the severity of FIRS.

Much remains to be understood regarding the differential impact of intraamniotic infection with differing microorganisms (either in isolation or simultaneously with one or more other microorganisms) on the systemic fetal inflammatory response; Lee and colleagues, for example, noted the isolation of some 11 species of microorganisms from the amniotic cavity and identified polymicrobial infections in 6 out of 89 cases (63). Similar findings suggesting that the involvement of a wide range of microbial species and a significant proportion of polymicrobial infections of the amniotic environment have been reported by both DiGiulio and Jones (17, 65).

How the presence of multiple immunomodulatory microbial agonists alters fetal inflammatory signaling *in utero* is still imprecisely understood. Based on the observations that (i) intrauterine inflammation is elevated in preterm compared to term labor; (ii) culture positive amniotic-fluid infection is associated with more severe fetal inflammatory responses; and (iii) polymicrobial infections are more common in labor at earlier gestations, one might be tempted to conclude that a polymicrobial infection would equate to a more severe inflammatory response. However, data, to date, suggest that the intrauterine inflammatory response is likely influenced by (at least) the magnitude and timing of microbial agonist exposure. The immunomodulatory effects of one microorganism, perhaps by down-regulating antimicrobial peptide expression as has been shown by *Ureaplasma* spp., may allow for additional microorganisms to establish *in utero* (66). It is also possible that the pathogen-specific inflammatory responses of different microorganisms combine to trigger multiple signaling pathways required to initiate preterm labor (67).

Findings from *in vitro* studies with preterm and term human monocytes, for example, suggested that *Ureaplasma urealyticum* exposure modified the pro-inflammatory response to a subsequent stimulation with *E. coli* LPS over a 24 h time course, and did so in a dose-dependent fashion. Exposure to a high dose (10^6 color change units) of *U. urealyticum* enhanced the release of TNF-α and IL-8, but not IL-6 and IL-10 (68). The authors speculated that the basis of the enhanced pro-inflammatory response was due to the differential suppression of regulatory cytokine (e.g., IL-10) expression. Evidence of an interaction between *U. parvum* and LPS is also provided by work in a sheep model of intrauterine infection. Chronic (70 days) but not acute (7 days) *U. parvum* exposure has been shown to inhibit pro-inflammatory signaling in the fetal lung (69). Surprisingly, acute *U. parvum* exposure was subsequently reported to have an LPS-inhibitory effect in the chorioamnion, suggesting that agonist, exposure timing, and tissue all impact inflammation deriving from intrauterine infection (70).

Data from clinical studies that suggest a fetal inflammatory response to direct stimulation by microbial agonist are supported by a number of studies undertaken in non-human primate, sheep, rabbit, and rodent models of human pregnancy. With the ability to control dose, type of exposure, and length of exposure, animal models of intrauterine infection have been important in advancing our understanding of the fetal inflammatory response to different microbial agonists.

Working with an ascending model of intrauterine infection in rabbits, Yoon et al. demonstrated that cervical inoculation of 10^3–10^4 colony forming units of *E. coli* resulted in white matter injury in pups euthanized 5–6 days after inoculation (71). Subsequent studies in a similar model system investigated the acute intrauterine responses to *E. coli* intrauteruine infection. Over a

30 h time course, progressive histologic inflammation was identified in the uterus, placenta, and lung (72). Interestingly, although mitotic activity in the pup brain was found to be retarded after 8 h post-inoculation, there was no evidence of brain inflammation or apoptosis suggesting that a potential lag in the transduction of inflammation from an infection of the amniotic fluid. The effects of intraamniotic *E. coli* LPS exposure have also been extensively characterized in the sheep using ultrasound-guided intraamniotic injections. LPS exposure has been shown to result in time and dose-dependent inflammatory responses in the chorioamnion, systemic fetal inflammation, changes in fetal lung development, and fetal brain inflammation (73–77).

Although *E. coli* and *E. coli* LPS serve as useful reagents for studying intrauterine inflammatory responses, *E. coli* infection in the setting of preterm delivery is comparatively uncommon. In contrast, the *Ureaplasma* spp., and especially *U. parvum*, are among the microorganisms most commonly identified by culture or PCR in cases of PTB. Intraamniotic inoculation of chronically catheterized macaques with clinical isolates of *U. parvum* serovar 1 or *M. hominis* resulted in significant increases in amniotic-fluid leukocytes within 24 h and amniotic-fluid TNF-α, IL-1β, Il-6, IL-8 between 48 and 72 h post-inoculation (78). Studies with group B streptococci in similarly catheterized macaques revealed increases in amniotic-fluid IL-1α and IL-6 at 15–18 h post inoculation, substantially earlier than that seen in response to intrauterine infection with *U. parvum*, suggesting that a differential inflammatory response to microbial agonist (78). Studies undertaken in the chronically infected sheep further underscore the pro-inflammatory role *U. parvum* in pregnancy; intrauterine *U. parvum* infection has been shown to be associated with a progressive chorioamnionitis, changes in fetal growth and fetal inflammation (79–81).

As summarized above, there is now ample evidence from clinical and animal studies to demonstrate that infection of the uterus is associated with intrauterine inflammation, and that the fetus is well equipped to respond to such infection with a robust pro-inflammatory response. Accordingly, it is of interest to determine the origins of this intrauterine inflammatory process in order to assist in our understanding of the pathological processes underpinning infection-associated PTB and our attempts to develop targeted interventions.

In many cases, it appears that fetal bacteremia is less common than colonization or infection of gestational tissues such as the placenta or the amniotic fluid. Recent studies in sheep, for example, demonstrated that fetal *U. parvum* bacteremia is uncommon despite the presence of viable microorganisms in the amniotic fluid (82). It is unclear, however, whether fastidious microorganisms such as *U. parvum* have the ability to replicate in amniotic fluid or if they are seeded into it following growth on amniotic-fluid exposed tissues.

A study investigating the acute fetal responses to intraamniotic *Candida albicans* infection demonstrated an absence of fungal RNA in the fetal ovine spleen in conjunction with positive amniotic-fluid cultures and the detection of *C. albicans* RNA in the fetal lung and skin (20). Similarly, in findings from the Alabama preterm birth study, placental cultures for *M. hominis* and/or *U. parvum* were positive in 63.4% of male fetuses. In contrast, only 27.6% of fetuses had positive cord blood cultures (83). For future studies, it will be of particular interest to determine how modifiable factors including gestational age, length of infection, and the infectious organism itself interact to influence the prevalence of fetal bacteremia in infection-associated PTB.

There is also evidence to suggest that pro-inflammatory microbial agonists in the amniotic fluid, such as LPS, does not passively diffuse across cell layers (84). These findings go some way to explaining the observation that while 1 μg/kg is considered a sub-lethal intravenous bolus dose of *E. coli* LPS in the sheep fetus, it is possible to inject in excess of 10 mg *E. coli* LPS into the amniotic fluid of pregnant ewes without causing fetal death (74, 85, 86). Interestingly, the intraamniotic administration of LPS from other Gram negative microorganisms associated with PTB has been shown to have a much more severe impact on fetal wellbeing, although it is unclear if this relates to a more toxic inflammatory response or enhanced ability to penetrate the fetal circulation. Studies in a sheep model of pregnancy, for example, have shown that *Porphyromonas gingivalis* LPS exposure results in much higher rates of fetal death than *E. coli* LPS (87). A recent analysis of human fetal membrane responses to PTB-associated microorganisms provides further evidence for an organism-specific and host-specific inflammatory effects deriving from the presence of microorganisms in the amniotic fluid. Peltier and colleagues demonstrated that PTB-associated pathogens including *E. coli*, *Gardnerella vaginalis*, Group B streptococci, and *Ureaplasma* spp. elicited differing pro-inflammatory responses in human fetal membrane explant studies. For example, *G. vaginalis* stimulated IL-1β and TNF-α production, whereas *U. parvum* elicited IL-10 and TNF-α expression but had no effect on the concentration of IL-1β (88). These data suggest that tissues directly exposed to the amniotic fluid are likely key in the propagation of amniotic-fluid inflammation in response to intrauterine infection and likely also contribute to systemic fetal inflammation. With this in mind, the remainder of this paper will discuss evidence for the contribution of the fetal lung, skin, and gastrointestinal tract to intrauterine inflammation.

CONTRIBUTION OF FETAL LUNG, SKIN, AND GASTROINTESTINAL TRACT TO INTRAUTERINE INFLAMMATION

Inflammation in the fetal lung in response to microorganisms in the amniotic environment has been a subject of significant interest for several decades and the primary focus of fetal inflammation in association with PTB (89). Broadly speaking, work in this area may be classified into two broad avenues of investigation: (i) the localized impact of intrauterine infection on the lung itself, including structural (90, 91), functional (92), and inflammatory adaptations (73, 76, 93); and (ii) the extra-pulmonary effects of inflammation in the fetal lung, including changes in fetal systemic inflammation (94–96), immunocyte reactivity (97–99), and amniotic-fluid inflammation (100).

The fetal lung has been shown to generate a pro-inflammatory response following exposure to a wide range of microbial agents including adenovirus (101, 102), *E. coli* LPS (73, 103, 104), *Ureaplasma* spp. (81, 105), and *C. albicans* (20), which is in keeping with studies suggesting that the developing fetal lung expresses a wide range of PRRs from early in gestation (29). Human lung

cells have also been shown to express complement factors, but not defensins, in response to endotoxin exposure (103, 106). Infection with *C. albicans* elicits an exceptionally vigorous inflammatory response in the fetal lung, but a much milder response in the chorioamnion (20). In comparison, the response elicited by *Ureaplasma* spp. is much more mild with to regards fetal lung inflammation (105). It is also hypothesized that signaling by pro-inflammatory cytokines plays a key role in driving lung maturation. Evidence in support of this model have been provided by studies in the sheep, wherein LPS-driven inflammation in the chorioamnion (occurring as early as 5 h after intraamniotic LPS injection) and lung (occurring 1–2 days after intraamniotic LPS injection) preceded lung maturation by as much as 5 days.

A number of cytokines have also been shown to exert differential effects on the fetal lung. Increases in a range of cytokines including IL-1, IL-6, IL-8, and TNF-α in the amniotic fluid and fetal lung are associated with intrauterine infection and preterm labor (107). Interestingly, work in a sheep model of pregnancy demonstrated that intraamniotic IL-8 exposure resulted in only small increases in bronchoalveolar lavage immunocytes and did not induce lung maturation (108). A similarly small response in the lung was identified following exposure to TNF-α (109).

In contrast, IL-1α and IL-1β both elicit marked pro-inflammatory and maturational effects on the fetal ovine lung, with IL-1α driving the strongest response (110). IL-1α has also been shown to elicit a dose-dependent increase in surfactant protein-A and protein-B mRNA expression in rabbits (111). Subsequent work by Sosenko et al. suggested that the pulmonary effects identified in association with intraamniotic IL-1α result from direct contact with the fetal lung (112). These observations may hint at a potential role for the lung in amplifying IL-1 inflammatory responses derived from the fetal membranes, skin, or gut, as well as acting as a transducer of intraamniotic inflammation to systemic fetal inflammation.

The role played by the lung in contributing to extra-pulmonary inflammation is of particular interest given the strong association between FIRS, PTB, and neonatal morbidities including bronchopulmonary dysplasia and white matter injury. Between 85 and 115 days' gestation, the ovine fetal lung produces lung fluid at a rate of 2–3 mL/kg/h and it is estimated that 50% of that volume is excreted into the amniotic fluid with a mixing time of 2–3 h (113, 114). As noted in seminal work by Tomoda et al., the fetus is estimated to swallow amniotic fluid at a substantial rate, with reported daily volumes varying between 200 and 1000 mL (115). In fetal sheep, *E. coli* LPS has been reportedly detected in the fetal stomach 2 days after intraamniotic exposure (116). As such, it seems likely that pro-inflammatory mediators excreted by the pulmonary epithelium would rapidly make their way into the amniotic fluid, where they could interact with immunocytes in the amniotic fluid, the fetal skin and membranes, and, after swallowing, the fetal gastrointestinal epithelium. Although the relative contributions of tissues amniotic-fluid inflammation remain unclear, it seems likely that the lung is an important source of inflammatory cytokines and white blood cells. Using polymyxin-B to bind and inhibit the inflammatory activity of intraamniotic *E. coli* LPS, Saito and colleagues demonstrated that a reduction in fetal lung mRNA expression was associated with significant reductions in cytokine protein concentration in the amniotic fluid (100).

In clinical studies, elevated white blood cell counts in the amniotic fluid are associated with intrauterine infection and an increased risk of spontaneous preterm delivery (117, 118). A significant majority of the leukocytes found in the amniotic fluid in association with intrauterine inflammation are of fetal origin (119). It is tempting to speculate that the lung is a likely source of leukocytes in the amniotic fluid, although it is possible that cells migrate across the gastrointestinal epithelium and developing skin in response to a chemotactic gradient. Indeed, Payne and colleagues have recently demonstrated the presence of clusters of basophilic cells in the apical layers of the developing fetal skin in response to experimental *C. albicans* infection (20). In human neonates, prolonged rupture of membranes during pregnancy has been associated with elevated white blood cells and IL-6 in bronchoalveloar lavage samples taken within 24 h of delivery (120). Similarly, infants with funisitis born prior to 28 weeks' gestation were found to have higher levels of $CD68^{+}$ cells in tracheobronchial aspirate fluid taken within 24 h of delivery than those without funisitis (121). Sheep infected with intraamniotic *U. parvum* serovar 3 were also shown to have statistically significant increases in bronchoalveolar neutrophils after 3 days of exposure (105).

Although the lung has been the major focus of fetal inflammatory responses to intrauterine inflammation, there is evidence to suggest that the fetal skin and gastrointestinal tract also generate a pro-inflammatory response following exposure to microbial agonists. In a fetal autopsy study, Kim and colleagues demonstrated fetal skin inflammation and TLR-2 regulation in association with microbial invasion of the amniotic cavity, concluding that dermatitis is a component of the FIRS (122). Subsequent studies in sheep have demonstrated that the intraamniotic injection of either *E. coli* LPS, *U. parvum* serovar 3 or *C. albicans* results in a pro-inflammatory response in the skin, with *C. albicans* eliciting the strongest response (20, 74, 79, 123). T-cells (present in the second trimester), mast cells (present from 12 weeks' estimated gestation), and antigen presenting cells (present from at least 9 weeks' estimated gestation) have been identified in the developing human dermis from early in fetal life (124). Fetal keratincoytes have been shown to respond directly to stimulation with microbial agonist and are known to express a wide range of antimicrobial peptides (55, 100, 125, 126). In the developing fetal gut, endotoxin exposure in preterm sheep is associated with disrupted structural development of the intestinal epithelium. Although an inflammatory response was not evident at 2 days after endotoxin administration, significant infiltrations of T-lymphocytes and myeloperoxidase cells were detected at 14 days-post exposure (116). These data suggest that a temporally different response in the fetal gut to inflammatory agonist when compared to more acute responses identified in the fetal lung and skin. Subsequent work, again in the fetal sheep, has suggested that a critical role for IL-1α in these gut-modifying processes, reminiscent of earlier data showing a similar role for IL-1α in the fetal ovine lung (110, 127).

With regards to systemic fetal inflammation, the available data suggest that the lung is likely a key mediator of transducing

inflammatory signaling from the amniotic fluid to the internal fetal organs and the fetal gastrointestinal tract (94, 96, 128). Although inflammation of the fetal lung has been demonstrated to induce systemic responses and cause inflammation in the fetal gut, exposure of the fetal skin and amnion to similar stimulation did not result in detectable inflammation in the lung or the gut (94, 96). However, it does appear that both the fetal skin and fetal gut play a smaller contributing role to the overall induction of FIRS (94, 128). Kramer et al. demonstrated a differential systemic fetal inflammatory response to acute *E. coli* LPS exposure, which was dependent on the fetal organs stimulated with agonist (128). Kemp and colleagues subsequently demonstrated that acutely exposing the fetal lung to *E. coli* LPS resulted in increased IL1-β, IL-6, and IL-8 mRNA in the fetal spleen, increased cord blood plasma MCP-1, and elevated cord blood white blood cell counts. Smaller changes were identified in response to fetal skin and amnion exposure, but not in response to acute gut exposure (94, 96). These data are also in keeping with previous studies that suggest a longer inflammatory response time for the fetal gastrointestinal tract compared to the skin or lung. Underscoring the complexity of intrauterine inflammatory signaling, these data also point to a differential temporal and spatial response to fetal inflammatory stimulation that likely varies with the microbial agonist in question.

CONCLUSION

As a function of PTB, the contribution of the fetus to intrauterine inflammation and FIRS is complex and, based on the evidence presented herein, likely dependent on a wide range of modifying factors. Evidence is available to suggest that gestational age at the time of infection, the nature of the infection itself (single organism vs. polymicrobial infection), the host's genetic background in addition to differences in specific tissue responses each combine to impact the origins and development of the intrauterine inflammation associated with PTB. It is clear, however, that the fetus is well endowed with the immunological armamentarium necessary to respond to microbial invasion of the amniotic fluid. Moreover, we now know that the fetus possesses the ability to recognize and respond to microbial agonist via elements of the innate immune system from comparatively early in gestation.

There is a compelling body of evidence from basic science and clinical studies to demonstrate that intrauterine infection is strongly associated with fetal inflammation. There is also excellent data to suggest that the intrauterine inflammation that is implicated in the precocious initiation of labor also plays a role in the development of a number of the congenital pathologies that are commonly identified in preterm infants, such as bronchopulmonary dysplasia and white matter injury. Accordingly, identifying the spatio-temporal origins of intrauterine inflammation, and how it might differ on an individual, case-by-case basis, is likely an important requirement in our efforts to develop treatments that prevent PTB while ensuring the continued development of a healthy fetus.

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Exploring preterm birth as a polymicrobial disease: an overview of the uterine microbiome

Matthew S. Payne* and Sara Bayatibojakhi

School of Women's and Infants' Health, The University of Western Australia, Perth, WA, Australia

Edited by:
Pietro Ghezzi, Brighton & Sussex Medical School, UK

Reviewed by:
Kai Fang, University of California Los Angeles, USA
Bo Shen, University of Illinois at Chicago, USA

****Correspondence:***
Matthew S. Payne, School of Women's and Infants' Health, The University of Western Australia, Perth, WA 6009, Australia
e-mail: matthew.payne@uwa.edu.au

Infection is a leading cause of preterm birth (PTB). A focus of many studies over the past decade has been to characterize microorganisms present in the uterine cavity and document any association with negative pregnancy outcome. A range of techniques have been used to achieve this, including microbiological culture and targeted polymerase chain reaction assays, and more recently, microbiome-level analyses involving either conserved, phylogenetically informative genes such as the bacterial 16S rRNA gene or whole shotgun metagenomic sequencing. These studies have contributed vast amounts of data toward characterization of the uterine microbiome, specifically that present in the amniotic fluid, fetal membranes, and placenta. However, an overwhelming emphasis has been placed on the bacterial microbiome, with far less data produced on the viral and fungal/yeast microbiomes. With numerous studies now referring to PTB as a polymicrobial condition, there is the need to investigate the role of viruses and fungi/yeasts in more detail and in particular, look for associations between colonization with these microorganisms and bacteria in the same samples. Although the major pathway by which microorganisms are believed to colonize the uterine cavity is vertical ascension from the vagina, numerous studies are now emerging suggesting hematogenous transfer of oral microbiota to the uterine cavity. Evidence of this has been produced in mouse models and although DNA-based evidence in humans appears convincing in some aspects, use of methodologies that only detect viable cells as opposed to lysed cells and extracellular DNA are needed to clarify this. Such techniques as RNA analyses and viability polymerase chain reaction are likely to play key roles in the clinical translation of future microbiome-based data, particularly in confined environments such as the uterus, as detection of viable cells plays a key role in diagnosis and treatment of infection.

Keywords: preterm birth, bacteria, virus, fungi, yeast, infection, amniotic fluid, placenta

INTRODUCTION

It has been well established that infection is a leading cause of preterm birth (PTB) and is highly associated with the deliveries that occur at the earliest gestations (1, 2). Although several theories have been proposed outlining the establishment of intra-uterine infections, the most widely accepted is that microorganisms residing in the vagina vertically migrate through the cervix, colonize the fetal membranes and then subsequently, the amniotic fluid (AF), placenta, and fetus (1). More recently, particularly in relation to bacteria colonizing the placenta, evidence has been presented suggesting hematogenous spread of organisms from the mother to the amniotic cavity (3–5).

For many years, studies have attempted to document these organisms, first using conventional microbiological culture and in the past two decades using a combination of culture, organism-specific polymerase chain reaction (PCR) assays and in the case of bacteria, 16S rDNA phylogenetics using a range of approaches including molecular cloning, denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism (TRFLP) analyses. These studies have contributed a wealth of information to our knowledge of what microorganisms colonize the uterine cavity. Recent advances in molecular biology that have seen the widespread use of next-generation sequencing (NGS) platforms for both amplicon and whole genome sequencing (WGS) have further enhanced our knowledge of these organisms, particularly those that represent very small proportions of a given microbial community.

This review aims to provide an overview of the total microbiome of the uterine cavity and discuss associations between specific organisms and negative pregnancy outcome. Some recent literature constantly use the term "microbiome" to describe bacterial microbiota, when in actual fact this term is really all encompassing and refers to microorganisms in general, including bacteria, viruses, yeasts, and fungi. As such, we will provide an overview of the bacterial, viral, and yeast/fungal microbiomes of the two main uterine compartments examined to date, the AF and placenta. In addition, we will discuss some of the limitations associated with current uterine microbiome data in terms of our ability to translate findings into clinical practice, as well as examining the potential implications of viewing PTB as a polymicrobial condition.

AMNIOTIC FLUID MICROBIOME

Since the discovery of bacteria in the AF of cesarean section pregnancies by Harris and Brown in 1927 (6), the previously held

belief that the fetus developed in a sterile environment has been challenged. Now, in the present day era of advanced molecular microbiological methodologies, we are well aware that numerous microbial organisms colonize the uterine environment (7, 8), many of which have been causally linked to PTB.

BACTERIA

Without a doubt, of all components of the uterine microbiome the greatest amount of data available relates to bacteria. Recent reviews by DiGiulio (7) and Mendz et al. (8) have provided a thorough overview of the major bacterial genera and species associated with AF colonization in cases of PTB. We have provided a summary of these and more recent bacterial microbiome studies in **Table 1**.

The most recent study documenting AF infection was conducted by Combs et al. (9), and examined 305 cases of women in spontaneous preterm labor with intact fetal membranes using a combination of enrichment culture and 16S rDNA cloning. Of the 305 cases, they reported the presence of bacteria in 30 AF samples, 26 of which they attributed to infection based upon elevated levels of interleukin-6 (IL-6) (>11.2 ng/mL) and 4 of which were deemed "colonizers" due to levels of IL-6 <2.6 ng/mL. The most common organisms identified were *Ureaplasma urealyticum* (11 cases), *Fusobacterium nucleatum* (5 cases), *Bacteroides ureolyticus* (4 cases), *Sneathia sanguinegens* (4 cases), *Ureaplasma parvum* (4 cases), and *Streptococcus agalactiae* (3 cases).

Interestingly, Combs et al. (9) also reported numerous cases of culture-positive, PCR-negative detection (65% positive by both, 16% by culture only, and 19% by PCR only), and concluded that the techniques are complementary and that neither can be relied upon 100% for detection of AF infection. A similar result was shown by DiGiulio et al. (14) where they reported six culture-positive samples that were negative by PCR and nine PCR-positive samples that were negative by culture. A potentially important consideration that may explain this disparity exists in the very small volume of AF used for DNA extraction in the DiGiulio et al. (14) study (0.2 mL). The amount of AF used by Combs et al. (9) in DNA extractions is not provided and neither study details the volumes of sample used in culture analyses, although DiGiulio et al. (14) state they centrifuged samples for culture and resuspended them in 1 mL of supernatant, so we assume the original volume was >1 mL in these cases. Considering the generally low titers of bacteria found in AF samples, for molecular detection it would appear to be beneficial to use as large a volume of AF as possible (at least 1 mL) in DNA extractions. This is reflected in the methodologies of Han et al. (15) and Markenson et al. (11), who extracted DNA from 1 to 2.5 mL volumes of AF, respectively. The authors of the first study were able to detect bacterial DNA via 16S rDNA PCR in 100% of culture-positive AF samples from cases of PTB. In addition, this study detected bacterial DNA in 17% of culture-negative AF samples and using molecular cloning, detected additional bacterial taxa in 9/16 culture-positive cases. The authors of the second study detected 30 PCR-positive cases of bacterial DNA in AF from 54 pregnancies with preterm labor compared to 5 cases of using only culture. In the case of large volumes of AF, bacterial cells could be pelleted in residual supernatant for subsequent DNA extraction. This would likely enhance the ability of PCR to detect organisms present in very low titers.

Overall, the most commonly associated organisms with AF infection and PTB include *U. parvum*, *U. urealyticum*, *Mycoplasma hominis*, *Gardnerella vaginalis*, *Peptostreptococcus* sp., *Enterococcus* sp., *Streptococcus* sp. (particularly *S. agalactiae*), *F. nucleatum*, *Leptotrichia* sp., *S. sanguinegens*, *Haemophilus influenzae*, and *Escherichia coli* (**Figure 1**). However, *Ureaplasma* sp. are by far the most commonly detected organism in AF from preterm pregnancies (23) and the greatest body of evidence exists suggesting a causal association between their presence in the AF and subsequent PTB (24). Of particular significance is the case–control study by Gerber et al. (25), who described a significant association between presence of *Ureaplasma* sp. DNA in second trimester AF and subsequent preterm labor. Similar associations have been described by Yoon et al. (26, 27) and Oh et al. (28) in case-specific studies. In contrast, within AF from women who delivered at term, detection rates for *Ureaplasma* sp. ranging from 0 (20) to 5.3% (25) have been described. A common colonizer of the vagina (24), this is believed to be the major reservoir for AF infection. However to date, no studies have been able to describe why only certain women vaginally colonized by *Ureaplasma* sp. deliver preterm and others do not. Recent work in a murine model by Racicot et al. (29) has offered a new viewpoint on this topic and will be discussed later in this review.

VIRUSES

During pregnancy, women are at a much greater risk of viral infection. For example, the mortality rate associated with influenza in pregnant women during the Spanish flu pandemic of 1918 was between 50 and 75% (30, 31). Unsurprisingly, numerous viral taxa have been previously described in the AF. Examples of these include rubella virus (32), varicella–zoster virus (VZV) (33), human immunodeficiency virus (34), adenovirus (35, 36), cytomegalovirus (CMV) (35, 36), herpes simplex virus (HSV) (36), human parvovirus (36), Epstein–Barr virus (EBV) (35), enterovirus (35, 37), and respiratory syncytial virus (35). All of these have been identified through either viral culture or targeted molecular assays as, unlike bacteria, at present there is no gene conserved throughout viral genera with sufficient variable regions that enables taxonomic classification. This factor dramatically limits our knowledge of the contribution of viruses to the AF microbiome.

Despite these difficulties, a small number of studies have examined the potential association between the presence of viral nucleic acids in the AF and subsequent PTB (**Figure 1**). The excellent review article by DiGiulio (7) summarized three such studies by Wenstrom et al. (36), Baschat et al. (35), and Miller et al. (38), all of which found a range of viral taxa in the AF of women, but did not report any association between the presence of any specific virus and negative pregnancy outcome.

More recently, Gervasi et al. (39) analyzed 729 mid-trimester AF samples for the presence of adenoviruses, HSV, VZV, human herpesvirus 6 (HHV6), CMV, EBV, parvovirus B19, and enteroviruses. They reported the presence of viral nucleic acids in 16/729 samples (2.2%) with HHV6 being the most prevalent (7 cases), followed by CMV (6 cases), parvovirus B19 (2 cases), and EBV (1 case). No

Table 1 | Overview of the major molecular[a] bacterial microbiome-based analyses of the uterine cavity.

Authors/year	Sample	Subjects	Organisms detected	Major findings
Jalava et al. (10)	AF[b]	20 cases of PPROM[b]; 16 controls (term)	**PPROM:** *Ureaplasma urealyticum*, *Haemophilus influenzae*, *Streptococcus oralis*, and *Fusobacterium* sp. **Controls:** no bacteria were detected	25% of samples were 16S rDNA positive for bacterial DNA. *U. urealyticum* was detected on two occasions
Markenson et al. (11)	AF	54 cases of PTL[b]	No sequencing of amplicons was conducted	55.5% of samples were 16S rDNA positive
Hitti et al. (12)	AF	69 cases of PTL with intact membranes	Group B Streptococci, *Enterococcus* sp., *Escherichia coli*, *Klebsiella pneumoniae*, *Mycoplasma hominis*, *Gardnerella vaginalis*, *Fusobacterium nucleatum*, *Bacteroides ureolyticus*, *Prevotella oulora*, *Clostridium* sp., and *Peptostreptococcus asaccharolyticus*	Bacteria were identified from 36% of culture-negative samples using PCR
Gardella et al. (13)	AF	69 cases of PTL	*Leptotrichia sanguinegens*, *F. nucleatum*, *U. urealyticum*, and an uncultured oral bacterium	16S rDNA PCR and sequencing are promising techniques to identify bacteria from culture-negative samples
DiGiulio et al. (14)	AF	166 cases of PTL	*M. hominis*, *Ureaplasma* sp., *Streptococcus agalactiae*, *Lactobacillus* sp., *Prevotella* sp., *F. nucleatum*, *Streptococcus mitis*, *uncultivated Bacteroidetes bacterium*, *Delftia acidovorans*, *Neisseria cinerea*, *Sneathia sanguinegens*, *Leptotrichia amnionii*, and an uncultured bacterium	17 women had positive results for bacterial 16S rDNA
Han et al. (15)	AF	46 cases of PTB[b]; 16 controls (term)	**PTB:** *L. sanguinegens*, *S. sanguinegens*, *B. ureolyticus*, *Citrobacter koseri*, *Bacteroides fragilis*, *F. nucleatum*, *Prevotella bivia*, *Shigella* sp., Clostridiales bacterium, *Bergeyella* sp., *Ureaplasma parvum*, *S. agalactiae*, *L. amnionii*, *M. hominis*, and *Peptostreptococcus* sp. **Controls:** no bacteria were detected	45% of AF samples were positive for bacterial 16S rDNA. The most abundant 16S rDNA sequence detected was *F. nucleatum* (33.3%)
Jones et al. (16)	FM[b] and PLAC[b]	26 cases of PPROM; 19 cases of PTL with intact membranes; 8 cases of indicated PTL; 21 controls (term)	**CS term:** no bacteria were detected **V term:** *U. parvum*, *Lactobacillus crispatus*, *Fusobacterium* sp., *Pantoea* sp., and *Eubacterium rectale* **CS indicated PTB:** *Fusobacterium* sp. **CS PTL with PROM:** *U. parvum*, *S. mitis* group, *Fusobacterium* sp., *Veillonella parvula*, *H. influenzae*, and *U. urealyticum* **V PTL with PROM:** *U. parvum*, *Fusobacterium* sp., *S. agalactiae*, *M. hominis*, *Atopobium vaginae*, *L. crispatus*, *E. coli*, *Peptoniphilus lacrimalis*, *Corynebacterium amycolatum*, and *U. urealyticum* **V PTL with intact membranes:** *U. parvum*, *Fusobacterium* sp., *S. agalactiae*, *S. mitis* group, *L. crispatus*, *H. influenzae*, *Oribacterium sinus*, *Veillonella* sp., *Peptostreptococcus* sp., *Enterobacter aerogenes*, *Corynebacterium aerogenes*, *G. vaginalis*, *Finegoldia magna*, *Peptoniphilus asaccharolyticus*, *Streptococcus anginosus*, and *B. ureolyticus*	PTL samples showed a higher prevalence and diversity of bacteria. Blood monocyte counts in PTL and PPROM groups that were positive for 16S rDNA were indicative of suppressed immunity. 30, 43, and 19% of samples were positive using broad-range 16S rDNA PCR, species-specific real-time PCR and a combination of both methods, respectively. 60% of PTL samples had multibacterial infection. The most commonly detected organisms were *U. parvum* followed by *Fusobacterium* sp.
DiGiulio et al. (17)	AF	52 cases of SGA[b] neonates	*Staphylococcus epidermidis* and *S. agalactiae*	Two bacteria positive samples were identified
DiGiulio et al. (18)	AF	62 cases of preeclampsia	*Lactobacillus iners*, *S. anginosus*, *Corynebacterium tuberculostearicum*, *Ureaplasma* sp., and *Sneathia/Leptotrichia* sp.	8% of samples were positive for bacterial DNA. *Ureaplasma* sp. *and Sneathia/Leptotrichia* were the most frequently detected bacteria in cases of MIAC.

(Continued)

Table 1 | Continued

Authors/year	Sample	Subjects	Organisms detected	Major findings
DiGiulio et al. (19)	AF	204 cases of PPROM	*Prevotella oris*, *Prevotella copri*, *Bacteroides* sp., *B. fragilis*, *Myroides* sp., *F. nucleatum*, *Fusobacterium* sp., *Leptotrichia* sp., *S. sanguinegens*, *L. amnionii*, *Dialister* sp., *Streptococcus* sp., *Streptococcus salivarius*, *S. agalactiae*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Staphylococcus equorum*, *Staphylococcus pettenkoferi*, *Staphylococcus* sp., *Lactobacillus delbrueckii*, *Lactobacillus gasseri*, *Coprobacillus* sp., *Peptostreptococcus* sp., *Filifactor alocis*, *Clostridiaceae* sp., *Clostridium hiranonis*, *Brachybacterium* sp., *Rothia dentocariosa*, *Bifidobacterium longum*, *Bifidobacterium pseudolongum*, *G. vaginalis*, *A. vaginae*, *Ureaplasma* sp., *M. hominis*, *Neisseria subflava*, *Kingella denitrificans*, *H. influenzae*, *Haemophilus haemoglobinophilus*, *Haemophilus parainfluenzae*, *Campylobacter* sp., and an uncultured bacterium	A 45% prevalence of MIAC in the study group was recorded. 44 bacterial species were identified using PCR. The most common organism detected was *Ureaplasma* sp.
Marconi et al. (20)	AF	20 cases of PTL and 20 controls (term)	**PTL:** *B. fragilis*, *P. bivia*, *L. amnionii*, *M. hominis*, and *U. urealyticum* **Controls:** *M. hominis*	40% of PTL and 5% of control cases were positive for MIAC
Wang et al. (21)	AF and CB[b]	36 cases of PTB, IAI[b] or EONS[b], and 8 controls (term)	**AF bacteria:** *E. coli*, *S. agalactiae*, *M. hominis*, *P. bivia*, *Lachnospiraceae* sp., *U. parvum*, *Peptoniphilus harei*, *S. sanguinegens*, *S. pneumoniae*, *B. ureolyticus*, *Bergeyella* sp., *S. mitis*, *L. monocytogenes*, *H. influenzae*, and *F. nucleatum* **CB bacteria:** *E. coli*, *S. agalactiae*, *F. nucleatum*, *M. hominis*, *U. parvum*, *Bergeyella* sp., and *S. sanguinegens* **Controls:** no bacteria were detected	31 and 18 bacterial species were identified in AF and CB, respectively. *E. coli* and *F. nucleatum* were the most frequently detected bacteria
Romero et al. (22)	AF	142 cases of PTL	*U. parvum*, *F. nucleatum*, *G. vaginalis*, *M. hominis*, *U. urealyticum*, *Acinetobacter junii*, *Sneathia* sp., *Pseudomonas* sp., *Aeromonas caviae*, *Moraxella osloensis*, *Staphylococcus aureus*, *Acidovorax* sp., *Lactobacillus* sp., *Pantoea dispersa*, and *Streptococcus* sp.	MIAC was present in 21% of cases. The most commonly detected bacteria was *U. parvum*
Combs et al. (9)	AF	305 cases of PTL	*B. ureolyticus*, *S. sanguinegens*, *F. nucleatum*, *G. vaginalis*, *H. influenzae*, *U. urealyticum*, *U. parvum*, *S. agalactiae*, *Bacteroides hemolyticus*, *L. monocytogenes*, *Bergeyella zoohelecum*, *Bergeyella* sp. *Staphylococcus hemolyticus*, and *L. amnionii*	MIAC was detected in 10% of AF samples
Aagaard et al. (3)	PLAC	320 pregnancies (preterm/term)	*E. coli*, *Escherichia* sp., *Prevotella tannerae*, *Bacteroides* sp., *Streptomyces avermitilis*, *Propionibacterium acnes*, *Rhodococcus erythropolis*, *Neisseria polysaccharea*, *Neisseria lactamica*, *Fusobacterium* sp., *Streptosporangium* sp., *Roseovarius* sp., *Rhodococcus* sp., *Paenibacillus* sp., *Klebsiella* sp., *Burkholderia* sp., and *Anaeromyxobacter* sp.	*E. coli* was the most commonly detected bacteria in the placenta. The placental microbiome is unique and harbors a variety of non-pathogenic commensal bacterial species. It is most closely related to the oral microbiome

[a]*Whole genome shotgun sequencing, broad-range16S rDNA, or a combination of broad-range 16S rDNA and targeted PCR assays;*

[b]*AF, amniotic fluid; FM, fetal membranes; PLAC, placenta; CB, cord blood; PTL, preterm labor; V, vaginal delivery; CS, Cesarean section; PPROM, preterm premature rupture of membranes; PTB, preterm birth; SGA, small gestational age; IAI, intraamniotic infection; EONS, early onset neonatal sepsis.*

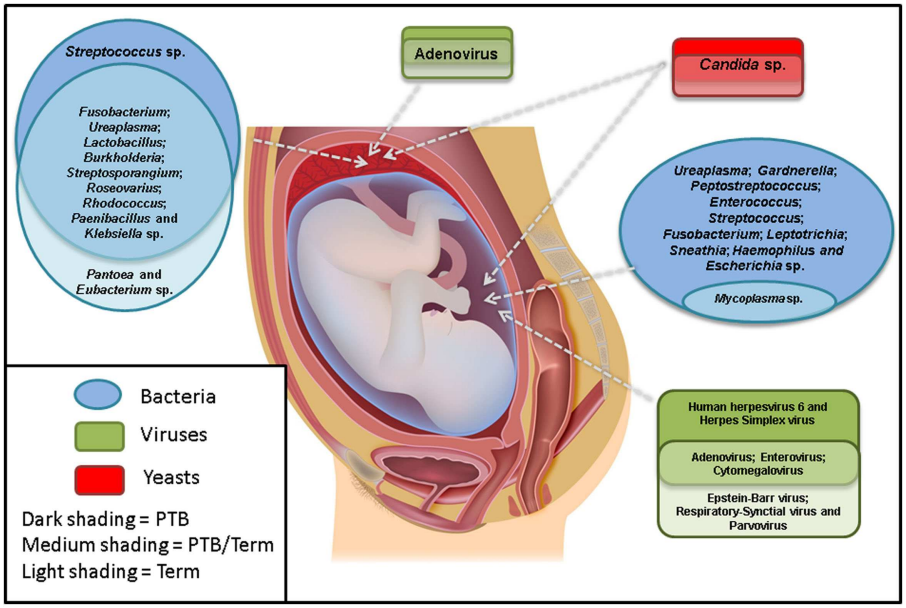

FIGURE 1 | The most commonly detected microorganisms in the amniotic fluid and placenta from preterm and term pregnancies. Studies were only included if there were well-defined preterm and/or term cohorts.

cases of HSV, VZV, enteroviruses, or adenoviruses were reported. The complete absence of adenoviruses in this cohort is in stark contrast to the earlier work of Wenstrom et al. (36) and Baschat et al. (35), who both reported adenoviruses as the most common amongst viral nucleic acid-positive AF samples (9/14 and 37/44 positive AF samples, respectively). Regardless, similar to previous studies, Gervasi et al. (39) also reported no significant association between the presence of any specific viral nucleic acid in mid-trimester AF and negative pregnancy outcome.

Romero et al. (22) utilized a novel molecular method that combined PCR with electrospray ionization time-of-flight mass spectrometry to examine AF from 142 women in preterm labor with intact membranes for the viruses, HSV-1 and -2, VZV, EBV, CMV, Kaposi's sarcoma-associated herpes virus, human adenoviruses, human enteroviruses, BK polyomavirus, JC polyomavirus, and parvovirus B19. Viral DNA was only identified in two cases, both of which were identified as enteroviruses.

Two additional recent studies examined any relationship between the presence of viral nucleic acid in AF and the clinical phenotype of preterm premature rupture of membranes (PPROM). The first of these was unable to detect any nucleic acid from HSV-1, HSV-2, adenovirus, adeno-associated virus-2, CMV, parvovirus B19, human papilloma viruses, and enteroviruses in the AF of 13 women with PPROM (40). Similarly, Bopegamage et al. (41) were only able to detect a single viral nucleic acid-positive AF sample from a cohort of 174 women with PPROM. In this case, the positive sample contained CMV DNA, but the study also tested for HSV, parvovirus B19, adenovirus, enterovirus, and human parechovirus.

Although these studies appear to paint a clear picture that no association exists between the presence of viruses and subsequent PTB, there are two important considerations to make. First, as mentioned above, our knowledge of viral taxa in AF is greatly limited by the lack of suitable viral phylogenetic markers similar to the bacterial 16S rRNA gene. At present, the only technology available to examine the viral microbiome that does not involve the use of targeted molecular assays is whole genome shotgun metagenomics. This is a rapidly expanding area in viral detection and identification and has resulted in the discovery of novel viral pathogens (42, 43). There is high potential for the application of this technology to examine the role of viral infection in cases of PTB. Second, if one is to adopt the view that PTB is actually a polymicrobial condition, as has been suggested in recent literature, then the view of whether viruses play a role can easily be revisited. Recent research by Racicot et al. (29) has added an interesting twist to this topic and will be discussed later regarding PTB as a polymicrobial disease.

FUNGI AND YEASTS

Similar to viruses, our knowledge of fungi and yeasts in the uterine cavity at present is largely limited to culture and targeted molecular assays. Unlike viruses, however, this is not through lack of suitable phylogenetically informative genes. In fact, in fungi and yeasts several such targets exist, the 18S, 5.8S, and 28S rRNA genes as well as the internal transcribed spacer regions (ITS1 and ITS2) (44). The major limiting factor of these targets compared to the bacterial 16S rRNA gene, however, is that the reference databases containing sequence identifications are significantly less populated than those for bacteria. In addition, the 18S rRNA gene is also present in the human genome, which presents a problem with NGS technologies in terms of the generation of unwanted amplicons utilizing sequencing reagents. This is likely to be of much more significance in samples with very small fungal/yeast content, where the levels of human DNA in an extract may greatly outnumber fungal/yeast DNA.

The only study to date that we are aware of which has employed a broad-range PCR approach to elucidating the fungal microbiome in AF is that of DiGiulio et al. (14). This study utilized the 18S–28S rRNA genes and only detected a single positive sample from 166 patients, which was identified as *Candida albicans*.

Although they are one of the most common organisms found in the vagina of pregnant women (45, 46) only a small number of studies have investigated *Candida* sp. as a potential source of AF infection and PTB. These have included both culture (47, 48) and molecular-based (14, 18) studies of women in preterm labor with intact membranes and women with PPROM, with prevalence rates varying between 0 and 1.2% for the first clinical phenotype and 0–5% for the second clinical phenotype. Numerous studies have also reported a distinct association between the presence of an intra-uterine device (IUD) during pregnancy and *Candida* sp. intra-uterine infection (7), with one study describing this phenomenon in 31.1% of pregnancies where an IUD was present vs. 6.3% where there was no IUD (49).

Unlike organisms more commonly associated with intra-uterine infection, the consequences of *Candida* sp. in the uterine cavity are typically severe. For example, Payne et al. (50) used a pregnant sheep model to demonstrate that colonization of the amniotic cavity by *C. albicans* causes severe uterine inflammation and subsequent fetal injury. Once acquired by a preterm neonate, *Candida* sp. colonization can rapidly progress to invasive candidiasis, a condition that is frequently associated with low birthweight, prematurely born infants. It has a high mortality and morbidity rate and is reported to be the second most common fatal infection associated with preterm infants (51).

At present the fungal/yeast microbiome of the uterine cavity is largely underestimated and our knowledge appears to be limited to *Candida* sp. (**Figure 1**). Microbiome investigations of other human body sites utilizing the aforementioned phylogenetically informative targets have revealed previously undescribed diversity, although levels are typically several magnitudes lower than that for bacterial studies (44). For example, in a study of the oral fungal/yeast microbiome, Ghannoum et al. (52) identified *Candida* species as the most common (present in 75% of participants), followed by *Cladosporium* sp. (65%), *Aureobasidium* sp., *Saccharomycetales* sp. (50% for both), *Aspergillus* sp. (35%), *Fusarium* sp. (30%), and *Cryptococcus* sp. (20%). Of more relevance to PTB, LaTuga et al. (53) detected fungal/yeast DNA in 7/11 stool specimens from extremely low birthweight infants. Genera identified included *Candida* sp., *Cladosporium* sp., *Clavisporas* sp., *Cryptococcus* sp., and *Saccharomyces* sp.

Our knowledge of fungi/yeasts that invade the uterine cavity is likely to substantially improve in future years through utilization of NGS technologies and conserved fungal/yeast genes. Comparison of such microbial communities with pregnancy outcome will allow an informed decision of the role that fungi/yeasts play in PTB.

PLACENTAL MICROBIOME

Bacterial colonization of the placenta has been reported previously on several occasions (**Table 1**). Although numerous studies have suggested colonization as a result of prior fetal membrane and AF infection, an increasing number of studies are reporting the presence of bacteria representative of the oral microbiota in the placenta and suggesting hematogenous transfer as the route of colonization. This will be discussed in detail later in this review.

BACTERIA

One of the best studies to truly document bacterial colonization of the placenta is that by Stout et al. (54), who hypothesized that the maternal basal plate of the placenta may be a reservoir for bacteria associated with negative pregnancy outcomes. In a study of 195 women, they reported Gram positive and negative intracellular bacteria of a range of morphologies (filamentous, cocci, rods, and spirochetes) in the basal plates from 27% of all placentas. In the case of preterm vs. term deliveries, there was a significant association identified between presence of bacteria in the basal plate and delivery at <28 weeks gestational age (GA) (54.5 vs. 26.7%), however, at preterm GAs >28 weeks GA, this significance was lost. Interestingly, the study also reported the presence of bacteria in placentas in the absence of clinical or pathologic chorioamnionitis, potentially indicating placental bacterial colonization through hematogenous mechanisms. Unfortunately, the authors did not go beyond Gram and cell morphology classification in this study, which would have been greatly enhanced with the use of laser capture micro-dissection to isolate specific cell morphologies and identify these using 16S rDNA techniques.

The most recent placental microbiome study applied cutting edge NGS methodologies to elucidate the bacterial microbiota of the placenta. Aagaard et al. (3) described a unique microbiome that bore similarity to oral taxa from non-pregnant subjects, specifically to the bacterial microbiota of the tongue, tonsils, and gingival plaques as previously described by the Human Microbiome Project consortium (55, 56). Using whole genome shotgun metagenomics, the most frequently detected sequences belonged to *E. coli* and the genus *Escherichia* sp; *Prevotella tannerae*, *Bacteroides* sp., *Streptomyces avermitilis*, *Propionibacterium acnes*, *Rhodococcus erythropolis*, *Neisseria polysaccharea*, *Neisseria lactamica*, and *Fusobacterium* sp. sequences were also detected in lower numbers. Of substantial interest amongst these sequence identifications is that *E. coli* appears to dominate placental bacterial communities. Aagaard et al. (3) suggested that the source of colonization may be infant meconium, a highly plausible theory considering recent studies showing the high abundance of *E. coli* in meconium from neonates (57, 58). Another potential source may be the maternal gut, where *E. coli* is a common resident. This would entirely depend on the ability of *E. coli* to cross the mucosal barrier of the intestine. A prime example of this is *Listeria monocytogenes*, which following passage across the intestine is able to spread hematogenously to various body sites, particularly the fetoplacental unit (57).

Aagaard et al. (3) also used a 16S rDNA approach to characterize the bacterial microbiota of a larger number of placental samples and looked for associations between these and PTB. Interestingly, they detected an enrichment of sequences associated with *Burkholderia* sp. in samples from women who delivered preterm. This genus contains known respiratory pathogens such as the *Burkholderia cepacia* complex. Other organisms detected included *Streptosporangium* sp., *Roseovarius* sp., *Rhodococcus* sp., *Paenibacillus* sp., *Klebsiella* sp., and *Anaeromyxobacter* sp. (**Figure 1**). These taxa are quite different to those described by Onderdonk

et al. (59), who reported positive cultures for 696/1365 placentas from pregnancies 23–27 weeks GA. The most commonly reported organisms were *Actinomyces* sp., *Streptococcus* sp., *Corynebacterium* sp., *E. coli*, *Lactobacillus* sp., *M. hominis*, *Peptostreptococcus* sp., *Prevotella bivia*, *Propionibacterium* sp., coagulase-negative Staphylococci, *Bacteroides* sp., *G. vaginalis*, and *Ureaplasma* sp.

Although the previously discussed study by Aagaard et al. (3), to our knowledge, represents the only "true" microbiome analysis of the placenta at present, numerous studies have documented a range of other bacteria in this organ. For example, a number of intracellular bacteria are known to colonize the placenta and are associated with negative pregnancy outcomes. These include *L. monocytogenes* (60), *Coxiella burnetii* (60, 61), *Chlamydia trachomatis* (60, 62), *Waddlia chondrophila* (60, 63), and *Parachlamydia acanthamoebae* (60).

A more recent study by Queiros da Mota et al. (64) reported 73 cases of positive bacterial culture from 376 placentas. Of these cases, 48 were described as monomorphic and half of the placentas with positive cultures were from preterm deliveries. They described the presence of a range of bacteria, dominated by Gram positive cocci and bacilli and Gram negative bacilli. A number of anaerobes of these same morphology were also present, particularly Gram negative bacilli. The most interesting aspect of this study, however, was the correlation between histological chorioamnionitis and placental bacterial culture. Of the 73 culture-positive cases, 28 occurred in the presence of chorioamnionitis, while 45 did not. This adds some support to the theory that not all cases of bacterial colonization are indeed infection and as suggested by Aagaard et al. (3) that the placenta may indeed harbor its own unique microbiome.

Our knowledge of the bacterial placental microbiome is likely to substantially improve in coming years with the increased application of NGS-based technologies. Data generated by such studies combined with detailed patient histories is likely to significantly enhance our knowledge of the role the placenta plays as a source of bacterial colonization and how this colonization impacts on pregnancy outcome.

VIRUSES

Only a small number of studies have attempted to document the presence of viruses in the placenta. The first of these was a study by Srinivas et al. (65) that looked at singleton pregnancies presenting with a spontaneous second trimester pregnancy loss secondary to PPROM, premature labor, or cervical insufficiency. The authors detected significantly more viral nucleic acid in cases (79%) compared to controls (second trimester induction of labor for congenital anomalies or maternal medical indications) (44%). The major viruses detected were CMV and HPV.

Several years later, Tsekoura et al. (66) examined 71 preterm and 122 full term placentas for the presence of adenovirus DNA and reported its presence in 40.8 and 20.5% of preterm and term cases, respectively (**Figure 1**). This was a significant finding. In addition, they also documented a significant increase in cases of histological chorioamnionitis in preterm adenovirus-positive placentas when compared to both preterm adenovirus-negative placentas and term adenovirus-positive placentas (75 vs. 36 vs. 19%, respectively).

Perhaps, the most important study looking at viruses in the placenta is that by Cardenas et al. (30), which outlined the importance of viral placental infection in a murine model. This study used murine herpesvirus-68 (MHV-68)-infected pregnant mice to show that viral infection of the placenta can elicit a fetal inflammatory response and that such an infection also may sensitize the mother to bacterial endotoxin and in turn, preterm labor. The authors injected LPS into MHV-68-infected mice in a dose that was known to have a modest effect on pregnancy outcome (20 μg/kg). All MHV-68/LPS animals subsequently delivered in <24 h post-LPS injection compared with only 29% of LPS-only animals. In addition, there was vaginal bleeding and a 100% fetal death rate observed in all MHV-68/LPS cases compared to none in LPS-only animals.

FUNGI AND YEASTS

As with the AF, with the exception of *Candida* sp., there is a complete dearth of information regarding the fungal/yeast microbiome of the placenta (**Figure 1**). There have been several case reports documenting placental *Candida* sp. infections, in particular those arising from cutaneous congenital candidiasis (67–70). This is an extremely rare disease (<100 published cases) that typically occurs secondary to *Candida* sp. chorioamnionitis. The phenotype is characterized by the presence of white microabscesses on the placenta and umbilical cord and a generalized rash on the infant shortly after birth (69).

PATHWAYS TO MICROBIAL COLONIZATION OF THE UTERINE CAVITY

The excellent review article by Goldenberg et al. (1) on the epidemiology and causes of PTB proposed four major routes of how microbial organisms are able to invade the uterine cavity. These were vertical ascension from the vagina; retrograde through the abdominal cavity, introduction through invasive procedures such as amniocentesis and hematogenously from the placenta. It has been well established that the major source of intra-uterine colonization is vertical ascension from the vagina (1), and this is largely believed to occur during the second trimester, although the actual timing is unknown and it is likely that this will vary between individual pregnancies.

The evidence currently supporting hematogenous spread of microbes however, is a contentious area that needs to be viewed carefully. There are increasing reports that bacteria, specifically those from the oral cavity, are able to spread hematogenously from the maternal bloodstream to the uterine cavity (5). This is further supported by apparent associations between periodontal disease and PTB (71, 72), although this association is also contentious with numerous studies (73), including a large randomized-controlled trial (74) finding that treatment of periodontal disease during pregnancy does not reduce the rate of PTB.

The best evidence supporting hematogenous spread of oral bacteria to the uterine cavity is provided through numerous studies by Han et al. The first of these was in a murine model where mice received an intravenous (IV) injection of live *F. nucleatum*. This subsequently spread to the uterus and resulted in negative pregnancy outcomes (75). Following this, Han et al. (4) attempted to show transfer of an uncultured *Bergeyella* sp. strain from the oral

cavity to the AF in a human case of PTB. The study identified the organism based upon its 16S-23S rDNA sequence and concluded that as the sequence homology was identical between the AF and sub gingival plaque sites that this demonstrated oral to AF transfer. Han et al. (76) then reported a case study of a woman with pregnancy-associated gingivitis who experienced an upper respiratory tract infection and subsequent stillbirth. *F. nucleatum* was isolated from both the placenta and infant and subsequent 16S–23S rDNA analysis of vaginal and rectal swabs failed to detect the presence of the organism. However, it was detected in the sub and supragingival plaques, and in the case of the subgingival plaque, the apparent identical clone was detected based upon sequence similarity. Unfortunately, the case study did not note the timeframe associated with still birth to collection of vaginal/rectal samples, which is important for validating the failure to detect *F. nucleatum* in these. These case studies offer the most robust information to date on potential oral-uterine bacterial transfer in humans. Further work by Fardini et al. (5) has shown potential transfer of a range of oral bacterial species to the murine placenta through IV inoculation; however, the method of detection in the placenta was DNA-based as opposed to culture. The reason that DNA-based studies such as these are contentious is that although they do indeed show the presence of microbial DNA in the uterine cavity that corresponds with that of species synonymous with the oral microbiota, they do not show the presence of viable microbial cells. Recently, it has been well publicized that cell-free fetal DNA is trafficked out of the placenta and into the maternal circulation, where it is readily detectable during pregnancy (77–81). Based upon this, it would also be plausible that lower molecular weight, microbial DNA can cross from the maternal bloodstream to the uterine cavity and vice-versa. Detection of microbial DNA in these samples at best demonstrates that such DNA can be spread from the maternal bloodstream to the uterine cavity. This said, the presence of microbial DNA in the uterine cavity alone may be enough to activate inflammatory responses that culminate in preterm labor.

Although work to date offers increasingly promising evidence that the oral microbiota can infect the uterine cavity through hematogenous transfer, further work is required to definitively uncover their role in intra-uterine infection. Due to inherent difficulties with culture of fastidious organisms present at these sites, it is increasingly important that molecular detection/characterization protocols are employed that represent the viable microbiota in these samples as opposed to lysed cells or free-circulating DNA. Such methodologies are discussed below.

MICROBIAL CELL VIABILITY, THE MICROBIOME, AND CLINICAL TRANSLATION

Although current research to elucidate the various microbiomes of the uterine environment have been limited to DNA-based approaches, the issue of how relevant DNA detection is on a clinical level has been present for many years. It has long been known that DNA is a stable molecule and can persist for weeks following microbial cell death (82). Wang and Levin suggested that the inability of DNA-based PCR assays to differentiate between non-viable and viable cells was a major limitation of this technology (83). Applying this to microbiome-level studies, which may be characterizing dynamic systems over several time points, detection of viable cells is critical to documenting microbial succession. In addition, in confined environments such as the uterus, where there is poor clearance of cellular material and particularly, in these scenarios following antibiotic usage; non-viable organisms and extracellular DNA can contribute significantly to molecular analyses (84).

Some studies have attempted to remedy this by utilizing the amplification of RNA instead of DNA, which degrades rapidly after cell death and in particular, using messenger RNA targets as this is a highly unstable molecule and is only produced by metabolically active cells (85–88). The major disadvantage to this approach, however, lies in the inherent difficulties associated with isolating RNA from samples, including the need for stringent sample storage conditions following sample collection, in addition to sample processing regimes to prevent RNA degradation (86). For example, RNA-degrading enzymes, ribonucleases (RNases), can rapidly degrade RNA if not promptly inhibited. The human skin is a prime example of how RNases can be accidentally introduced into samples (89).

In terms of using RNA for microbiome characterization, this instability is the major limitation, as even minor degradation of nucleic acid can potentially result in loss of characterization of the total viable microbial community in a given sample, especially that of organisms present in low cell titers.

A potential solution that addresses the issue of cell viability in DNA-based methodologies and may be highly applicable to microbiome-level studies is that of viability PCR (vPCR). This technology utilizes membrane-impermeable dyes, either ethidium monoazide (EMA) (90) or the more recent and preferred propidium monoazide (PMA) (91). Samples are pre-treated with the chosen dye, which is unable to cross an intact cell wall. In cases, where the integrity of the microbial cell wall has been lost, the dye is able to intercalate into the cell's DNA, which results in covalent cross-linkage after exposure to strong visible light. Cross-linked DNA is subsequently blocked from PCR amplification in downstream analyses (86). This technology has been applied to bacteria (92, 93), fungi (94), viruses (95), yeasts (96), and protozoa (97) on many previous occasions and has also been used in both environmental (98) and clinical (84) microbiome analyses. A detailed review of this technology is provided by Fittipaldi et al. (86).

However, although vPCR certainly has the potential to yield clinically relevant microbiome data, careful validation is first needed for some of the key microorganisms associated with PTB. In particular, organisms of the Class Mollicutes, including all *Ureaplasma* and *Mycoplasma* sp. do not possess a true bacterial cell wall. Their nucleic acid is instead protected by a triple layered membrane and its permeability to EMA or PMA is currently unknown. An additional consideration that is highly relevant to microbiome studies is that surrounding the buffer used to resuspend swab-collected samples. It is very important that the buffer itself does not result in cell lysis. For example, an alkaline pH may result in significant loss of viability of *Lactobacillus* sp. cells from a vaginal swab.

PRETERM BIRTH: A POLYMICROBIAL DISEASE?

Many studies, particularly since the increase in NGS-based microbiome work, have emphasized the importance of assessing PTB as a polymicrobial disease, a large number of which are summarized in **Table 1**, in addition to the review by DiGiulio et al. (7). However, in the context of most of these studies, the word polymicrobial is used to imply the presence of two or more bacterial species. A more appropriate term to describe such an infection would be "polybacterial. " In a disease context, the word "polymicrobial" is best used to describe diseases involving multiple infectious agents (99). As such, a polymicrobial infection may entail the initial presence of a virus, which creates a favorable environment for a secondary bacterial or fungal infection or vice-versa.

Evidence of the importance of viewing polymicrobial disease in this way is provided by Racicot et al. (29) who recently conducted an elegant study demonstrating how a viral infection during pregnancy may compromise the antibacterial defenses of the cervix, prompting a secondary bacterial infection of the uterine cavity. These authors demonstrated that the cervix in mice shows resistance to bacterial infection with *E. coli* during pregnancy, but not in non-pregnant animals. Extending this further, they replicated the same experiment using the most commonly observed organism from preterm pregnancies, *Ureaplasma* sp., and reported the same result. Having previously shown that infecting pregnant mice with a virus, MHV-68, predisposes the animals to the effects of bacterial endotoxin, but viral infection itself does not induce preterm labor (30), they went on to test whether a systemic viral infection could alter the ability of the cervix/uterine cavity to resist bacterial infection. Following an intraperitoneal injection of MHV-68 into pregnant and non-pregnant mice, they showed that 7 days post-injection the virus was observed at similar concentrations in the spleen of both pregnant and non-pregnant animals, but was only present in the cervix of pregnant mice. They subsequently suggested that pregnancy may render the cervix susceptible to a viral infection. After administering *Ureaplasma* sp. intravaginally to MHV-68-infected and non-infected pregnant mice, they reported significantly higher amounts of *Ureaplasma* sp. nucleic acid in the decidua and lymphoid aggregates of MHV-68-infected mice compared to non-infected. The authors suggested that a viral infection during pregnancy can alter the ability of the female reproductive tract to defend against an ascending bacterial infection (29).

Although this work was carried out in mice and potentially may not apply to humans due to physiological differences in the cervix and pregnancy in general, it still provides substantial evidence that future microbiome studies of the uterine cavity need to not only focus on bacteria, but also other organisms including viruses and fungi/yeasts, and document any relationship between these and negative pregnancy outcomes.

CONCLUSION

Our knowledge of the microbiome of the uterine cavity has been greatly enhanced since the widespread use of molecular microbiological techniques, particularly 16S rDNA phylogenetics, which have uncovered numerous bacterial taxa not previously described. Bacteria, particularly *Ureaplasma* sp. and *Fusobacterium* sp. appear to be most significantly associated with negative pregnancy outcomes when present in the uterine compartment. Although viruses are also present and on their own do not appear to be significant, when combined with a bacterial infection they may contribute significantly to PTB. Viral infection of the placenta, however, does appear to be associated with negative pregnancy outcomes. Our knowledge of fungi/yeasts that colonize the uterine cavity is currently limited to yeasts, specifically *Candid*a sp. Further research effort is required to characterize the fungal microbiome of the uterine cavity using conserved fungal/yeast genes. These data combined with existing data on bacteria and viruses are likely to shed further light on the polymicrobial nature of intra-uterine infections.

Although current microbiome-based studies have contributed valuable data to our knowledge of intra-uterine infection, the application of these data to clinical scenarios is currently limited due to cell viability issues surrounding DNA-based analyses. Future microbiome-based studies, especially those attempting to document hematogenous spread of viable microbial cells from various body sites to the uterine cavity, should adopt molecular approaches that either:

(1) Utilize RNA-based characterization of a given microbial community using known conserved genes (for instance, the 16S rRNA gene in bacteria), coupled with strict sample collection and processing regimes so as to inhibit the activity of RNases.
(2) Maintain current DNA-based characterization approaches, but implement vPCR procedures to inhibit amplification of DNA from non-viable cells.

These approaches are likely to be of particular relevance if/when microbiome-based NGS approaches are introduced into clinical diagnostic laboratories.

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The maternal serological response to intrauterine *Ureaplasma* sp. infection and prediction of risk of pre-term birth

Demelza J. Ireland* and Jeffrey A. Keelan

School of Women's and Infants' Health, The University of Western Australia, Perth, WA, Australia

Edited by:
Delmiro Fernandez-Reyes, Brighton and Sussex Medical School, UK

Reviewed by:
Lisa Mullen, Brighton and Sussex Medical School, UK
Florence Burté, Newcastle University, UK

****Correspondence:***
Demelza J. Ireland, School of Women's and Infants' Health, Faculty of Medicine, Dentistry and Health Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia
e-mail: demelza.ireland@uwa.edu.au

Pre-term birth (PTB) associated with intrauterine infection and inflammation (IUI) is the major cause of early PTB less than 32 weeks of gestation. *Ureaplasma* spp. are common commensals of the urogenital tract in pregnancy and are the most commonly identified microorganisms in amniotic fluid of pre-term pregnancies. While we have an understanding of the causal relationship between intra-amniotic infection, inflammation and PTB, we are still unable to explain why vaginal *Ureaplasma* sp. colonization is tolerated in some women but causes PTB in others. It is now known that placental tissues are frequently colonized by bacteria even in apparently healthy pregnancies delivered at term; usually this occurs in the absence of a significant local inflammatory response. It appears, therefore, that the site, nature, and magnitude of the immune response to infiltrating microorganisms are key in determining pregnancy outcome. Some evidence exists that the maternal serological response to *Ureaplasma* sp. colonization may be predictive of adverse pregnancy outcome, although issues such as the importance of virulence factors (serovars) and the timing, magnitude, and functional consequences of the immune response await clarification. This mini-review discusses the evidence linking the maternal immune response to risk of PTB and the potential applications of maternal serological analysis for predicting obstetric outcome.

Keywords: antibody, immune response, intrauterine infection, pre-term birth, predictive marker, *Ureaplasma* spp.

INTRODUCTION

It is estimated globally that approximately 12 million babies are born pre-term each year, making the prevention of pre-term birth (PTB) one of the highest priorities for international obstetric research (1). Intrauterine infection (IUI) and subsequent inflammation of the extra-placental membranes (chorioamnionitis) accounts for approximately 40% of all spontaneous PTBs and is the major cause of early PTB (<32 weeks of gestation). IUI is typically "silent" (undiagnosed) until the onset of pre-term labor at which point it is often too late for treatment as chorioamnionitis is well established, the risk of fetal inflammatory response syndrome (FIRS) is high (2), and tocolysis is ineffective. Identifying women at risk of infection-associated PTB sufficiently early in pregnancy to allow therapeutic intervention would be a significant advance in the prevention of PTB.

Traditional thinking associates IUI with the ascension of bacteria from cervicovaginal fluid, resulting in intra-amniotic infection and immune stimulation within the otherwise sterile intrauterine environment (3). It is now clear, however, that the placenta and extra-placental membranes can no longer be considered strictly sterile (4, 5). Instead, they are home to a unique microbiome of non-pathogenic commensals; the presence of which is normal and not associated with early delivery or adverse pregnancy outcomes (4, 6). Histological and immunological analysis of intrauterine tissues and fluids suggests that the nature and magnitude of inflammatory response associated with bacterial colonization may be key in determining obstetric outcome (6–8).

Recent placental microbiological studies have reignited debate regarding the role of differential virulence (9), poly-microbial interactions (10), host genetics (11), and immune factors (12) in determining obstetric outcome. While attention has primarily been placed on defining the local immune response to bacteria within placental tissues, the role and significance of the maternal systemic immune response to the infection has been largely neglected. Yet, studies conducted at the end of the last century strongly suggested that the maternal immune response to commensal microorganisms found in the urogenital tract in pregnancy – in particular the *Ureaplasma* species – may provide us with important clues as to why some women are at risk of adverse pregnancy outcomes while the majority are not.

In this mini-review, we discuss in detail the somewhat contradictory evidence relating to the presence and nature of maternal antibodies to *Ureaplasma* sp. and their significance in determining and predicting obstetric outcome. A specific focus is placed on the potential clinical utility of serological analysis in the identification of women at elevated risk of PTB.

UREAPLASMA AND PTB

Ureaplasma spp. are generally considered commensal microorganisms (13–16) and are classified into two species and 14

distinct serovars (SV). SV1, SV3, SV6, and SV14 belong to *U. parvum* species and the remaining ten SV to *U. urealyticum* (17). *Ureaplasma* sp. commonly colonize the urogenital tract of both males and females (18, 19). Vaginal colonization rates can vary greatly in non-pregnant women (up to 70%) (20, 21) and women with uncomplicated pregnancies [2.7–70%; reviewed in Ref. (22)]. *Ureaplasma* spp. are some of the most frequently identified microorganisms in placental tissues and amniotic fluids (AF) from pre-term deliveries (23–25). Colonization of the placenta with *Ureaplasma* sp. has been demonstrated to be an independent risk factor for chorioamnionitis [odds ratio (OR), 11.27; 95% CI, 5.09–24.98] (7). Detection rates in AF vary from 0% to 19% in early mid-trimester (26–28), to 2–80% at pre-term labor (26, 29) and 18–100% with pre-labor premature rupture of membranes (PPROM) (26, 30). A meta-analysis of 22 studies found a significant association between the presence of *Ureaplasma* sp. in the vagina and AF with PTB (22). However, *Ureaplasma* sp. colonization in the urogenital tract and AF is a relatively common finding in pregnant women and alone it is not sufficiently predictive of PTB to be clinically useful (8).

The reasons why commensal *Ureaplasma* sp. cause ascending IUI leading to PTB in only a subset of women are still unknown but are likely to be complex and multifactorial (**Figure 1**). SV-specific virulence has been proposed as an important determinant of risk of adverse outcome. Of the two *Ureaplasma* species, *U. parvum* is the most commonly isolated species from clinical samples (31), with two of its SV – SV3 and SV6 – associated with worse pregnancy outcomes (32–34). Unfortunately, most studies of IUI do not differentiate between *Ureaplasma* SV so data on the relationship between SV prevalence and risk are lacking. Poly-microbial interactions may also be significant as diagnosis of abnormal bacterial flora or bacterial vaginosis (BV) (i.e., a high Nugent score) is also a risk factor for PTB (35), independent of *Ureaplasma* sp. colonization status. It is worth noting that poly-microbial colonization of the amniotic cavity is common in pre-term deliveries, with around half of all infected AF containing two or more microorganisms (23). Co-colonization of the vagina with a *Ureaplasma* sp. and another genital *Mycoplasma* sp. has been reported to be associated with more severe adverse pregnancy outcomes compared to colonization with a single organism (36). Competence of the cervical barrier to microbial ascension is also likely to play a role in determining risk of PTB. A short cervix in pregnancy has been associated with increased risks of microbial colonization (37) and spontaneous PTB (38, 39).

Finally, maternal/fetal immunological tolerance or competence is a likely determinant of obstetric outcome associated

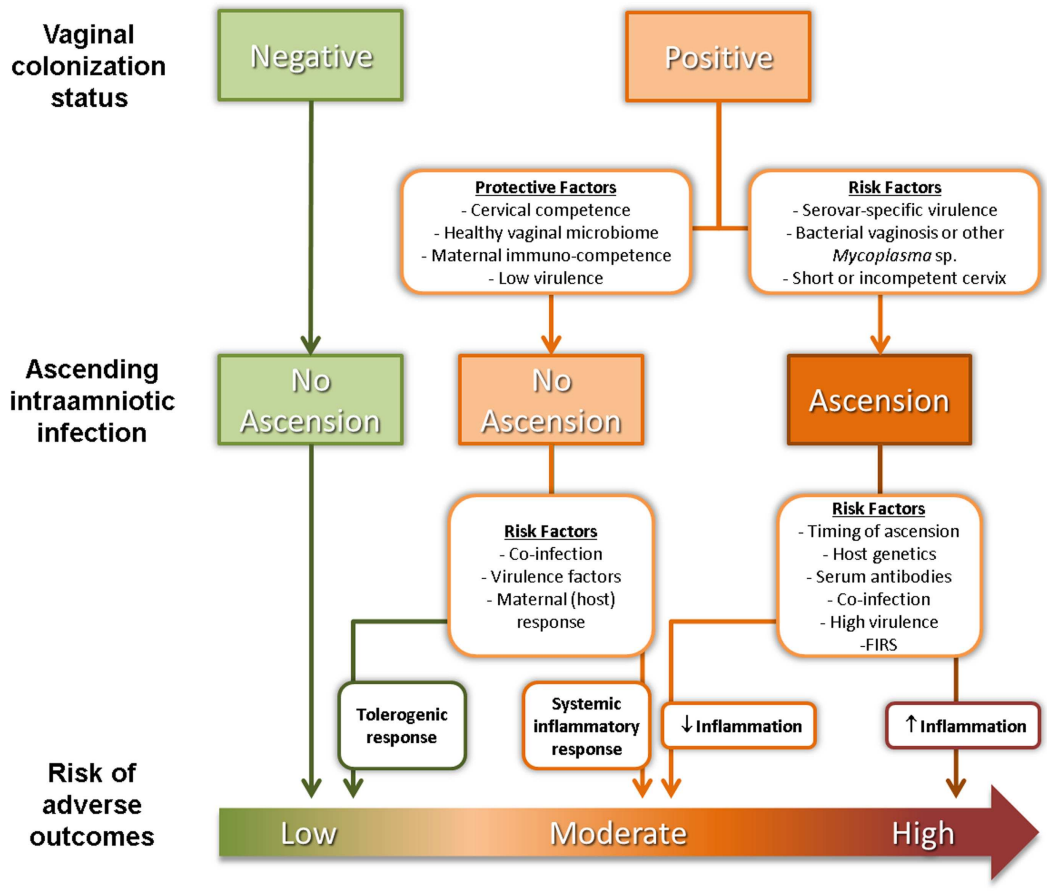


FIGURE 1 | Factors affecting ascension of vaginal *Ureaplasma* sp. and risk of adverse pregnancy outcomes.

with colonization by *Ureaplasma* sp. and other microorganisms. Surface-exposed lipoproteins of *Ureaplasma* sp. activate the pro-inflammatory transcription factor NF-κB through TLR ligation (40, 41), although exposure of intrauterine tissues to *Ureaplasma* sp. does not generally trigger a robust inflammatory response. Nevertheless, intra-amniotic infection with *U. parvum* has been causally linked to chorioamnionitis, FIRS and PTB in animal models (42–44). This is consistent with observations from clinical studies showing that chorioamnionitis/funisitis is more likely in pregnancies infected with *Ureaplasma* sp. as opposed to other bacteria (7). On the other hand, most women with vaginal colonization and local inflammatory response will not deliver preterm (24, 45). Combs, Gravett (8), proposed a model of IUI comprising of five subgroups: (1) microbial colonization plus inflammation (AF IL-6 >11.3 ng/ml); (2) severe inflammation with no detectable microorganisms (AF IL-6 >11.3 ng/ml); (3) mild inflammation with no detectable microorganisms (AF IL-6 of 2.6–11.2 ng/ml); (4) microbial colonization with no inflammation; and (5) absence of infection or inflammation. This more complicated scenario highlights the need to accurately stratify women at risk of PTB associated with IUI.

MATERNAL ANTIBODIES AGAINST *UREAPLASMA* FOR THE PREDICTION OF PTB

ANTI-*UREAPLASMA* SERUM ANTIBODIES IN PATHOGENESIS OR PROTECTION FROM DISEASE

Specific antibodies may be produced in response to a bacterial infection and detected in the serum (seropositivity). Antibodies act to neutralize bacterial toxins, facilitate opsonization, and together with the complement system work toward clearance of the infection. Antibodies generated against surface-exposed *Ureaplasma* sp. epitopes have been identified and shown to inhibit *Ureaplasma* sp. metabolism (46) and support complement mediated *Ureaplasma* sp. clearance (47) suggestive of a protective effect. Interestingly, individuals with the greatest number/intensity of anti-*Ureaplasma* antibody bands by immunoblot appeared to have higher *Ureaplasma* sp. killing ability (47). These data are in contrast with other studies which report that the presence of anti-*Ureaplasma* antibodies in colonized pregnant women are associated with worse pregnancy outcome [reviewed in Ref. (26); see Anti-*Ureaplasma* Antibodies as Biomarkers for IUI and PTB below]. In these circumstances, it is likely that seropositivity is pathologic rather than protective, possibly acting via an exacerbation of inflammatory processes triggering PTB (48). This concept is supported by data from a pregnant sheep model of intra-amniotic *Ureaplasma* sp. infection, in which increased intrauterine inflammation was detected in sheep in which maternal anti-*Ureaplasma* IgG was also detected (9). Alternatively, it may be the absence of a specific antibody that confers protection from PTB. The key to unraveling this confusion is the identification of antigen–antibody characteristics associated with a given pregnancy outcome to determine whether it is antibody abundance (i.e., titer), nature of the target (i.e., virulence factor), onset of response (time of exposure), or capacity to induce microbial lysis and destruction that is associated with risk of PTB.

ANTI-*UREAPLASMA* ANTIBODIES AS BIOMARKERS FOR IUI AND PTB

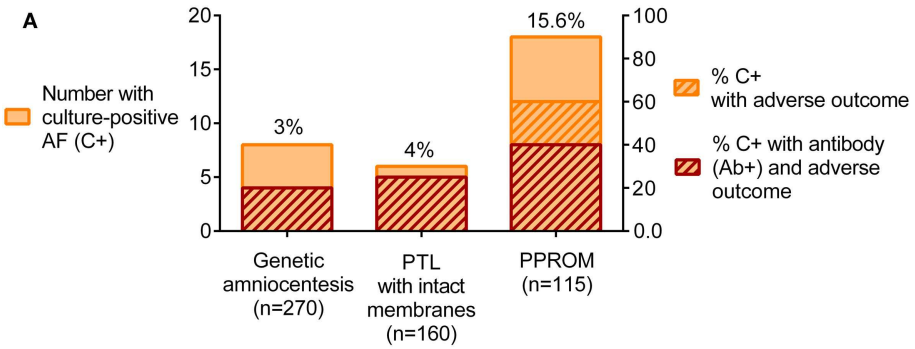
In the 1980–1990s, several research groups investigated the antibody response to *Ureaplasma* sp. as a biomarker for intra-amniotic or urogenital infections (49–51). A review on the topic of assessment of antibodies for identification of intra-amniotic infection with *Ureaplasma* sp. during pregnancy was published in 1994 by Shulamith Horowitz (26). He reviewed the literature and correlated cervical and intra-amniotic *Ureaplasma* sp. colonization rates with the presence of antibody and pregnancy outcome. Specifically, he found that a greater percentage of women had adverse pregnancy outcomes if they had positive *Ureaplasma* sp. AF cultures and elevated serum anti-*Ureaplasma* antibody titers at either genetic amniocentesis, or pre-term labor with/without PPROM (defined as fetal loss, stillbirth, pre-term delivery, or low birth weight) (**Figure 2A**) (26). Odds ratios and relative risks for adverse pregnancy outcome in culture-positive (C+) /antibody-positive (Ab+) women vs. C+/antibody-negative (Ab−) women were calculated based on these data and are presented in **Figure 2B**. Despite some discrepancy between studies, the detection of anti-*Ureaplasma* IgG in maternal sera together with AF colonization was predictive of an increased risk of developing pregnancy complications [OR: 81.00 at genetic amniocentesis ($p = 0.04$); OR: 24.56 ($p = 0.04$); and RR: 2.31 ($p = 0.02$) at PPROM]. Despite these encouraging findings, this line of research has not matured over the proceeding decades for the reasons outline below.

FACTORS THAT HAVE LIMITED THE CLINICAL TRANSLATION OF MATERNAL ANTIBODY DETECTION FOR PREDICTION OF PTB

Unfortunately, routine amniocentesis for the purpose of diagnosing intra-amniotic colonization with *Ureaplasma* sp. is not clinically feasible due to the procedure-associated risk of spontaneous miscarriage (RR 1.60; 95% CI 1.02–2.52) (52). Furthermore, the rates of *Ureaplasma* sp. colonization of AF in mid pregnancy are actually very low (22, 26, 27). As such, consideration has been given to the ability of maternal antibodies alone to stratify pregnant women for risk of PTB.

SEROCONVERSION IN RESPONSE TO *UREAPLASMA* IS COMMON

Early studies varied in their reported rates of seroconversion. ELISA based studies reported anti-*Ureaplasma* IgG seropositivity rates of 50–85% in culture-positive individuals and 6–15% in culture-negative individuals (46, 53, 54). However, these studies reported positive cutoffs based on maximum detection in culture-negative cohorts, assuming culture-negative individuals to be seronegative (53, 54). Others have since shown by immunoblot that seroconversion is more common, with greater than 80% of sera from healthy non-pregnant individuals (47) recognizing at least one *Ureaplasma* sp. antigen (IgG response). As the seroconversion rate is high in both colonized and non-colonized individuals, several studies used a method of serum dilution for assigning positivity to sera with elevated titers (46, 55). It is still unclear whether differences between patients with slightly elevated vs. very high levels of antibodies are clinically significant. As colonization is so common, it is most likely that seropositivity in non-colonized individuals represents a persistent antibody to a cleared infection. Nevertheless, high seroconversion rates mean that detection of



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	Time of sampling		
	Genetic amniocentesis	PTL with intact membranes	PPROM
OR	81.00	33.00	24.56
(95%CI; p-value)	(1.30-5046.71; p=0.04)	(0.44-2470.77; p=0.11)	(1.11-542.43; p=0.04)
RR	9.00	3.67	2.31
(95%CI; p-value)	(0.64-126.85; p=0.10)	(0.33-40.93; p=0.29)	(1.12-4.78; p=0.02)

FIGURE 2 | Pregnancy outcome in relation to culture-positive (C+) AF and anti-*Ureaplasma* antibodies (Ab+) in maternal serum.
(A) *Ureaplasma* sp. detected by culture technique. Antibodies determined by ELISA and semi-quantitation of antibody titer; adverse pregnancy outcome defined as fetal loss, stillbirth, pre-term delivery, and low birth weight. Genetic amniocentesis was performed at 16–20 weeks of gestation; no gestational age provided for the PTL and PPROM groups. **(B)** Odds ratios (OR) and relative risks (RR) for adverse pregnancy outcome in culture-positive/antibody-positive (C^+Ab^+) vs. C^+Ab^- were calculated for each group. Data extracted and adapted from Horowitz et al. (26).

IgG antibody responses to whole cell lysates or isolated membrane antigens is unlikely to be clinically useful.

SEROVAR-SPECIFIC *UREAPLASMA* ANTIGENS AND CROSS-REACTIVITY

U. parvum SV3 and SV6 are most commonly associated with worse pregnancy outcome (32–34). The possibility of a SV-specific antibody response to *Ureaplasma* sp. was proposed following the identification of apparent SV-specific antigens. For example, an 85 kDa protein in SV1, a 71 kDa protein from SV3, 61 kDa and 55 kDa proteins from SV6, and an 88 kDa protein from SV14 have all been identified (56). However, there is extensive antigen cross-reactivity among all SV with the evidence for SV specificity strongest in individual cases. It is likely that the multiple-banded antigen (MBA) is a key virulence factor for *Ureaplasma* sp. (57), containing both SV-specific and conserved/cross-reactive epitopes (58). The MBA, a surface-expressed diacylated lipoprotein, is recognized by TLRs 1, 2, 6 (40), and 9 (41) and has a molecular weight of approximately 70–90 kDa (59), but migrates as a symmetrical ladder pattern between 55 and 75 kDa on immunoblot due to conformational folding (58). The *mba* gene exists as a single copy within the *Ureaplasma* sp. genome (60). The precise immunogenic MBA epitopes are still unknown but are considered to be within the size-variable and surface-exposed C-terminal region (58). Mutations in the *mba* gene have been proposed to play an important role in helping the organism evade the host immune system (60) with multiple mechanisms proposed (58–61).

Serum from *Ureaplasma* sp. colonized individuals detect multiple and likely closely related antigens forming the ladder seen on immunoblots. The identity of specific immunodominant epitopes and their role in differential pathogenesis in pregnancy remains unclear and is further complicated by the variability of the MBA. A number of cases where mother-fetus dyads appeared to be colonized with different SV and generated different antibody responses (as determined by ELISA) have been observed (62). It needs to be remembered that *Ureaplasma* spp. are fastidious organisms, and without careful collection and appropriate culture protocols their presence may have been missed (63). Molecular methods, in combination with routine culture, are now preferred for the detection and speciation of *Ureaplasma* sp. in clinical samples (64).

The lack of robust and reliable techniques to differentiate between SV, the diversity of responses among individuals and the cross-reactivity between SV have significantly compromised the ability to use antibody responses beyond the simple determination of seroconversion. Clarification of the relationship between SV-related antigens, microbial virulence, and differential systemic immune responses will be required to resolve the present impasse.

FETAL ANTIBODY RESPONSES TO *UREAPLASMA* AND OBSTETRIC OUTCOME

While our emphasis has been on the maternal immune response as a determinant of obstetric outcome, fetal systemic responses can undoubtedly contribute to progression to PTB (65). *In utero* exposure to *Ureaplasma* sp. is commonly associated with increased incidence of neonatal complications including bronchopulmonary dysplasia, intraventricular hemorrhage, necrotizing enterocolitis, and pneumonia (66–69). Fetal production of antibody against *Ureaplasma* sp. has been reported (70–72), with antibody detected at birth in neonates as young as 22–27 weeks of gestation. Moreover, antibody titer appears to correlate negatively with neonatal outcome. IgM has been detected in the fetus/neonate consistent with an initial stage of immune recognition (62). In the sheep model, anti-*Ureaplasma* IgG has also been detected in fetal serum, with one case reported where the antibodies in maternal and

fetal sera recognized different antigens (9). Furthermore, fetuses which developed systemic *Ureaplasma* sp. infection [culture-positive cerebrospinal fluid (CSF)] were found in ewes with the lowest numbers of MBA variants in AF and the highest monocyte count in the chorioamnion (61). Thus, monitoring maternal responses alone may fail to identify the fetuses at most risk *in utero*. Unfortunately, acquiring blood antenatally for determination of fetal seroconversion is not a practical approach for assessing risk of PTB.

CELLULAR RESPONSES TO *UREAPLASMA*

Ureaplasma sp. have been shown to stimulate pro-inflammatory responses in fetal membranes (73), choriodecidual explants (74), and preterm and term cord blood (75). However, studies of white blood cell responses to *Ureaplasma* sp. in pregnancy have not proven to be useful to date, with no differences in responsiveness observed in leukocytes from women at risk of PTB compared to those with normal pregnancies (12). Reyes et al. explained differential pathogenesis of *Ureaplasma* sp. in urinary tract infections (UTIs) by demonstrating the presence of two distinct immune cell profiles (76). Asymptomatic UTIs were characterized by minimal monocytic and lymphocytic infiltration, less tissue damage and increased IFN-γ, while complicated UTIs were associated with greater concentrations of pro-inflammatory cytokines, extensive inflammation and predominantly a neutrophilic response. No comparable data from pregnant women have been published to date. Recently, unconventional lipid antigens such as those present in *Ureaplasma* sp. (9, 57), and microbe-derived vitamin B metabolites (77), have also been shown to stimulate T cell responses vital for microbial clearance (78, 79). However, the role of these unconventional T cells in the response to *Ureaplasma* sp. in the context of pregnancy immunity and PTB risk remains unknown and the role of the systemic immune cell responses to *Ureaplasma* sp. exposure in pregnancy remains relatively unexplored.

CONCLUDING REMARKS

Despite advances in our understanding of the causal relationships between intrauterine infection and PTB, major research questions remain unanswered: is there a subpopulation of patients in which *Ureaplasma* sp. colonization correlates with worse disease outcome? Does the site of colonization determine disease outcome? Are there pathogenic and non-pathogenic SV determining disease outcome? Is there a specific aspect of the maternal immune response to *Ureaplasma* sp. infection that influences risk of PTB?

Research on specific *Ureaplasma* sp. SV and virulence factors have failed to yield conclusive results. Despite the earlier clinical promise of antibody-based predictive approaches (26, 62), the assessment of maternal or fetal seropositivity to identify women for prophylactic treatment has not made it into clinical practice. It is unclear when and where exposure to *Ureaplasma* sp. antigens takes place in pregnancy and when/how commensal colonization becomes an infection. As such, it is difficult to define the role of antibodies in pathogenesis. Antibody cross-reactivity among the *Ureaplasma* SV and lack of stringent proof of epitope specificity have also limited attempts to use an individual's antibody response to make a SV-specific diagnosis of infection or predict an outcome.

Before an antibody-based immunological test can be considered as part of a routine antenatal screen, future studies must address technical and scientific issues surrounding the detection and antigen characterization of antibodies to *Ureaplasma* sp. (41, 46, 47, 54, 58). Key issues to be addressed include: (i) need for the identification of the colonizing SV; (ii) requirement for the detection of a SV-specific or global antibody response; (iii) characterization of immunodominant epitopes; (iv) defining the difference between commensal colonization and infection; (v) determining when and where *Ureaplasma* sp. are first immunologically detected; and (vi) characterizing the kinetics and magnitude of the antibody response in relation to pregnancy outcome. Based on current knowledge, it seems that the detection and measurement of uncharacterized maternal antibodies against *Ureaplasma* sp. has limited predictive value for identifying women at elevated risk of infection-driven PTB.

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Oxidative stress damage as a detrimental factor in preterm birth pathology

Ramkumar Menon*

Department of Obstetrics and Gynecology, School of Medicine, The University of Texas Medical Branch, Galveston, TX, USA

Edited by:
Jeffrey A. Keelan, University of Western Australia, Australia

Reviewed by:
Gaurav K. Gupta, Harvard Medical School, USA
Walter Balduini, Università degli studi di Urbino Carlo Bo, Italy

****Correspondence:***
Ramkumar Menon, Department of Obstetrics and Gynecology, School of Medicine, The University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1062, USA
e-mail: ram.menon@utmb.edu

Normal term and spontaneous preterm births (PTB) are documented to be associated with oxidative stress (OS), and imbalances in the redox system (balance between pro- and antioxidant) have been reported in the maternal–fetal intrauterine compartments. The exact mechanism of labor initiation either at term or preterm by OS is still unclear, and this lack of understanding can partially be blamed for failure of antioxidant supplementation trials in PTB prevention. Based on recent findings from our laboratory, we postulate heterogeneity in host OS response. The physiologic (at term) and pathophysiologic (preterm) pathways of labor are not mediated by OS alone but by OS-induced damage to intrauterine tissues, especially fetal membranes of the placenta. OS damage affects all major cellular elements in the fetal cells, and this damage promotes fetal cell senescence (aging). The aging of the fetal cells is predominated by p38 mitogen activated kinase (p38MAPK) pathways. Senescing cells generate biomolecular signals that are uterotonic, triggering labor process. The aging of fetal cells is normal at term. However, aging is premature in PTB, especially in those PTBs complicated by preterm premature rupture of the membranes, where elements of redox imbalances and OS damage are more dominant. We postulate that fetal cell senescence signals generated by OS damage are likely triggers for labor. This review highlights the mechanisms involved in senescence development at term and preterm by OS damage and provides insight into novel fetal signals of labor initiation pathways.

Keywords: oxidative stress, preterm birth, premature rupture of fetal membranes, inflammation, oxidative damage, senescence, senescence-associated secretory phenotype

The World Health Organization recently estimated the global preterm birth (PTB) rate for singleton gestation at 9.6% (1). The PTB rate has increased in the United States by as much as 30% during the last 25 years despite advances in medical care (1, 2). Twenty-eight percent of all neonatal deaths (deaths within the first 7 days of life) that are not related to congenital malformations are due to PTB (1, 2). The most common phenotype of PTB is *spontaneous* PTB of unknown etiology. Approximately 60% of PTBs are spontaneous, and 30–40% of these are preceded by preterm premature rupture of the fetal membranes (pPROM) (3–10). The current management of preterm labor and pPROM is based largely on inhibiting uterine contractions (7, 11–25). This approach has not been successful, as such interventions are usually performed too late in the process to succeed. A second problem with the current management of preterm labor is that only women who have clear risk factors (abnormally short cervixes) or a history of PTBs are targeted for interventions designed to prevent PTB (26–30). The vast majority of PTBs occur in women who are considered low-risk because they are either on their first pregnancies or have only had term births previously (31–37). Although the rate of PTB is lower in these women (3–5%), they make up the largest volume of clinical practice. Simple interventions that can be applied to this group are likely to have the largest impact on PTB rates. Knowledge gaps in current literature about causality and causally linked pathways make it difficult to provide appropriate or personalize interventions based on the specific risk profile of an individual (6).

Risk factors of PTB and pPROM can be classified into two major categories, static and dynamic. As shown in **Figure 1**, all the risk factors outlined in the outermost layer can be called static risk factors as they are unlikely to change during the course of pregnancy. Independently or in combination, these static risk factors can either predispose or cause the dynamic risk factors that are commonly diagnosed as clinical risks or pathologies associated with adverse pregnancy outcomes. Epigenetic changes that are independent of DNA base variations generated by complex interactions between various risk factors during pregnancy can also contribute to dynamic clinical risks by altering expression of certain genes. These changes can transition between static and dynamic risks. Static and dynamic risk factors produce pathways and pathophysiologies depicted in the inner circle with a unique biomarker profile contributing to labor-inducing changes, resulting in PTB or pPROM. The final effector pathways culminating in labor and delivery include inflammation and oxidative stress (OS). In normal pregnancies, these are generated by various fetal and maternal factors that signal the end of pregnancy. In PTB, the maternal–fetal signals and their causal origins are still unclear as they arise from complex etiologies and redundant pathways.

Inflammation is a well-studied pathophysiology of both PTB and pPROM (38–40). This review is an attempt to shed some light on one of the under-studied mechanistic pathways: OS damage to intrauterine tissues and how it may impact pregnancy outcomes.

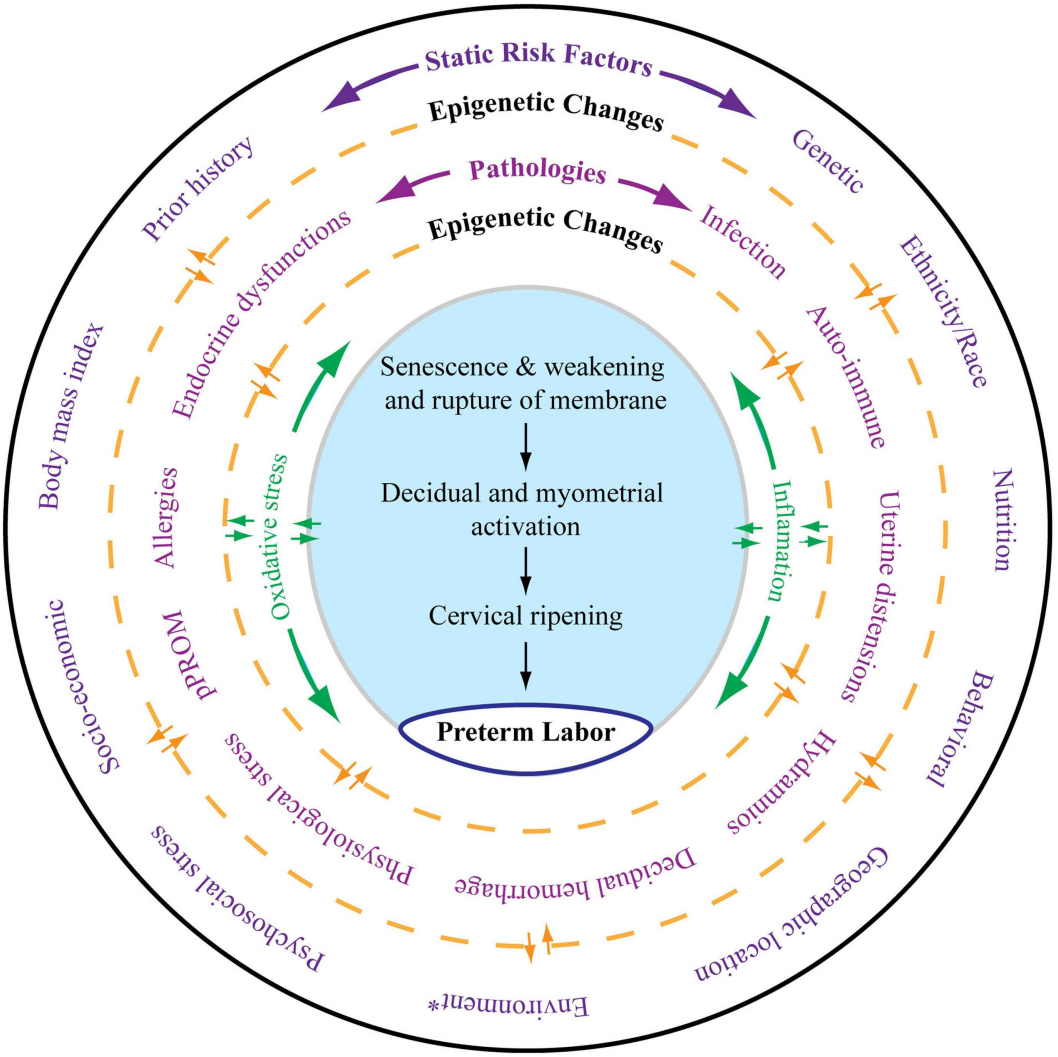

FIGURE 1 | As depicted in this figure, preterm labor (the innermost circle) is an end result of multitudes of complex interacting pathologies and pathophysiologic pathways. The external layer (the outermost circle) shows static risk factors, including epidemiologic and genetic risk factors, that can lead to multiple disease processes as depicted in the middle circle. Epigenetic markers can be dynamic, and that complex interaction between the host environment and risk factors can produce epigenetic changes, which can lead to diseases contributing to final effecter pathways (the blue shaded area). Various diseases may also cause epigenetic changes in the genes of preterm labor pathways. Spontaneous preterm labor leading to preterm birth is a complex syndrome comprising multiple diseases, each of which can be independent initiators of labor-inducing pathways. All these disease processes can trigger inflammation and oxidative stress (OS). However, the extent and biomolecular characteristics of inflammation and OS are dependent on the type of risk that initiated the process, their complex interactions with the genetic–epigenetic system, and the disease that they cause. The final terminal pathways that involve inflammation and OS trigger labor-inducing signals from fetal membranes and decidua and cause cervical ripening, myometrial contractions, and membrane rupture, resulting in preterm labor and delivery. *Any exogenous factor that can impact pregnancy outcomes. Many of the static risk factors in the outer circles are also called environments.

INFLAMMATION, A WELL-DOCUMENTED FEATURE OF LABOR AND DELIVERY

Labor and delivery at term and preterm have an underlying pathophysiology marked by inflammatory mediators. Preterm labor is hypothesized to be driven by overwhelming inflammation that eclipses fetal uterotonic signals of organ maturity. Approximately 50% of PTBs and 70% of pPROMs are associated with intra-amniotic infection (IAI) and inflammation. Histological and microbiological findings indicate that focal infection and inflammation may play a significant role in the pathogenesis of PTB and pPROM. Inflammatory changes that precede PTB – such as leukocyte activation, increased inflammatory cytokines and chemokines, and collagenolysis of the extracellular matrix metalloproteinases (MMPs), resulting in loss of membrane structural integrity, myometrial activation, and cervical ripening – are well documented by various experimental and clinical studies (9, 38, 41). Recent studies have reported that the heterogeneity in the inflammatory response

(cytokines/chemokines, toll-like-receptors, and their interactions) is associated with IAI and PTB risk factors (42–45). PTB and pPROM are well-documented host response diseases in which overwhelming immune activation can trigger labor-associated changes. The biomolecular markers that trigger labor-associated changes are different, and the difference is attributed to both epidemiologic and clinical risk factors.

SIGNIFICANCE OF OS AND OXIDATIVE DAMAGE

Of these risk factors, IAI, behavioral factors (cigarette smoking, alcohol intake, and drug use), obesity, malnutrition or antioxidant-deficient diets, physiologic and psycho-social stressors, environmental pollutants, genotoxic agents, and geographic location are some of the most common and well-studied risk factors of both PTB and pPROM (3, 10). The role of inflammation induced by these risk factors in PTB pathophysiology is well studied and multiple biomarkers are reported to be associated with the adverse outcome. OS, characterized by generation of reactive oxygen species (ROS), is an inseparable component of inflammation. Generation of ROS as a part of the aerobic energy building process is inevitable and is well balanced (redox status) by an array of enzymatic and non-enzymatic antioxidant systems (46–50). Redox balance is maintained through the production and subsequent elimination of ROS. Cells are able to protect themselves against OS by the finely tuned regulation of redox status through endogenous enzymes, antioxidants, and other cellular mechanisms. At low levels, ROS, often generated in biological systems, is essential for cell division and survival, cell signaling, inflammation and immune functions, autophagy, and stress response (50–52). However, an overwhelming redox imbalance compromises a biological system's ability to detoxify these highly reactive molecules or to repair any damage caused by them (53). Therefore, the former is termed "OS" and the latter "oxidative damage" (see Glossary) (54). Oxidative damage due to ROS generation has been linked to the development of adult diseases, including cardiovascular disease, cancer, chronic inflammation, and neurologic disorders. The two main sources of ROS in human cells are: (1) mitochondrial and (2) non-mitochondrial. Mitochondria generate ROS as a byproduct of the electron transport chain during respiration, and the rate of ROS production is proportional to the rate of mitochondrial respiration. The ROS production rate is thus higher when metabolic rates are high (55, 56). A vast majority of ROS in the human body is produced by mitochondria, and mitochondria are the primary sources of superoxide production other than phagocytic cells during innate immune defense.

Figure 2 depicts the mechanism of ROS generation, antioxidant system, and potential damage by ROS that can cause damage during pregnancy complications. The figure also models the potential of ROS in generating inflammation and damage to cellular elements that can generate pathways leading to pPROM or PTB. This figure represents only a few key mediators of ROS generation and does not include all known endogenous antioxidants.

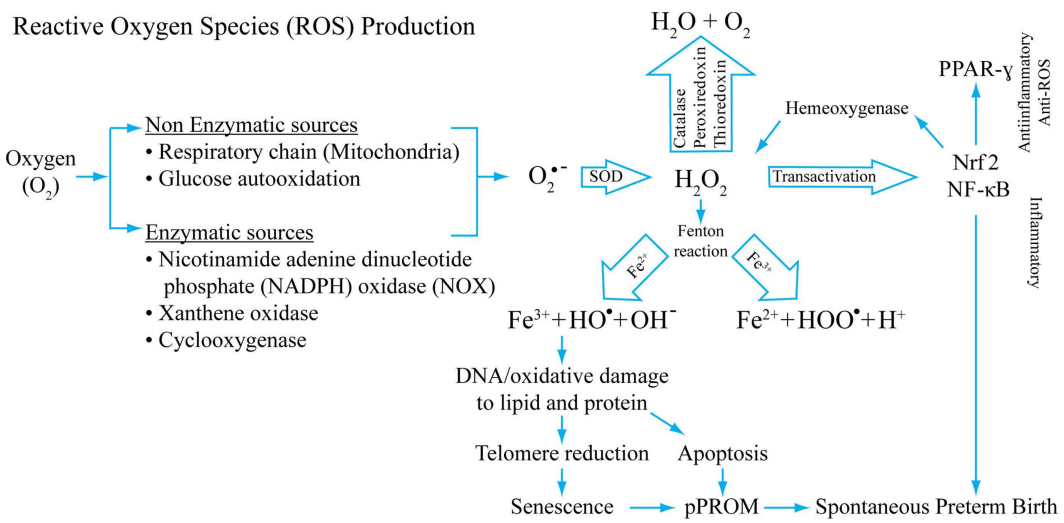

FIGURE 2 | A model of reactive oxygen production, its inactivation by antioxidant systems, and potential consequences of redox imbalances. Superoxide radicals are generated during normal cellular respiration (mitochondrial) and also during infection following phagocytosis. Non-enzymatic and enzymatic mechanisms generate super oxide radicals (O_2- from O_2) that are converted into H_2O_2 by superoxide-dismutase (SOD). H_2O_2, a reactive oxygen species (ROS), is reduced to water by several different enzymes, mainly by catalase. Hydroxyl and/or superoxide radicals are created from H_2O_2 by a Fenton reaction that includes Fe^{3+}. H_2O_2 is a potential transactivator and can activate a master transcription factor NF-κB, resulting in activation of several proinflammatory genes including many uterotonic genes. The Hydroxyl and/or superoxide radicals can cause DNA, protein, and lipid damage. DNA damage as detailed in this review can lead to telomere reduction and senescence. DNA damage of the cell can also cause apoptosis. One of the transactivator proteins is a nuclear factor of erythroid 2-related factor 2 (Nrf2). When activated in response to ROS, this protein can transactivate Hemeoxygenase-1 that can also neutralize H_2O_2, providing a regulatory mechanism. Our studies have shown that Nrf2 can cause anti-inflammatory PPAR-γ activation. Depending on the dose and type of ROS, inflammatory and/or anti-inflammatory/antioxidant pathways may be initiated. This model shows that OS-associated pathologic events result in spontaneous preterm birth and pPROM. This is not a universal model for all OS-related pathologies. Damage to cellular elements has been well documented in cancers and other diseases.

HETEROGENEITY IN ANTIOXIDANT FUNCTIONS

Generation of ROS is tightly regulated by an array of enzymatic and non-enzymatic ways to keep redox balance. This balance in humans is maintained by a well-balanced antioxidant system that is responsible for homeostatic balance, which maintains physiologic functions but prevents oxidative damage. Antioxidants, in general, are substances that decrease the severity of OS by forming less active radicals or by quenching damage created by free-radical chain reactions. They can slow down or prevent damage to body cells and lower the risk of infection by improving immune function. Antioxidants can be classified into three major categories: (1) low molecular antioxidants [glutathione (GSH), vitamins C and E, bilirubin, and urate], (2) enzymes that neutralize free radicals [superoxide-dismutase, catalase, glutathione peroxidase (GPx), DT diaphorase, and peroxiredoxin], and (3) non-enzymatic proteins (thioredoxin, glutaredoxin, and metllothionines). Antioxidants can also be classified as primary and secondary. Primary antioxidants are endogenous enzymes [e.g., super oxide dismutase (SOD), catalase, and GPx] acting at the site where free radicals are formed. Secondary antioxidants are exogenous molecules obtained through food (e.g., Vitamins A, C, E, carotenoids). As detailed in **Table 1**, regardless of primary or secondary, endogenous or exogenous status, antioxidants have specific functions.

WHY ANTIOXIDANT SUPPLEMENTATION FAILS IN PREVENTING ADVERSE PREGNANCY OUTCOMES?

A healthy pregnancy is characterized by a stable balance between ROS and antioxidants (46, 47, 49). Redox imbalance is an underlying pathologic feature of many pregnancy complications. All of the PTB and pROM risk factors detailed above are capable of causing redox imbalance, leading to the production of superoxide, hydrogen peroxide, hydroxyl ions, and nitric oxide that can damage collagen matrix and consume antioxidant defenses. These events can trigger uterine contractions (labor), leading to PTB. Based on this imbalance theory, antioxidant clinical trials have been primarily carried out to compensate for deficiencies and to maintain redox balances. However, these trials expected to reduce OS and improve pregnancy outcomes have met with minimal success (36, 57–61). Numerous reasons can be cited for the failure of these trials, but the following primary factors are highlighted:

1. OS-induced tissue injury or damage is the likely pathology and trigger of several downstream effects like initiation of labor or membrane rupture. OS damage generates a vicious cycle of events (enhanced inflammation, cell death, and tissue destruction), and antioxidants do not regulate these events and are unlikely to impact OS damage. Therefore, it is unlikely that reversing OS through antioxidant supplementation would reduce the risk of adverse pregnancy outcomes.
2. Clinical trials are conducted based on the generalized theory that OS is an underlying pathology. This theory is unlikely; this may be the case for a limited subset of subjects with specific exposures with ROS as an initiator of downstream events.
3. Biomarker screenings for ROS are not done prior to intervention to determine the type of ROS or to assess the amount of ROS-associated damage. Even the type of biomarker measures may not be reflective of risk exposure or the extent of ROS because these biomarkers may represent an end stage indicative of tissue damage and not necessarily risk.
4. Like inflammation, ROS is not a homogeneous phenomenon. Different tissues and cells, cytoplasmic and nuclear membranes, and the contents of different organelles (inner and outer membranes of mitochondria) may react differently to various stressors, producing distinct patterns of ROS. The antioxidants used in trials may curtail a particular ROS pathway or production of a specific free radical, but depending on the type of risk factor (e.g., cigarette smoke vs. poor nutrition), the ROS pathway and antioxidant requirement may be different.
5. Reactive oxygen species-associated damage can be any of the major functional elements mentioned earlier, and the degree of damage is likely dependent on the site, risk factors, and types of triggers that initiate an ROS-mediated response.

Table 1 | Antioxidants and their potential roles associated with reactive oxygen species (ROS) mediated damage.

Antioxidants	Potential role
Super oxide dismutase (SOD) Glutathione peroxidase (GPx) Catalase (Cat) Peroxiredoxin	Direct protection against ROS
Glutathione Vitamin C	Non-specific reduction
Vitamin E β-Carotene	Protection against lipid peroxidation
Transferrin Lactoferrin Ferritin Metallothionein (MT)	Metal sequestration
DNA repair enzymes Macroxyproteinases Glutathione transferase (GST) Thioredoxins (Trx)	Repair enzymes

AMBIGUITY IN ANTIOXIDANT AND OS DAMAGE MARKER ANALYSIS

Many studies have reported antioxidant deficiencies as a causal factor associated with adverse pregnancy outcomes. In the wake of a failed antioxidant supplementation trial, my laboratory conducted a cross-sectional study examining two antioxidant enzymes (SOD1 and catalase) and two OS damage markers (F2-Isoprostane – lipid peroxidation product – and 8-OxodG – DNA damage). Banked maternal plasma samples were collected from subjects at the time of delivery admission for either a term labor ($n = 19$), preterm labor ($n = 18$), or pPROM ($n = 13$). An ELISA-based analysis was performed using these samples. If each of the markers is analyzed independently, multitudes of interpretations can be derived. For example, if SOD was the only analyte tested, as shown in **Figure 3A**, SOD was higher at term and reduced significantly in PTB and pPROM. These data are normally interpreted as heightened OS at

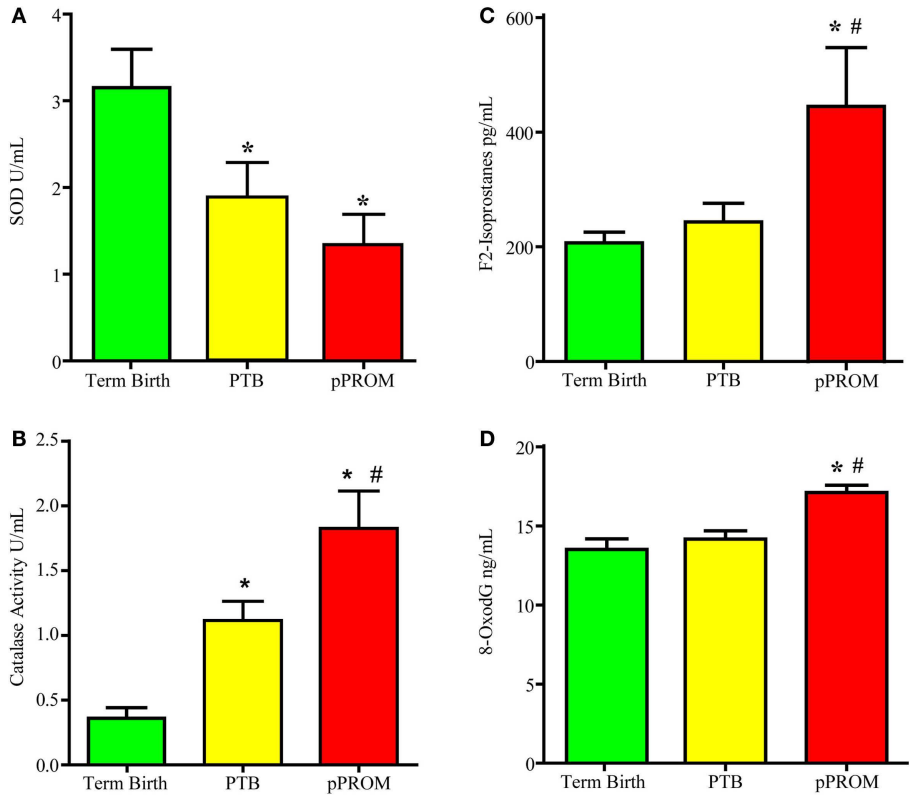

FIGURE 3 | Maternal plasma levels of two antioxidants super oxide dismutase (SOD) and catalase and two OS markers, 8-oxodG and F2-Isoprostanes. **(A)** Antioxidant enzyme Super oxide dismutase concentrations. **(B)** Antioxidant enzyme catalase concentrations. **(C)** Lipid peroxidation product F2-Isoprostane concentration. **(D)** bDNA oxidation product 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) concentration. $^{*}P < 0.05$ compared to term birth; $^{\#}P < 0.05$ compared to PTB.

pPROM, followed by PTB, and none at term birth. If catalase was the only marker analyzed using the same samples (**Figure 3B**), term birth has the highest OS, followed by PTB, and none at pPROM, which highlights the ambiguity associated with singe marker analysis of OS. Even examining the two enzymes together only provides conflicting data regarding OS during pregnancy. This interpretation did not take metabolic demands, prooxidant levels, the status of other antioxidants, or existing damage due to ongoing OS into consideration. It is important to note that measurement of OS in biological fluids may not be a true reflection of cellular level OS as it is difficult to measure. Redox balance is a coordinated function of multitudes of molecules, and examining antioxidants alone may not be sufficient to gage the intensity of OS. However, examining the OS-induced damage can generate better conclusions regarding OS than examining antioxidant levels alone. **Figures 3C,D** demonstrates the evidence for OS-induced generation of DNA and lipid peroxidation products (8-OxodG and F2-Isoprostanes, respectively). Our analysis showed that maternal plasma OS damage is higher in pPROMs than in both normal term births and PTBs. To note, the damages are minimal at term birth although labor and delivery are documented to have higher OS. It is expected that at term labor and delivery the metabolic demands are very high, and mature fetuses and fetal tissues of the intra uterine cavity generate OS and OS-induced signals from cellular damages. However, at term, a normal physiologic redox balance is fully functional and thus minimizes the OS-induced damage to cellular elements. Risk-induced OS and overwhelming damage generated signals are unlikely to be controlled by cellular or organ-level antioxidant capacity, leading to premature activation of signals for PTB or pPROM.

CAN OS DAMAGE LEAD TO ADVERSE PREGNANCY OUTCOMES?

Oxidative stress and OS-induced damage can produce a spectrum of genetic, metabolic, and cellular responses, and prooxidants exert their effects on cellular elements – namely lipids, proteins, and nucleic acids – disrupting their expression, structure, and function (46, 54, 62). Peroxidation of proteins leads to the loss of the sulfhydryl groups and linking of carbonyl groups with the side chains of other amino acids (63, 64). Although these proteins are normally proteolytically cleared from the system, under heightened ROS, oxidized proteins do not undergo proteolysis but rather accumulate as long hydrophobic bonds that affect cell function (63).

Cell membrane phospholipids are always a target of ROS activity (48). The peroxidized cell membrane stiffens, loses its selective permeability, and loses its integrity. Oxidized cell membranes are also susceptible to action by phospholipase enzymes that can

activate a series of enzymatic and non-enzymatic breakdowns of oxidized phospholipids. One of the non-enzymatic by-products of this lipid peroxidation is F2-Isoprostane, which is considered the biomarker of ROS (65).

Reactive oxygen species causes the most lethal damage on DNA. This damage can result in single- or double-strand breaks, interchanging of sister chromatids, crosslinking of DNA to DNA or protein, or base modifications (66–68). Although all four bases of DNA are susceptible to ROS-mediated alterations, hydroxylated nucleotide 8-hydroxy-deoxyguanine (8-OHdG) was for the first time referred to as a major product of oxidative DNA damage (see Glossary), and high concentrations of 8-OHdG in biological fluids are considered a biomarker of ROS (69–80). Damages in DNA are constantly repaired, and simply measuring 8-OHdG levels is not sufficient to measure the extent of ROS; however, higher concentrations of 8-OHdG clearly indicate an underlying pathology that cannot be ignored.

DOCUMENTATION OF OS DAMAGE IN PTB, pPROM, AND NORMAL TERM BIRTH

This section of the review explains some of the ongoing basic research studies designed to address OS-induced damages and how they differentially associate with PTB, pPROM, and normal term birth (81, 82). Briefly, we examined amniotic fluid samples for OS markers (lipid peroxidation – F2-Isoprostanes) and OS-induced 3-nitrotyrosine modified proteins (3-NT) in fetal membranes from PTB, pPROM, and term birth. Both PTB and pPROM had higher amniotic fluid F2-Isoprostanes than term birth. Cigarette smoking during pregnancy was associated with higher F2-Isoprostanes, regardless of pregnancy outcomes, and infection (in PTB). It was also associated with higher F2-Isoprostanes, compared to PTB with no infection. 3-NT staining, a marker of protein modification by OS, was more similar between term birth and pPROM than PTB.

In an earlier study, we reported changes associated with telomere length, a marker of aging, and OS. Telomeres are DNA–protein complexes, consisting of 5–15 kbp of repetitive DNA sequences, located at the ends of the chromosomes. They are essential for chromosome stability and cell survival, protecting them from end-to-end fusion and degradation. With each cell division, telomeres are shortened, and once a critical shortening is attained, division cycles halt and cell senescence (see Glossary) is triggered. Thus, telomere lengths serve as a valid marker of a cell's biologic age. OS can induce single and double DNA strand breaks, which are detrimental to telomere shortening. Therefore, telomere shortening can be caused by natural aging or pathologically induced premature aging due to OS.

Interestingly, telomeres are shorter in early pPROM (<34 weeks) than in gestational age-matched PTB with intact membranes. However, telomere lengths are similar between early pPROMs and term births (>40 weeks), and it is suggestive of a similarity between the two phenotypes of pregnancy (83). Several such similarities in molecular and histologic markers exist between pPROM and normal term birth that lead us to hypothesize that pPROM may have early aging fetal tissues that may be prompting rupture and delivery.

We have examined likely causes and mechanisms of telomere shortening in fetal tissues. The next sections of this review will describe how OS-induced damages and tissue level changes may generate signals of parturition during normal pregnancies and how they may be pathologic in pPROM and a subset of PTB.

OS-INDUCED DNA DAMAGE AND ITS CONSEQUENCES

As detailed in previous sections, OS causes changes in DNA bases, especially Guanine. We have tested the generation of 8-OxoG formation in fetal amnion cells in response to OS. **Figure 4** provides experimental evidence for DNA damage and its likely consequences. The OS response was induced in laboratory conditions using water soluble cigarette smoke extract (CSE) that is a known OS producer and a well-known risk factor of adverse pregnancy outcomes. Primary amnion cells obtained from placentas from normal term births, not Cesarean births, were used for this study (84). When exposed to CSE, amnion cells produced ROS (**Figure 4A**). This ROS production was down-regulated by antioxidant N-acetyl cysteine treatment, confirming OS production by amniocytes. These amnion cells also demonstrated significant DNA damage (**Figure 4B**), and the DNA lesions contained 8-OxoG (**Figure 4C**). High 8-OxoG levels may explain the shortened telomere length observed in a prior report, as these repetitive sequences are guanine rich and susceptible to ROS (83). To verify the shortening of telomeres, we treated the cells with CSE. CSE-induced telomere reduction was visible after 96 h compared to untreated controls, confirming that OS-inducing risk factors may cause oxidation of DNA that can lead to telomere attrition (**Figure 4D**). Telomere uncapping occurs when cells have critically shortened telomeres or when telomere-protective factors are impaired (85). Loss of telomere function can induce cell cycle arrest and senescence. One of the consequences of DNA damage and telomere attrition is also formation of DNA damage foci (DDF) in cells.

We have also analyzed the consequences of DNA damage and telomere attrition in amnion cells. DNA damage is a regular event during growth, and it is precisely fixed or repaired by multitudes of DNA repair mechanisms (86–92). However, persistent DNA damage in response to overwhelming OS or in response to OS-inducing risk factors, as often seen during adverse pregnancies, causes DNA breaks followed by the phosphorylation of the histone H2AX, which is a variant of the H2A protein family known as γ-H2AX. Analysis of amnion cells that lost telomere fragments after exposure to CSE revealed generation of DDF (**Figure 4E**). Signals arising from cells with DDF will eventually determine the fate of the affected cell through multitudes of pathways activating several cell cycle regulatory molecules (93–97). Analysis of multiple cell cycle regulatory factors revealed activation of p38 mitogen activated protein kinase pathway (p38MAPK) in amnion cells in response to CSE exposure (**Figure 4F**). p38MAPK activation by phosphorylation of its catalytic site residues, threonine-180 and tyrosine-182, promotes cell cycle arrest and cellular senescence by targeting the expression of proteins of SP (**Figure 4G**) (98, 99). Activation of p38MAPK to phosphorylated p38MAPK (pp38MAPK) by SP was prevented by antioxidant NAC confirming the influence of OS in p38MAPK activation. It is interesting to note that we did not see activation of another pro-senescence protein p53 in amnion cells treated with CSE although this has been reported in maternal decidual cells in animal models of pregnancy by SK Dey's group.

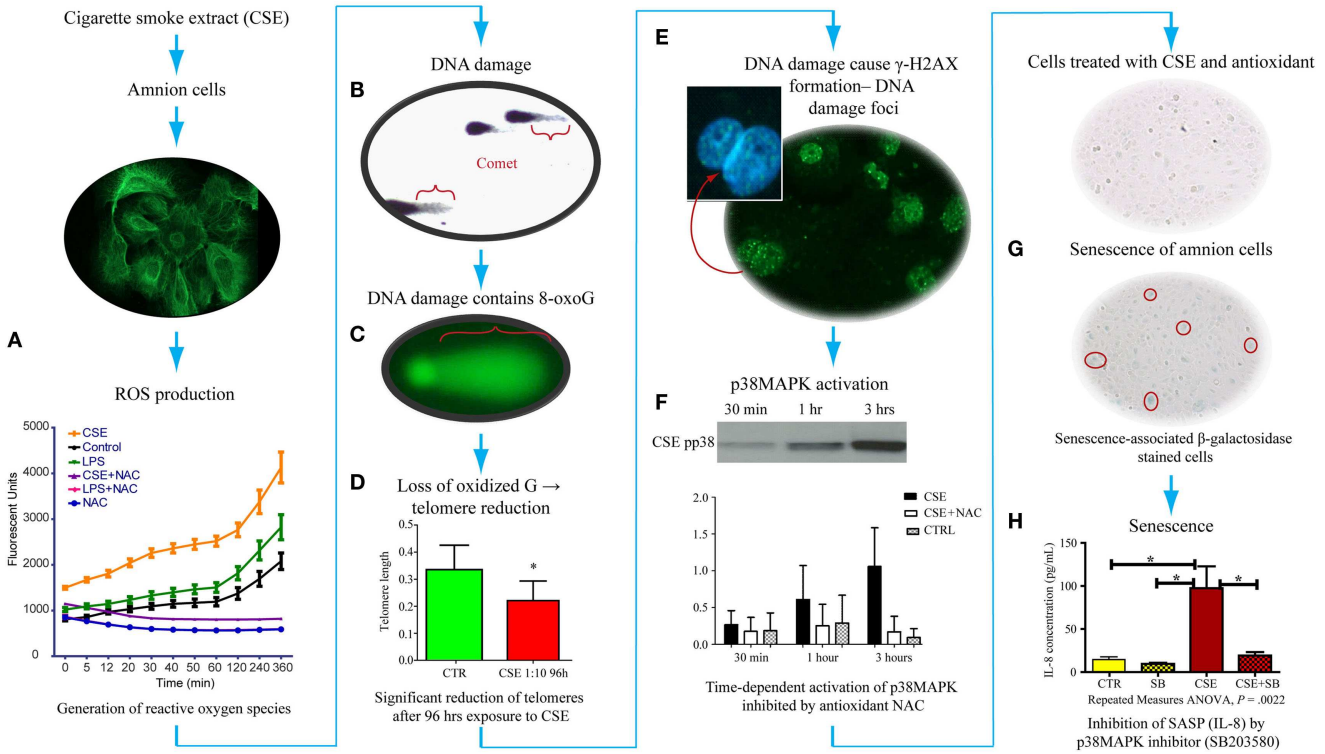

FIGURE 4 | Proposed pathways of senescence activation in human amnion primary epithelial cell cultures. **(A)** Amnion cell cultures established from normal term Cesarean section placental membranes (cytokeratin stained) were exposed to cigarette smoke extract (CSE), and reactive oxygen species (ROS) generation was measured. ROS kinetics were monitored using 2′-, 7′-dichlorodihydrofluorescein diacetate (H2DCFDA) as an indicator for ROS in cells. Changes in DCF fluorescence were expressed as arbitrary fluorescence units. CSE-induced ROS generation was inhibited by N-acetyl cysteine. LPS also induced ROS but at a much lower level than CSE. **(B)** We performed two separate experiments to document: (1) DNA damage (Comet assay-silver staining) and (2) 8-OxoG production (FLARE) in amnion cells for Comet and FLARE. Amnion cells were harvested after treatment with CSE and immobilized in a layer of low melting point agarose on the FLARE slide. We demonstrated Comet formation after CSE treatment of amnion cells, confirming DNA damage with CSE. **(C)** Fragment length analysis using repair enzyme (FLARE) assays showed a similar Comet formation that confirmed 8-oxoG production, one of the most lethal biomarkers, in response to CSE, indicating 8-oxoG:OGG1 complex activity. **(D)** Exposure of these cells to ROS (by CSE) led to reduction of telomere length ($P < 0.05$), confirming a similar observation reported in fetal membranes from pPROM. **(E)** Telomere reduction by ROS can cause DNA damage foci (DDF) formation, indicating chronic OS damage. Amnion cells showed phosphorylated histone B protein (g-H2AX). **(F)** These amnion cells exposed to CSE also demonstrated a time-dependent activation of p38MAPK that persisted for 96 h. This activation was inhibited by NAC, confirming ROS-mediated effect. **(G)** p38MAPK (a pro-senescence protein) caused development of senescence-specific b-galactosidase staining in cells, confirming senescence of amnion epithelial cells after CSE exposure. Senescence development led to the production of one of the cytokines associated with senescence-associated secretory phenotype (SASP). **(H)** We confirmed that this is a SASP or p38-mediated effect when IL-8 was down-regulated after treating amnion cells with CSE and p38MAPK inhibitor (SB203580).

ACTIVATION OF SENESCENCE IN FETAL CELLS BY p38MAPK

p38 Mitogen activated kinase is a pluripotent molecule and one of its functions is to activate senescence (aging) of the cell. Senescence is characterized by irreversible arrest of cell growth (99–103). Irreversible growth arrest at the G1 phase of the cell cycle is a major characteristic of senescence. Unlike apoptosis, these cells persist, alter their function, and change the tissue environment, inducing a unique signature of inflammatory markers similar to those seen in PTB and pPROM (104, 105). The amniochorionic cells divide at a rapid rate throughout gestation to accommodate the increasing demands of the intrauterine contents. This proliferative activity is a normal process that is expected to continue even at term (106). Overwhelming OS forces cells to undergo transformation either as malignant or senescence (107). Hostile pregnancy environments promote senescence of membrane cells and activation of an inflammatory condition that we propose causes adverse pregnancy outcomes as seen predominantly in early pPROM and a subset of PTBs complicated by chronic OS. We were able to document senescence of amniotic cells exposed to CSE using senescence-associated β-Galactosidase (SA-β-Gal) staining (**Figure 4G**). The number of SA-β-Gal positive cells was significantly higher after CSE treatment than untreated control amnion cells, confirming the development of senescence phenotype. Morphologic examination using transmission electron microscopy revealed features of senescence in fetal membrane cells, characterized by enlarged cells with senescence-associated heterochromatic foci, enlargement of organelles, and particularly endoplasmic reticulum and mitochondria. Senescing cells are also reported to produce a unique inflammatory signature known as senescence-associated secretory phenotype (SASP) (see Glossary) (104, 108, 109). SASP markers

include cytokines, chemokines, growth factors, angiogenic factors, MMPs, and tissue inhibitors of matrix degrading enzymes, adhesion molecules, receptors, and receptor antagonists. Many SASP factors are reported as inflammatory mediators of pregnancies, and therefore it is not surprising to see more of them during preterm and term labors. Treatment of amnion cells with CSE and p38MAPK inhibitors (SB203580) substantially reduced IL-8 to the level of controls (**Figure 4H**). IL-8 generation as a part of CSE treatment and controlling its production through p38MAPK pathways suggests that the inflammatory process associated with term labors, preterm labors, and pPROMs can also arise in the absence of infections, and it is likely generated by senescing cells of the placenta and fetal membranes. Based on these findings and ongoing research in the laboratory, I propose that the source of inflammation is not just invading immunocytes, but it is likely that senescing fetal cells generate signals of parturition at term. In pPROM or PTB, a pathologic trigger resulting in OS can cause premature fetal cell senescence and SASP signaling onset of labor or cause rupture.

DIFFERENCES BETWEEN PTB AND pPROM; CAN OS DETERMINE OUTCOMES?

As described in previous sections, there are several similarities between early pROM and term birth. Similarly, several salient differences exist between PTB and pPROM although they have epidemiological and clinical similarities. Many of the similarities and differences are listed in **Table 2**. Inflammation is an underlying factor in PTB and pPROM; however, the extent of OS damage may be higher in pPROM, especially those that are early (<34 weeks of gestation). OS and inflammation are inseparable events, and biomarkers executing these responses are linked. It is also important to note that PTB is not devoid of any of the markers or indications listed in **Table 2**. In comparison with pPROM, the degree of OS and proteolysis seems to be more minimal in PTB than in pPROM. These findings are limited to early PTB and pPROM. Data are more inconclusive in cases between 34 and 37 weeks of gestation, late PTB, and pPROM. Based on the data used in *in vitro* models and *in situ* findings, pPROM seems to result from chronic OS whereas PTB results from acute OS. Chronic and overwhelming OS can cause telomere attrition, DDF formation, p38MAPK activation, fetal cell senescence, and SASP whereas acute OS may be sustainable by cells' ability to resist OS-induced damages but still develop an inflammatory environment that is different than SASP. The former is expected to cause pPROM, and the latter may likely lead to PTB. In light of this evidence, it is also possible that infection is not the causal factor for a large subset of pPROMs and PTBs. Non-infectious cellular atrophies due to OS or OS-induced senescence cause sterile inflammation that can generate an immunocompromised intrauterine setting. A recent report by Romero et al. supports the concept of sterile intra-amniotic inflammation (110). When a sterile inflammatory milieu is created, it may provide an ideal environment for microbial invasion or activation of resident microbial flora in which case infection is likely secondary to an underlying pathology.

The delineation of PTB and pPROM pathways depends on several factors and are not limited to the: (1) type and load of risk factors, (2) antioxidant status of subject, (3) gestational age, (4) overall immune status of the individual, or (5) genetic, epigenetic, and other socio-demographic factors. Based on the data described above, a new model of PTB and pPROM delineating pathways are described in **Figure 5**. These cases can be divided into subsets based on OS/inflammation and OS/inflammation-induced damages; therefore, overlap between subsets are expected and rupture or lack of rupture in PTB may depend on an individual's profile as listed in the above paragraph. OS-induced DNA damage can cause telomere uncapping; however, depending on the strength of ROS and redox imbalance, pathways can arise in a telomere-dependent and independent way. Telomere-dependent pathways will result if base excision repair mechanisms fail to restore the damaged segments and the cells proceed to undergo cell cycle arrest and eventual cell death. Data from our laboratory demonstrate classic signs of telomere attrition and telomere damage-induced changes (8-oxoG accumulation, γ-H2AX, and nuclear membrane structure related Lamin B loss) in pPROM membranes or in amniotic fluid (data not shown). Many of these factors are either minimal or non-existent in a majority of PTB cases. Telomere-independent pathways can arise where DNA damage is minimal or where base excision repair mechanisms are functional and able to rebuild the damaged segments. During this process, 8-OxoG generated due to oxidative damage of telomere segments or G bases in other parts

Table 2 | Markers studied in various maternal–fetal compartments and their similarities and differences between PTB with intact membranes and preterm premature rupture of the membranes (pPROM).

Biomarker	Sample	Indication	pPROM vs. PTB
F2-IsoP	Amniotic fluid	Oxidative stress	Higher in pPROM
3-NT staining	Fetal membranes	Oxidative stress	Higher in pPROM
Salivary proteases	Maternal saliva	Proteolysis	Higher in pPROM
MMPs/TIMPs	Amniotic fluid	Proteolysis	Higher in pPROM
Cytokines (IL-1, TNF, IL-6, IL-8)	Amniotic fluid	Inflammation	No difference
Telomere length	Fetal DNA and fetal membranes	Senescence	Higher in PTB
p38MAPK	Fetal membranes	Senescence	Higher in pPROM
p53	Fetal membranes	Senescence	Higher in pPROM
8-OxodG	Maternal plasma	DNA damage	Higher in pPROM
8-Oxoguanine glycosylase (OGG1)	Fetal membranes	DNA damage repair	Higher in PTB
Ras-GTPase	Fetal membranes	Intracellular signaling	Higher in PTB
Fas/Fas L	Fetal membranes	Apoptosis	Higher in pPROM

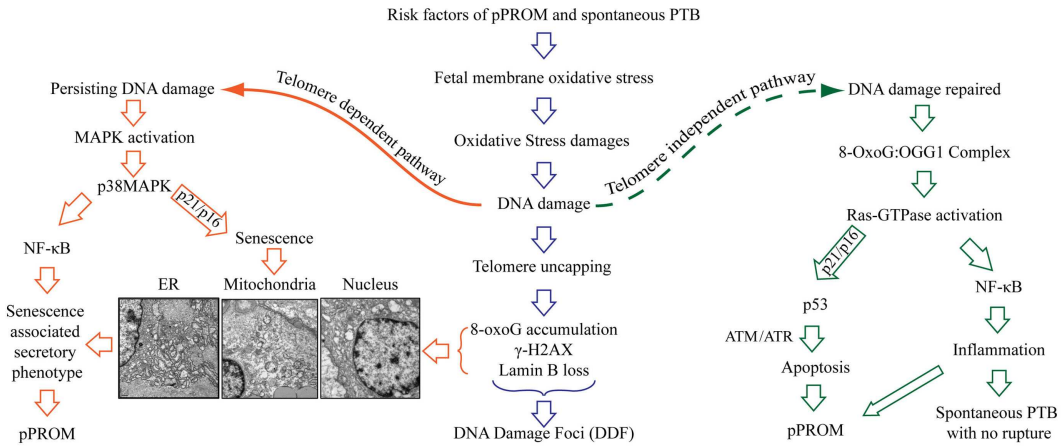

FIGURE 5 | Oxidative stress-induced pathways of pPROM and spontaneous PTB with intact membranes. Risk factors of pPROM and PTB – like infection, obesity, poor nutrition, stress, behavioral risk factors (cigarette smoking, drinking, and drug abuse) – can cause oxidative stress (OS) in human fetal membranes that leads to OS-induced damages to all major cellular elements (lipids, proteins, and nucleic acid). Damages to DNA are especially lethal to fetal membranes and placental cells because they lead to oxidation of guanine base, resulting in mutagenic 8-OxoG formation. Telomeres are rich in G bases, and oxidation G bases lead to telomere attrition as a part of damage repair [base excision repair (BER)]. The pathway of pPROM and PTB may be delineated at this stage based on the type of risk and the type of OS response: overwhelming OS (chronic OS with smoking, alcohol use, obesity, poor nutrition, etc.) vs. infection/inflammation-related acute OS by fetal cells or immunocytes. Overwhelming OS can lead to persistent DNA damage and telomere reduction, 8-OxoG formation, resulting in DNA damage foci (DDF) formation. Telomere-dependent pathway – when OS is too high to be controlled by antioxidant mechanisms of the intrauterine tissues, especially fetal membranes, a telomere-dependent pathway arises where either OS itself or damaged DNA and other cellular elements can trigger p38MAPK activation either through NF-κB activation or direct cellular senescence. This is characterized by rounded, swollen organelles, and nuclear condensates. Additionally, senescing cells generate a unique set of biomarkers (senescence-associated secretory phenotype – SASP). SASP is characterized by cytokines, chemokines, matrix metalloproteinases, growth and angiogenic factors, and eicosanoids, all of which are involved in promoting labor. Telomere-independent pathway – in this pathway, oxidized Gs are excised from the damaged region by 8-oxoguanine glycosylase (OGG1) as a part of BER. 8-OxoG:OGG1 complex then activates Ras-GTPase and either promotes p53-mediated apoptosis or NF-κB-mediated inflammation. The exact mechanism of this switch is still unclear. Apoptosis is previously linked to pPROM. The latter pathway of NF-κB-mediated inflammation can be linked mostly to PTB with intact membranes.

of DNA are repaired by a specific enzyme called 8-Oxoguanine glycosylase (OGG1) (88, 111–113). Recent findings suggest that 8-OxoG:OGG1 complex can cause Ras-GTPase activation, resulting in inflammation (114–116). Fetal membrane analysis of OGG1 mRNA expression also revealed more OGG1 in PTB than in pPROM, suggesting that DNA repair is more active in PTB than in pPROM due reduced availability of OGG1 (117). In unpublished findings, we also noticed higher Ras-GTPase activation in fetal membranes from PTB than pPROM. Although we have not confirmed this descriptive data through mechanistic studies, it is also possible that telomere-independent activation of DNA damage repair may cause two separate events as shown on the right side of **Figure 5**.

8-OxoG:OGG1 complex (DNA damage repair) can cause Ras-GTPase activation culminating in either antitumor p53 (proapoptotic) activation, resulting in pPROM, or culminating in master transcriptional factor NF-κB activation, resulting in inflammation without apoptosis. Earlier reports have shown evidence of apoptosis in fetal membranes from pPROMs through p53 pathways (118–123). Therefore, it is likely that both apoptosis and senescence may be seen in pPROM and will be a subset (based on exposure and host response). It is also important to note that we did not document active p53 in membranes from pPROM in our studies reported a decade ago. However, we did see effector caspase activation suggesting that apoptosis and senescence may both have independent roles in pPROM outcomes. NF-κB activation and its contribution are well reported by several investigators (124–130). NF-κB activation can occur by multitudes of risk factors' specific signaling routes in PTB and pPROM or by maternal–fetal endocrine signals of initiation of parturition at term. DNA damage repair associated signals may also cause NF-κB activation resulting in PTB and pPROM, but this is not a well-studied mechanism in PTB and pPROM.

CAN FETAL CELL SENESCENCE TRIGGER TERM BIRTH?

Senescence of fetal membrane cells are very pronounced in term (>40 weeks) deliveries but not in term Cesarean sections when the patient is not in labor. Fetal membranes and placentas experience considerable OS at term prior to initiation of labor, which can lead to p38MAPK activation similar to that seen in pPROM membranes, causing senescence and further enhancement of SP and SASP. Fetally derived SASP factors are signals of maturation and can promote labor at term. The inflammatory milieu observed at term is of non-infectious origin, and senescence/SASP is likely one of the factors contributing to this phenomenon. Ongoing work in our research laboratories will further elucidate this mechanism, and understanding this phenomenon will improve our knowledge of the labor process and likely allow us to develop screening and diagnostic markers and identify intervention targets based on OS leading to p38MAPK and senescence.

SUMMARY

Oxidative stress is an inevitable component of pregnancy, and it is tightly regulated until labor and delivery. OS build-up at term due to an increasing demand from the fetus and heightened physiologic stress promotes senescence. Fetal tissues, placentas, and maturing fetuses generate signals of aging prompting labor and delivery. These signals we report here as SASP are mediators of uterotonic activities. These SASP signals may very well be regulated or influenced or in tandem with already reported biologic, endocrine regulated mechanisms of labor triggers (131–135). Risk factors of adverse pregnancies may cause premature aging of fetal tissues, triggering pathological mechanisms (specifically pPROM and in a subset of PTB with high OS damage as demonstrated by our data) that may result in premature activation of labor and/or rupture of the membranes. Reduction of adverse outcomes requires better characterization of biomolecules and pathways to understand their precise roles in triggering premature aging.

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GLOSSARY

OXIDATIVE DAMAGE

Reactive oxygen species generated due to various physiologic and pathologic processes can lead to damage of major cellular elements, including DNA, RNA, proteins, and lipids, causing dysfunctional molecules that impact cellular integrity. Oxidative stress damage is associated with several disease processes and is also seen in the natural aging process.

OXIDATIVE DNA DAMAGE

Reactive oxygen species often target the DNA and cause oxidatively damaged DNA base lesions. Guanine is most prone to have oxidative damages, primarily 8-oxo-7, 8-dihydroguanine (8-oxoG). Telomeres, the chromosomal protective caps, are rich in Guanine bases, and they succumb to oxidative damage. Telomere attrition, seen in certain pregnancy complications, denotes oxidative stress during pregnancy complications.

SENESCENCE

Senescence or biologic aging involves the phenomenon of irreversible arrest of cell growth. Unlike apoptosis, these cells persist, alter their function, and change the tissue environment, inducing a unique signature of inflammatory markers. Placental and fetal tissues at term experience intensified oxidative stress and cellular damage, resulting in senescence. Senescent fetal cells can be considered as fetal signals of maturation to initiate labor.

SENESCENCE-ASSOCIATED SECRETORY PHENOTYPE

Cells undergoing senescence produce a distinctive tissue microenvironment that transforms these cells into inflammatory cells characterized unique biochemical markers. These markers include cytokines, chemokines, growth and angiogenic factors, matrix degrading enzymes, and inhibitors among other classes of proteins. Fetal cell senescence can cause senescence-associated secretory phenotype (SASP) production with uterotonic properties and acts as signals of labor initiation.

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Drugs to block cytokine signaling for the prevention and treatment of inflammation-induced preterm birth

Pearl Y. Ng†, Demelza J. Ireland *† and Jeffrey A. Keelan

King Edward Memorial Hospital, School of Women's and Infants' Health, University of Western Australia, Perth, WA, Australia

Edited by:
Masaaki Murakami, Hokkaido University, Japan

Reviewed by:
Hideki Ogura, Yale University, USA
Yasunobu Arima, Osaka University, Japan

****Correspondence:***
Demelza J. Ireland, King Edward Memorial Hospital, School of Women's and Infants' Health, University of Western Australia, 374 Bagot Road, Subiaco, Perth, WA 6008, Australia
e-mail: demelza.ireland@uwa.edu.au

† Pearl Y. Ng and Demelza J. Ireland have contributed equally to this work.

Preterm birth (PTB) at less than 37 weeks of gestation is the leading cause of neonatal morbidity and mortality. Intrauterine infection (IUI) due to microbial invasion of the amniotic cavity is the leading cause of early PTB (<32 weeks). Commensal genital tract *Ureaplasma* and *Mycoplasma* species, as well as Gram-positive and Gram-negative bacteria, have been associated with IUI-induced PTB. Bacterial activation of Toll-like receptors and other pattern recognition receptors initiates a cascade of inflammatory signaling via the NF-κB and p38 mitogen-activated protein kinase (MAPK) signaling pathways, prematurely activating parturition. Antenatal antibiotic treatment has had limited success in preventing PTB or fetal inflammation. Administration of anti-inflammatory drugs with antibiotics could be a viable therapeutic option to prevent PTB and fetal complications in women at risk of IUI and inflammation. In this mini-review, we will discuss the potential for anti-inflammatory drugs in obstetric care, focusing on the class of drugs termed "cytokine suppressive anti-inflammatory drugs" or CSAIDs. These inhibitors work by specifically targeting the NF-κB and p38 MAPK inflammatory signaling pathways. Several CSAIDs are discussed, together with clinical and toxicological considerations associated with the administration of anti-inflammatory agents in pregnancy.

Keywords: chorioamnionitis, cytokine suppressive anti-inflammatory drugs, intrauterine inflammation, intrauterine infection, NF-κB inhibitors, preterm birth

INTRODUCTION

Preterm birth (PTB), delivery prior to 37 weeks of gestation, is estimated to affect 5–15% of pregnancies worldwide (1) and remains the leading cause of morbidity and mortality of neonates (2) and the second largest direct cause of death in children under 5 years (3). There are many pathological pathways which can lead to PTB, including intrauterine infection (IUI), uterine ischemia, uterine over-distension, abnormal allogeneic recognition and allergic reactions, cervical disease, and endocrine disorders (4). Whilst interventions such as progesterone therapy and ultrasound cervical monitoring are now utilized in many high-risk clinics in developed countries, they are not specific to a particular PTB etiology (5). IUI and inflammation has been casually linked to early PTB and account for approximately 40% of all spontaneous PTB (6, 7). Microbial invasion of the amniotic cavity, most commonly with ascending vaginal microorganisms, activates pattern recognition receptors (PRRs) which induce the production of pro-inflammatory mediators leading to the premature activation of labor and ultimately PTB [reviewed in Ref. (8)]. Neonatal health and development is further compromised if chronic exposure of the fetus to these inflammatory mediators results in fetal inflammatory response syndrome (FIRS) (9, 10). It is likely that optimal pregnancy outcomes will come from the development of therapeutic strategies that are cause-specific and targeted to women at risk and likely to benefit from the treatment.

There is growing interest in therapeutic interventions that target the inflammatory labor cascade by blocking the production of pro-inflammatory mediators or up-regulating anti-inflammatory mediators and/or the exogenous administration of anti-inflammatory or pro-resolution mediators. In their review of anti-inflammatory agents for the prevention of labor, Rinaldi et al. (8) concluded that progesterone was the most likely compound to progress into mainstream clinical use. Although progesterone treatment reduces the incidence of preterm delivery (11), its ability to block inflammatory signaling associated with infection is unclear. In a previous review, we concluded that while the use of anti-inflammatory agents for the treatment and/or prevention of PTB appears promising, pre-clinical studies demonstrating clear benefits and lack of toxicity are needed (12). This mini-review will revisit the benefits and risks of administration of anti-inflammatory drugs in obstetric care, focusing specifically on the emerging class of drugs termed "cytokine suppressive anti-inflammatory drugs" or CSAIDs. These inhibitors, when administered in conjunction with an effective antibiotic regimen,

Abbreviations: ATP, adenosine triphosphate; COX, cyclooxygenase; CSAID, cytokine suppressive anti-inflammatory drug; FIRS, fetal inflammatory response syndrome; IκB, inhibitor of NF-κB; IKK, IκB kinase; IL, interleukin; IRAK, IL-1R-associated kinase; IUI, intrauterine infection; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MCP, monocyte chemoattractant protein; MKK, MAPK kinase; MMP, matrix metalloproteinase; NAC, *N*-acetyl cysteine; NBDI, NEMO binding domain inhibitor; NEMO, NF-κB essential modulator; NF-κB, nuclear factor κB; NSAID, non-steroidal anti-inflammatory drug; OxZnl, 5z-7-oxozeaenol; PAMP, pathogen-associated molecular pattern; PTB, preterm birth; PTL, preterm labor; PG, prostaglandin; SSZ, sulfasalazine; TAK, TGFβ activated kinase; TGF, transforming growth factor; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF, TNF receptor-associated factor.

have the potential to both prolong pregnancy and improve neonatal outcomes.

INTRAUTERINE INFECTION AND INFLAMMATION

One in four preterm infants is born to a mother with IUI (13). These infections commonly arise via the ascending route of infection where bacteria from the cervicovaginal fluid ascend, breach the cervical barrier, colonize the amniotic fluid, and invade fetal membranes and in some cases the fetus (14). IUI also occurs via hematogenous dissemination with trans-placental transfer (15). Chorioamnionitis is the hallmark feature of IUI and is typically diagnosed after delivery by histopathology as the infiltration of leukocytes into the fetal membranes; histological chorioamnionitis severity is positively correlated with intra-amniotic infection, fetal inflammation, and poorer pregnancy outcomes (16, 17). While bacteria are the major organism responsible for chorioamnionitis, viruses and yeast are also capable of causing intrauterine inflammation.

Genital *Mycoplasma* and *Ureaplasma* species are some of the most commonly isolated organisms from amniotic fluid in cases of infection-induced PTB (7), although the appearance of these, and numerous other bacteria (7, 18), in amniotic fluid does not necessarily denote causation (19). Evidence suggests that the extent of bacterial colonization, route of infection, and the stimulatory capacity of the bacteria all play key roles in the activation of maternal and fetal pro-inflammatory signaling cascades which induce production of pro-inflammatory cytokines (e.g., IL-1β and TNF-α) and chemokines (e.g., IL-8 and MCP-1), which in turn promote prostaglandin (PG) production and myometrial contractility, ripening of the cervix, and degradation of the fetal membrane extracellular matrix leading to preterm labor (PTL) (20). The importance of cytokine and chemokine signaling in the pathogenesis of infection-induced PTL is well established and has been thoroughly reviewed in Ref. (14, 21, 22). Microorganism-specific pathogen-associated molecular patterns (PAMPs) are sensed by trans-membrane PRRs, e.g., Toll-like receptors (TLRs) (23, 24), with ligation resulting in recruitment of adaptor proteins [IL-1R-associated kinase (IRAK)1, IRAK4, and TNF receptor-associated factor (TRAF6)] and activation of TAK1 kinase (**Figure 1**). TAK1 then mediates the phosphorylation and activation of the IκB kinase complex (IKK), which comprises of two catalytic subunits (IKKβ and IKKα) and a regulatory subunit IKKγ (25). The IKK complex phosphorylates IκB-α, targeting it for degradation, allowing NF-κB heterodimers to dissociate and translocate to the nucleus to drive inflammatory gene expression (26). TAK1 kinase can also phosphorylate and activate the mitogen-activated protein kinases (MAPKs), MKK3 and MKK6 that subsequently activate p38 MAPK (27). Although there is some evidence that p38 MAPK is involved in intrauterine inflammatory activation of fetal membranes (28), the exact mechanism of activation in gestational tissues and pregnancy is unknown and likely varies according to the nature of the stimulatory agent.

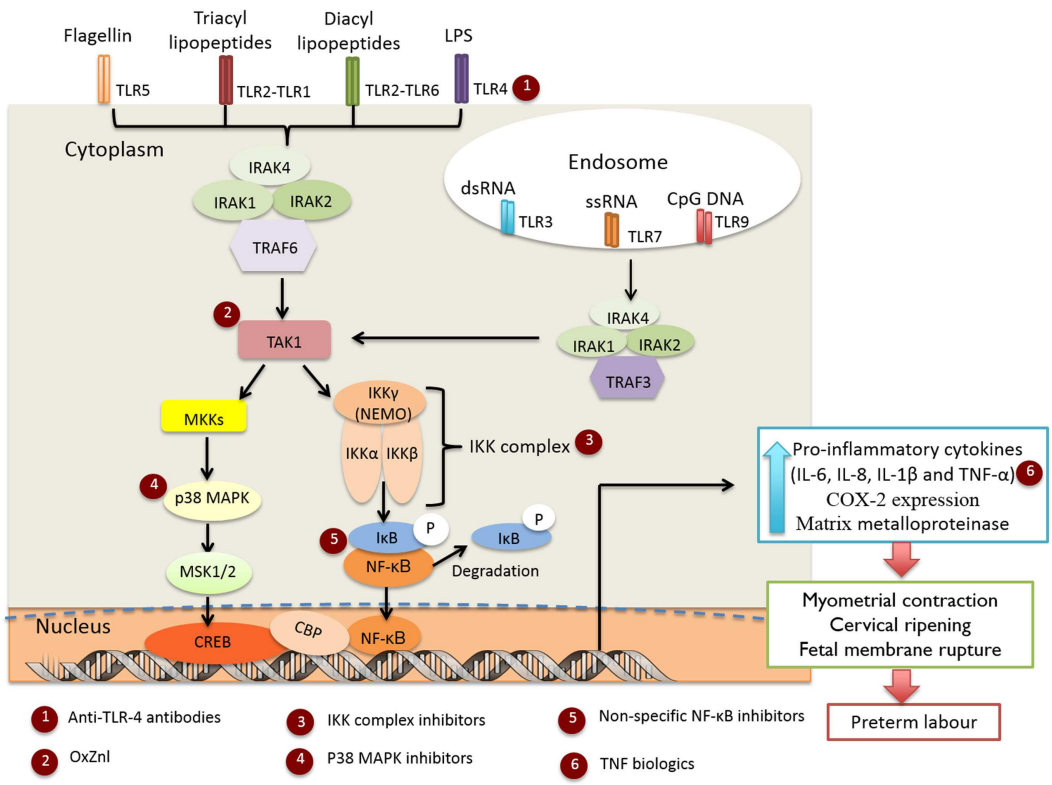

FIGURE 1 | Infection-induced preterm labor triggered by activation of TLR-mediated NF-κB and p38 MAPK inflammatory signaling cascades. Targets for the selected anti-inflammatory agents are indicated in red circles.

TARGETING PRO-INFLAMMATORY SIGNALING FOR PREVENTION OR TREATMENT OF PTB

Antibiotic treatment is routinely given to women presenting with PTL (29, 30). However, it is not the infection but the subsequent inflammation that initiates PTL and is primarily responsible for adverse neonatal outcomes. The use of non-steroidal anti-inflammatory drugs (NSAIDs) to inhibit PG synthesis provided initial evidence that the use of anti-inflammatory drugs may help to delay PTB (31, 32). However, significant pregnancy complications and adverse fetal side effects have been associated with their use (33) as summarized in Table S1 in Supplementary Material. The following sections consider a number of promising alternative anti-inflammatory agents with potential for use in preventing inflammation-driven PTB.

NON-SPECIFIC NF-κB INHIBITORS

N-acetyl cysteine (NAC) is a non-specific free radical scavenger and NF-κB inhibitor (34–36) (**Figure 1**, indicated by red circle at position 5). Treatment of fetal membranes with NAC has been shown to inhibit lipopolysaccharide (LPS) and γ-irradiation killed *E. coli*-induced inflammation (37, 38). NAC has been tested clinically in pregnancy, but has not progressed into mainstream clinical use (39) and the clinical findings have not yet been replicated in other studies. Sulfasalazine (SSZ), a salicylate drug that blocks NF-κB activation by directly inhibiting the IKK kinases (40), is well tolerated and approved for use in pregnancy, with no discernible increase in risk of fetal congenital defects, morbidity, or mortality (41). SSZ treatment has been shown to reduce both LPS-induced placental inflammation in an explant model (42), and the incidence of preterm delivery in a mouse model of PTL (43). However, increased levels of chorionic apoptosis has also been reported in a human membrane model after SSZ exposure (20 h), suggesting that prolonged treatment may result in eventual membrane degradation and loss of function and structural integrity (42).

TLR4 ANTAGONISTS

TLR4 activation by LPS is the most commonly used IUI model and accordingly TLR4 antagonism has been assessed for therapeutic potential (**Figure 1**, red circle at position 1). Studies of TLR4–LPS inhibition using a monoclonal anti-TLR4 antibody found treatment effective *in vivo* in reducing pro-inflammatory mediator (TNF-α, IL-8, and PGE_2) production in amniotic fluid (44), and the incidence of LPS-induced PTB (45). Alternate TLR4 antagonists include eritoran tetrasodium (46) and TAK-242 (47), neither of which have been examined in this context. IUI and inflammation can be triggered by a range of PAMPs, while TLR4 antagonism is only appropriate in cases of Gram-negative bacteria-induced PTL.

TNF-α BIOLOGICS

Conflicting reports exist regarding the efficacy of anti-TNF-α antibodies to decrease the incidence of PTB in murine models (48, 49). Drugs blocking the production of pro-inflammatory TNF-α are used in pregnancy (50, 51), but the complexity of cytokine interactions associated with PTL suggests that targeting individual cytokines may not be the most optimal therapeutic intervention (**Figure 1**, red circle at position 6). Interestingly, clinical studies have reported that maternal administration of antibody-based TNF-α biologics (e.g., infliximab) persist in the neonatal circulation for many weeks after birth (52) and may therefore dampen both intrauterine and fetal inflammation protecting the fetus from the adverse sequelae of IUI and inflammation. There is little evidence for congenital abnormalities with the use of anti-TNF-α therapy during pregnancy (53), but high levels in fetal circulation may increase risk of neonatal infection. The consequences of such treatments for the developing immune system need to be fully considered.

CSAIDs: A NOVEL CLASS OF ANTI-INFLAMMATORY DRUGS

As a class of compounds, CSAIDs specifically target the NF-κB and p38 MAPK signaling pathways to inhibit cytokine-mediated events with demonstrated efficacy in a range of animal models (54–56). These agents are now being examined for their potential to be more effective and selective than NSAIDs for the inhibition of inflammation-driven PTB, as they directly target signaling molecules leading to the activation of the NF-κB and p38 MAPK inflammatory cascades without interfering with the constitutive/homeostatic roles of prostanoids (**Table 1** and **Figure 1**). Importantly, depending on the route of administration and placental transfer properties, CSAIDs may have the potential to block intra-amniotic and fetal inflammation, thereby protecting the fetus from the adverse sequelae of exposure to inflammatory mediators.

p38 MAPK inhibitors

The first p38MAPK inhibitor investigated in human extraplacental membranes was SKF-86002, a potent inhibitor of p38 MAPK and less potent inhibitor of cyclooxygenase-2 (COX-2) and 5-lipoxygenase activity (75). This led to research into the use of similar inhibitors, which selectively bind to the adenosine triphosphate (ATP) site of p38 MAPK, to block the placental production of pro-inflammatory cytokines (**Figure 1**, red circle at position 4). Lappas et al. (28) reported that treatment of LPS-stimulated human fetal membranes with SB202190 inhibited the release of IL-6, TNF-α, and PGs, whilst we demonstrated that SB239063 inhibited the production of IL-6, TNF-α, and PGE_2 at both the maternal and fetal faces of human fetal membranes stimulated with γ-irradiation-killed *E. coli* (38). This suggested that p38 MAPK may be a useful pharmacological target for prevention of PTL; however, caution is warranted as MAPKs are also involved in many aspects of cell function and signaling, including placental growth and differentiation (59, 60).

IKK complex inhibitors

A short, membrane-permeable NEMO-binding domain inhibitor (NBDI) peptide that spans the IKKβ NEMO-binding domain disrupting interaction between NEMO and IKKβ (76) (**Figure 1**, red circle at position 3), is effective in ameliorating inflammatory responses in ear swelling (77) and colitis (63) mouse models. Recently, NBDI was also shown to inhibit LPS and *Ureaplasma parvum*-induced PGE_2 production in ovine gestational membranes (38) but not γ-irradiation-killed *E. coli*-induced pro-inflammatory responses in *ex vivo* human fetal membranes (38); differences in binding affinity or endogenous protease activity in human fetal membranes may explain differential efficacy observed.

Table 1 | Cytokine suppressive anti-inflammatory drugs (CSAIDs) with potential for the prevention or treatment of PTB.

CSAID	Formula (molecular weight, kDa)	Mode of action	Anti-inflammatory effects in IUI and other models of inflammation	Potential side effects
SKF-86002	$C_{16}H_{12}FN_3S$ (297.4 kDa)	Inhibits p38 MAPK, COX-2, and 5-LO enzymes (57)	↓ IL-1β and PGE_2 production by LPS-stimulated human fetal membranes (58), ↓ IL-1β from endotoxin-stimulated human macrophages (58)	Downstream MAPK inhibitory effects on placental growth and differentiation (59, 60)
SB202190	$C_{20}H_{14}FN_3O$ (331.3 kDa)	Selectively binds to the ATP-binding pocket of p38α and β isoforms (61)	↓ IL-6, TNF-α, PGE_2, and $PGE_{2\alpha}$ production by LPS-stimulated human fetal membranes (28), ↓ IL-6, IL-8, PGE_2, and PGF2α secretion in macrophage-exposed annulus fibrosis, cells in response to TNF-α (62)	Downstream MAPK inhibitory effects on placental growth and differentiation (59, 60)
SB239063	$C_{20}H_{21}N_4O_2F$ (368.4 kDa)	Selectively binds to the ATP-binding pocket of p38α and β isoforms (61)	↓ IL-6, TNF-α, and PGE_2 production by γ-irradiation-killed *E. coli* stimulated human fetal membrane Transwell model (38)	Downstream MAPK inhibitory effects on placental growth and differentiation (59, 60)
NBDI	Synthetic peptide corresponding to the NEMO amino-terminal alpha-helical region (3780.4 kDa)	Binds to IKKβ NEMO-binding domain and inhibits kinase activity by disrupting the interaction of IKKβ with NEMO (63)	↓ PGE_2 production in LPS and *Ureaplasma parvum*-stimulated ovine gestational membrane Transwell model (38), ↓ IL-6, TNF-α, and IL-1β production and expression in a dose-dependent manner in a mouse model of inflammatory bowel disease (63)	Inhibition of NF-κB constitutive activity resulting in non-specific toxicity (64)
Parthenolide	$C_{15}H_{20}O_3$ (248.3 kDa)	Binds to IKKβ and inhibits kinase activity by covalent modification of the cysteine 179 reside in the kinase activation loop (65)	↓ Inflammatory gene expression and production in primary choriodecidual cells (66), ↓ LPS-induced IL-6 and TNF-α in a mouse model (67), ↓ TNF-α and COX-2 expression in TNF-α-stimulated human urothelial cells (68)	Inhibition of NF-κB constitutive activity resulting in non-specific toxicity (64)
TPCA-1	$C_{12}H_{10}FN_3O_2S$ (279.9 kDa)	Selectively binds to the ATP pocket of the IKKβ kinase (69)	↓ Inflammatory gene expression and production in primary choriodecidual cells (66), ↓ TNF-α and PGE_2 in fetal side of human fetal membrane Transwell model (38), ↓ IL-8 and PGE_2 LPS ovine gestational membrane explant model (38), ↓ PGE_2 in a 2-day LPS ovine pregnancy model (70), ↓ IL-1β-induced MMPs expression and NF-κB nuclear translocation in corneal fibroblasts (71), ↓ IL-1β, IL-6, TNF-α, and IFN-γ in mouse model of collagen arthritis (69)	Inhibition of NF-κB constitutive activity resulting in non-specific toxicity (64)
OxZnl	$C_{19}H_{22}O_7$ (362.4 kDa)	Selectively binds to the ATP pocket of TAK1 kinase (72)	↓ PGE_2 production by LPS-stimulated ovine model of pregnancy (70), ↓ COX-2 production in mouse model of picryl chloride-induced ear swelling (72), ↓ IL-1-induced COX-2 production in mouse embryonic fibroblast (72), ↓ CD40L-induced IL-6, MCP-1, and ICAM1 in mouse model of vascular injury (73)	Downstream inhibitory effects on MAPK activity involved in cell differentiation and apoptosis (74)

OxZnl, 5z-7-oxozeaenol; ATP, adenosine triphosphate; COX, cyclooxygenase; CSAID, cytokine suppressive anti-inflammatory drug; IFN, interferon; IL, interleukin; IUI, intrauterine infection; IKK, IκB kinase; LPS, lipopolysaccharide; MMP, matrix metalloproteinase; MAPK, mitogen-activated protein kinase; MCP, monocyte chemoattractant protein; NBDI, NEMO-binding domain inhibitor; NEMO, NF-κB essential modulator; NF-κB, nuclear factor κB; PG, prostaglandin; TAK, TGFβ-activated kinase; TNF, tumor necrosis factor.

TPCA-1 (69) and parthenolide (65) are specific IKKβ inhibitors (**Figure 1**, red circle at position 3) shown to inhibit LPS-induced inflammation and NF-κB nuclear translocation in primary choriodecidual cells (66). Unlike SSZ, no impairment of cell viability, apoptosis, or expression of anti-apoptotic genes was detected in these studies (66). Addition of TPCA-1 to the fetal compartment of a human fetal membrane Transwell model was also reported to inhibit γ-irradiation-killed *E. coli*-induced TNF-α and PGE_2 production in the fetal compartment, and to a lesser extent in the maternal compartment (38). TPCA-1 also blocked LPS- and *U.*

parvum-induced IL-8 and PGE_2 production in an ovine gestational membrane model (38), demonstrating that this pharmacological strategy is likely to work across a wide range of microbial stimuli and different PAMPs. Significantly, in an ovine model of LPS-induced chorioamnionitis, intra-amniotic administration of TPCA-1 was found to inhibit production and accumulation of PGE_2 in amniotic fluid and leukocytosis of the fetal membranes (70). However, at the doses employed, no significant changes in amniotic fluid or fetal circulating cytokine concentrations were observed.

TAK1 inhibitors

The TAK1 kinase complex is unique to the TLR-mediated activation pathway and offers an excellent pharmacological target within the p38 MAPK and NF-κB pro-inflammatory signaling cascades to block premature activation of labor, without the downstream effects of IKK inhibition on constitutive NF-κB activity (**Figure 1**, red circle at position 2). The upstream location of TAK1 also suggests that blockade of activity is likely to exert broad-spectrum anti-inflammatory effects against the range of microbes and stimuli associated with IUI and inflammation. Gene deletion of TAK1 impairs IKK and NF-κB activity, subsequently blocking pro-inflammatory cytokine release and expression (78). To date, availability of pharmacological TAK1 inhibitors is extremely limited; 5z-7-oxozeaenol (OxZnl), a resorcyclic acid lactone and selective inhibitor of TAK1 kinase, appears to be the most promising (79) and has been shown to inhibit pro-inflammatory mediator production in murine fibroblasts (72), primary cortical neurons (80), dermal fibroblasts (81), and ear swelling models (72). In an ovine model of LPS-induced chorioamnionitis, we recently demonstrated intra-amniotic treatment with OxZnl to reduce amniotic fluid levels of PGE_2 and fetal membrane leukocyte infiltration (70), although intra-amniotic cytokine levels were not altered. Whilst these studies suggest that TAK1 kinase inhibitors might be an effective approach to prevent inflammation-induced PTL, caution is warranted as TAK1 is also involved in MAPK signaling which regulates cell function and signaling, including apoptosis and differentiation (74). Further studies are required to fully define the role of TAK1 in pregnancy and the clinical potential of TAK1 inhibitors.

CONSIDERATIONS FOR THE CLINICAL TRANSLATION OF CSAIDs

MODE OF DRUG DELIVERY: SIDES EFFECTS AND EFFICACY

In the context of PTB prevention, therapeutics should ideally eliminate the microorganism from the amniotic cavity, block the ensuing cytokine cascade that drives release of PGs and matrix metalloproteinases (MMPs), prevent the onset of PTL, and minimize risk of FIRS. While prophylactic antibiotic trials have not been overly encouraging, there have been some successes (82) and exciting new antibiotics such as solithromycin hold great promise (83). However, therapies that deal solely with the infection, without suppressing inflammation, are unlikely to achieve maximal benefit. The NAC clinical trial of Shahin et al. (39) demonstrated that maternal administration of CSAIDs (following antibiotics to treat bacterial vaginosis) could prevent PTB and improve neonatal outcomes, although it should be noted that the concentrations of NAC achieved in amniotic fluid and fetal blood following maternal administration were not determined.

Given the wide range of genes controlled by NF-κB, the inhibition of NF-κB activation by CSAIDs could have unwanted side effects. Maternal administration may inhibit NF-κB-dependent innate immune defenses, increasing susceptibility to infections (84). Caution is also warranted regarding the possibility of non-specific fetal toxicity. Observations that p38 MAPK null mice are non-viable (57) highlight the need to investigate the safety and toxicity of p38 MAPK inhibitors during pregnancy. While there are no published studies on the teratogenic effects of IKK inhibitors, complete inhibition of NF-κB activation by IKKβ gene deletion ($IKK\beta^{-/-}$) resulted in embryonically lethal uncontrolled apoptosis in the liver of mice (64). Heterozygous $IKK\beta^{+/-}$ embryos developed with normal livers, despite approximately 50% reduction in IKKβ activity, suggesting that modest inhibition in the fetus may be tolerated (64). The pharmacodynamic profile of TPCA-1 appears promising, and the lack of toxicity *in vitro* and *in vivo* suggests that this could be a useful therapeutic approach for the treatment of PTL. Intra-amniotic treatment with competitive ATP protein kinase inhibitors TPCA-1 (IKKβ inhibitor) and OxZnl (TAK1 inhibitor) in an ovine model of LPS-induced chorioamnionitis showed that the CSAIDs were well tolerated by the fetus, at least in the short-term, with no obvious changes in birth weight or fetal liver function observed (70). This suggests that modest reduction in the activity of upstream kinases IKKβ and TAK1 is unlikely to result in the complete suppression of NF-κB activity and non-specific toxicity. Clearly, such concerns are drug- and dose-dependent, requiring extensive and longer-term safety studies before clinical introduction.

Alternatively, it is possible to deliver anti-inflammatory agents directly to the amniotic cavity via ultrasound guided intra-amniotic injection. This route will likely enhance efficacy by delivering the minimal effective dose to target tissues and minimizing unintended exposures and side effects. Depending on the compound, delayed clearance from the amniotic cavity may in fact enhance efficacy and allow single-dose therapy. Recently, we reported that the anti-inflammatory effects of TPCA-1 and SB239063 administered to the amniotic face in a human fetal membrane model were primarily restricted to the fetal compartment, suggesting a lack of trans-membrane transfer (38). The potential benefits of amniotic drug delivery must always be counterbalanced by an assessment of the risks. The procedure-associated risk of spontaneous miscarriage following second trimester amniocentesis is low, with a recent large study finding a non-significant 0.6% increase in miscarriage compared to controls over a 15-year period (85). How this compares to the risks of a third trimester intra-amniotic injection is not known, although the risks at later gestations are likely to be lower than at 12–20 weeks. Nevertheless, it would be prudent that intra-amniotic treatment be given selectively to women in whom a significant benefit from CSAID therapy can be expected.

IDENTIFICATION OF WOMEN AT RISK: SHORT CERVIX AND INFLAMMATION

Intrauterine infection is often chronic and usually asymptomatic until the presentation of PTL, at which time it is often too late to

treat and the fetus has been irreversibly exposed (86). The early identification of women at high risk of adverse pregnancy outcome associated with IUI, before the presentation of clinical symptoms, is challenging but also key for the successful prevention of PTB and improvement of neonatal outcomes. Analysis of amniotic fluid/cervicovaginal fluid cytokine levels or microbial status have been explored to identify women at an elevated risk of PTB (87), but have lacked specificity and/or sensitivity. Sonographic studies have reported that a short cervix (cervical length $\leq$25 mm) is associated with intra-amniotic inflammation, and patients with this condition are at increased risk of adverse pregnancy outcome (88, 89). Gomez et al. (90) reported that women with a cervical length of $\leq$15 mm between 22 and 30 weeks of gestation have a higher rate of microbial invasion of the amniotic cavity (43 vs. 3.9%; $p < 0.05$), and were more likely to deliver spontaneously before 35 weeks of gestation (66.7 vs. 13.5%; $p < 0.01$). These studies suggest that assessing sonographic cervical length may be a useful predictor of risk of microbial invasion of the amniotic cavity and intra-amniotic inflammation (89).

SUMMARY AND CONCLUSION

Infection and inflammation is the leading cause of PTB, but antenatal antibiotic treatment has had limited success at preventing PTB or improving neonatal outcome (30, 91). Newer macrolide antibiotics such as solithromycin, with greater efficacy and better trans-placental passage, may prove in time to be more effective (83, 92). We propose that a combination of anti-inflammatory therapy and effective antibiotics will be required to combat IUI and reduce the associated inflammatory responses leading to PTL and adverse fetal sequelae. Intra-amniotic delivery offers significant advantages in terms of dose reduction, localized site of action, and reduction in potential side effects. CSAIDs, novel compounds that specifically target cytokine signaling pathways, have anti-inflammatory actions in both human fetal membranes *in vitro* and animal models of IUI. These compounds have the potential to be safer and more effective than less selective inhibitors as they target key molecules involved in the pro-inflammatory signaling cascades that prematurely trigger labor. Issues regarding maternal and fetal toxicity, mode of drug delivery, off-target side effects, and appropriate identification of women requiring treatment remain to be addressed. Based on our current appreciation of the importance of IUI and inflammation in the etiology of PTB, the identification and treatment of pregnant women at risk of IUI with effective cytokine signaling inhibitors holds great promise for the prevention of PTB and improvement of neonatal outcomes.

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SUPPLEMENTARY MATERIAL

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Is there a role for probiotics in the prevention of preterm birth?

Siwen Yang[1,2], Gregor Reid[3,4], John R. G. Challis[1,5], Sung O. Kim[3,4], Gregory B. Gloor[6] and Alan D. Bocking[1,2]*

[1] *Department of Physiology, Obstetrics and Gynecology, University of Toronto, Toronto, ON, Canada*
[2] *Lunenfeld Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON, Canada*
[3] *Department of Microbiology and Immunology, Western University, London, ON, Canada*
[4] *Lawson Health Research Institute, London, ON, Canada*
[5] *Department of Obstetrics and Gynecology, The University of Western Australia, Perth, WA, Australia*
[6] *Department of Biochemistry, Western University, London, ON, Canada*

Edited by:
Jeffrey A. Keelan, The University of Western Australia, Australia

Reviewed by:
Christopher John Griffin, Government of Western Australia, Australia
Anna Maria Viola Forsberg, Linköping University, Sweden
Lisa C. Hanson, Marquette University, USA

****Correspondence:***
Alan D. Bocking, University of Toronto, c/o Mount Sinai Hospital, 60 Murray Street, Box 43, 6th Floor, Room 6-1017, Toronto, ON M5T 1X5, Canada
e-mail: abocking@mtsinai.on.ca

Preterm birth (PTB) continues to be a global health challenge. An over-production of inflammatory cytokines and chemokines, as well as an altered maternal vaginal microbiome has been implicated in the pathogenesis of inflammation/infection-associated PTB. *Lactobacillus* represents the dominant species in the vagina of most healthy pregnant women. The depletion of *Lactobacillus* in women with bacterial vaginosis (BV) has been associated with an increased risk of PTB. It remains unknown at what point an aberrant vaginal microbiome composition specifically induces the cascade leading to PTB. The ability of oral or vaginal lactobacilli probiotics to reduce BV occurrence and/or dampen inflammation is being considered as a means to prevent PTB. Certain anti-inflammatory properties of lactobacilli suggest potential mechanisms. To date, clinical studies have not been powered with sufficiently high rates of PTB, but overall, there is merit in examining this promising area of clinical science.

Keywords: probiotics, preterm birth, infection and inflammation, cytokines, vaginal microbiome, bacterial vaginosis

INTRODUCTION

The etiology of preterm birth (PTB) is multifactorial: 50% of the cases are idiopathic while 20–40% are disease specific or medically indicated deliveries such as pre-eclampsia or fetal growth restriction (FGR), which require delivery (1, 2). The remaining 25–30% of PTB can be attributed to intrauterine infection and/or inflammation (1, 2). Microorganisms can invade the uterus through the fallopian tube in a retrograde fashion from the abdominal cavity, hematogenously via the placenta or ascending through the cervix and vagina (3).

MECHANISM OF INFLAMMATION AND INFECTION-ASSOCIATED PRETERM LABOR

Microorganisms can reach the maternal intrauterine tissues through any mucosal surface and secrete phospholipase A2 to act on membrane phospholipids and form unesterified arachidonic acid (AA). The AA is converted into endoperoxide products and subsequently into primary prostaglandins (PGs; PGE2, PGF2a) by PGH synthase-2 and isomerases, respectively. Alternatively, some microbes secrete endotoxins, such as lipopolysaccharides (LPS), which specifically bind toll-like receptor 4 (TLR4) and activate the nuclear factor κ light-chain-enhancer of activated B cells (NFkB) pathway to induce pro-inflammatory cytokine and chemokine gene expression in the intrauterine tissues (amnion, chorion, and decidua), macrophages, and endothelial cells (4, 5). Pro-inflammatory cytokines interact with each other as well as with PGs in a feed-forward cascade, hence amplifying the inflammatory response (6, 7). Furthermore, pro-inflammatory cytokines enhance the expression of matrix metalloproteinase (MMPs), which are zinc-dependent enzymes that catalyze the degradation of collagen constituted-extracellular matrix of the cervix, fetal membrane, placenta, and uterus (8–11). Elevated levels of MMP-9 in the maternal plasma, and MMP-3 and MMP-8 in the amniotic fluid are associated with preterm labor (PTL) and/or microbial invasion of the amniotic cavity (11–13).

Bacteria and viruses can also cross an intact chorioamniotic membrane and induce intra-amniotic inflammation, a condition termed the fetal inflammatory response syndrome (FIRS). Elevated interleukin (IL)-6 and LPS-binding proteins are observed in the umbilical cord blood in FIRS-affected preterm neonates (14–17). Pathogenic microorganisms such as *Ureaplasma urealyticum* and *Mycoplasma hominis* have been isolated from the umbilical cord blood of very preterm newborns (18). Intrauterine infection can also lead to activation of the fetal hypothalamic–pituitary–adrenal (HPA) axis giving rise to increased cortisol biosynthesis and decreased metabolism of maternal cortisol to inactive cortisone by 11β-hydroxysteroid dehydrogenase-2 in the placenta (19). Sustained stimulation of placental corticotropin releasing hormone by fetal cortisol leads to an increase in PG production (20). PG in turn promotes a positive feed-forward loop that comprise an increase in the expression and production of gap junctions such as connexin 43 and pro-inflammatory cytokines including IL-6 and

tumor necrosis factor alpha (TNFα) (20). Together, they promote synchronous and forceful myometrial contractions and PTL.

In short, microbes are well known for their involvement in PTL. In order to understand the origins of these organisms, studies have been undertaken on many sites in the reproductive tract, particularly the vagina.

ALTERED VAGINAL MICROBIOME AND PTB

The vaginal microbiota composition is dynamic throughout a woman's life. Before puberty, it is dominated by anaerobic bacteria (21). Rising estrogen levels at puberty lead to an increase in mucosal glycogen production whose metabolized substrates support vaginal colonization with lactobacilli (21, 22). This is one reason for the vagina to be highly colonized by lactobacilli during reproductive years and pregnancy (23). At menopause, lactobacilli abundance decreases coinciding with a reduction in circulating estrogen (24–26).

Gram-positive lactobacilli are facultative anaerobic bacteria, whose adherence to the vaginal mucosal epithelia appears to form an important line of defense against pathogens (27). There is no definitive "normal" vaginal microbiota, but in the vast majority of pregnant healthy women, lactobacilli dominate (23, 28, 29). Several important aspects of the vaginal microbiota have been uncovered recently, particularly by sequencing PCR-amplified universal 16S ribosomal DNA (rDNA): (1) the healthy vaginal microbiota is dominated by a few *Lactobacillus* species (30); (2) the detection of *Lactobacillus iners*, *Atopobium vaginae*, and bacterial vaginosis-associated bacteria 1, 2, and 3 (BVAB), is apparent in women with BV (30–32). Due to some variations within sequencing techniques, selection of suitable PCR primers, and sufficient depth, future studies may yet reveal more important profiles of healthy versus infected women (33, 34).

Although relatively few 16S DNA studies have been used with samples from pregnant women, indications are that the microbiota does fluctuate during this time. Some researchers have suggested that there are up to five different community state types (CSTs) of bacteria, clusters generated based on similarity in vaginal bacterial composition, in asymptomatic pregnant and non-pregnant women (23, 35). Three of the CSTs (I, II, III) are dominated by *Lactobacillus,* namely *L. iners, L. crispatus*, and *L. jensenii* and/or *L. gasseri*. Two others, CST IV-A and CST IV-B have low relative abundance of *Lactobacillus* spp. and are composed of *Peptoniphilus*, *Anaerococcus*, *Corynebacterium*, *Finegoldia*, and *Prevotella* (CST IV-A), and *Atopobium, Sneathia*, *Gardnerella*, *Ruminococcaceae, Parvimonas*, and *Mobiluncus* (CST IV-B) (23). Such studies have suggested that the vaginal microbiota composition of pregnant women has a higher abundance of *L. vaginalis, L. crispatus, L. gasseri*, and *L. jensenii,* but lower CST IV-B bacteria, and is more stable than non-pregnant women (23, 28), with *L. crispatus*, promoting stability (36). This remains to be verified, but it may be due to hormonal changes. With advancing gestational age, the relative abundance of *Lactobacillus* spp. increases while that of anaerobe or strict-anaerobe microbial species decreases (37).

Bacterial vaginosis is essentially a polymicrobial dysbiosis, characterized by an alteration in the endogenous vaginal microflora with an absent or decreased proportion of lactobacilli and dominance of *G. vaginalis, Prevotella bivia, Mobiluncus sp., Mycoplasma hominis*, and *A. vaginae* (23, 35, 38, 39). Aerobic vaginitis (AE) is an inflammatory condition in which organisms, such as *Escherichia coli* and *Staphylococcus aureus* dominate (40). In many clinical units, the diagnosis of BV involves using a Gram stain Nugent scoring system with or without the Amsel criteria (a vaginal pH >4.5, an amine fishy odor when vaginal fluid is mixed with potassium chloride, the presence of clue cells) (41, 42). A Nugent score of 7–10 seen microscopically as a near absence of rod shaped lactobacilli and high abundance of pathogenic morphotypes is considered BV (42). However, the reliability of the Nugent score has recently been questioned (29). Indeed, sequencing of the vaginal microbiota of women with BV reveals a diverse array of bacteria, including the presence of *L. iners* (32, 43, 44). Improvement in diagnostic accuracy for BV can be accomplished by using a DNA level of $\geq 10^9$ copies/mL for *G. vaginalis* and $\geq 10^8$ copies/mL for *A. vaginae* (45).

The prevalence of BV can vary between populations, but it remains common during pregnancy, where it is associated with a 40% increase in the risk of PTB (46). Women with an abnormal vaginal flora in their first trimester of pregnancy have a higher risk of delivering preterm (39). Although an earlier Cochrane Review (47) suggested that antibiotic treatment of abnormal vaginal flora (intermediate flora or BV) before 20 weeks of gestation may reduce the risk of PTB, a recent Cochrane Review concluded that antibiotic treatment of BV does not reduce the risk of PTB, regardless of when (before 20 weeks or after 20 weeks of gestation) the treatment is given (48). Some of these organisms possess sialidase activity, which has been associated with an increased risk of PTB (49). Sialidases are hydrolytic enzymes that play a role in down-regulating the innate response by degrading immunoglobin-A (IgA), and it has been used in some diagnostic kits for this reason. Higher LPS concentrations, mostly from *P. bivia* (50), and the concentrations of pro-inflammatory cytokines IL-1β, IL-6, and IL-8 have been found to be elevated in the cervico-vaginal fluid of pregnant women with BV (51). The elevation in vaginal pH above 4.5 is a feature of BV, and this displaces *L. crispatus*, but not *L. iners*, which has adapted to upregulate genes for carbohydrate metabolism (52).

In African American and Hispanic women, a higher abundance of *Mycoplasma* and lower abundance of BVAB3 is associated with an increased risk of PTB in the second trimester (53). This is unlikely due to race *per se*, but rather cultural and social aspects. Other pathogens, such as *Leptotrichia, Sneathia*, BVAB1, and *Mobiluncus* spp. appear in higher abundance prior to 16 weeks gestation in women with a previous history of PTB and who deliver preterm (54). Yet, such findings are not universal, and other studies, albeit small, have reported no difference in the vaginal microbial composition between women who have a spontaneous PTB and those who deliver at term (37, 55).

Future microbiome studies should focus on the functionality of organisms in the vagina, uterus, and perhaps even the placenta (56). This should include the use of metabolomic analysis to help understand how the vaginal microbiome may influence the risk of PTB.

ROLE OF IMMUNE-MEDIATORS IN PTL

The balance of pro and anti-inflammatory cytokines, produced by CD4+ T helper (Th) cells, is important in predicting pregnancy outcomes. In early pregnancy, a modest Th1 pro-inflammatory environment promotes successful implantation and placentation (57). As pregnancy progresses, there is a predominance of Th2 anti-inflammatory cytokines including IL-4 and IL-10, which maintain uterine quiescence (57). Disruption of the Th1/Th2 balance favoring the predominance of Th1 pro-inflammatory cytokines such as IL-1, IL-6, and TNFα may be responsible for some cases of PTL (7). Chemokines, such as IL-8, chemokine ligand (CCL)-2, 3, 4, and 5 attract decidual leukocytes and lead to the recruitment of additional pro-inflammatory cytokines that amplify the inflammatory cascade (58, 59). In the choriodecidua, levels of CCL2, 3, 4, and 5 are increased in women undergoing PTL both with and without infection when compared to women at term not in labor (59). In the amniotic fluid, levels of IL-1β, IL-6, IL-8, TNFα, CCL3, 4, and 5 are elevated in women with threatened PTL, especially in the presence of intra-amniotic infection, as are IL-1β, IL-6, IL-8, TNFα, and CCL2 in the cervical fluid (60–65). IL-6 is increased in the umbilical blood of infants born to mothers with chorioamnionitis (65–67). Furthermore, IL-1β, IL-6, and IL-8 concentrations are increased in maternal plasma women with preterm premature rupture of the membranes and chorioamnionitis (64, 68).

Anti-inflammatory cytokines maintain pregnancy quiescence by inhibiting the production of pro-inflammatory cytokines and PGs (69, 70). IL-10 expression in the placenta is lower in women who give birth preterm with chorioamnionitis compared to samples obtained from women who underwent elective terminations in their second trimester of pregnancy (71). The same has been observed in women in term labor with chorioamnionitis compared to women at term not in labor (71). Amniotic fluid concentrations of IL-10 are not different between preterm and term delivery, while cervico-vaginal levels of IL-4 and IL-10 are often below the level of detection using current assays (72, 73). Data regarding the role of maternal plasma IL-10 in mediating PTB remain conflicting. Some studies report decreased plasma IL-10 concentrations with PTB compared to term (1), whereas others have found an association between elevated plasma IL-10 with an increased risk of pre-eclampsia or intrauterine growth restriction, which may in turn lead to PTB (74). Overall, the positive and negative predictive values of any single specific cytokine or chemokine for PTB is limited (75) although the examination of interactions with a multifactor dimensionality reduction analysis between multiple cytokines within the maternal–fetal compartments, rather than a single cytokine, may better predict the risk of PTB (76).

PREBIOTICS AND PROBIOTICS FOR PREVENTION OF PTB

Prebiotics are indigestible food ingredients such as dietary fiber, resistant starch, and oligosaccharides. They confer health benefits by "causing significant changes in the composition of the gut microflora with increased and reduced numbers of potentially health-promoting bacteria and potentially harmful species, respectively" (77, 78). The prebiotics galacto-oligosaccharide (GOS), fructo-oligosaccharides (FOS), and lactulose have been shown to provide substrates for the growth of lactobacilli and bifidobacteria, suggesting that they may contribute to the beneficial effects of probiotics. Prebiotics also possess immune-regulatory functions (79–81) and in particular immune-saccharides are known to induce activation of the innate immune system (81). Prebiotic FOS increases the level of IL-27 concentrations in human milk, which may help prevent the onset of allergic disorders in their children (82). There is anecdotal evidence to suggest that prebiotic-containing food may reduce the risk of PTB (83). Of interest, one study reported that dried fruits and garlic that contained antimicrobial and prebiotic compounds were associated with a reduced risk of spontaneous PTB (84).

Probiotics are defined as "live microorganisms, which when administered in adequate amounts, confer a health benefit on the host" (85). A number of meta-analyses of clinical trials with probiotics have confirmed that probiotics are both safe and effective for the treatment and/or prevention of numerous infectious and/or inflammatory diseases (86–89). *Lactobacillus* and *Bifidobacterium* are the most commonly studied probiotics. Supplementation with *Bifidobacterium lactis* in preterm infants reduces pathogenic *Enterobacteriaceae* and *Clostridium spp.* counts (90). Bifidobacteria are present in large abundance in the intestinal flora, but they can also be detected in the vagina. Probiotic lactobacilli play a potential beneficial role in human reproduction and maintenance of healthy urinary and reproductive tracts (91).

The use of antibiotics to treat BV in non-pregnant and pregnant women remains the method of choice, unchanged in many decades, and still too often ineffective. Metronidazole and clindamycin, by far the most used agents, do not restore vaginal lactobacilli abundance, which may account for relapses in some women; and prolonged use promotes the development of drug resistance (27, 92). The need for new treatment for BV that restores microbiota homeostasis and acidity without undesirable side effects has led investigators and patients to study probiotics. Human studies have provided evidence that probiotic lactobacilli can reduce BV recurrence and increase lactobacilli abundance in the vagina of pregnant and non-pregnant women (93–95). The use of lactobacilli as an adjuvant therapy has also shown promise in lowering BV recurrence rates (92). Indeed, the adjunctive use of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 with metronidazole has been shown to improve actual cure of BV (96, 97).

Probiotic intervention in pregnancy is generally acceptable with good compliance among pregnant women (98). A recent meta-analysis of randomized clinical trials demonstrated that the use of probiotics *Lactobacillus* and *Bifidobacterium* during pregnancy had no effect on the incidence of Cesarean section, birth weight, or gestational age (99).

Oral administration of 10^9–10^{11} colony-forming units (cfu) of lactobacilli is the standard dose believed to be required for passage through the intestine and subsequent improvement of gut and vaginal health (27, 93, 100, 101). There are many variables that influence vaginal colonization by lactobacilli including glycogen level, substances used in vaginal washing, the use of antibiotics, and the ability of lactobacilli to produce substances such as hydrogen peroxide (102–104). Bodean et al. (92) reported that oral administration of *L. acidophilus* and *L. bifidus* was more effective than the vaginal route in reducing BV occurrence in antibiotic-treated

non-pregnant women. However, the probiotic composition of the oral capsule was different from the vaginal capsule (*L. rhamnosus, L. acidophilus, S. thermophilus*, and *L. bulgaricus*) in that study, and the mechanism seems unclear. Furthermore, the treatment duration was longer for patients who received the oral capsule than those who received vaginal capsules (92). An advantage of the oral route is that it may reduce pathogen ascendance from the rectum to perineum and vagina, while a concern of the intravaginal approach for some women may be the more invasive instillation of microbes.

A number of mechanisms whereby lactobacilli defend against pathogens in the vaginal environment have been described, albeit mostly from *in vitro* studies. These include the production of antimicrobial substances, competitive exclusion with pathogenic bacteria and fungi, acidification of the vaginal area, and modulation of the immune system (40). Endogenous lactobacilli maintain the vaginal pH <4.5 by metabolizing glycogen secreted by vaginal mucosal epithelia and produce lactic acid, which is a potent microbicide against potential reproductive tract infections (105, 106). The acidic environment of a healthy vagina creates a hostile environment for BV-associated pathogens while favoring lactobacilli growth (105, 107). It may also help to prevent viruses, such as HIV, from infecting the host (108, 109).

The anti-inflammatory property of lactobacilli has been shown to be important in the control of mucosal and systemic inflammation (110). *L. rhamnosus* GR-1 supernatant (GR-1 SN) enhances IL-10 and colony-stimulating factor 3 (CSF3) production in mouse macrophages (111). In primary human placental trophoblast cells, GR-1 SN increases IL-10 and CSF3 production via JAK/STAT and MAPK pathways, down-regulates LPS-induced TNFα output through c-Jun-N-terminal kinases (JNKs) inhibition, and increases the expression of the PG metabolizing enzyme PGDH in a sex-dependent fashion (112–114). When administered intra-peritoneally to pregnant mice, GR-1 SN reduces LPS-induced PTB in association with a decrease in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines in maternal plasma and the amniotic fluid (115).

The effect of lactobacilli on the immune system and their vaginal colonization ability can be species/strain specific. In the mouse gut, *L. plantarum* and *L. rhamnosus* GG exacerbate inflammation and the development of dextran sulfate sodium (DSS)-induced colitis while *L. paracasei* is protective (116). In the human vagina, *L. rhamnosus* GR-1 and *L. reuteri* RC-14 but not the intestinal probiotic *L. rhamnosus* GG persist up to 19 days (117). Intra-vaginal instillation of *L. rhamnosus* GR-1 has been shown to upregulate some antimicrobial activity in premenopausal women (118). A combination of *B. bifidum, B. infantis, L. acidophilus, L. casei, L. salivarius,* and *Lactococcus lactis* has been reported to provide a wider antimicrobial spectrum, better stimulation of IL-10 production, and suppression of pro-inflammatory cytokines in cultured human peripheral blood mononuclear cells compared to the individual strains (119). A combination of the bacteriocin-like inhibitory substances (BLIS) from the *L. rhamnosus L60* and *L. fermentum L23* can reduce the growth of group B streptococcal isolates obtained from pregnant women more effectively than each *Lactobacillus* strain alone (120).

Lipoteichoic acid (LTA) on the cell surface of lactobacilli can also stimulate macrophages to secrete immune-mediators. Improved anti-inflammatory activity in a murine model of colitis *in vivo* has been observed when LTA is removed or substituted (121–123). *L. rhamnosus* GR-1 supernatant reduces LPS-induced PTB and associated systemic and intrauterine inflammatory cytokines in pregnant mice (115). The supernatant of lactobacilli also has anti-inflammatory properties in cultured human placental trophoblast cells, decidual cells, monocytes, and macrophages (112–114, 124, 125). In human decidual cells challenged with *E. coli*, supernatant of *L. rhamnosus* CNCM I-4036 was found to be more effective than the live bacteria counterpart in the suppression of pro-inflammatory cytokine production (126). These studies imply that administration of supernatant from lactobacilli may promote desirable effects and represent an alternative for the prevention and/or treatment of inflammatory disorders such as some cases of PTB. The identification of these bioactive metabolite(s) remains to be achieved.

Future clinical studies should consider not only the sample size and design but also the appropriate probiotic strain(s), dose and duration of treatment, and route of administration. Until a sufficiently large study is performed in which the rate of PTB is high enough to note a reduction due to an intervention (127), we can only say that currently, the administration of a few probiotic strains is safe for use in pregnancy and shows promise in conferring health benefits, of which potentially reducing the risk of PTB is one.

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REVIEW
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Advances in the Prevention of Infection-Related Preterm Birth

Ronald F. Lamont[1,2]*

[1] Research Unit of Gynecology and Obstetrics, Department of Gynecology and Obstetrics, Institute of Clinical Research, Odense University Hospital, University of Southern Denmark, Odense, Denmark, [2] Division of Surgery, University College London, London, UK

Infection-related preterm birth (PTB) is more common at early gestational ages and is associated with major neonatal mortality and morbidity. Abnormal genital tract microflora in early pregnancy predicts late miscarriage and early PTB. Accordingly, it is logical to consider antibiotics as an intervention. Unfortunately, the conclusions of systematic reviews and meta-analyses (SR&MAs) carried out in an attempt to explain the confusion over the heterogeneity of individual studies are flawed by the fact that undue reliance was placed on studies which: (a) had a suboptimal choice of antibiotic (mainly metronidazole) or used antibiotics not recommended for the treatment of bacterial vaginosis (BV) or BV-related organisms; (b) used antibiotics too late in pregnancy to influence outcome (23–27 weeks); and (c) included women whose risk of PTB was not due to abnormal genital tract colonization and hence unlikely to respond to antibiotics. These risks included: (a) previous PTB of indeterminate etiology; (b) low weight/body mass index; or (c) detection of fetal fibronectin, ureaplasmas, Group B streptococcus or *Trichomonas vaginalis*). While individual studies have found benefit of antibiotic intervention for the prevention of PTB, in meta-analyses these effects have been negated by large methodologically flawed studies with negative results. As a result, many clinicians think that any antibiotic given at any time in pregnancy to any woman at risk of PTB will cause more harm than good. Recently, a more focused SR&MA has demonstrated that antibiotics active against BV-related organisms, used in women whose risk of PTB is due to abnormal microflora, and used early in pregnancy before irreversible inflammatory damage has occurred, can reduce the rate of PTB. This review presents those data, the background and attempts to explain the confusion using new information from culture-independent molecular-based techniques. It also gives guidance on the structure of putative future antibiotic intervention studies.

Keywords: infection, antibiotics, bacterial vaginosis, preterm labour/labor, preterm birth

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***Correspondence:**
Ronald F. Lamont
rflamont@aol.co.uk

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Abbreviations: AF, amniotic fluid; BMI, body mass index; BV, bacterial vaginosis; CDC, Centers for Disease Control and Prevention; CSAIDS, cytokine suppressive anti-inflammatory drugs; CVC, clindamycin vaginal cream; FDA, Food and drug Administration; GBS, group B streptococcus; HIV, human immunosuppressive virus; HPV, human papilloma virus; HSV, herpes simplex virus; IA, intra-amniotic; IL, interleukin; IV, intravenous; LPS, lipopolysaccharides; MMP, matrix metalloproteinases; PG, prostaglandin; PPROM, preterm prelabor rupture of the membranes; PTB, preterm birth; RCT, randomized controlled trial; SPTL, spontaneous preterm labor; SR&MA, systematic review and meta-analysis.

THE IMPORTANCE OF PRETERM BIRTH

In high-income countries, preterm birth (PTB), particularly at early gestations, is the major cause of death and handicap in neonates (1–3). Babies born at 22, 24, and 26 completed weeks of gestation have an infant mortality rate of 54, 21, and 2%, respectively, and a rate of survival without major morbidity at 365 days of 0.02, 14.1, and 45.9%, respectively (2). Approximately 65% of babies born between 22 and 26 completed weeks of gestation will die on the labor ward or in the neonatal intensive care unit and at 30-month follow-up, around 50% will be handicapped and in 50% of these, the handicap will be severe. Accordingly, at 2.5 years of age only 12–13% will be alive and intact (3). In the UK, the cost of hospital readmissions in the first five and 10 years of life is 20 times greater for those babies born before 28 completed weeks of gestation compared to those born after 37 completed weeks (4). In 2007, the Institute of Medicine calculated that the annual cost associated with PTB in the USA was $26.2 billion comprising medical costs for the baby ($16.9 billion), labor and delivery costs for the mother ($1.9 billion), early intervention programs for children with disabilities and developmental delays from birth to age 3 years ($611 million), special education services ($1.1 billion), and lost work and pay for those born preterm ($5.7 billion) (5). It has been demonstrated that between 23 and 26 completed weeks of gestation, each day of prolongation of pregnancy increases the survival rate by 3% (6).

INFECTION AS A CAUSE OF PRETERM BIRTH

Spontaneous preterm labor (SPTL) leading to PTB is now recognized as a syndrome caused by a number of pathological processes leading to activation of the common terminal pathway of parturition (7). The etiology of SPTL is multifactorial but there now exists abundant evidence that local or systemic infection or inflammation is a major cause, particularly of early PTB (8–10). This involves *inter alia*, prostaglandins (PGs), proinflammatory chemokines and cytokines, as well as pattern recognition receptors known as toll-like receptors (11, 12). In addition, the relationship between infection and PTB changes as pregnancy progresses. Infection in late PTB (34–36 weeks) is unusual but is present in most cases in which PTB occurs before 30 weeks gestation (13). In addition, the earlier in pregnancy at which PTB occurs, the more likely it is to be due to infection (14, 15). Between 26 and 34 completed weeks of gestation, women admitted in SPTL are more likely to have abnormal genital tract microflora and chorioamnionitis compared to women delivered electively at the same gestational age for feto-maternal indications (16–18). Compared with term birth, the prevalence of maternal endometritis, chorioamnionitis and neonatal infection is much more common following PTB (10, 13, 19, 20). The gestational age association of acute chorioamnionitis shows a dramatic reduction from 94.4% at 21–24 weeks to 39.6% (25–28 weeks), 35.4% (29–32 weeks), 10.7% (33–36 weeks), and only 3.8% at 37–40 weeks (21).

THE PREDICTION OF INFECTION-RELATED PRETERM BIRTH

As pregnancy progresses, the genital tract microflora becomes progressively more benign such that by term; the vaginal microflora poses no significant threat to the fetus as it passes through the birth canal (22). Although all births before 37 completed weeks of gestation are defined as preterm, PTB before 32 weeks gestation (2% of all births) accounts for most of the neonatal mortality and morbidity (23). Accordingly, if screening and treatment begins at gestations beyond 24 weeks, the opportunity to prevent late miscarriage and very early PTB is lost. The main cohort studies from Europe, North America, and Indonesia (24–33) and three case control studies from the USA, Sweden, and Australia (34–36) have used different methodologies to examine the association between abnormal genital tract microflora either in the form of bacterial vaginosis (BV) or the presence of BV-associated organisms and adverse outcomes of pregnancy. The majority of these studies show a statistically significant association between abnormal genital tract microflora and late miscarriage and PTB. Furthermore, the degree of risk is greater the earlier in pregnancy at which abnormal microflora was detected (24). A positive screening test for abnormal genital tract microflora at 26–32 weeks gestation is associated with a statistically significant 1.4- to 1.9-fold increased risk of PTB (27, 29–32). In contrast, a positive result from screening in the second trimester is associated with a 2.0- to 6.9-fold increased risk of an adverse outcome (25–28). In a longitudinal study of women in Indonesia, women with BV in early pregnancy had a 21% risk of an adverse outcome, compared to only 11% of those who developed the condition later in pregnancy (27).

Abnormal Genital Tract Microflora

Due to the polymicrobial nature of vaginal microflora, the definition of what is normal or abnormal genital tract microflora is very difficult. Normal vaginal microflora is assumed to be present in the absence of disease. Disease results from the interplay between microbial virulence, numerical dominance, and the innate and adaptive immune response of the host. Disease is assumed to be absent if the woman is asymptomatic, and there are no clinical signs of vaginal infectious morbidity. Abnormal vaginal microflora may occur (a) because of a sexually transmitted infection; (b) colonization by an organism which is not normally a constituent part of the vaginal microbial community such as *Haemophilus influenzae*, or *Listeria monocytogenes*; (c) due to increased virulence or overgrowth of an organism that is normally a constituent part of the vaginal microflora, e.g., *Escherichia coli*; or (d) BV.

The Bacterial Vaginosis Syndrome

Disordered vaginal microflora sometimes now referred to as "dysbiosis" is most commonly due to BV – a polymicrobial condition, characterized by a significant decrease in the quantity or quality of lactobacilli in association with a 1,000-fold increase in the number of other potentially pathogenic organisms such as *Gardnerella vaginalis*, *Mycoplasma hominis*, *Mobiluncus* species, and other anaerobic organisms. Since many of the organisms

associated with BV are quite fastidious, and since BV is a quantitative rather than a qualitative change in vaginal microflora, qualitative or semiquantitative culture techniques are unhelpful for diagnosis. Accordingly, the diagnosis of BV requires quantification of vaginal microbiota (37). With the introduction of culture-independent techniques, more sensitive and specific ways of diagnosing BV may be developed (38–40). BV affects almost a third of women (41). In gynecological practice, BV has been found to be associated with the acquisition of STIs such as chlamydia, gonorrhea, trichomoniasis, and viral infections (HIV, HSV, and HPV), plus a range of morbidities including postabortal sepsis, infertility, pelvic inflammatory disease, and posthysterectomy vaginal cuff infections. In pregnancy, BV has been associated with PTB, preterm prelabor rupture of the membranes (PPROM), early, late, and recurrent miscarriage, and postpartum endometritis (24). If BV is detected early in pregnancy, it is associated with a five to sevenfold increased risk of SPTL and PTB (25, 26). A longitudinal study in pregnancy has demonstrated that only 2% of women who did not have BV in the second trimester will develop BV by 34 weeks. In contrast, 50% of women who had BV in the second trimester will still have BV at 34 weeks (42).

New Information from Culture-Independent, Molecular-Based Techniques

Recent evidence from cultivation-independent molecular-based techniques has demonstrated that BV is not a single entity but a syndrome (the BV syndrome) of different sub-types with different etiologies, different microbial communities, and hence different responses to antibiotics and, in all likelihood, different subsequent phenotypical outcomes from normal term birth to late miscarriage, very early PTB, PPROM, or preterm stillbirth (38). Such new information clarifies why the etiology remains unknown, why the microbiology of BV differs from case to case, and why the response to antibiotics remains inconsistent. It would also explain why the phenotypic outcome of pregnancy differs from case to case, ranging from a normal outcome to a very early PTB or late miscarriage. A better understanding would help to limit the administration of antibiotics for the prevention of infection-related PTB to those antibiotics that are known to be effective in women with objective evidence of abnormal vaginal microflora and use these antibiotics early in pregnancy before inflammation and tissue damage has occurred (43).

ANTIBIOTICS FOR THE PREVENTION OF INFECTION-RELATED PRETERM BIRTH

This review reflects the use of prophylactic antibiotics used early in pregnancy for the prevention of PTB and does not cover the management of PPROM, which may be a cause, or a result of infection and probably a combination of the two. The literature pertaining to the use of antibiotics following PPROM is legion and so may be the subject of a separate review.

We know that abnormal vaginal colonization in early pregnancy is predictive of PTB (24, 25, 27–36). Accordingly, it is logical to consider the use of antibiotics for the prevention of infection-related PTB. Unfortunately, antibiotic studies have chosen different: (a) risk groups; (b) diagnostic methods; (c) degrees of abnormal vaginal colonization; (d) antibiotic dose regimens and routes of administration; (e) women with different host susceptibilities and hence host response; (f) gestational age at time of treatment; (g) outcome parameters; and (h) definitions of success (44–62). Understandably, the results of such studies are conflicting. A number of systematic reviews and meta-analyses (SR&MAs) of these studies have been conducted and updated (63–74). However, SR&MAs are retrospective analyses of pooled data that are only as good as the quality of studies included (75). Due to the aforementioned limitations of the studies published to date, the conclusions derived from these SR&MAs are also limited and should not be used to provide guidelines or make recommendations for the use or change of practice (75). Similarly, until recently, none of the SR&MAs on the use of antibiotics for the prevention of infection-related PTB has simultaneously addressed the optimal choice of agent, the choice of patient, and the timing of intervention. If antibiotic intervention is to be successful in reducing the incidence of PTB, these antibiotics (a) should be active against those organisms known to be associated with PTB, (b) should only be used in women with abnormal genital tract microflora, and (c) should be used early in pregnancy before infection and inflammation have had an opportunity to cause irreversible damage which will inevitably lead to SPTL and PTB.

Choice of Antibiotic

The Centers for Disease Control and Prevention (CDC) do not recommend erythromycin or coamoxiclav for the treatment of BV. Their recommendation is to use either metronidazole or clindamycin, orally or vaginally (76). Like macrolide antibiotics, clindamycin has anti-inflammatory properties (77–81) and has a broader range of activity against BV-related organisms such as species of *Mobiluncus* and the genital mycoplasmas (82–87). Metronidazole and other nitro-imidazoles are inactive *in vitro* against BV-associated organisms such as *M. hominis*, *G. vaginalis*, *Ureaplasma urealyticum* (88, 89), and *Atopobium vaginae* (90, 91). In addition, they have little or no activity against other aerobic organisms such as *Staphylococcus aureus* or species of *Streptococci.* However, metronidazole has a similar treatment success rate as clindamycin *in vivo* (76). This suggests one or both of two possible mechanisms. Firstly, *in vivo*, BV-related organisms may be sensitive to the hydroxy-metabolite of metronidazole. Alternatively and more likely, metronidazole acts indirectly by destroying anaerobes which provide nutrients to other BV related organisms such as *G. vaginalis* or *A. vaginae* (92). Molecular-based studies have indicated a far greater diversity of microorganisms associated with BV than has been evident from culture-dependent techniques (38). These organisms form different communities that may be anaerobe dominated or *G. vaginalis* and *A. vaginae* dominated. Other abnormal subtypes may be due to mixed organisms or perhaps due to a subtype caused by sexual transmission (38). Accordingly, it is possible that those sub-types of BV in which anaerobes are dominant are more successfully treated by metronidazole. In contrast, in other subtypes where anaerobic organisms are not dominant, metronidazole may be less effective. Finally, clindamycin may be active against both metronidazole-sensitive sub-types but also against

a wider range of BV sub-types with different microbial communities. It should be noted that while *M. hominis* is extremely sensitive to clindamycin, *U. urealyticum* is only weakly sensitive to clindamycin (87, 93).

Effect on Lactobacilli

When comparing clindamycin with metronidazole, the case for metronidazole and against clindamycin is often given that metronidazole conserves vaginal lactobacilli, whereas clindamycin destroys them. However, phage virus colonization of lactobacilli is associated with BV, and it has been postulated that diet acquired phage viruses, may be induced to become lytic by a factor related to sexual activity, or alternatively that *Lactobacillus* phages may be directly inoculated into the vagina from sexual partners (94). If phage virus colonization of lactobacilli is present, metronidazole may be perpetuating rather than curing BV, whereas the opposite would be true with clindamycin.

PREMEVA1 Trial

In 2013, an abstract presented orally to the Society for Maternal–Fetal Medicine was published on-line. http://dx.doi.org/10.1016/j.ajog.2013.10.036. The PREMEVA1 trial was a French multicentre randomized controlled trial comprising 2,869 low-risk women randomized to receive clindamycin or placebo before 15 weeks' gestation. In the placebo group, late abortion/very preterm spontaneous delivery rate (12–32 weeks) did not differ significantly between the clindamycin and placebo groups. Requests for details of the study have elicited no response. Accordingly, at the time of completion of this manuscript, no peer-reviewed, full-study report could be found on any of the appropriate search engines. This being the case, the risk of bias cannot be assessed and, until the details of the study are fully available, it is difficult to comment on the significance of the findings and these should not be used to influence guidelines.

Route of Administration of Clindamycin

The choice between oral clindamycin or clindamycin vaginal cream (CVC) to treat abnormal genital tract microflora/BV in pregnancy needs to be addressed. Vaginal administration is the most direct and efficient route of administration of antibiotic to the site of the heaviest bacterial load. In contrast, we know that BV is associated with subclinical endometritis (95). Accordingly, if vaginal microorganisms have already gained access to the choriodecidua, they may not be treatable by CVC, and systematic therapy may be necessary. To the authors knowledge, no study has studied the simultaneous combined use of CVC and oral clindamycin.

The Potential for Newer Macrolide Antibiotics

More data are now available on azithromycin and a new antibiotic, solithromycin, that may be considered candidate antibiotics in future intervention studies. In a SR&MA, macrolides and clindamycin administered during the second trimester of pregnancy were associated with a reduction in the rate of PTB (96). Second trimester metronidazole used alone was associated with an increased risk of PTB in a high-risk population. Like many other SR&MAs, studies were included where the risk of PTB was positive fetal fibronectin, urogenital mycoplasma infection, previous PTB of unqualified phenotype, or prepregnancy weight of <50 kg. In addition, while azithromycin and clarithromycin were included in the search, the only macrolide included was erythromycin (96). In a RCT of interconceptional antibiotics to prevent PTB, neither azithromycin nor metronidazole was of any benefit in reducing the subsequent rate of PTB (97).

Two recent studies from Malawi tested the effect of prophylactic azithromycin on the subsequent rate of PTB. In a high-risk population, routine prophylaxis with azithromycin showed no benefit, but this population was unselected (high risk of poor pregnancy outcome but not specifically PTB), and no objective evidence of infection-related risk of PTB was sought (98). Also in Malawi, a RCT of intermittent treatment of maternal malaria and reproductive tract infection with monthly sulfadoxine-pyrimethamine plus two doses of azithromycin was associated with a significant reduction in PTB and low birth weight (99).

Solithromycin is a new antibiotic that is highly potent against ureaplasmas and mycoplasmas and other antibiotic resistant organisms. In an animal study, combined intra-amniotic (IA) and intravenous administration of solithromycin resulted in effective concentrations of solithromycin in amniotic fluid (AF) and maternal and fetal plasma, leading the authors to conclude that solithromycin may have promise in future for the prevention of PTB (100). Subsequent studies showed that a 4-day course of solithromycin eradicated IA *Ureaplasma parvum* infection in the same sheep model (101).

Choice of Patient

Women with BV (Nugent score 7–10) respond better to clindamycin than women with intermediate microflora (Nugent score 4–6). Accordingly, prophylactic antibiotics to prevent infection-related PTB should only be given to women with objective evidence of abnormal vaginal colonization such as BV (37). Without such evidence, treatment may disrupt, rather than treat, abnormal microflora. In many antibiotic intervention studies, the indication for administration of prophylactic antibiotics was previous PTB. There is no doubt that previous PTB is a known risk factor for subsequent PTB (102, 103). However, a previous PTB may have been for feto-maternal indications, such as antepartum hemorrhage and fulminating pre-eclampsia. Such indications would not place a subsequent pregnancy at risk of infection-related PTB and consequently are unlikely to benefit from antibiotic prophylaxis. Such studies (104), have been erroneously cited as evidence that antibiotics have no role in the prevention of PTB (105). Only a small portion of such women (even those with BV) may be at risk for PTB. Better diagnostic or predictive methods are required to improve our ability to identify those women who would be most likely to benefit from treatment with antibiotics.

Pharmacogenetics

Pharmacogenetics is also an important consideration (106). Metronidazole used in mainly Black or Hispanic women in North America has not shown benefit (52). In contrast, in predominantly White North European women, five studies using clindamycin have shown benefit (43). Black or Hispanic women may have a genetic predisposition to mount a damaging

inflammatory response to the challenge of BV, while predominantly white Northern European women do not. Alternatively, in predominantly white Northern European women, the inflammatory response may be sufficiently less rigorous to allow time for antibiotic therapy to be of benefit. Finally, it may be that some women with a prior PTB have a genetically non-infectious risk of PTB under which circumstances their propensity to deliver preterm will exist with or without BV and hence would not be expected to respond to antibiotic treatment. These studies and the findings of racial differences in the vaginal microbiome highlight the importance of tailoring antibiotic treatment approaches for different racial groups and controlling for race in clinical trials.

Treatment of Symptomatic or Asymptomatic Pregnant Women with BV

Symptomatic pregnant women with BV should be treated even if they are at otherwise low risk of PTB. The management of asymptomatic women with BV who do not have other risk factors such as a previous BV-related PTB is less well accepted. Since BV is an independent risk factor for PTB, one could argue that any woman with BV (symptomatic or asymptomatic) is at a significant twofold increased risk of PTB, if BV is detected at or beyond 24 completed weeks of gestation. This is the same risk associated with smoking which is considered significant enough to merit intervention. In contrast, if BV is detected before 16 weeks gestation, there is a five to sevenfold increased risk of PTB (see the Section on The Prediction of Infection-Related Preterm Birth). If one relies on previous PTB as a risk factor for subsequent PTB in women with BV, it is essential to record the phenotype of that previous PTB. If the previous PTB was iatrogenic, because of twins, APH, or pregnancy-induced hypertension/preeclampsia, it may not be relevant in women with BV. Similarly, if the previous PTB was unexplained apart from a maternal weight <50 kg or a BMI <18 kg/m^2 then the detection of BV may be irrelevant. As more information becomes available from molecular-based, cultivation-independent techniques, the identification of sub-types of BV, the different etiologies of each, the different microbiology, the different response to antibiotics, and the different phenotypic outcomes may address this concern.

Timing of Antibiotics

Abnormal genital tract microflora in early pregnancy, even if this reverts to normal, is still associated with late miscarriage and PTB (44) suggesting that whatever damage is done by infection and inflammation, this occurs early and persists (44, 107–113). If antibiotics are used late in pregnancy when inflammatory tissue damage may have already occurred, and there are already irreversible changes in the cervix, myometrium, decidua, placenta, and extraplacental membranes, then antibiotics are unlikely to be of benefit. Accordingly, concern has been expressed that under these circumstances, antibiotics may cause more harm than good (114–120). Hence, it may be argued that antibiotics should be used early in pregnancy before infection/inflammation can cause irreversible damage that ultimately leads to SPTL and PTB. In their recommendations for the treatment of BV in pregnancy, the CDC treatment guidelines advise the use of oral or vaginal metronidazole or oral clindamycin (76). However, it was noted that the late administration of CVC up to 32 weeks gestation was associated with subsequent adverse outcomes, such as low birth weight and neonatal infection (28, 48, 51). As a result, the guidelines recommend that CVC should only be used in the first half of pregnancy (76).

Gene–Environmental Interaction

Many diseases like PTB are due to a combination of genetic susceptibility and environmental exposure. A woman may have the environmental exposure (BV), but if she does not have the genetic susceptibility (gene polymorphism) to mount a damaging inflammatory response then little harm may occur. Conversely, a woman may possess the gene polymorphism to mount a damaging inflammatory response, but if she does not have environmental exposure (BV) then damage may not occur. However, when both susceptibility and exposure are present, the risk of an adverse outcome will be increased, and this is referred to as the gene–environmental interaction (121). Abnormal vaginal microflora or infection leads to adherence, invasion, and host inflammatory response. That response may be appropriate resulting in tissue repair and healing. Alternatively, the response may be exaggerated (hyper-response) resulting in tissue damage from increased production and release of proinflammatory cytokines. Conversely, the response may be inadequate (hyporesponse) leading to overwhelming infection. Both a hyper-response and a hypo-response may result in mortality and morbidity due to tissue damage. If antibiotics are used late in this process, it may not be possible to prevent irreversible tissue damage, morbidity, and mortality. In contrast, if antibiotics are used early, before tissue damage occurs, this damage might be prevented. Accordingly, the earlier the gestational age at which clindamycin is administered to women with objective evidence of risk of infection-related PTB, the more likely it is to be able to demonstrate a reduction in the rate of PTB (43).

Potential for the Use of Anti-inflammatory Agents as Adjunctive Treatment

The potential for adding to antibiotics an anti-inflammatory agent which targets the NF-κB and p38 MAPK (cytokine suppressive anti-inflammatory drugs [CSAIDs]) that block cytokine signaling for the prevention and treatment of inflammation-induced PTB shows promise and has been comprehensively reviewed elsewhere (122). In an ovine model, IA administration of a single dose of CSAID suppressed the lipopolysaccharide-induced IA inflammatory response with minimal fetal effects (123). Several animal model studies have shown additional benefit of antibiotic cotreatment with anti-inflammatory agents. In the rhesus monkey, following IA inoculation of *U. parvum*, azithromycin plus dexamethasone and indomethacin was able to prolong pregnancy and prevent advanced fetal lung injury (124). Similarly, to determine whether treatment with ampicillin/dexamethasone/indomethacin (AMP/DEX/INDO) delayed PTB induced by IA Group B streptococcus (GBS) inoculation in rhesus monkeys, ampicillin alone eradicated GBS but uterine activity, AF cytokines, PGs, and matrix metalloprotein (MMP)-9 remained elevated. In contrast,

the combination of AMP/DEX/INDO suppressed interleukin-1β, TNF-α, PGE_2, and $PGF_{2\alpha}$ but did not alter MMP expression or chorioamnionitis. The combination of AMP/DEX/INDO suppressed inflammation and significantly prolonged gestation (125).

Rescreening and Retreating with Antibiotics

In many antibiotic intervention studies there has been inconsistency of rescreening and retreatment in which persistent or recurrent BV occurs in ~10–30% (43). Using stringent diagnostic criteria (BV on Nugent score together with all four elements of Amsel's clinical composite criteria), 70.8% of women who received CVC were cured/improved at 20–24 days post-treatment compared to only 12% in the placebo group. Recurrence rates in those CVC patients successfully treated were ~6% at 6 weeks postbaseline and 10% at 28–34 weeks. Of the 29.2% of women who failed to respond to the first 3-day course of CVC and who were therefore retreated with a 7-day course of CVC, 32.6% and 51.2% were cured/improved at 20–24 days postretreatment and at 28–34 weeks gestation, respectively (126). Accordingly, rescreening and retreating in pregnancy may be helpful since an initial course of CVC cured or improved BV in 88% of women, and a second course some 3–6 weeks later was still able to cure or improve BV in 50% of those who still had the condition (127).

CRITICAL REVIEW OF THE LITERATURE ON THE USE OF ANTIBIOTICS TO PREVENT INFECTION-RELATED PRETERM BIRTH

As discussed earlier, the majority of the SR&MA that consider the use of antibiotics for the prevention of PTB inappropriately merged clindamycin and metronidazole studies together rather than considering them separately. Those studies that initially considered clindamycin and metronidazole studies separately then erred by combining the two antibiotics when considering the gestational age at treatment (63, 66, 74). While the majority of studies included in the SR&MA comprised women with objective evidence of BV, two meta-analyses included studies where the risk-status or entry criteria was measured by other parameters unrelated to BV. These included parameters, such as positive fetal fibronectin test, previous PTB, and detection of GBS, *U. urealyticum*, or trichomonas (66, 74). Using these SR&MAs, it can be concluded that if inappropriate antibiotics are used at late gestations, in women without objective evidence of abnormal vaginal bacterial colonization, there is no benefit with respect to the prevention of infection-related PTB. However, concerns have been expressed that if these SR&MAs are not interpreted carefully, they will be erroneously cited as evidence that any antibiotic, given to any pregnant woman, at any gestational age will be unhelpful in preventing PTB. For this interpretation, caution has been urged (114–120, 128–130). Two large studies are regularly cited in SR&MA as evidence that antibiotics are of no benefit for the prevention of PTB: the National Institutes for Child Health and Human Development (NICHD)/Maternal Fetal Medicine Network Units (MFMU) study (52) and the ORACLE II study (131, 132). Despite their faults (see below), these studies markedly outweigh all other studies in SR&MA and hence strongly influence conclusions.

The NICHD/MFMU (2000) Study

This study screened 29,626 women (52) of which 6,540 were positive solely for BV without other conditions, such as trichomoniasis. From these, the recruitment was low with only 1,936 (29.6%) randomized to receive either metronidazole or placebo. Of the 4,604 exclusions, 999 were excluded for reasons recorded as "other." Most SR&MAs classify this study as having been in a "low-risk population" yet 85% of the population was either Black or Hispanic. As a part of the methodology, up to 8 weeks could elapse between screening and initiation of treatment. During this delay, the grade of microflora on Gram stain changed in 25% of women (115). Metronidazole was administered as a once only 2 g oral dose and unsurprisingly, vomiting occurred in a high percentage of women. Under such circumstances, the 2 g oral dose was repeated 2 days later. With no objective measure of compliance, such as metronidazole blood levels, the number of women who took the repeat course remains undocumented. Importantly, there was an inexplicable 37% placebo effect while BV remained in 22% of the metronidazole group and 63% of the placebo group at 1 month. This suggests confounding by factors, such as a lack of effectiveness of metronidazole in the treatment group or spontaneous resolution in the placebo group. Finally, treatment was started late in pregnancy with 44% treated after 20 weeks gestation, and no women were treated before 16 weeks gestation.

ORACLE II Study

The ORACLE II trial is commonly cited as evidence that antibiotic treatment does not prevent PTB. More accurately, the ORACLE II trial should be cited as demonstrating that if inappropriate antibiotics are given to women too late in pregnancy with no objective evidence of abnormal vaginal microflora then they are ineffective in preventing infection-related PTB. Accordingly, in any SR&MAs related to the use of antibiotics to prevent infection-related PTB, the Oracle II study should be excluded; sadly, this is not the case and due to the numbers involved the study has a strong weighting such that the positive results of other studies are negated. Erythromycin and coamoxiclav were used in this study (132) because of the perceived importance of *Ureaplasmas* in neonatal infectious morbidity (133), but neither is recommended for the treatment of BV (76). It is notable that erythromycin, while being effective against *Ureaplasma* spp., exhibits minimal passage across the placenta and hence does not reach effective concentrations in AF. Hence it is not effective in eradicating intrauterine *Ureaplasma* infections. Women with known infections were excluded, and the trial protocol required no objective evidence of abnormal vaginal colonization for the diagnosis of BV (37). Without objective evidence of abnormal vaginal microflora, at least 60% and probably more at this late gestation were not in infection-related SPTL. There are also serious concerns about the accuracy of diagnosis of SPTL. Only 50% of cases required tocolytics and around 90% of women were still undelivered after

48 h. Approximately 85% of women remained undelivered by 7 days and the mean gestational age at delivery was 38 weeks. The timing of administration of antibiotics was also questioned since the intervention occurred after SPTL had begun. Finally, in the 7-year follow-up report of the ORACLE II study (131), the assessment of cerebral palsy has been questioned. The assessment only applied to the two-thirds of cases that were recruited from the UK rather than the Republic of Ireland, and the assessment of cerebral palsy was based upon telephone calls to the parents, and in some cases, parent completed postal questionnaires rather than an objective, structured neurobehavioral assessment by skilled healthcare professionals.

Cochrane Systematic Review (Updated 2013)

The recently updated Cochrane Review (63, 74) is already being cited as evidence that antibiotics are unhelpful for the prevention of PTB. The updated review (74) is extensive and contains data from 21 trials and reports 57 different analyses. These numbers are necessary because the included studies used different risk groups, diagnostic methods, degrees of abnormal microflora, antibiotic dose regimens and routes of administration, host susceptibilities, host response, gestational age at time of treatment, outcome parameters, and definitions of success (44–53, 55–58) resulting in different results. However, in contrast to the systematic review reported below (43), the Cochrane Review (74) includes studies which used antibiotics that are not recommended for the treatment of BV and importantly did not consider the effect of pharmacogenetics (106) as outlined in the Section above "Choice of patient." In addition, the review included women with a previous PTB of non-infectious etiology. It did not differentiate between clindamycin and metronidazole when assessing the benefit of treatment before 20 weeks gestation. The review selected studies of pregnant women with either "BV" or "intermediate microflora" without considering that these are different entities with differing rates of response to antibiotics (134). Finally, the review did not include recent evidence of the benefit of rescreening and retreating BV in pregnancy (127) and the title of the Review was "the treatment of bacterial vaginosis in pregnancy" and should not have been used to comment on the prevention of preterm birth.

AJOG Systematic Review 2011

To address the deficiencies of existing SR&MA with respect to the optimal choice of agent, the choice of patient, and the timing of intervention, we performed a SR&MA of clindamycin use before 22 weeks gestation in women with abnormal genital tract microflora (43). The hypothesis of the review was that previous SR&MA on the use of antibiotics used prophylactically for the prevention of PTB or their individual studies were flawed by the fact that undue reliance was placed on studies in which suboptimal antibiotics (mainly metronidazole) were used. They were also flawed by the fact that antibiotics were used too late in pregnancy to influence outcome (23–27 weeks gestation) and used in women whose risk of PTB was not due to BV but due to some other markers not directly related to infection. Conversely, the hypothesis of the SM&MA was that antibiotics that are active against BV or BV-related organisms that are appropriately used in women whose risk of PTB is due to abnormal genital tract colonization and that are administered early in pregnancy before irreversible inflammatory damage occurs can reduce the rate of PTB. The primary outcome of the studies included in this SR&MA was spontaneous PTB at <37 completed weeks gestation and late miscarriage. These were chosen because they were used in most meta-analyses that evaluated preventative strategies for PTB. In the meta-analysis, the RR for delivery <33 weeks was 0.44 (95% CI: 0.41–1.41; nine versus four cases), but due to the low numbers this was not statistically significant. Although the reduction was consistent with the beneficial effect of clindamycin seen in the later gestational age groups, further research is required to confirm efficacy at lower gestations. The SR&MA demonstrated that when clindamycin was compared to controls, administration before 22 weeks gestation to women with objective evidence of abnormal genital tract microflora was associated with a significant reduction in the rates of PTB and late miscarriage by 40 and 80%, respectively.

Secondary Outcome Variables

The secondary outcome variables demonstrated that of those infants born preterm, low birth weight occurred in 20% of those who received clindamycin compared to 80% of those who received no treatment ($P < 0.009$). There was also a 32.5-day difference in the mean prolongation of pregnancy in favor of clindamycin compared with no treatment ($P < 0.024$) (62). In women with the highest Nugent Score of 10, late miscarriage and PTB occurred in 5.4% of those who received clindamycin compared to 35.7% of those who received placebo (60). Finally, the rate of late miscarriage or PTB was 28% in those women with persistent BV compared to 10% in those in whom BV was cured (OR = 2.9; 95% CI = 1.3–5.2), and the rate of late miscarriage or PTB was 15% in women with cured but recurrent BV, compared to only 2% in those women whose BV was cured with no recurrence (OR = 9.3; 95% CI = 1.6–53.5) (54).

SAFETY OF AND RESISTANCE TO ANTIBIOTICS IN PREGNANCY

The safety of antimicrobials in pregnancy has recently been reviewed (135). A common response to the case for antibiotic use to prevent infection-related PTB is that we already use antibiotics too frequently in pregnancy. However, few can cite local/personal audit of such practice. Accordingly, we reported a large, population-based study comprising nearly 1 million Danish women which demonstrated that >40% received antimicrobials at some stage during pregnancy (136). We felt that this might be an underestimate because Denmark, like other Nordic countries, is cautious about the use of antibiotics in pregnancy. In addition, the Registry used included only antibiotics obtained by prescription in the community. Our response would be that by employing a more focused approach to the use of antibiotics for the prevention of infection-related PTB we would, in effect, be reducing the indiscriminate use of antibiotics already demonstrated. The development and introduction of new antibiotics has declined markedly and drug-resistant bacteria are more common in hospitals and the community. In 2013, a report by the CDC

reported that >2,000,000 people each year suffer from antibiotic resistant infections and >23,000 die as a result. Unfortunately, the number of new drugs to replace ineffective antibiotics is not adequate to meet current needs, and many major pharmaceutical companies have abandoned development of new antibiotics, focusing instead on new, long-term medications, such as statins and antihypertensives that produce greater profits. As an incentive for manufacturers to develop new antibiotics, in 2012, the Generating Antibiotic Incentives Now (GAIN) legislation was signed into US law as a part of the FDA Safety and Innovation Act. This legislation extends by 5 years the exclusivity period during which time those antibiotics that treat serious or life-threatening infections can be sold without generic competition. Drugs that fall under the GAIN provisions receive fast track and priority review status and undergo an expedited regulatory approval process with the FDA (137).

Neonatal Gut Microbiome and Atopic Disease

New information from The Human Microbiome Project using cultivation-independent, molecular-based techniques has revolutionized our understanding of the vaginal microbiome in pregnancy and the non-pregnant state (38). The immune system is primed *in utero* and modified after birth. Accordingly, the use of antibiotics during pregnancy or the neonatal period may cause disruption of the developing neonatal gut microbiome, resulting in a failure of maturation of the immune response and the subsequent development of asthma, allergy, and atopic disease (138–141). This has led to new initiatives such as the Neomune Project publicly funded by the Danish Council for Strategic Research whose objective is to develop new diet and gut microflora treatments for new born infants.

EVIDENCE-BASED MEDICINE

Guidelines issued by professional bodies, such as the Royal College of Obstetricians and Gynecologists, use a systematically developed standardized methodology (http://www.rcog.org.uk/guidelines) and a standardized grading scheme for the classification of evidence levels and grades of recommendations. Other organizations or governing bodies that produce guidelines use very similar methodology and schemes. The highest classification of evidence is 1++, which is defined as "high-quality meta-analyses, systematic reviews of randomized controlled trials or randomized controlled trials with a very low risk of bias." The highest grade of recommendation is A which is defined as "At least one meta-analysis, systematic review or randomized controlled trial rated as 1++ and directly applicable to the target population; or a systematic review of randomized controlled trials or a body of evidence consisting principally of studies rated as 1+ directly applicable to the target population and demonstrating overall consistency of results." Those who provide guidelines and recommendations base these on *some* SR&MAs of *some* RCTs as a result of which they may claim to be "evidence based." SR&MAs are only as good as those studies included and if the questions asked about the populations, interventions, and outcomes are wrong or misdirected they should not be used to make recommendations or provide clinical guidelines (75). The Cochrane database is often the default of many clinicians looking for information, yet Cochrane is not without its faults, particularly in the area of PTB (142). Most SR&MAs of antibiotics for the prevention of PTB (Cochrane or otherwise) ask the question "In women at risk of PTB (population), do antibiotics (intervention) reduce the rate of PTB (outcome)." It is not surprising, therefore, that the updated Cochrane review of 2013 (74) contained data from 21 trials and required 57 different analyses because the included studies used different risk groups, diagnostic methods, degrees of abnormal microflora, antibiotic dose regimens and routes of administration, host susceptibilities, host response, gestational age at time of treatment, outcome parameters, and definitions of success (44–53, 55–58) resulting in different results. In contrast to other SR&MAs, in the AJOG SR&MA (43), the question was much more focused: "In pregnant women at risk of PTB of infectious etiology (population) does clindamycin administered before 22 weeks gestation (intervention) reduce the rate of PTB or late miscarriage (outcome)."

FUTURE RESEARCH

Even if the evidence based data is insufficient for some, they must at least accept that clinical equipoise exists (the ethical basis for medical research that involves assigning patients to different treatment arms of a clinical trial) and support a definitive randomized controlled trial. The choice of antibiotics (a) should be active against those organisms known to be associated with PTB, (b) should only be used in women with abnormal genital tract microflora, and (c) should be used early in pregnancy before infection and inflammation can cause irreversible tissue damage which will inevitably lead to SPTL and PTB. Such a study should contain genomic, transcriptomic, proteomic, and metabolomic studies to assess the vaginal microbiome, the vaginal milieu created by different microbiomic communities, and the host response of the individual to each sub-type of microbiome and milieu. PTB *per se* is only a surrogate for neonatal outcome. Detailed neonatal outcome data with appropriate long-term follow-up as well as the number of days gained from treatment to delivery should be the primary outcome parameters. Finally, different phenotypical outcomes of SPTL and PTB, such as late miscarriage, extreme PTB around the limits of viability, PPROM, late PTB, preterm stillbirth, and SPTL with intact membranes with or without vaginal bleeding should be considered. This is because the combination of different vaginal microbial communities, different vaginal milieu, and different host response may result in a range of phenotypic outcomes from normal term delivery to preterm stillbirth or severe morbidity associated with extremely premature birth.

CONCLUSION

The earlier in pregnancy at which PTB occurs, the more likely this is to be due to infection (14). The earlier in pregnancy at which abnormal genital tract colonization is detected, the greater

is the risk of an adverse outcome like late miscarriage or PTB (24). Abnormal vaginal microflora in early pregnancy, even if this resolves, is still associated with an adverse outcome (44) suggesting that whatever damage is caused by infection, this occurs early and persists. Accordingly, if antibiotics are to be used to prevent infection-related PTB these should be administered early. New evidence from molecular-based culture-independent studies of the vaginal microbiome (38) indicates that across the range of different microbial communities or sub-types of BV the bacteria detected are more likely to respond to clindamycin than metronidazole. Finally, treatment on the basis of the previous PTB should be predicated by some measure of infective etiology. Antibiotic treatment on the basis of previous PTB of unknown etiology or other risk factors for PTB unrelated to abnormal genital tract microflora should be discouraged. While individual studies have found benefit of antibiotic intervention for the prevention of PTB, in meta-analyses, these effects have been negated by large methodologically flawed studies with negative results. While (rightly) SR&MAs of efficacy focus on primary outcome parameters, the benefits associated with secondary outcomes are important and should not be ignored (*vide supra* Secondary outcomes) (43). At worst, equipoise exists with respect to the early use of clindamycin for the prevention of infection-related PTB. If a further, hopefully definitive trial is deemed necessary, this should be of a design and contains molecular omic data which will give a greater understanding of the underlying systems biology and mechanisms involved so that the same mistakes of previous flawed studies are not repeated. In the meantime, the use of antibiotics in pregnancy for the prevention of PTB should be restricted to those who are most likely to benefit.

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REVIEW
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A New, Potent, and Placenta-Permeable Macrolide Antibiotic, Solithromycin, for the Prevention and Treatment of Bacterial Infections in Pregnancy

Jeffrey A. Keelan, Matthew S. Payne, Matthew W. Kemp, Demelza J. Ireland and John P. Newnham*

King Edward Memorial Hospital, School of Women's and Infants' Health, University of Western Australia, Perth, WA, Australia

Intrauterine infection–inflammation is a major cause of early preterm birth and subsequent neonatal mortality and acute or long-term morbidity. Antibiotics can be administered in pregnancy to prevent preterm birth either prophylactically to women at high risk for preterm delivery, or to women with diagnosed intrauterine infection, prelabor rupture of membranes, or in suspected preterm labor. The therapeutic goals of each of these scenarios are different, with different pharmacological considerations, although effective antimicrobial therapy is an essential requirement. An ideal antibiotic for these clinical indications would be (a) one that is easily administered and orally bioactive, (b) has a favorable adverse effect profile (devoid of reproductive toxicity or teratogenicity), (c) is effective against the wide range of microorganisms known to be commonly associated with intra-amniotic infection, (d) provides effective antimicrobial protection within both the fetal and amniotic compartments after maternal delivery, (e) has anti-inflammatory properties, and (f) is effective against antibiotic-resistant microorganisms. Here, we review the evidence from clinical, animal, and *ex vivo/in vitro* studies that demonstrate that a new macrolide-derived antibiotic – *solithromycin* – has all of these properties and, hence, may be an ideal antibiotic for the treatment and prevention of intrauterine infection-related pregnancy complications. While this evidence is extremely encouraging, it is still preliminary. A number of key studies need to be completed before solithromycin's true potential for use in pregnancy can be ascertained.

Keywords: macrolide antibiotics, intrauterine infection, prelabor rupture of membranes, *Ureaplasma*, *Mycoplasma*, pregnancy

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****Correspondence:***
Jeffrey A. Keelan
jeff.keelan@uwa.edu.au

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INTRODUCTION

Preterm infants are at high risk of adverse outcomes, including both acute and long-term disability and death (1–4). Evidence from multiple clinical and animal studies suggests that the majority of early preterm deliveries (before 34 weeks' gestation) arise as the result of intrauterine infection and inflammation (5, 6), although causation is hard to prove in any individual case.

Ascending intrauterine infection occurs when bacteria residing in the vagina ascend and breach the cervical barrier, colonize and invade the fetal membranes and amniotic fluid (AF) – and sometimes infect the fetus itself (7, 8). When a vigorous inflammatory response ensues (typically manifested as histologic chorioamnionitis), this may trigger preterm labor and delivery (7, 9, 10). Microbial colonization of the amniotic cavity without a significant inflammatory response rarely manifests as a cause of preterm delivery (11–13).

In order to successfully prevent intra-amniotic infection-associated preterm birth and associated neonatal sequelae, an effective antibiotic therapy needs to be a core component of any pharmaceutical solution. Ideally an antibiotic administered antenatally for preterm birth prevention should (a) be easily administered and orally bioactive; (b) have a favorable adverse effect profile in pregnancy (devoid of reproductive toxicity or teratogenicity); (c) exhibit efficacy against the wide range of microorganisms known to be commonly associated with intra-amniotic infection; (d) be able to provide effective antimicrobial protection within both the fetal and amniotic compartments after maternal delivery; (e) possess anti-inflammatory properties; and (f) be effective against antibiotic-resistant microorganisms.

In this review, we discuss the key properties, benefits, and potential obstetric and perinatal applications of the novel antibiotic *solithromycin*. We suggest that solithromycin is the first antibiotic that may meet all of the above-mentioned criteria and as such has the potential to represent an exciting and major new advance in obstetric and perinatal medicine. We highlight the key indications where solithromycin may be of most benefit, and identify areas where further research is needed in order to facilitate the introduction of solithromycin into obstetric practice.

INTRAUTERINE INFECTION, ANTIBIOTICS, AND PRETERM BIRTH

Intrauterine infection and inflammation play a well-recognized role in the etiology of spontaneous preterm labor and birth, particularly in early preterm deliveries or those complicated by preterm prelabor rupture of membranes (PPROM) (6, 9, 12). A large number of microorganisms have been implicated in the etiology of preterm birth, including many organisms commonly found in normal vaginal microbiota as well as conditions associated with vaginal dysbiosis (**Figure 1**) (14–16). Some of the bacteria that commonly cause infection-driven preterm birth are common Gram-positive bacteria frequently found in the reproductive tract of pregnant women; some are more closely associated with oral microbes, while others are often found in women with abnormal vaginal microbiota [bacterial vaginosis (BV)] and/or are associated with reproductive tract infections (14, 17–19). In many preterm deliveries, multiple bacteria are present in the amniotic cavity (14, 17, 20). The incidence of confirmed intra-amniotic infection in preterm deliveries varies according to a variety of factors, including race and gestational age. In a recent analysis, Romero et al. reported that AF bacterial colonization rates are around 10–15% in preterm births overall, approaching 30% in extreme preterm births delivered before 30 weeks' gestation (12). In an earlier review of the topic, DiGiulio described a frequency of AF infection ranging from 15 to 50%, with pregnancies complicated by PPROM having a similar infection rate (14). Intra-amniotic inflammation (with or without infection) is considerably more common, and increases more markedly with decreasing gestational age at delivery (12, 21, 22).

Bacteria of the class Mollicutes, in particular the "genital mycoplasmas," such as *Ureaplasma parvum*, *Ureaplasma urealyticum*,

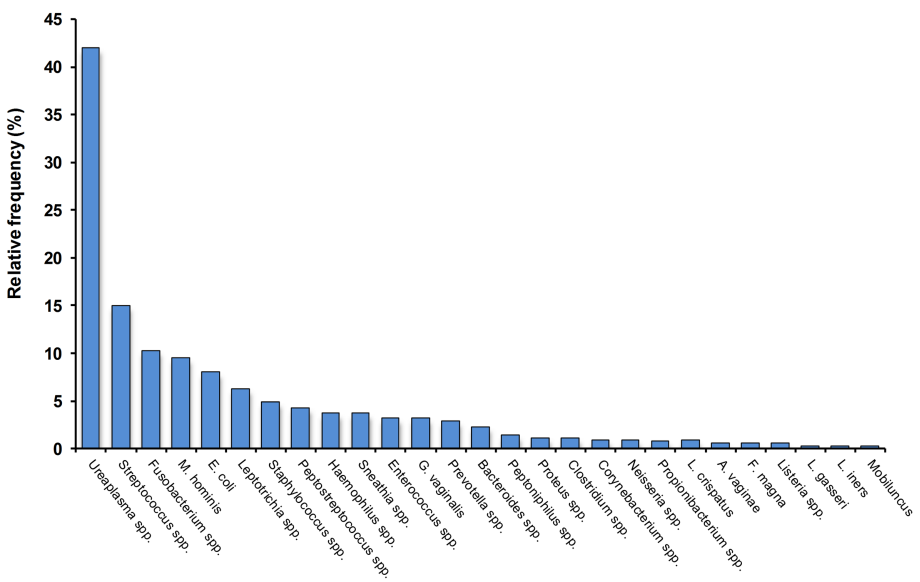

FIGURE 1 | Relative frequency of colonization by different bacteria of the amniotic cavity in preterm deliveries with intact membranes and intra-amniotic infection. Note that more than one bacterium are frequently detected. Data, compiled from Ref. (16–19), are indicative only and will vary according to clinical and demographic characteristics, plus methodological differences.

and *Mycoplasma hominis*, are the most common group of microorganisms isolated from the amniotic cavity of preterm deliveries (9, 14). Vaginal colonization rates of these organisms in pregnant women ranges from 35 to 90% for *Ureaplasma* spp. and 5–75% for *Mycoplasma hominis* (23). Dual colonization with both microorganisms is approximately fourfold more common in women with preterm vs. term deliveries (23, 24). Most studies with a preterm birth endpoint have reported a significant association with intrauterine *Ureaplasma* sp. colonization and preterm birth (25); studies of AF and placental tissues obtained from preterm deliveries show a clear link between *Ureaplasma* colonization, a vigorous inflammatory response, and preterm delivery (24–29).

The clinical evidence is supported by experimental studies consistent with causality (30). Using a pregnant sheep model (31), we reported that intra-amniotic injection with *Ureaplasma parvum* resulted in chronic chorioamnionitis accompanied by pro-inflammatory cytokines in the AF and enhanced lung maturation. Experiments in Rhesus macaques have shown that intra-amniotic *Ureaplasma* sp. injection also drives intrauterine cytokine and prostaglandin production, preterm labor, and chorioamnionitis, replicating the disease pathogenesis and ontogeny observed in human pregnancy (32, 33). Together, these and other studies have shown that robust intrauterine inflammation sufficient to cause preterm birth can be induced by *Ureaplasma* sp. colonization of the amniotic cavity (25). However, it is important to note that around half of all preterm deliveries with intra-amniotic infection contain bacteria other than the genital Mycoplasmataceae, and a large number of bacterial species have been associated with inflammation-driven preterm birth (14, 17, 18, 34).

A number of clinical trials of maternal antibiotic administration have been performed to attempt to prevent or treat intrauterine infection with the aim of reducing the rates of preterm birth and associated neonatal morbidities. As discussed in detail in this series by Lamont (35), some recent meta-analyses have concluded that antibiotic treatment of BV does not prevent preterm birth or improve neonatal outcomes (36–41). Metronidazole and clindamycin are the two most studied antibiotics. It should be noted here that conventional treatment of BV results in relatively high recurrence rates (42–44), and that the antibiotics commonly used to treat BV show only weak activity against *Mycoplasma hominis* (erythromycin, azithromycin, metronidazole) or *Ureaplasma* spp. (metronidazole, clindamycin) (14). High concentrations of these antibiotics may be required for efficacy that may not be achievable with standard oral doses due to their comparatively low oral bioavailability or adverse effects profile.

However, there are some studies that suggest that prophylactic antibiotic administration can be effective – if given before 20 weeks' gestation (35). This is presumably because antimicrobial therapy is most effective and beneficial when administered prior to colonization of the amniotic cavity (45, 46). A retrospective study of clindamycin treatment of women with genital mycoplasmas at high risk of preterm birth found a small but significant reduction in preterm birth rates and neonatal complications (47). In addition to clindamycin, azithromycin may also be effective. In non-human primates, Grigsby and colleagues showed that 10 days of high-dose maternal azithromycin treatment delays preterm labor induced by experimental intra-amniotic *Ureaplasma* spp. infection and prevents fetal inflammatory response (32). We recently showed in our ovine model that a 4-day course of azithromycin-delivered maternally (10 mg/kg i.v.) eradicated intra-amniotic *Ureaplasma parvum* infection (48). Surprisingly, there are only two clinical studies of macrolide treatment of vaginal *Ureaplasma* spp. colonization on pregnancy outcome, the results of which are inconclusive (49, 50).

In addition to difficulties surrounding diagnosis of infection and the appropriate selection of antibiotics, a fundamental reason for the lack of success of antibiotic trials for preterm birth prevention may lie in the limitations of the antibiotics employed. While macrolide antibiotics, such as erythromycin and azithromycin, are considered effective in treating important microorganisms, such as *Ureaplasma* spp., and are generally free of serious maternal and fetal side effects, their potency against genital mycoplasmas is not high, and there is growing prevalence of antibiotic resistance in these organisms (23). Studies have shown that maternal erythromycin administration is largely ineffective in eradicating intrauterine infection (39, 51, 52). This is likely due to poor transplacental passage of macrolides, estimated to be only 2–4% (53, 54). We previously showed in our pregnant sheep model that maternal macrolide administration fails to deliver effective levels of antibiotic to either the fetal circulation or the amniotic cavity (55) and does not eradicate intra-amniotic *Ureaplasma parvum* infection (52). Human studies confirm that the degree of maternal-to-fetal (M:F) passage of macrolides, such as erythromycin and azithromycin, is low and variable (53, 54), while the extent of maternal-to-amniotic transfer is only marginally greater. Antibiotics with better maternal-amniotic-fetal transfer properties and enhanced potencies against key bacterial pathogens are required to eliminate intra-amniotic infection and prevent significant neonatal morbidity and mortality.

SOLITHROMYCIN: PHARMACODYNAMICS AND ANTIMICROBIAL PROPERTIES

Solithromycin, a fourth-generation macrolide derived from clarithromycin, is a novel fluoroketolide antibiotic being developed by Cempra Inc. (Chapel Hill, NC, USA) for the treatment of community-acquired pneumonia and a variety of other indications (56, 57). It exhibits broad-spectrum activity against Gram-positive and some Gram-negative organisms, including many that are resistant to other macrolide antibiotics (58–67). It is acid stable (63) and has excellent oral bioavailability (~70%), superior to the approved macrolides (68–70). Solithromycin also demonstrates excellent tissue uptake and accumulation, important when considering its activity in tissues infected with intracellular pathogens, such as *Ureaplasma* sp. (70, 71).

Like other macrolides, solithromycin contains a 14-atom lactone ring structure and selectively binds to the peptide exit tunnel of the bacterial ribosome, blocking subunit assembly, and mRNA translation and protein synthesis (72). It has three key structural features that distinguish it from first- and second-generation macrolides: a keto group replacing the cladinose moiety (hence, the origin of the class name "ketolide"), a fluoro group at the C2 position of the lactone ring, and an aryl–aryl side

chain at C11–C12. Deletion of the cladinose structure renders the molecule insensitive to methylation-dependent resistance. Hydrogen bonding via the amino-phenyl headgroup of the C11,C12 side chain is primarily responsible for solithromycin's high-affinity bacterial ribosomal binding properties, while the fluoro group enhances binding in some macrolide-resistant strains. Ketolides are generally less susceptible than macrolides to bacterial efflux pumps, enhancing their efficacy in some species. In addition, there is some evidence that solithromycin selectively blocks translation of specific polypeptides, which confers additional antimicrobial efficacy over traditional macrolides (72).

Solithromycin has been shown to exhibit excellent activity against many of the microbial species known to be associated with infection-associated preterm delivery (**Table 1**), including *Ureaplasma* spp., *Mycoplasma* spp., Group B streptococci, staphylococci, and *Chlamydia trachomatis* (59, 61, 65–67, 73, 74). Indeed, although solithromycin has not yet been tested on all relevant microorganisms, from the established efficacy profile of its parent drug clarithromycin, it is likely that solithromycin will be effective against all bacteria known to be associated with intra-amniotic infection. We recently showed that its potency against *Ureaplasma* spp. is ~30 times greater than azithromycin *in vitro* (75). Importantly, no strains of *Ureaplasma* were resistant to solithromycin, and both *Ureaplasma parvum* and *Ureaplasma urealyticum* were susceptible, with overall MIC_{90} values 125 ng/ml (compared to 2000 ng/ml for azithromycin).

Solithromycin has been shown to be well tolerated and relatively free of adverse effects, with the most frequent complaint being GI disturbance and nausea that has not been dose-limiting (57, 69, 78, 79). It is not extensively metabolized in humans and is eliminated essentially unchanged via biliary excretion; furthermore, its plasma pharmacokinetics are not altered in patients with mild and moderate renal impairment (80). Two large global Phase 3 trials in community-acquired bacterial pneumonia have recently been completed using both oral and intravenous solithromycin administration (57). The standard oral regimen for solithromycin is 800 mg on day 1 followed by 400 mg daily for 4 days (78). However, a recent clinical trial demonstrated that a single 1000 mg dose of solithromycin eradicates *Neisseria gonorrhoeae* infection at oral, rectal, and genital sites (79).

TRANSPLACENTAL PHARMACOKINETICS OF SOLITHROMYCIN: SIGNIFICANCE AND IMPLICATIONS

Critically, unlike preexisting macrolides, there is strong evidence that the M:F passage of solithromycin is comparatively efficient. We have shown in an *ex vivo* perfusion model that solithromycin readily crosses the human placenta and reaches effective concentrations in the fetal compartment (81). The M:F transfer ratio is around 0.4–0.6 for the human placenta, while in the pregnant sheep model the M:F transfer ratio of solithromycin was 0.3–0.5 – more than 10-fold greater than erythromycin and azithromycin at similar doses (5–10 mg/kg) (**Figure 2**) (82). Although ovine and human placentation differ, the similarity in apparent M:F transfer between the species (53) suggests that, in this case, the sheep is a good model to investigate biodistribution of this and other macrolides. Our data would indicate that a daily oral regimen of around 10 mg/kg would maintain effective antimicrobial protection to the fetus, achieving fetal plasma concentrations of several hundred nanogram/milliliter (**Figure 3A**) (82). Levels are likely to increase further with

TABLE 1 | Comparison of antimicrobial efficacy (MIC_{50} and MIC_{90} values) of solithromycin vs. four other relevant antibiotics against a range of important bacteria.

Organism (number of strains)	Solithromycin		Macrolides[a]		Levofloxacin		Penicillins[b]		Doxycyclin		Reference
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	#
Streptococcus pneumoniae (1363)	<30	120	<250	>2,000(Er)	1,000	1,000	<30	2000(P)			(67)
Streptococcus pyogenes (124)	60	500	8,000	>64,000(Az)	500	1000	15	15(AC)			(76)
Streptococcus agalactiae[c] (GBS)[R] (62)	30	125	>8,000	>8,000(Az)							(59)
Streptococcus agalactiae[c] (GBS)[S] (10)	8	15	<125	<125(Az)			32	47(P)			(59)
Staphylococcus aureus (4729)	60	>4000	>2,000	>2,000(Er)	<500	>4,000	1,000	>2,000(O)			(67)
Coagulase-neg Staph. (CoNS) (862)	60	>4,000	>2,000	>2,000(Er)	4,000	>4,000	>2,000	>2,000(O)			(67)
Haemophilus influenzae (150)	1,000	2,000	1,000	4,000(Az)	<500	<500	<1,000	2,000(AC)			(67)
Neisseria gonorrhoeae (246)	125	250	500	8,000(Az)			1,000	16,000(A)			(65)
Chlamydia trachomatis (10)	250	250	125	125(Az)					60	60	(77)
Mycoplasma pneumoniae (38)	0.03	0.125	0.25	0.5(Az)	500	500			125	250	(74)
Mycoplasma hominis (13)	4	8	2,000	4,000(Az)	250	500			125	8,000	(74)
Mycoplasma genitalium (40)	<1	1,000	8	>8,000(Az)					250	1,000	(61)
Ureaplasma urealyticum[d] (10)	8	31	2,000	4,000(Az)	500	1,000			1,000	16,000	(74)
Ureaplasma parvum[d] (10)	8	16	2,000	4,000(Az)	500	2,000			8,000	16,000	(74)

[a](Er) erythromycin; (Az) azithromycin.

[b](P) penicillin G; (AC) Amoxicillin–clavulanic acid; (O) oxacillin; (A) ampicillin.

[c]R, macrolide resistant; S, macrolide susceptible.

[d]More recent data from the analysis of 100 strains of Ureaplasma spp. (U. parvum and U. Urealyticum combined) suggest that the solithromycin MIC_{90} is 125 ng/ml and 2000 ng/ml for azithromycin (75).

Solithromycin data are highlighted in the shaded text.

multiple doses, such that the standard dosing regimen is likely to achieve effective levels in the fetus and intra-amniotically, but this requires experimental confirmation. The key structural features responsible for solithromycin's dramatically enhanced placental permeability are not known.

Importantly, we have also shown in sheep that significant solithromycin levels in the amniotic cavity (the primary site of infection in this context) are achieved after maternal administration (**Figure 3A**) (82); repeat daily dosing is likely to achieve even higher concentrations due to delayed clearance from AF, and thereby provide enhanced protection against less sensitive organisms (82). The pharmacokinetic profile in the group receiving an intra-amniotic bolus of solithromycin (**Figure 3B**) showed that high concentrations were achieved and maintained for over 48 h, with a half-life estimate of 16.5 h. However, it should also be pointed out that this route of administration failed to achieve therapeutic levels in either the maternal or fetal circulation (82), highlighting the need for concurrent maternal administration.

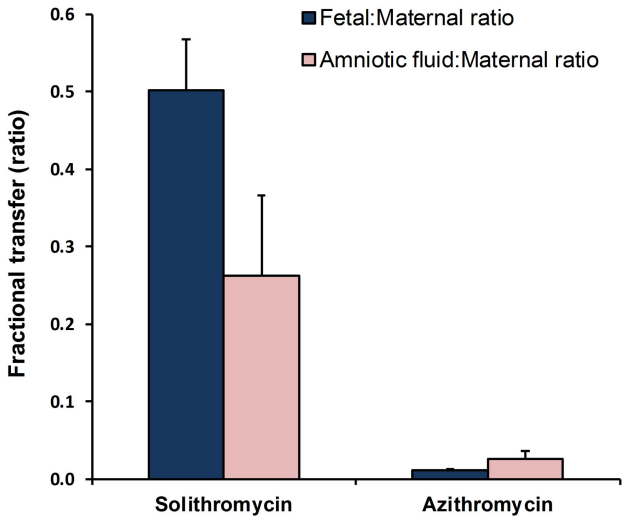

FIGURE 2 | Comparisons of maternal-to-fetal and maternal-to-amniotic transfer efficiency of solithromycin vs. azithromycin in the pregnant sheep model (10 and 5 mg/kg, respectively). Data are mean ± SD; taken from Refs. (55, 82), respectively.

REPRODUCTIVE AND DEVELOPMENTAL TOXICITY ASPECTS OF SOLITHROMYCIN

Macrolides (clarithromycin being the exception due to evidence of teratogenic effects in animal studies) are considered safe to use in pregnancy and have been administered to pregnant women for decades. Two recent, large studies of pregnancies in Canada and Israel (each including more than 100,000 women studied over a 10-year timeframe) reported no evidence of congenital malformations (including cardiac abnormalities) associated with antenatal exposure to erythromycin, azithromycin, or clarithromycin (first-, second-, and third-generation macrolides) (83, 84). Exposure during the third trimester was not associated with increased risk of perinatal mortality, low birth weight, preterm birth, or low apgar scores (83). The Israeli study also found no evidence of increased risk of pyloric stenosis (83), a complication reported in several studies to be associated with perinatal and postnatal exposure of infants to macrolides during the first few weeks of life (85, 86). It is important to note that exposure to macrolides in pregnancy (excluding the peri-partum period) was not associated with a similar increased risk (83, 85). The topic was recently discussed by de Vries and Ludvigsson et al., who pointed out some potential causes of the disparities in the literature (87); the event is rare even if the association is correct (88).

A potential concern around increased risk of congenital heart disease and maternal erythromycin exposure (odds ratio 1.92, 95% CI: 1.37–2.68) was raised in a study by Kallen (89), again

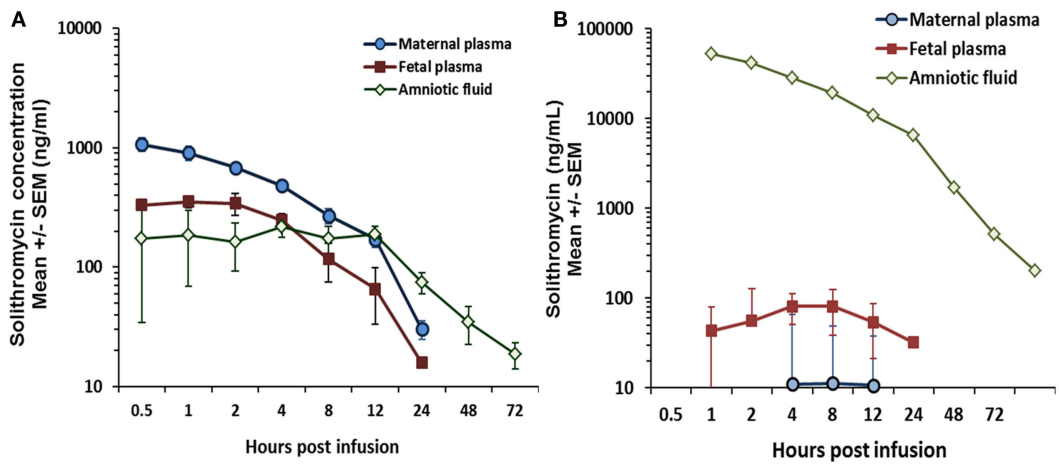

FIGURE 3 | (A) Biodistribution of solithromycin in pregnant sheep, showing concentrations in the maternal, fetal, and amniotic fluid (AF) compartments after maternal intravenous administration (10 mg/kg); **(B)** Plasma and AF concentration data in the same model after intra-amniotic injection (1.4 mg/kg fetal weight); azithromycin and solithromycin data taken from Ref (82).

using data from the Swedish Birth Register. However, a later study of over 13,000 pregnancies (280 of which were exposed to macrolides) failed to find evidence to support such an association (90). Subsequently, the risks of antenatal macrolide exposure on pyloric stenosis and congenital cardiac malformations were specifically examined in a study of almost 5000 infants born with major congenital defects over a 20-year period by Lin et al. (91). These investigators found no evidence of an association between incidence of these complications and macrolide exposure, regardless of trimester of exposure, including exposure to erythromycin specifically (91). Collectively, these studies support the conclusion that macrolides are safe to administer in pregnancy and any risks to the mother and fetus are extremely low or non-existent.

Nevertheless, if solithromycin is to be administered in pregnancy, it needs an excellent safety profile, particularly in light of its ability to cross the placenta (unlike other macrolides) that could theoretically increase the potential for adverse fetal effects. Studies to date show that its tolerability is high and its side-effects profile is favorable compared to other antibiotics. A phase 2 comparator study in adults showed that at the standard oral dose the most common adverse event was diarrhea (4.7%) followed by flatulence and nausea (1.6% each); no cardiac or neurological side effects were reported and the adverse event rate was significantly lower than in patients taking levofloxacin (78). In a series of phase 1 pharmacokinetic/safety studies, no clinically significant effects were seen, although transient increases in liver enzyme levels were observed in 40% of subjects receiving 600 mg daily over 5 days (68).

Available data suggest that the risks of developmental toxicity and/or teratogenesis with solithromycin are extremely low. The effects of orally administered solithromycin on male and female fertility and early embryonic development to implantation have been evaluated in rats and rabbits (Cempra Inc.: unpublished findings on file). No changes were noted in estrus cycles or sperm parameters in the Segment I study at the maximum dose tested (220 mg/kg). In Segment II studies, some maternal toxicity was observed at the highest dose (decreases in body weight and food consumption and treatment-related clinical signs). The "no observed adverse effect limit" for developmental toxicity was 110–220 mg/kg. No evidence of a teratogenic effect on the fetuses was evident in any treatment group.

Solithromycin has also been evaluated in three *in vitro* genetic toxicology assays and was not mutagenic or clastogenic in any of these assays. In our recent sheep studies, solithromycin-treated animals exhibited no clear evidence of hepatotoxicity (48), although the studies were not designed to specifically address toxicity. Detailed, long-term safety studies have not been carried out in this model.

An emerging concern relating to the use of antibiotics during pregnancy relates to potential adverse effects mediated by perturbation of the maternal–neonatal microbiome by, among other things, antibiotics (92–95). There is now strong evidence that maternal antibiotic exposure can cause neonatal dysbiosis and influence perinatal microbiome development, with potentially significant effects on many developmental processes (96, 97). However, it remains to be seen how dose, timing, and duration of exposure, not to mention the nature of the antibiotic itself, impacts upon these effects. It will be important to assess the effects of maternal solithromycin treatment on both the maternal microbiome (gut, vagina, and other sites) and the infant microbiome and correlate these with developmental outcomes, growth, and the incidence of allergic (98) and metabolic disorders (99, 100). At this point in time, there are no data on solithromycin's effects on the gut microbiome. However, due to its efficient absorption by the upper GI tract, it is expected that solithromycin's effects on lower GI tract microflora (typically sampled for microbiome studies) will be markedly less than other macrolides. To address this question, and to assess the short-term and longer-term effects of solithromycin administration on maternal microbiota, a series of studies are planned for the near future.

ANTI-INFLAMMATORY PROPERTIES OF SOLITHROMYCIN

It is now widely accepted that fetal and intra-amniotic inflammation, which occurs as a consequence of intrauterine infection or exposure to non-infection inflammatory agents, must be prevented in order to protect the fetus and maximize the benefits of antenatal/perinatal antimicrobial treatment (4, 101, 102). A number of pharmacological strategies have been evaluated by different researchers in order to achieve this effectively and safely. We have focused on the use of cytokine suppressive anti-inflammatory drugs (CSAIDs), in particular agents that block inflammatory signaling via NF-κB and p38MAPK pathways; an overview of these studies is presented in a companion article in this series (101). A key issue of such approaches, however, is the mode of delivery and the prevention of side effects. When given maternally, the dose administered must be large enough to ensure that the drug achieves effective anti-inflammatory concentrations in the amniotic cavity, but not sufficiently high to cause maternal toxicity or off-target side effects. To address this problem, and also overcome the lack of permeability of some agents across the human placenta, we have investigated intra-amniotic delivery of agents. We have been able to demonstrate benefits of this approach with several agents in animal and *ex vivo* models (103–105). However, while this mode of delivery has some clear benefits, including the ability to achieve quite high drug concentrations in the amniotic cavity without risk of significant maternal or fetal exposure (where fetal drug uptake is low), it also has drawbacks in that any maternal inflammation remains untreated. This limitation may represent a lost opportunity to improve pregnancy outcomes in a sub-group of women.

In this context, solithromycin may provide additional pharmacological benefits as it is also an effective anti-inflammatory agent. As it would be given maternally, and crosses the placenta relatively efficiently, solithromycin therapy may be able to achieve the benefits of inhibiting both maternal and intrauterine inflammation in addition to its antimicrobial actions – depending upon the dose administered. In a key publication, Kobayashi et al. reported that solithromycin exhibits significant NF-κB-mediated anti-inflammatory effects (reduced

cytokine and matrix-metalloproteinase (MMP)-9 expression) in human monocytes and peripheral blood mononuclear cells at concentrations ~10–40 μM (106, 107). Importantly, the anti-inflammatory effects were ≥10-fold more potent than erythromycin, clarithromycin, or azithromycin. The structural features responsible for the anti-inflammatory properties of macrolides have not been identified, although the macrocyclic ring is likely to be crucial (106); the specific structural characteristics responsible for solithromycin's enhanced anti-inflammatory properties are unknown. *In vivo* suppression of neutrophilia and MMP-9 activity in a mouse model was also achieved with solithromycin treatment (100 mg/kg) after exposure to a non-infectious inflammatory stimulus (107). The mechanism of action appears to be, in part, a combination of effects on HDAC2 activity (enhanced) and Akt phosphorylation (inhibited), via increased protein phosphatase PP2A activity (106). Solithromycin also has significant effects on NF-κB activity, probably mediated through enhanced dissociation of IκBα from p65/RelA, as has been demonstrated for other macrolides (107–110). These findings have stimulated interest in the use of solithromycin administration for treatment of chronic obstructive lung disease, asthma, and non-alcoholic steatohepatitis, with a series of investigational studies now underway.

In an *in vitro* study of human placental tissues, we confirmed the anti-inflammatory properties of solithromycin in human placentas, reporting inhibition of pro-inflammatory cytokine production by the antibiotic; however, this effect was only observed at relatively high concentrations (≥33 μg/ml, or ~40 μM) at which a decline in cell viability was observed in this model (81). Effects of a similar magnitude and potency were also observed in (maternal) decidual cells. Furthermore, data from the pregnant sheep model also support an anti-inflammatory effect of solithromycin in pregnancy. We previously reported that solithromycin, delivered maternally (10 mg/kg i.v.), decreases the levels of mRNA expression of IL-1beta, IL-6, IL-8, and *MCP2* in fetal skin of *Ureaplasma parvum*-exposed animals (48). No significant effects on the inflammation scoring of lung or chorioamnion were observed in this study, although some non-significant trends were observed toward reductions in lung cytokine expression, inflammatory histology, and cord blood white blood cell count (48).

These findings raise questions as to whether a sufficiently high dose of the antibiotic can be given to achieve anti-inflammatory benefits without toxicity. Our studies, assuming that they can be extrapolated to the pregnant woman, would suggest that large amounts of solithromycin would need to be given maternally to suppress placental and intra-amniotic inflammation, running the risk of placental toxicity and possibly other adverse effects. Administration via the intra-amniotic route by ultrasound-guided injection would be able to achieve the levels necessary to exert significant anti-inflammatory effects within the amniotic cavity without maternal or placental exposure. This may be an advantageous strategy in some clinical situations, in which the fetus is at risk and particularly rapid antimicrobial and anti-inflammatory therapy is required. Appropriate randomized clinical trials in pregnancy would be required to ensure that the benefits outweigh the potential risks of the intervention.

CONCLUSIONS, APPLICATIONS, AND FUTURE RESEARCH DIRECTIONS

We believe solithromycin has exciting potential for the treatment of intrauterine infections, prevention of preterm birth, and also treatment of perinatal and postnatal infections. Its pharmacodynamic and pharmacokinetic properties are ideally suited to these applications, and our data on transplacental passage and AF accumulation suggest that this antibiotic may represent a major advance in antimicrobial therapy in pregnancy.

There are three main obstetric scenarios where solithromycin therapy may be particularly beneficial. The first is in the prophylactic treatment of asymptomatic women at high risk of preterm birth in the first half of pregnancy. The strategy requires the ability to identify women who are at risk of intrauterine infection and, thus, target them for solithromycin treatment (35). Prognostic indications, in addition to standard clinical risk factors, could be abnormal vaginal microbiota or presence of particular microbial profiles or species (15, 16, 111–113), or a short cervix with evidence of inflammatory changes (114–116). The second situation is in women with PPROM. In these pregnancies, macrolides have been shown to have significant benefits in terms of neonatal outcomes (117); with its far superior efficacy profile and ability to treat the fetus *in utero*, it is likely that solithromycin would be much more beneficial than erythromycin for this indication. Finally, solithromycin may be effective in improving neonatal outcomes in women presenting with preterm labor and intact membranes, providing both antimicrobial and anti-inflammatory benefits to the fetus prior to delivery. In all of these scenarios, co-administration with a more potent anti-inflammatory agent may further improve outcomes. Clinical trials to explore all of these applications are warranted, once pharmacokinetic studies have been conducted to establish safe and effective dosing regimens in early, mid, and late pregnancy. Assessment of the short- and long-term effects of antenatal solithromycin therapy on the vaginal, gastrointestinal, and neonatal microbiomes would also be warranted prior to trials of its therapeutic effectiveness in pregnancy.

AUTHOR CONTRIBUTIONS

JK conceptualized the article and prepared the first draft, including the figures and tables. MP, MK, DI, and JN contributed to various sections of the text and provided input, comment, and changes to the entire manuscript.

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