Retinopathy of prematurity (ROP), a leading cause of childhood blindness worldwide, is strongly associated with gestational age and weight at birth. Yet, many extremely preterm infants never develop ROP or develop only mild ROP with spontaneous regression. In addition, a myriad of other factors play a role in the retinal pathology, one of which may include the early gut microbiome. The complications associated with early gestational age include dysbiosis of the dynamic neonatal gut microbiome, as evidenced by the development of often concomitant conditions, such as necrotizing enterocolitis. Given this, alongside growing evidence for a gut-retina axis, there is an increasing interest in how the early intestinal environment may play a role in the pathophysiology of ROP. Potential mechanisms include dysregulation of vascular endothelial growth factor and insulin-like growth factor 1. Furthermore, the gut microbiome may be impacted by other known risk factors for ROP, such as intermittent hypoxia and sepsis treated with antibiotics. This mini-review summarizes the literature supporting these proposed avenues, establishing a foundation to guide future studies. (Am J Pathol 2023, 193: 1683—1690; https://doi.org/10.1016/j.ajpath.2023.01.013)

Retinopathy of prematurity (ROP), a vasoproliferative disease of the retina, is a leading cause of childhood blindness worldwide. The condition initially stems from downregulation of vascular endothelial growth factor (VEGF) secondary to local retinal hypoxia, compounded by loss of maternal insulin-like growth factor 1 (IGF-1) and disruption in other growth factors, leading to incomplete vascularization of the retina. This premature interruption in angiogenesis from increased oxygen tension relative to the intrauterine environment stalls retinal vascular development. Eventually, the metabolic demands of the poorly vascularized retina and subsequent local retinal hypoxia trigger a VEGF-mediated vasoproliferative phase in which aberrant neovascularization occurs. This is compounded by increases in IGF-1 as the infant matures. Although treatment is largely successful, in some cases, the disease may progress to retinal detachment and blindness. Clinically, gestational age and birth weight remain the two most significant risk factors for ROP, with current screening guidelines by the American Association for Pediatric Ophthalmology set at ≤30 weeks and ≤1500 g, respectively. In addition, high oxygen tension and fluctuations in oxygen saturation remain independent risk factors. However, most infants with these traditional risk factors never develop severe ROP, and other factors remain incompletely understood.

In other areas of neonatology, the gut microbiome has emerged as a significant player in the pathogenesis of neonatal conditions common among patients with ROP, such as necrotizing enterocolitis (NEC) and bronchopulmonary dysplasia (BPD). Located primarily in the intestines, the microbiota consists of trillions of microorganisms, including bacteriophages, eukaryotes, bacteria, archaea, and fungi. This commensal community originates at birth and evolves constantly throughout life through both endogenous and exogenous influences. These include diet, antibiotic use, environmental exposures, and genetics, alongside host adaptive and innate immune systems. In preterm infants, this complex interface is perturbed.
Through 16S rRNA-sequencing and shotgun metagenomics, Claud et al\textsuperscript{13} demonstrated a distinct deviation in microbial development between preterm infants with NEC and preterm healthy counterparts. This builds on their previous work highlighting the close relationship between the microbiome and preterm infant health, particularly through intrauterine inflammation, epigenetic changes, and intestinal epithelial differentiation.\textsuperscript{12–15} As a result, there is increasing appreciation of the early microbiome’s impact on neonatal pathologies, with growing interest in similar linkages in ROP.

The gut microbiome has extensive cross talk with various distal organs, including the eye. Perturbation in the fragile microbial community, known as gut dysbiosis, has been associated with a wide range of pathologies. Numerous studies have assessed the associative and causative roles played by the gut microbiome in these disease states. These studies include the recent identification of a gut-retina axis and associations with age-related macular degeneration, diabetic retinopathy, and retinitis pigmentosa.\textsuperscript{16–18} With the identification of this axis, alongside the dynamic and rapidly evolving nature of the perinatal microbiota and its relationship with other neonatal conditions, evidence has begun to emerge linking ROP with the gut microbiome and metabolome, the collection of small-molecule metabolites within an organism, including amino acids, fatty acids, and lipids. We hypothesize that this linkage between gut dysbiosis and ROP is driven primarily by dysregulation of VEGF, likely as a result of increased intestinal permeability and systemic inflammation (Figure 1).

This review summarizes the current clinical and preclinical findings in this novel yet expanding field. Although many findings remain largely exploratory and associative in nature, they provide a foundation for future generation of hypotheses and identification of more causative connections. This will hopefully highlight areas with potential for both future screening and therapeutic purposes.

The Developing Microbiome and Preterm Gut Dysbiosis

Although some studies, including those in the Neonatal Oxygenation Prospective Meta-analysis Collaboration, suggest an inverse relationship between NEC and ROP,\textsuperscript{19} other evidence points to NEC as a significant risk factor for ROP.\textsuperscript{20} For instance, secondary analysis of the Postnatal Growth and Retinopathy of Prematurity study showed that infants with early surgical NEC ($n = 356$) were at significantly increased risk of developing ROP and severe ROP compared with control counterparts.\textsuperscript{21} This relationship is further supported by cohort analyses showing mutually reduced rates of NEC and ROP among infants following exclusive human milk-based feeding protocols.\textsuperscript{22} Previous literature has also identified several mechanisms by which the microbiome may shape NEC pathophysiology, including deviations in microbial composition and activation of proinflammatory pathways through expression of microbial-associated molecular patterns.\textsuperscript{23} Given this possible association between NEC and ROP, and the link between NEC and the microbiome, these studies support the hypothesis that the neonatal gut microbiome is involved in the pathogenesis of ROP.

Compared with its adult counterpart, the neonatal gut microbiome is relatively simple, with communities of fungi, archaea, and eukaryotes accompanying a predominantly bacterial population.\textsuperscript{24} Although there has been recent debate, current literature continues to reaffirm the notion that acquisition and colonization of this microbial community begins at birth with overall sterility \textit{in utero}.\textsuperscript{25} Yet, the composition is still maternally influenced, particularly during vaginal vertical transmission—a process that is altered by cesarean delivery, after which greater abundance of \textit{Staphylococcus} and other skin-dwelling microbes is seen.\textsuperscript{26} Evidence has remained equivocal regarding an association between mode of delivery and ROP.\textsuperscript{20} Other factors that have been shown to influence early microbial composition of the microbiome include maternal health,\textsuperscript{27} gestational age at birth,\textsuperscript{28–30} and diet (ie, breast milk or formula).\textsuperscript{24,26} Compared with formula-fed counterparts, human milk oligosaccharides in breastfed infants have been shown to increase abundance of beneficial \textit{Bifidobacterium} strains and attenuate pathogenic species, possibly linked to decreased ROP rates among breastfed preterm infants.\textsuperscript{22,32,33} This beneficial effect of human milk on ROP may be mediated by the microbiome.

Gestational age at birth, one of the most important risk factors for ROP, also plays a significant role in neonatal gut dysbiosis. Like impairments in development and an immature immune system, preterm-related gut dysbiosis reflects longer exposure in the hospital environment, frequent use of antibiotics, and introduction of feeding tubes with associated biofilms.\textsuperscript{20} Very low birth weight preterm infants with gut dysbiosis are at increased risk of neonatal sepsis, another known risk factor for ROP.\textsuperscript{20,34} These factors lead to not only delays in colonization but also distinct intestinal microbial populations characterized by decreased \textit{Firmicutes}, \textit{Bifidobacterium}, and \textit{Lactobacillus} alongside increased \textit{Clostridium difficile} and groups of \textit{Proteobacteria}.\textsuperscript{26} Balancing this unique profile is an overall nonrandom, patterned maturation of the microbiome. Through 16S rRNA sequencing, it has been shown that the intestinal environment transitions through a succession of bacterial classes from \textit{Bacilli} to \textit{Gammaproteobacteria} to \textit{Clostridia}, an order that remains stable regardless of preterm age but varies in pace depending on the aforementioned preterm-associated factors.\textsuperscript{29}

Characteristic changes in timing and composition of the microbiome among preterm infants are associated with neonatal pathologies, including BPD and NEC.\textsuperscript{11} In a recent single-center retrospective analysis, stool from preterm neonates showed a strong association between low acetic acid
levels and increased odds of developing BPD. Given its dependence on nutritional intake and microbial composition, acetic acid and other short-chain fatty acids provide a functional representation of metabolic activity—and corresponding pathologic divergences—within the infantile microbiome. Histopathologic studies of intestinal tissue from infants with NEC suggest that the condition stems from dysregulated cycles of microbe-induced inflammation and immune response with excessive toll-like receptor-4 signaling. The fact that NEC is not reproducible in germ-free mice but recapitulated with fecal microbiota transplantation further supports the notion that early gut dysbiosis plays a significant role in neonatal disease. In contrast to the equivocal association between NEC and ROP, BPD is consistently associated with severe ROP. Although early gestational age represents a potential confounding factor among these relationships, the associations between the microbiome and each of these diseases of prematurity warrant further investigation for a similar connection between ROP and the microbiome.

### Gut Microbiome Composition and ROP

It has been suggested that preterm infants diagnosed with ROP exhibit significant differences in microbiome composition in regard to both diversity and taxonomic abundances. Skondra et al identified an enrichment of...
Enterobacteriaceae at 28 weeks postmenstrual age among infants who later developed type 1 ROP compared with matched controls. This was observed alongside a decrease in several metabolic pathways, including amino acid metabolism, oxidative phosphorylation, and benzoate degradation. Subsequently, Westaway et al. characterized the gut microbiome of probiotic-treated premature infants, assessing variations in gut flora between admission and discharge. The 16S rRNA sequencing of meconium and fecal samples showed that infants with ROP displayed significantly reduced z diversity, a marker of species diversity and evenness. In another study, microbiomes in patients with ROP at admission were significantly enriched in Staphylococcus species, which has been shown to interfere with tissue revascularization and vascular repair.  

Although the associations between the gut microbiome and ROP in these studies do not necessarily imply causation, the consistent findings across these individual studies strengthen the hypothesis that gut dysbiosis, marked by decreased diversity and enrichment in pathogenic bacterial species, may play a consequential role in the future development of ROP. The z diversity, a common indicator of microbial health, is reduced in not only ROP but also age-related macular degeneration, another vasoproliferative disease of the retina. Although the exact significance is unknown, this reduction in diversity may unmask the pathogenic contributions of rarer resident species, a less acknowledged component that can have disproportionate effects. More important, Enterobacteriaceae species, particularly proinflammatory Escherichia coli strains, have been shown to up-regulate VEGF, which is implicated in the second phase of ROP with neovascularization and the potential for retinal detachment. Such perturbations in the gut microbiome have previously been shown to have a significant impact on the retinal and retinal pigment epithelium/choroidal transcriptomes. For instance, VEGFC is differentially expressed in the retinal pigment epithelium/choroid of a germ-free murine model, suggesting a link between microbial composition, VEGF expression, and potentially ROP progression. However, despite this potential relationship, changes in local oxygen status remain the predominant driving factor behind VEGF expression in the retina. Another potential mechanistic link between the gut microbiome and retinal oxygenation involves increased intestinal permeability secondary to gut dysbiosis. This has been proposed to result in increased systemic circulation of pathogen-associated molecular patterns with associated endotoxemia. It would be reasonable to hypothesize that this para-inflammatory response exacerbates retinal oxygen tension and subsequently ROP progression.  

Despite these distinct findings, a recent meta-analysis suggests that probiotic supplementation does not seem to attenuate the risk of ROP in preterm infants. However, given the heterogeneity of probiotic supplantations between randomized control trials, the meta-analysis may overgeneralize conclusions or bury significant comparative nuances between single-strain and multistrain probiotic preparations. Furthermore, data from the study’s analyzed randomized control trials are themselves limited and gathered primarily from reported secondary outcomes. Additional studies need to be done to clarify the relationship between probiotic treatment and ROP.  

Gut Microbiome, IGF-1, and ROP  

Considering the outlined role of VEGF in ROP, and the possible connection between VEGF and the microbiome, it is important to consider key regulators of its activation. IGF-1, an anabolic hormone integral to postnatal growth and development, partially controls activation of VEGF through p44/42 mitogen-activated protein kinase. For example, it is widely established that rapidly declining and persistently low levels of the hormone in preterm infants are associated with development of ROP, with an inverse correlation in severity. This is supported by multiple prediction models connecting poor postnatal weight gain, a proxy for low IGF-1, with ROP, as well as studies associating aggressive parenteral nutrition with reduced risk of ROP. This association has led to several clinical and preclinical studies assessing potential therapeutic targets. For instance, i.v. infusion of recombinant human IGF-1 with its binding protein in extremely preterm infants did not reduce the risk of developing ROP, but decreased incidence of severe BPD. Meanwhile, among oxygen-induced retinopathy (OIR) mice, a common animal model for ROP, exogenous administration of IGF-1 led to faster maturation and decreased rates of OIR development. Factors that influence endogenous levels of the growth factor, such as the microbiota, could be considered among possible causes. Murine studies have demonstrated a strong relationship between the gut microbiome and IGF-1, with decreased levels in germ-free mice that were rescued by reconstitution with conventional microbiota from specific pathogen-free mice.  

Specific species that have shown induction of the hormone include Gram-positive bacteria, as evidenced by attenuation with oral vancomycin, as well as Lactobacillus—a probiotic previously discussed to be decreased in hypoxia-associated neonatal rat models. Despite the strong connection between ROP and IGF-1, and the substantial relationship between microbial health and IGF-1, linkage between the gut microbiota and IGF-1 activity in distant tissues, such as the eye, remains conjectural. Among potential unifying factors are metabolites produced by microbial species, specifically short-chain fatty acids. This is supported by increased circulation of IGF-1 following short-chain fatty acid supplementation in antibiotic-treated mice compared with control counterparts. Another proposed link is the regulation of local VEGF expression in the retina by systemic circulation.
of IGF-1. Previous literature has shown that IGF-1 regulates VEGF signaling specifically during retinal neovascularization.\(^63,67\) It seems possible that perturbations in the microbiome may be reflected by the presence of both unique metabolites and retinal vascularization activity associated with IGF-1.

**Metabolomics, Microbiome, and ROP: Biomarkers and Mechanistic Insights**

The impact of the microbiome on distal levels of IGF-1 highlights the close relationship between microbiota function and host metabolomics. This is further seen in disease states such as ROP, in which studies have recently shown to include a unique metabolic profile that can potentially be used for screening and therapeutic purposes. For instance, in an OIR rat model, Lu et al.\(^68\) identified arginine and proline metabolism pathways as having significant associations with VEGF pathways and the development of ROP. This preclinical study was reaffirmed by the previously described study by Skondra et al.\(^37\) which showed differences in not only arginine and proline metabolism but also histidine metabolism and degradation of valine, leucine, and isoleucine among patients with ROP. Other studies at the clinical level have shown that compared with control counterparts, patients with ROP have increased plasma citrulline, arginine, and aminoacids alongside reduced creatine levels.\(^59\) One prospective study has shown that low concentrations of the signaling lipid, sphingosine-1-phosphate, are strongly related to severe ROP.\(^60\)

Beyond the potential for novel screening biomarkers, the identification of these differential metabolites sheds light on potential pathways by which ROP may develop. For instance, excessive arginine metabolism has been tied to ROP because of increased uncoupling of nitric oxide synthase secondary to decreased l-arginine supply. This results in increased formation of superoxide, which reacts with nitric oxide to form the toxic oxidant peroxynitrite and increase oxidative stress in retinal cells.\(^61\) Arginine metabolism and its systemic availability is dependent in part on gut microbiome composition.\(^62\) Previous studies have shown that sphingosine-1-phosphate is positively associated with Klebsiella overgrowth, a bacterial population that is also up-regulated in the gut microbiota of preterm patients.\(^63,64\) Taken together, these findings suggest multiple metabolic pathways by which the gut microbiome may participate in the pathogenesis of ROP. However, many of the discussed prospective clinical studies are limited in sample size, restricted to single centers, and not controlled for confounding factors, such as diet and medication or oxygen therapies. More still needs to be done to elucidate the precise significance and mechanism behind these proposed associations.

**Intermittent Hypoxia and Gut Dysbiosis**

Neonatal intermittent hypoxia (IH), or episodic hypoxia, may represent another possible link between ROP and the microbiome. IH is a frequent occurrence in preterm neonates and a significant risk factor for ROP.\(^65\) Although its impact on oxidative stress has been well characterized, IH has also recently been shown to play a role in early gut dysbiosis with a significant reduction in abundances of commensal species. In one murine model, IH-exposed mice exhibited a greater abundance of *Firmicutes* and lower abundances of *Bacteroidetes* and *Proteobacteria*.\(^66\) Although this appears to contradict the microbial profile previously described in OIR mice, it is important to note the contextual differences in mouse model/exposure and, instead, consider the connection between hypoxia and gut dysbiosis as a whole. In a rat model exposed to intermittent hypoxia, Bodkin et al.\(^56\) and Morita et al.\(^67\) demonstrated that the neonatal microbiome had a reduction in *Lactobacillus*, a probiotic that has shown protective effects in retinal pathologies. Although the associations between IH and the microbiome alongside IH and ROP do not necessarily connect ROP with the microbiome, the compositional shifts in microbial populations secondary to intermittent hypoxia may play a pathologic role in ROP development or progression.

**The Role of Bile Acids**

Evidence for the presence of a gut microbiota—bile acid axis has been previously described and may also help elucidate the link between the gut microbiome and ROP. Following synthesis in hepatocytes, bile acids are released into the gastrointestinal tract and interact with host microbial communities, inducing varying levels of bile stress. In response, the dynamic composition of the host gut microbiome adapts and plays a significant role in controlling the global bile acid pool through metabolism of tauro-β-muricholic acid, a farnesoid X receptor antagonist.\(^68\) Previous studies have investigated the use of bile acid agents as avenues of therapeutic intervention, with ursodeoxycholic acid (UDCA) and tauroursodeoxycholic acid emerging as pharmacologic agents with extensive biological activity and potential protective effects. Beyond their reported protective benefits against photoreceptor degeneration and ganglion cell death,\(^69\) UDCA and tauroursodeoxycholic acid have also been identified as potential therapeutics in ROP. OIR mouse pups showed attenuated pathologic neovascularization, decreased OIR-induced blood-retinal barrier dysfunction, and decreased levels of oxidative stress when treated with UDCA.\(^70\) Furthermore, the secondary bile acid inhibited VEGF-STAT3 pro-angiogenic and propermeability effects in an ischemic-dependent manner, with anti-angiogenic effects localizing to avascular areas of the retina and sparing normal revascularization in the central retina. Interestingly,
these protective effects were specific to UDCA and did not extend to its derivatives, glycoursodeoxycholic acid and taurosodeoxycholic acid.

Given the interplay between bile acids and host microbiome, the protective effects exhibited by UDCA in an OIR animal model suggest that the gut microbiome may play a role in the pathogenesis of ROP. This hypothesis is supported by data showing gut microbiota—bile acid dysregulation in OIR mice. Specifically, cecal samples from the animal model showed increased levels of unconjugated bile acids alongside a microbial composition marked by increased Proteobacteria and decreased Firmicutes and Bacteroidetes. These results were further associated with decreased serum IGF-1, which is decreased in patients with ROP. Considering the high activity of bile salt hydrolases in Firmicutes and Bacteroidetes, the reduction of these microbial populations in OIR mice may play a pathogenic role in ROP and explain the protective effects of i.p. UDCA administration. However, the OIR model does not fully recapitulate ROP pathogenesis, and compositional changes in the microbiome and metabolome may be a consequence rather than a driver of the pathologic state. Future studies are needed to clarify the precise role of these associated changes.

**Neonatal Sepsis and Antibiotic Use**

Neonatal sepsis, a blood infection that occurs in infants within 90 days postpartum, has been variably tied to increased risk of ROP development. One common route of neonatal sepsis involves translocation of intestinal bacteria. Given the delay in sequential colonization in preterm neonates with a predominance of pathogenic species, as described earlier, premature infants are at greater risk of developing sepsis and related pathologies. Regarding management, the use of prolonged empirical antibiotics showed negative outcomes in the incidence of late-onset neonatal sepsis, suggesting it may impair endogenous protective microbiota. Similarly, the association between neonatal sepsis and increased risk of ROP may be driven in part through dysbiosis of the microbiome.

**Early Ocular Surface Microbiome**

Although extensive work has been done to characterize the gut microbiome and its associated systemic pathologies, it is important to briefly address emerging data regarding the early ocular surface microbiome. 16S rRNA sequencing revealed that the ocular microbiota of newborns is comprised primarily of Proteobacteria, Actinobacteria, and Firmicutes phyla, with a relative reduction in Proteobacteria and an increase in Bacteroidetes following antibiotic prophylaxis. Although relatively paucibacterial, the ocular surface microbiome has been associated in maturity with several ophthalmic diseases, including keratitis and dry eye syndrome.

**Conclusion**

While its relationship to the gut microbiome is less characterized than other retinal pathologies, such as age-related macular degeneration, recent literature suggests that ROP may also have an important connection to the intestinal environment. Although current studies remain largely associative, confirmatory testing, such as recapitulation of pathology through fecal microbiota transplantation, can help elucidate the significance of specific findings. Nevertheless, from microbiota composition and hypoxia-induced perturbations to metabolite biomarkers and gut microbiota—bile acid dysregulation, there are several emerging connections that justify further study to elucidate their precise roles and potentially pave the way for more timely screening techniques and effective therapeutic interventions.

**References**

10. Frazer LC, Yakah W, Martin CR: Decreased acetic acid in the stool of preterm infants is associated with an increased risk of bronchopulmonary dysplasia. Nutrients 2022, 14:2412


