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Maternal influences on fetal microbial colonization and immune development

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Abstract

While critical for normal development, the exact timing of establishment of the intestinal microbiome is unknown. For example, although preterm labor and birth have been associated with bacterial colonization of the amniotic cavity and fetal membranes for many years, the prevailing dogma of a sterile intrauterine environment during normal term pregnancies has been challenged more recently. While found to be a key contributor of evolution in the animal kingdom, maternal transmission of commensal bacteria may also constitute a critical process during healthy pregnancies in humans with yet unclear developmental importance. Metagenomic sequencing has elucidated a rich placental microbiome in normal term pregnancies likely providing important metabolic and immune contributions to the growing fetus. Conversely, an altered microbial composition during pregnancy may produce aberrant metabolites impairing fetal brain development and life-long neurological outcomes. Here we review the current understanding of microbial colonization at the feto-maternal interface and explain how normal gut colonization drives a balanced neonatal mucosal immune system, while dysbiosis contributes to aberrant immune function early in life and beyond. We discuss how maternal genetics, diet, medications, and probiotics inform the fetal microbiome in preparation for perinatal and postnatal bacterial colonization.

MICROBIAL COLONIZATION *IN UTERO*

Although critically important for normal development of the intestinal mucosal immune system (1), the exact timing of bacterial colonization of the intestinal flora is unknown. Microbial invasion of the amniotic cavity has been well described in women with preterm rupture of membranes (2). In fact, intrauterine infection is considered the most common cause of spontaneous preterm birth (3). However bacteria can also be cultured from chorioamniotic tissue using sterile procedures during cesarean sections in cases of preterm labor with *intact* membranes, indicating a possible transmission of bacteria from the maternal blood stream into the amniotic cavity (3). Recently, the combination of bacterial culture techniques with culture independent molecular methods revealed that the amniotic cavity harbors a greater diversity of microbes than previously suspected (4), including uncultivated, previously uncharacterized taxa (5). In this cohort of pregnant women with

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intact membranes, a strong inverse correlation exists between intra-amniotic detection of bacteria via culture or polymerase chain reaction and gestational age at delivery. The type and number of bacteria in the amniotic cavity likely are important for pregnancy outcomes since amniotic fluid concentrations of white blood cells and interleukin-6 were highest when bacteria were detected by both culture and polymerase chain reaction.

Historically, *Ureaplasma* species of the Firmicutes phylum were implicated as culprits of perinatal infection and severe neonatal morbidity and mortality (6). *Ureaplasma* has been isolated from amniotic fluid as early as 16–20 wk gestation and its presence in the chorioamnion alone or in the company of other microbes has been strongly associated with histologic chorioamnionitis and preterm birth (7,8). Though originally suspected to ascend from the genitourinary tract secondary to maternal colonization, hematogenous transmission via placental infection or at delivery through an infected birth canal are considered an additional source of fetal and perinatal exposure (9). The exact role of *Ureaplasma* spp. in chorioamnionitis and adverse pregnancy outcomes remains controversial since up to 60–80% of women are colonized and only a small percentage of pregnancies develop histologically proven chorioamnionitis (10,11). In addition, clinical studies in which women were treated for *Ureaplasma* before and after birth demonstrated no differences in neonatal outcomes (11). Compelling data has recently emerged associating *in utero Ureaplasma* exposure with increased rates of bronchopulmonary dysplasia, intraventricular hemorrhage, and necrotizing enterocolitis (12–14). Causal relationships and exact timing of *Ureaplasma* exposure in relation to the postnatal sequelae require further investigation. As may be the case for *Ureaplasma* exposure, some degree of bacterial colonization *in utero* may underlie normal fetal development. Because not all microbes cause preterm labor, only specific bacteria within fetal membranes, other areas of the feto-maternal unit or even the fetal gastrointestinal tract may drive adverse pregnancy outcomes during critical developmental windows (15). It remains elusive whether “pathologic” bacterial colonization is always a primary factor in premature birth.

It is possible that maternal immune factors play an important role in bacterial invasion of the amniotic cavity and/or promote an inflammatory cascade resulting in preterm labor (16). For example, prostaglandin E_2 expression is a potent negative regulator of innate immunity in the human placenta and has been shown to foster bacterial dissemination (17). Surprisingly, little is known about the innate immune capacities of intact fetal membranes, which serve as the final barrier between the external environment and the incubating fetus. Innate immune responses including antimicrobial peptide expression, such as human beta defensins (hβds), were studied *ex vivo* in human extraplacental membranes after inoculation with live *Streptococcus agalactiae* (GBS) (18). After GBS inoculation, hβd expression increased concomitant with markedly diminished recovery of GBS from fetal membranes. This robust innate immune response inhibited subsequent GBS colonization of the placenta and fetus. Additional studies are necessary to further examine the role of the maternal immune response in fetal microbial colonization and outcomes of pregnancy.

Despite an appreciation for *in utero* bacterial colonization in the setting of preterm labor and preterm rupture of membranes, the amniotic environment has traditionally been considered sterile in normal term pregnancies. Though this paradigm is conceptionally attractive, it’s

long-held dogma is nevertheless surprising considering that maternal microbial transmission is a universal phenomenon in the animal kingdom and recently has been thought to play a critical role in evolutionary development (19,20). In addition to a potential “signature” fetal microbiome, a vibrant placental microbiome has been uncovered with the application of whole metagenomic shotgun sequencing. In studies with human placental tissue, investigators revealed a vibrant placental microbiome harboring specific metabolic functions (21). The overlap between the metabolic functions of the placenta and the developing fetus are largely unknown.

Multiple recent studies employing meconium as a proxy for *in utero* bacterial communities suggest that bacterial transmission from the mother to the fetus is a regular occurrence during human pregnancies. For example, traditional culture of meconium portions in 21 healthy term newborns obtained within 2 h of birth and prior to feeding revealed a diverse group of Gram-positive and Gram-negative bacteria (22). The most commonly isolated genus was *Enterococcus* (17 samples, 80%), followed by *Staphylococcus* (11 samples, 52%). Another piece of evidence stems from studies comparing the microbiome between meconium and sequentially collected fecal samples in neonates. When meconium collected from 14 preterm infants was evaluated and compared with postnatal fecal samples collected prospectively, the meconium microbiota was significantly different than that of feces obtained within the first week of life (23). Bacilli and other Firmicutes were the main bacteria groups detected in meconium while Proteobacteria dominated fecal samples. Culture-dependent techniques showed that *Staphylococcus* predominated in meconium, while *Enterococcus*, together with Gram-negative bacteria such as *Escherichia coli*, *Escherichia fergusonii*, *Klebsiella pneumoniae*, and *Serratia marcescens*, was more abundant in fecal samples. More severe prematurity correlated with an increased detection of microbiota in meconium and a microbial community present in amniotic fluid and rich in proinflammatory cytokines (24). Specific phyla were more abundant at lower gestation, however, the exact mechanism of these potentially immunoreactive bacteria in triggering a fetal inflammatory response syndrome followed by preterm birth remains to be studied.

Recently, we detected an unprecedented microbial diversity in intestinal tissue samples obtained directly from the operating room during resection for anatomic gut anomalies shortly after birth and prior to initiation of feeds in infants delivered via cesarean section (25). Diversity measures such as Simpson’s inverse index, Shannon-Wiener, and Chao 1 estimator were statistically significantly higher in the small intestinal mucosa compared to parallel collected fecal samples, suggesting a “top-to-bottom” pattern of microbial colonization that likely occurred *in utero*.

THE FETAL MICROBIOME AND IMMUNE DEVELOPMENT

Perhaps exposure to various microbial antigens in the human fetus explains the relative early development of a functional immune system. For example, $CD4^+$ and $CD8^+$ T cells can already be identified toward the end of the first trimester in the human fetus (26). More recently, Michaëlsson *et al*. (27). discovered that fetal T cells are highly responsive to stimulation and require control by an abundant and functional pool of $FOXP3^+$ T regulatory (Treg) cells. Interestingly, fetal Treg cells appear to have been informed by substantial

numbers of maternal cells that crossed the placenta and induced antigen-specific tolerance (28).

Maternal mononuclear blood cells carry whole bacteria or their genetic material at higher frequency and diversity during pregnancy (29). As some of these bacterial signatures are represented in infant feces, it is fascinating to speculate that *in utero* transfer of maternal microbial antigens during fetal development enables a balanced immune response in the newborn to the rapidly developing microbiome postpartum. This is especially intriguing in the context of bacteria known to be beneficial for immune regulation, such as *Bifidobacteria* and *Lactobacilli* (30,31). DNA from both of these probiotic bacteria has been detected repeatedly in the human placenta (32). This proposed balanced immune response to commensal flora in the fetus is in stark contrast to immune dysregulation observed after *in utero* exposure to potentially pathogenic bacteria. For example, colonization with *Ureaplasma* spp. may alter the mucosal and/or systemic immune milieu of the fetus and newborn and constitute a risk factor for adverse postnatal outcomes such as necrotizing enterocolitis, a common and often fatal complication of prematurity (14). It is not clear if bacterial products directly affect organ system development or if inflammatory responses at the feto-maternal interface lead to immunological imbalances and priming for harmful responses to postnatal triggers (33–35).

MATERNAL INFLUENCES ON FETAL INTESTINAL COLONIZATION

In this section, we describe how genetic predisposition, diet, medications, and other environmental influences shape the maternal microbiome, which informs the fetal microbiome in preparation for perinatal and postnatal bacterial colonization (Figure 1).

Maternal Genotype

In order to define the effects of maternal genotype on the fetal and neonatal microbiome, environmental and fetal genetic influences have to be controlled for. Murine models have been particularly informative by transplanting pups to dams with different genetic origins in order to dissociate mouse pups from the influences of the microbiota from their respective families. In such studies, mice from different genetic lines that were born together after transplantation to a new dam had similar microbiota (36). This suggests a new maternal effect that is introduced by the birth mother.

While human twin studies have provided equivocal data on how host genetics may influence microbial assemblage (37–39), several single-gene studies have demonstrated a strong effect of specific host genes on microbial composition and several of these gene candidates are listed in Table 1. As one would expect, the majority of these single gene effects are observed with genes that regulate immune function and metabolism.

Maternal Diet and Medications

Maternal diet seems to be a significant contributor to healthy pregnancy outcomes and prevention of premature birth (40). Specifically, consumption of probiotic containing food components may reduce the risk for spontaneous preterm delivery (41) and childhood allergic diseases (42). In a randomized trial in Finland a few years ago, pregnant mothers

were given *Bifidobacterium lactis*, in combination with *Lactobacillus rhamnosus* GG (LGG) or placebo for 14 d before elective cesarean section at full term (43). Microbial DNA in amniotic fluid and placenta was associated with changes in innate immunity gene expression in placenta and meconium. Novel probiotic bacterial-derived proteins, LGG p75 (75 kilodaltons) and p40 (40 kilodaltons), are particularly promising as maternal therapies during pregnancy (44). In murine models of inflammatory bowel disease, they suppress cytokine-induced colonic epithelial apoptosis and injury. While other live probiotics have been associated with bacteremia in the very young (45), these soluble proteins do not pose the same risks yet still have potent anti-inflammatory activity at the level of the intestinal mucosa. Manipulation of the maternal microbiota with dietary supplements such as p40 and p75 could potentially temporize *in utero* fetal inflammatory responses triggered by chorioamnionitis and restore intestinal microbial homeostasis at the feto-maternal interface.

Antibiotics are one of the few medications whose effects on the maternal and fetal microbiomes have been studied (46). When given to pregnant dams, an altered neonatal gut microbiome, reduced intestinal host defense and increased risk for neonatal sepsis were observed. This was thought to be due to decreased transfer of bacteria during and/or shortly after delivery. However, it is likewise possible that maternal antibiotics limit bacterial gut colonization of the fetus even before birth resulting in anomalous immune priming.

A recent study demonstrates how high-fat maternal diet alters the offspring microbiome in primates (47). Therefore, while a shift in the intestinal microbiome during pregnancy due to hormonal or other factors may influence maternal and possibly fetal metabolism, conversely changes in pregnancy diet may shape the maternal and neonatal microbiome.

Maternal Microbiome

Transfer of bacteria from the pregnant mother to the fetus is universal in the animal kingdom. In vertebrates, one common example is transmission of *Salmonella* bacteria from contaminated chicken eggs. In an elegant experiment, Jiménez *et al.* (22) orally inoculated pregnant mice with genetically labeled *Enterococcus* that had been previously isolated from the breast milk of a healthy woman. The labeled bacteria were retrieved after term cesarean section delivery of pups from the internal meconium. However, labeled bacteria were not detected in the pups of control animals that had not been inoculated.

The exact mechanism of materno-fetal bacterial transfer is unknown. However, at least in mammals, the maternal fecal microbiome appears to shape the microbiota in the fetal intestine, and therefore, the maternal gastrointestinal tract is the most likely source (48–50). Bacterial translocation from the intestine to the maternal blood stream and from there to other organ systems is increased during pregnancy and lactation (29). Although not yet clear which process is active during human pregnancy, multiple pathways have been described for migration of luminal bacteria through the intact small intestinal epithelium. These include dendritic cells (51–53), microfold (M) cells (54), and goblet cells (55). In addition to transmigration of intact organisms, molecules such as polysaccharide A (PSA), produced by *Bacteroides fragilis*, influence the mucosal immune structure. PSA induces generation of $FOXP3^+$ Treg cells in the intestinal mucosa, which are critical for tolerance to food antigens and prevention of inflammatory complications in the gut (56–58). Supplementation with

Lactobacillus rhamnosus or *Bifidobacterium lactis* probiotics during pregnancy modulated the fetal immune response and enhanced immunoprotective components in breast milk (59). Exposure of the fetal intestine to commensal organisms or their products in swallowed amniotic fluid may be an important contributor to gut maturation. Indirect effects on the fetal microbiome may be achieved by other forms of maternal exposures such as gluten-free diet. Gluten-free diet fed during pregnancy changed gut microbiota in both mothers and offsprings resulting in shifts to a less proinflammatory immunological milieu in the gut and pancreas (60).

Aagard's recent study, detailing a robust placental microbiome, suggests a different mode for transmission of bacteria to the fetus. The investigators found that the placenta microbiome resembled microbiome in an adult human's mouth more than the vaginal, skin, gut, or other body microbiomes. However, comparisons between placental tissue and oral microbiota were not made between the same individuals and all oral samples were collected from nonpregnant patients. Given the shifts in the bacterial metabolic machinery (61) and systemic and mucosal immune functions (62) during pregnancy, the findings on the origin of the placenta microbiome require further validation in a cohort of pregnant women.

Maternal Environment

Immune stimulation such as during maternal viral infections can possibly also alter the intestinal microbiome during pregnancy resulting in potentially long-lasting consequences for the offspring that are not limited to the digestive system. One example of where immune activation and aberrant maternal flora affect fetal brain development and determine life-long neurological outcomes was recently reported in an elegant study by Hsiao *et al.* (63). In this study, when pregnant mice were injected with polyinosinic:polycytidylic acid (poly(I:C)) mimicking viral infection, their offspring exhibited autism-like behavior and neuropathology. In this model, maternal immune activation also reduced gut barrier integrity in offspring and significantly altered their microbial composition and metabolites. Oral treatment with *Bacteroides fragilis* corrected gut permeability, microbial composition, and improved autism-like behavior in pubs born to mothers with a history of immune activation during pregnancy. This study suggests that altering fetal exposure to microbial antigens can cause persistent microbiome changes with enduring consequences. Regulating the potentially adverse microbial metabolism after inflammatory exposures during pregnancy may be a powerful approach in the future to reduce the risk of chronic diseases.

Delivery Mode

Comprehensive studies of the succession of the microbiota immediately after delivery include those described by Dominguez-Bello *et al.* (64) in a cohort of maternal-newborn dyads. Infants born vaginally acquired bacterial communities resembling their own mother's vaginal microbiota, while those delivered by cesarean section harbored bacterial communities similar to organisms found on maternal skin surfaces. Longitudinal fecal sampling reveals the striking persistence of delivery-imparted changes in the gut microbiota at 4 mo (65) and up until 2 y of age (66), including loss of bacterial richness and diversity in infants born via caesarean delivery. While health implications from inheritance of such divergent microbiota remain largely unknown, an association between cesarean sections and

obesity in offsprings has been reported (67). In addition, while the fetus was assumed to be sterile in this study, fetal microbial colonization, as described above, may be setting the stage for intestinal microbial homeostasis or dys-biosis prior to delivery.

CONCLUSION

While long accepted in the animal kingdom, routine transmission of bacterial antigen from mother to fetus during normal human pregnancy remains to be confirmed. While evidence is accumulating that microbial programming begins *in utero* and is a central component for the development of a balanced mucosal immune system in the newborn, the current research is preliminary and more studies are needed. The specific types of bacteria along with their products and communication with maternal and fetal host factors likely determine the fetal immune composition as well as immediate and long-lasting health outcomes. Fetal programming is substantially modulated during and after birth by mode of delivery, perinatal antibiotics, and type of feeding. In addition, maternal and fetal host genetic and environmental factors may play a role and our understanding of these complex interactions is currently insufficient. More knowledge in this area could inform future clinical trials of microbial manipulation of the maternal diet as a possible strategy to prevent adverse pregnancy outcomes and to foster the development of a healthy microbiome in the offspring (68).

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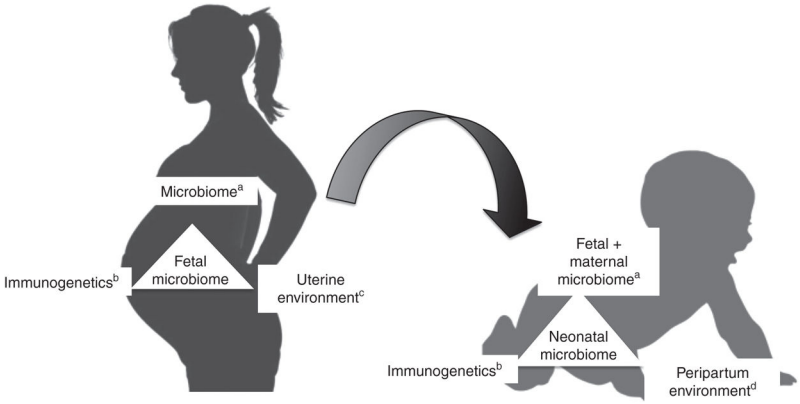

Figure 1.

Maternal influences on fetal microbial colonization and immune development. Maternal-fetal transmission of microbes is an evolutionary preserved phenomenon in the animal kingdom and likely an important mechanism for the development of a balanced immune system in the human fetus (19,69–71). (**a**) Maternal-fetal bacterial transfer is poorly understood and multiple mechanisms have been proposed (22,40–44,72). (**b**) Fetal and maternal host immunogenetic factors play a role in assemblage of the fetal and neonatal microbiome. Specific genes of interest are listed in Table 1 and reviewed by Spor *et al*. (73). (**c**) Ascending infection and ensuing chorioamnionitis have a major impact on the uterine environment and subsequent fetal development, including the intestinal microbiome (5,24). (**d**) Delivery mode (63–65) and diet (68,74) strongly influence early life microbial colonization. Other life events, especially introduction of antibiotics and solid food, are associated with major shifts in the microbiota composition (75). Despite taxonomic variation, the functionality of the newborn intestinal microbiota converges towards his/her mother's microbiome, supporting the early and sustained role of the maternal environment on health (76).

Table 1

Single genes and their respective gene products associated with modulating intestinal microbial colonization

Gene	Role of gene product	Impact on gut microbiome and associated diseases
IgA genes	Immunoglobulin A is an antibody that plays a critical role in mucosal immunology. IgA does not cross the placenta and must be generated by the fetus. It is also an important immunologic component in breast milk.	IgA has a central role in maintaining intestinal homeostasis. Transition from a neonatal to a mature microbiota in mice is in part regulated by induction of a γ-Proteobacteria-specific IgA response (77). Humans with IgA deficiency have a greater tendency towards developing celiac and inflammatory bowel diseases (78).
Defensins	Class of antimicrobial peptides produced by the host that are important for mucosal immune defense.	Without activated α-defensin, mice have fewer Bacteroidetes and Firmicutes in the small intestine (79). A reduction in gene copy number for both α- and β-defensins has been associated with Crohn's disease (80,81).
HLA genes	Human leukocyte antigen (HLA) genes on chromosome 6 encode cell-surface antigen presenting proteins triggering downstream immune responses, including antibody production.	Inheritance of specific HLA-DQ genotypes explains 40% of the genetic predisposition to Celiac disease (82). In patients at high risk of Celiac disease based on specific HLA-genotypes, fecal microbial composition showed differences in the abundances of the *Bacteroides* and *Prevotella* genera (69).
MEFV	The gene product, pyrin, interacts with the cytoskeleton of certain white blood cells to control inflammation.	This gene has been associated with familial Mediterranean fever. In fecal studies, individuals with MEFV mutations have distinct microbiomes depending on their allele carrier status. Patients with familial Mediterranean fever have less microbial richness (70).
MYD88	The myeloid differentiation primary response 88 (MYD88) adaptor protein senses bacterial products and is critical in innate immunity signaling through interactions with Toll-like receptors.	In the absence of MYD88, innate responses to pathogens are compromised. In murine cecal samples, MYD88 deficient mice had different abundance of several bacterial families (Lactobacillaceae, Rikenellaceae, and Prophyromonadaceae) compared to wild-type mice (71).
NOD2	The nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is a key intracellular pattern recognition receptor.	Significant intestinal microbiota shifts are associated with NOD2 composite genotype in specific subsets of patients with Crohn's disease and ulcerative colitis (72).

Adapted from ref. (73).