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Review article

Retinopathy of prematurity: A review of pathophysiology and signaling pathways

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ABSTRACT

Retinopathy of prematurity (ROP) is a vasoproliferative disorder of the retina and a leading cause of visual impairment and childhood blindness worldwide. The disease is characterized by an early stage of retinal microvascular degeneration, followed by neovascularization that can lead to subsequent retinal detachment and permanent visual loss. Several factors play a key role during the different pathological stages of the disease. Oxidative and ni-

Abbreviations: AA, arachidonic acid; ADAM, "a" disintegrin and metalloproteinase; Akt, protein kinase B; Ang, angiopoietin; APE1/Ref-1, apurinic/apyrimidinic endonuclease 1/reduction-oxidation factor 1; BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; DHA, docosahexaenoic acid; eNOS, endothelial nitric oxide synthase; EPO, erythropoietin; GPCR, G protein-coupled receptor; GPR91, G protein-coupled receptor 91; HIF, hypoxia-inducible factor; IGF-1, insulin-like growth factor-1; IGFBP3, insulin-like growth factor-binding protein 3; IL-1, interleukin 1; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NADPH, nicotinamide-adenine-dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; NOX, nicotinamide-adenine-dinucleotide phosphate oxidase; Nrf2, nuclear factor erythroid 2-related factor 2; OIR, oxygen-induced retinopathy; PDGF, platelet-derived growth factor; PEDF, pigment epithelial-derived factor; PHD, prolyl hydroxylase domain; PLGF, placental growth factor; PI3K, phosphatidylinositol 3-kinase; PUFAs, polyunsaturated fatty acids; ROP, retinopathy of prematurity; ROS, reactive oxygen species; RPE, retinal pigment epithelium; Sema, semaphorins class; shRNAs, short-hairpin RNAs; siRNAs, small interfering RNAs; STAT3, signaling transducer and activator of transcription 3; TIMPs, tissue inhibitors of metalloproteinases; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

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trosative stress and inflammatory processes are important contributors to the early stage of ROP. Nitric oxide synthase and arginase play important roles in ischemia/reperfusion-induced neurovascular degeneration. Destructive neovascularization is driven by mediators of the hypoxia-inducible factor pathway, such as vascular endothelial growth factor and metabolic factors (succinate). The extracellular matrix is involved in hypoxia-induced retinal neovascularization. Vasorepulsive molecules (semaphorin 3A) intervene preventing the revascularization of the avascular zone. This review focuses on current concepts about signaling pathways and their mediators, involved in the pathogenesis of ROP, highlighting new potentially preventive and therapeutic modalities. A better understanding of the intricate molecular mechanisms underlying the pathogenesis of ROP should allow the development of more effective and targeted therapeutic agents to reduce aberrant vasoproliferation and facilitate physiological retinal vascular development.

1. Introduction

First described in 1942 as retrolental fibroplasia, retinopathy of prematurity (ROP) is a retinal neovascular disorder impacting 30–50% of very low birth weight preterm infants, making it one of the most prevalent causes of blindness and childhood visual impairment worldwide.[187,202,306] Of those affected by ROP, 25–30% develop serious eye complications, including severe ametropia, strabismus, abnormalities of retinal function, and, in the most severe cases, blindness.[187] Advances in neonatal care have helped improve the survival rate of preterm infants, resulting in an increased number of preterm infants at-risk for ROP.[202]

ROP is a multifactorial disease, with short gestational period, low birth weight, and hyperoxia being the most frequently associated factors.[349] Its pathogenesis has been widely studied in humans and animal models.[142] Development of human retinal vasculature begins during the 16th week of gestation and concludes at the 40th week.[94] Therefore, preterm infants exhibit incomplete development of the retinal vasculature and a peripheral avascular zone.[94] In addition, an increase in oxygen bioavailability at birth exposes preterm infants to a relatively hyperoxic environment that, coupled with the infant's immature antioxidant system, leads to oxidative stress.[24] The relative hyperoxia also inhibits the expression of hypoxia-inducible factor (HIF) and vascular endothelial growth factor (VEGF), disrupting the growth of retinal blood vessels.[62] During phase 1 of ROP, retinal microvascular degeneration occurs, associated with an arrest in the progressive vascularization of the peripheral retina.[93] In phase 2, these vascular changes result in retinal ischemia and trigger the release of growth factors leading to abnormal intravitreal neovascularization.[93]

Several multicenter clinical trials conducted over the past 70 years have failed to find an ideal oxygen saturation range that prevents ROP in Phase 1 without increasing morbidity and mortality, and high-oxygen treatment in Phase 2 did not show any benefit.[78,271] The insights resulting from these studies, however, have significantly improved the clinical management of ROP.[78] Although the ideal oxygen saturation range remains unknown, data from randomized clinical trials suggest that maintaining an oxygen saturation range of 90–95% appears to be safer than 85–89%.[75] Another insight from these trials is that rigorous management to avoid fluctuations in oxygen saturation is important in reducing the risk of ROP.[78] More clinical trials are needed to optimize oxygen therapy and help prevent ROP.[78]

Currently, laser photocoagulation and ablative cryotherapy are the primary treatments for ROP. They work by destroying the avascular retina that produces the growth factors responsible for neovascularization; however, neither of these therapies target the main mechanisms of pathological neovascularization.[23,78] Both treatments can reduce the incidence of blindness, but often do not improve visual acuity and have potential adverse side effects, namely inflammation, myopia, peripheral vision loss, and scar induction.[61,154] Of the 2 treatments, laser photocoagulation is more convenient to administer and results in less pain and inflammation, and relatively few systemic complications. It is the standard therapy, especially for ROP stage 3 with plus disease in zone 2.[23] Recent studies have shown promising results for antiangiogenic therapy with anti-VEGF agents. One of them, ranibizumab, was approved for the treatment of ROP in the European Union in 2019, but has yet to be approved for this specific use by the US Food and Drug Administration.[78] While studies have shown anti-VEGF to be the most effective treatment for severe ROP (zone I),[257] reports of recurrent intravitreal neovascularization presenting as late as 60 weeks post-menstrual age,[167] long-term defects in visual acuity or size of the visual field, disorders in photoreceptor functions, and the possibility of adverse outcomes in other organs during the neonatal period, remain a concern.[11,118,146]

In almost 8 decades of clinical and laboratory research, advances have been made in clarifying the pathogenesis of ROP; however, a better comprehension of ROP pathogenesis and the mechanisms that regulate angiogenesis may lead to more effective and targeted therapeutic agents for the prevention and treatment of severe ROP while preserving physiological retinal angiogenesis. We focus on existing information on mediators involved in the pathogenesis of ROP and its signaling pathways. Based on current knowledge of molecular mechanisms, we describe new potentially preventive and therapeutic modalities that can constitute important lines of investiga-

tion. We begin with a description of the animal models used to study the pathogenesis of ROP, followed by normal and pathological retinal vascularization and, lastly, the molecular mechanisms involved in ROP that constitute our main objective, highlighting potential therapeutic targets.

2. Animal models of oxygen-induced retinopathy

To avoid the ethical and safety concerns inherent in experimentation with eyes of human preterm infants, animal models of oxygen-induced retinopathy (OIR) are typically used to study the pathophysiology of ROP and evaluate potential treatments.[141] Animals such as mice, rats, and cats vascularize their retinas after birth, resulting in a retinal vascular development similar to preterm infants in eyes obtained at term in these animals[349]; however, a limitation to all animal models is that they cannot fully simulate complications that may arise after birth in preterm infants.[141]

In one of the most common models,[119,140] the mouse OIR model designed by Smith,[348] newborn mice are exposed to a high oxygen (75%) environment from postnatal day 7–12,[130] causing vaso-obliteration of capillaries already developed in the central retina. They then return to ambient air to develop OIR that leads to vasoproliferation into the vitreous at the junctions of the vascularized and avascular central retina.[140] One advantage of the mice model is the ease of genetic manipulation which facilitates the study of the molecular mechanisms of ROP.[231]

Penn's rat model of fluctuating oxygen (50/10 OIR model) is another commonly used OIR model.[119,140] The oxygen-controlled environment is changed from 50% to 10% oxygen, every 24 hours, from birth to postnatal day 14. This leads to a delay in development of the peripheral avascular retina followed by vasoproliferation at the junction of vascularized and avascular retina; similar to what is seen in human preterm infants.[140] This model is the most representative of human ROP.[119,140] Unlike mice, genetic manipulation of rats is complicated, making analysis of molecular mechanisms more challenging. Newer techniques emerged to address this limitation.[141] One such technique involves the introduction of short-hairpin RNAs or genetic mutations through gene therapy to silence RNA through small interfering RNAs or knockout specific genes.[141] For example, lentivirus has been used to link cell specific promotors with short-hairpin RNAs to target specific types of cells in the retina, resulting in the knockdown of gene products in the targeted cells only.[181] This new technique has been useful to determine the effects of angiogenic signaling on pathological and physiological retinal angiogenesis.[30,181]

In another OIR animal model newborn beagles are exposed to 100% oxygen up to postnatal day 4, causing a delay in physiological retinal vascular development and vasoproliferation.[237] The eyes of the beagle puppy have a size close to that of human preterm infants, and this translational aspect is useful when testing pharmaceutical agents to treat human ROP.[142]

The phases of ROP in animal models of OIR are similar to those of human ROP. Human ROP is divided into two phases, which are subdivided into five stages. Phase 1 of human ROP (stages 1 and 2) is defined by delayed physiologic retinal vascular development and corresponds to mouse and rat model phase 1. The stage 3 of phase 2 of human ROP is defined by neovascularization and corresponds to phase 2 of the mouse and rat models.[240,358] While part of phase 2, the final two stages of ROP in humans (stages 4 and 5) are sometimes considered a pseudo-phase 3. They are characterized by fibrovascular changes with retinal detachment.[141,237,249] This "phase-3″ is absent from the mouse and rat models, as neither model reproduces the retinal detachment seen in human ROP; however, the beagle model shows some characteristics of the advanced stages of human ROP, specifically the retinal folding seen in stage 4.[142,249]

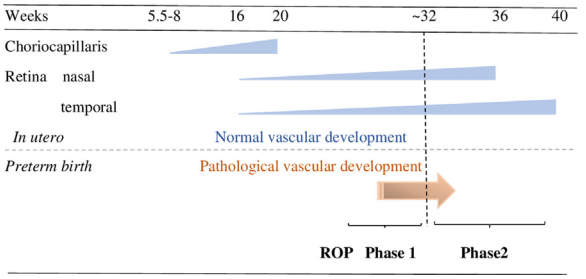

Figure 1 – Timeline of normal vascular development versus pathological vascular development (ROP phases) by weeks of gestational age. The development of the choriocapillaris starts between 5.5 and 8 weeks and is completed at 20–22 weeks. Retinal vascularization starts at around 16 weeks. Retinal blood vessels grow radially from the optic disc towards the ora serrata. Vascularization of the nasal retina is completed at around 36 weeks and that of the temporal retina at 40 weeks. The transition between phase 1 and phase 2 of ROP generally occurs around 32 weeks.

3. Normal and pathological retinal vascularization

3.1. *Retinal vascularization*

Nutrients and oxygen are supplied to the retina via two-vessel systems: the choroidal circulation that receives the largest blood flow (65–85%) and nourishes the outer part of the retina (photoreceptors) and the retinal circulation, responsible for a smaller part of the flow (20–30%) and supplies the inner layers.[13] The choroidal circulation is complete at around 20 weeks of gestation (Fig. 1).[36] The development of retinal blood vessels in humans begins at around 16 weeks of gestation and spreads from the middle of the optical disc outward.[173] The nasal retina is vascularized at 36 weeks of gestation and the temporal retina at around 40 weeks.[94] For this reason, preterm infants present incompletely vascularized retinas, with the area of the peripheral avascular zone dependent on the gestational age.[173,349]

Vascular development occurs in two phases: vasculogenic and angiogenic.[172] The vasculogenic phase consists of the formation of new blood vessels from bone marrow-derived angioblasts and is usually seen during embryogenesis.[55,105,172]

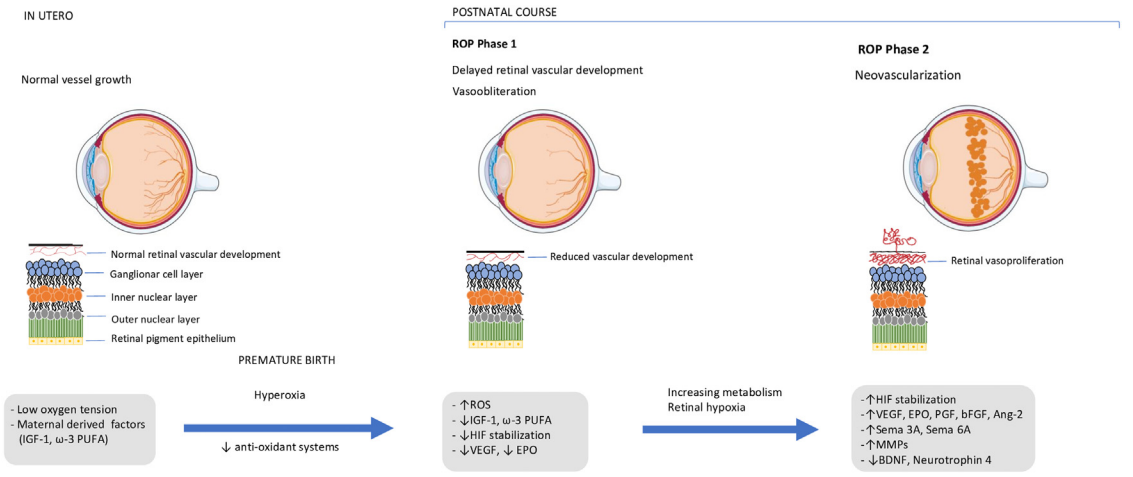

Figure 2 – Schematic representation of the main events implicated in the pathogenesis of retinopathy of prematurity. Abbreviations: Ang-2 = angiopoietin 2; BDNF = brain-derived neurotrophic factor; bFGF = basic fibroblast growth factor; EPO = erythropoietin; HIF = hypoxia-inducible factor; IGF-1 = insulin-like growth factor-1; MMPs = matrix metalloproteinases; ω3-PUFA = omega-3 polyunsaturated fatty acids; ROP = retinopathy of prematurity; ROS = reactive oxygen species; Sema = semaphorin; VEGF = vascular endothelial growth factor. Images adapted from Servier Medical Art by Servier are licensed under a Creative Commons Attribution 3.0 Unported License.[18]

The angiogenic phase is characterized by the development of new blood vessels that bud from existing blood vessels.[105,172] Angiogenesis is driven by physiological hypoxia.[172]

During fetal development, relative tissue hypoxia acts as a stimulus for HIF to trigger the transcription of angiogenic genes to produce growth factors, such as VEGF, its analog placental growth factor (PLGF), and the proangiogenic erythropoietin (EPO).[223] Tissue hypoxia that stimulates angiogenesis is not thought to be necessary for the vasculogenic period.[55,120] Maternal-fetal interaction in utero provides unique factors and ideal oxygen levels to stimulate the growth of the retinal vasculature.[381]

3.2. *ROP phases*

ROP progresses in 2 phases: the vascular attenuation phase (phase 1) and the fibrovascular proliferative phase (phase 2).[93,173] The first phase is vaso-obliterative and characterized by an interruption and delay in retinal vascular growth associated with microvascular degeneration.[93] It occurs because preterm infants are exposed to higher oxygen tension after birth compared to that *in utero*,[24] eliminating the physiological hypoxia.[89] The hyperoxia leads to a downregulation of VEGF, as well as an increase in vaso-obliteration of immature retinal capillaries through the actions of oxidative stress and intertwined inflammation.[82,88,94] During this phase, levels of HIF-1, VEGF, insulin-like growth factor-1 (IGF-1), and EPO are all decreased (Fig. 2).[154]

The loss of blood vessels in an increasingly metabolically active retina causes it to become gradually hypoxic.[154] To ensure adequate perfusion, an overproduction of growth factors, particularly VEGF, induce the growth, differentiation, and migration of endothelial cells.[300] This leads to abnormal growth of new blood vessels at the junction between the vascular and avascular retina, corresponding to the vasoproliferative phase of ROP (phase 2).[350] This phase occurs at around 32–34 weeks of postmenstrual age.[24,349] The transition between phases 1 and 2 corresponds more closely to postmenstrual age than to postnatal age,[284] however, this association may not be observed in extreme prematurity.[16] These new blood vessels fail to reperfuse the avascular retina. Instead of growing into areas of need, they grow chaotically into the vitreous and can lead to the development of a fibrous scar that can cause retinal detachment and lead to vision loss.[61] This critical phase of ROP (stages 4–5) occurs most frequently around 34–36 weeks of postmenstrual age[161]; however, the timing of the ROP phases can be modified by exposure to very high concentrations of oxygen.[331] Prenatal factors, such as inflammatory processes and chorioamnionitis, can also affect intrauterine retinal neurovascular development and predispose the fetal retina to severe ROP.[410] Understanding ROP phases and their causes can allow the identification of the ideal postnatal environment for the immature preterm infant.[154]

The International Classification of ROP was originally published in 1984,[10] expanded in 1987, and revisited in 2005[308] and 2021,[65] and describes 5 stages of ROP. ROP initially appears as a fine demarcation line between the vascular and avascular retina – stage 1. It then progresses, elevating this junction into a ridge – stage 2. These first two stages may regress spontaneously and are considered as initial or mild ROP; however, the pathological vasculature can continue to grow outside the retinal plane, leading to the vascular stage – stage 3. These neovessels are fragile and can bleed into the vitreous, causing fibrotization and traction, ultimately resulting in the retinal detachment that defines fibrovascular stage – stages 4 and 5. Retinal detachment may have permanent blindness as a possible consequence.[140]

3.3. *The role of retinal and choroidal blood flow*

As a result of the incomplete retinal vascularization in preterm infants, retinal oxygenation depends mainly on

choroidal circulation, which seems to play a fundamental role in the pathogenesis of ROP.[73] Preterm infants are subject to rises in blood oxygen tension that, in the absence of a fully developed autoregulatory control of ocular blood flow, may result in an increase of retinal oxygenation.[136] An elaborate genetic and epigenetic interplay between prostanoids and nitric oxide (NO) in vasomotor regulation leads to the absence of vascular autoregulation and excessive oxygen delivery to the eyes.[134]

Choroidal blood flow and retinal blood flow are autoregulated over a narrow range of perfusion pressure in the newborn, partly due to insufficient constriction but mainly from high perinatal levels of prostaglandins, such as prostaglandin D2 and prostaglandin E2 produced by cyclooxygenase (COX), and NO produced by NO synthase (NOS).[2,135] NO is a potent signaling molecule in blood vessels and its increased formation from endothelial cells activates guanylate cyclase in the underlying smooth muscle cells. This leads to the generation of cyclic guanosine monophosphate that induces vasodilation and masks the effect of constrictors involved in autoregulatory responses in newborns.[136,137] The effects of various prostaglandins are NO-dependent,[1] and specific prostaglandins regulate the expression and activity of endothelial NOS (eNOS) in ocular blood vessels.[87] NOS inhibition improves choroidal blood flow response to hyperoxia, stabilizes oxygen supply, and prevents the hyperoxia-induced increase in retinal peroxidation.[136]

In preterm infants, the increase in carbon dioxide tension (hypercapnia) is another factor that contributes to the interruption of regulation of retinal blood flow and choroidal blood flow.[57] Because of the Bohr effect, as the pressure of carbon dioxide increases, the oxygen dissociation curve is shifted to the right, allowing more oxygen to be delivered, increasing its negative effect on the developing retina.[379] During sustained hypercapnia, the increase in prostaglandin E2induces the expression of eNOS that releases NO and further reduces ocular blood flow autoregulation.[57] Hypercapnia has been associated with ROP in humans and OIR animal models.[379]

4. Oxidative and nitrosative stress, reduced anti-oxidative reserve

Oxidative stress is a consequence of an imbalance in the generation and quenching of reactive oxygen species (ROS) and has been implicated in the pathophysiology of ROP.[140] With high oxygen consumption,[252] constant exposure to light and rich content of easily oxidable long-chain polyunsaturated fatty acids (PUFAs), retinal tissue is prone to lipid peroxidation and highly susceptible to oxidative damage by ROS.[63,225] Hyperoxia, inflammatory response due to hypoxia-reperfusion, infection, long-term parenteral nutrition, blood transfusions and increased levels of non-protein-bound iron produce high levels of ROS.[298] Fluctuations in oxygen saturation appear to be more damaging than sustained hyperoxia.[31]

Qanungo and coworkers demonstrated a notable increase in the production of enzymatic and nonenzymatic antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase in the late stage of gestation.[304] Therefore, preterm infants present a relative deficiency in antioxidant systems and low levels of vitamin E and ascorbic acid to counterbalance ROS increase.[298]

ROS, namely superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$)[92] are by-products from normal aerobic metabolism that activate signaling pathways.[123,320] The mitochondrion is the main intracellular source responsible for the production of superoxide radical, though some enzymes also have a role in ROS generation, namely nicotinamide-adenine-dinucleotide phosphate (NADPH) oxidase (NADPH oxidase/ NOX) and NOS.[8,317] In experimental OIR models, several isoforms of NOX, including NOX1,[409] NOX2,[424] and NOX4,[396] are implicated in the production of ROS that interfere with peripheral retinal vascularization and are involved in later intravitreal neovascularization.[321] Retinal vascular obliteration, seen in the first phase of ROP, is thought to be partly due to endothelial cells apoptosis induced by oxidative stress[320,382] which is also associated with delayed retinal vascular development in models of ROP.[123,320]

In addition to hyperoxia, hypoxia that develops after hyperoxia damages newly developed capillaries can also lead to the activation of NOX and NOS.[397] The enhanced NOS is dysfunctional (uncoupled) and contributes to oxidative stress (Fig. 3).[89] Saito and coworkers found that activation of NOX and Janus kinase (JAK)/signaling transducer and activator of transcription 3 (STAT3), involved in pathways of apoptosis, is triggered in hypoxia-exposed retinal microvascular endothelial cells.[321] In the 50/10 OIR model, the increased NOX activity induced by supplemental oxygen caused intravitreal neovascularization mediated by JAK/STAT3 activity.[321] Inhibition of NOX activity with apocynin reduced the percentage of the area of intravitreal neovascularization to the total retinal area, but there was no decrease in VEGF, suggesting that NOX can also act independently from VEGF.[321]

Saito and coworkers also demonstrated that inhibition of NOX led to a reduction in apoptosis and avascular retina in an animal model of ROP, but reduced vasoproliferation was only observed if the hypoxic stimulus for angiogenesis was limited when pups were placed into supplemental oxygen.[142,320] This showed that ROS may activate signaling of angiogenesis, indirectly through NOX activation[321] or directly through VEGF.[92]

Bursts of superoxide generated by leukocytes are important to combat invading microorganisms, and this may be particularly important in the immune-suppressed preterm infant.[142] Therefore, NOX inhibition can have undesirable consequences such as sepsis and necrotizing enterocolitis.[39,185] In addition, studies do not support the use of certain antioxidants such as n-acetyl cysteine in preterm infants.[352] While antioxidants may be effective in quenching external ROS, they may not be able to access intracellular oxidative signaling mechanisms.[397] To find safer potential therapies, it is useful to study the signaling cascades activated by ROS that mediate the pathological characteristics of ROP.[397]

Nuclear factor erythroid 2-related factor 2 (Nrf2), a cytoprotective transcription factor, is upregulated in response to oxidative stress.[138] Heme oxygenase-1, a gene regulated by Nrf2, catalyzes heme degradation and, in response to oxidative stress, is upregulated to protect cells from ROS.[138] Previous studies have suggested the modulation of Nrf2 and Heme oxygenase-1 expression by the phosphatidylinositol 3-kinase

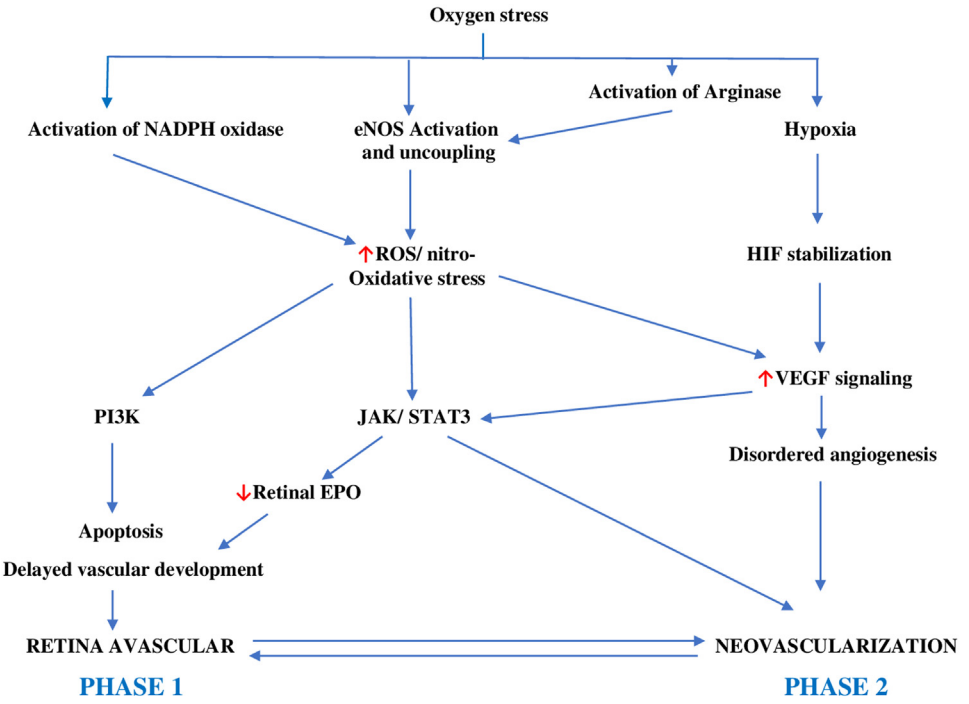

Figure 3 – Diagram showing the relationship between oxygen stress and retinopathy of prematurity. In the early life of preterm infants, oxygen stress leads to activation of NADPH oxidase, eNOS and arginase. The retinal hypoxia that occurs due to fluctuating oxygen tensions or when hyperoxia injures newly developed capillaries, triggers the overproduction of ROS and HIF stabilization. The signaling pathways activated by ROS/ nitro-oxidative stress, and HIF-1α contribute to pathogenesis of retinopathy of prematurity. Abbreviations: eNOS = endothelial nitric oxide synthase; EPO = erythropoietin; JAK = Janus kinase; HIF = hypoxia-inducible factor; PI3K = phosphatidylinositol 3-kinase; ROS = reactive oxygen species; STAT3 = signaling transducer and activator of transcription 3; VEGF = vascular endothelial growth factor.

(PI3K) / protein kinase B (Akt) and extracellular signal- regulated kinase (ERK) pathways.[86,201] Recently, Dong and coworkers demonstrated in experimental studies that polypyrimidine tract-binding protein-associated splicing factor induced the expression of Nrf2 and Heme oxygenase-1 via PI3K/Akt and ERK signaling, resulting in the elimination of intracellular ROS and suppression of the development of pathological vascularization.[86] Protein-associated splicing factor appears to be a potential antioxidant capable of regulating pathological retinal angiogenesis.[86]

4.1. *Nitric oxide and endothelial nitric oxide synthase*

NO is a gas-signaling molecule,[89] synthesized by NOS enzyme catalysis in which L-arginine is converted to L-citrulline using molecular oxygen and reduced NADPH as co-substrates.[50] NO binds to the only known NO receptor, the enzyme guanylate cyclase.[71] There are three main isoforms of NOS in vertebrates: neuronal NOS (nNOS/ NOS I), inducible NOS (iNOS/ NOS II), and eNOS (NOS III).[361,374] The isoform iNOS is usually expressed following the exposure to proinflammatory stimuli[121] and is a hallmark molecule of the pro-inflammatory M1 macrophages.[418] NO generated by eNOS has the function of vasodilation, among other important vascular effects, while nNOS is expressed in neurons, modulates neurogenesis and some neurophysiological functions, and regulates vascular tone.[107,121]

Among all isoforms, eNOS is the most abundant enzyme in vascular endothelial cells.[374] NO produced by eNOS is crucial for the induction of angiogenesis.[127] VEGF activates eNOS through the Akt signaling pathway, and Akt-dependent eNOS phosphorylation appears to play a key role in angiogenesis.[107,127] The contribution of eNOS-derived NO to VEGF-induced vascular permeability[273] and to promoting endothelial cell survival and migration is also well documented.[263,351] Recently, Ninchoji and coworkers demonstrated that NO causes vascular hyperpermeability by destabilizing the adherens junctions of endothelial cells.[273] Inhibition of eNOS in mice stabilized the adherens junctions of endothelial cells in vascular tufts, decreasing vascular leakage and, consequently, reducing pathological neovascularization without affecting the general blood perfusion of the retina.[273] These findings show that it is possible to prevent vascular leakage through pharmacological inhibition of NO production.[273] In another recent study, Smith and coworkers showed that eNOS controls the polarization of endothelial cells, influencing VEGF-induced migration and the sprouting of angiogenesis in a mouse model of OIR.[351] The results of this study indicate that eNOS inhibition increases endothelial cell polarization and redirects revascularization of the avascular retina, preventing misdirected vessels from growing into the vitreous

body.[351] Further studies are needed to assess the potential of eNOS to prevent retinal neovascularization and as a strategy for vascular regeneration of the ischemic retina.[351] Combination therapy of NO inhibitors and anti-VEGF, given at low but still effective doses, may prove to be beneficial for vascular retinopathies.[273] Local administration of NOS inhibitors may be necessary to avoid adverse effects of systemic administration, such as hypertension or other complications associated with vasoconstriction.[273]

NO can have protective and proangiogenic properties in the eye with different effects on OIR depending on the retinal redox state.[28] Oxidative stress may convert eNOS from a NO-producing enzyme to a dysfunctional enzyme that generates O_2^{-}.[89] This process is referred to as NOS uncoupling.[89] Beauchamp et al showed in an OIR model that eNOS expression and activity increases when the redox state is shifted towards an oxidative environment.[28] In this circumstance the oxygen reduction by eNOS is uncoupled from the formation of NO and eNOS produces less NO that can react with ROS, resulting in the generation of highly reactive nitrogen species.[89,123] Reactive nitrogen species can elicit several modifications of macromolecules and lead to nitrosative stress.[67] NO reacts with superoxide to form peroxynitrite ($ONOO^-$), an important mediator of hyperoxia-induced vaso-obliteration, resulting in microvascular degeneration.[89] Excessive production of reactive nitrogen species can result in harmful effects such as lipid peroxidation, DNA damage, and superoxide dismutase inactivation.[225,279] An increase in oxidative stress results in increased NO degradation due to nitrosative stress, ultimately leading to endothelial dysfunction.[107] The important role of nitrosative stress in ROP was demonstrated by reducing the severity of oxygen-induced retinal microvascular degeneration in mice after genetic ablation[40] and pharmacological inhibition of eNOS.[194]

In summary, in retinal hypoxia, a greater retinal eNOS expression increases NO production and, consequently, vasodilation and angiogenesis.[317] In the early stages of OIR, vasodilation induced by NO represents a compensatory mechanism to reduce vascular obliteration.[317,376] The subsequent improvement in ocular blood flow induces the production of ROS.[376] Furthermore, although NO is essential for rapidly initiating angiogenesis, it plays a critical role in retinal endothelial cells, increasing vascular permeability and pathological neovascularization.[351] The evidence described above allow us to conclude that further research is needed to assess the potential of regulating NO production by eNOS in the prevention of vascular retinopathies, including ROP. In addition, in an OIR mouse model, iNOS modulated the activity of HIF-1 via PI3K/Akt signaling and VEGF expression, presenting another potential form of intervention.[144]

4.2. *Interaction between free-radicals and prostanoids*

Isoprostanes are primarily produced by free radical-mediated oxidation of arachidonic acid (AA).[301] Under oxidizing conditions, the production of isoprostanes exceeds that of COX-derived prostaglandins and may contribute to microvascular injury in ROP, as they induce the production of thromboxane A2 which has cytotoxic effects.[165] Beauchamp and coworkers demonstrated in an OIR model that inhibition of COX and thromboxane A2 synthase selectively restricted retinal oxygen-induced vaso-obliteration.[27] Cis- to trans-isomerization of AA by nitrative stress resulting in trans- AA (TAA) formation was associated with vaso-obliteration and retinal endothelial degeneration in the model.[21] The formation of the antiangiogenic and proapoptotic thrombospondin-1[194] via activation of long-chain fatty acid receptor GPR40 is responsible for the endothelial cells toxicity induced by trans-AA.[162]

Phospholipase A2 enzymes can be activated in response to physiological stimuli or to oxidative stress and hypoxia.[25] These enzymes hydrolyze fatty acids of membrane phospholipids and can lead to the release of AA, platelet activation factor, and lysophospholipids.[397] The platelet activation factor is a pro-inflammatory mediator that contributes to microvascular damage to the retina, with its cytotoxic effects mediated mainly by thromboxane A2.[26] Barnett and coworkers suggest that phospholipase A2 and its downstream signaling is associated with both phases of ROP in OIR models, either independently or in association with the activation of VEGF signaling in vascular endothelial cells.[25]

5. Arginase

Arginase belongs to the ureohydrolase enzyme family, with two isoforms encoded by two different genes.[269] Arginase 1, the cytosolic isoform, is mainly expressed in the liver, where it has a central role in the urea cycle.[261,269] Arginase 2, the mitochondrial isoform, is expressed in extrahepatic tissues, especially the kidney.[341] Both isoforms are also present in the brain, retina, and other tissues.[341]

Arginase converts L-arginine to urea and ornithine. The production of hepatic urea is crucial for the detoxification of ammonia.[367] L-ornithine is metabolized by ornithine aminotransferase to form proline needed for collagen synthesis and by ornithine decarboxylase to form polyamines that enhance cell differentiation and proliferation.[311,367] The metabolism of L-arginine by arginase also results in the formation of glutamate.[269] Arginase has an important role in wound repair and has been implicated in neuroprotection and neural regeneration via the production of polyamines.[95,191,213]

Preterm infants tend to have low levels of arginine and glutamine due to the inability to maintain endogenous synthesis of these semi-essential amino acids.[378] Experimental studies show a significant contribution of these amino acids to retinal vascular function.[108,199,270,341] In an OIR mouse model, intravitreal neovascularization and vascular hyperpermeability were reduced by supplementary treatment with arginine and glutamine.[270]

Arginase activity and expression are increased by inflammatory processes and ROS states.[19,54] When arginase activity is increased, it can compete with NOS for the common substrate L-arginine, causing NOS to become uncoupled, resulting in a decrease of NO production and contributing to nitrosative stress.[367] For this reason, arginase can regulate the function of the three isoforms of NOS, eNOS, iNOS, and nNOS.[108]

Arginase 1 upregulation in endothelial cells decreases NO, resulting in decreased endothelial cell-dependent vasorelaxation, and ultimately leading to reduced blood flow and sub-

sequent ischemia[110,269]; however, the expression of arginase 1 in immune cells can decrease NO production by iNOS, reducing oxidative stress and inflammation.[110,340] In fact, arginase 1 is considered a marker of antiinflammatory M2 macrophages.[340] Fouda and coworkers showed that intravitreal treatment with arginase 1 reduces retinal neurovascular degeneration in wild-type mice after ischemia-reperfusion injury.[110] This study also demonstrated the importance of arginase 1 in macrophage polarization towards a reparative phenotype allowing neurovascular recovery.[108,199,270,341] In a translational study, Fouda and coworkers also demonstrated that the systemic administration of the pegylated arginase 1 (polyethylene glycol linked to recombinant human arginase) dampens the inflammatory response of macrophages and markedly protected against neurovascular injury after retinal ischemia-reperfusion injury.[109] Pegylated arginase was also reported to cross the blood-retinal barrier and its penetration was enhanced by impairment of the blood-retinal barrier.[109]

While studies have demonstrated a protective role of arginase 1, other studies provide evidence that arginase 2 plays a role in retinal damage. Elevated arginase 2 levels have been associated with retinal neurovascular degeneration in models of ischemic retinopathy through mechanisms involving increased oxidative stress, glial activation, and changes in polyamine pathways.[340] The catabolic products of polyamine oxidation and glutamate can lead to oxidative stress and DNA damage that can cause cell damage.[269] Shosha and coworkers suggested that arginase 2 is a downstream target of NOX2.[341] The superoxide produced from activated NOX2, along with increases in other ROS after ischemia/reperfusion, may play an important role in arginase 2 upregulation, leading to cell death and ischemia/reperfusion-induced neurovascular degeneration.[341]

L-citrulline is a precursor to L-arginine via the L-arginine recycling pathway; however L-citrulline has been shown to have an inhibitory effect on arginase.[332,333] This inhibitory effect may further increase NO production by providing more L-arginine to the NOS pathway.[332] Shatanawi and coworkers demonstrated that supplementation of L-citrulline reduces arginase activity and increases nitric oxid plasma levels in patients with type 2 diabetes.[332] In humans, oral L-arginine treatment is impaired by metabolism in the gut and liver. L-citrulline, the precursor of L-arginine, is not subject to presystemic elimination, and for this reason, oral L-citrulline supplementation increases the plasma concentration of L-arginine more effectively.[327] L-citrulline is available in oral and intravenous forms and has been used safely in clinical trials with infants undergoing cardiac surgery and in children with sickle cell disease and mitochondrial disease.[91,346,400] A clinical trial aims to evaluate the safety profile, efficacy and adequate dosage of enteral L-citrulline supplementation in preterm infants (NCT03649932). The investigators intend to use the information from this study to conduct a randomized placebo-controlled trial to assess the role of L-citrulline supplementation to treat pulmonary hypertension associated with bronchopulmonary dysplasia in preterm infants.

In summary, more studies are needed to evaluate the arginase pathway as a therapeutic target to treat oxidative stress-related retinopathy. The development of methods for cell-specific targeting and specific inhibitors of arginase isoforms could facilitate progress in this area.[340] Specific delivery of pegylated arginase 1 to microglial cells/macrophages may be an option to limit inflammation, avoiding potentially harmful effects on vascular endothelium.[340] Pegylated arginase 1 is being clinically tested in patients with melanoma and advanced hepatocellular carcinoma and appears to be safe and well tolerated.[108,199,270,83,341,423] While the role of arginase in ROP is clearer than before, many aspects still need to be explored. For example, the mechanisms of arginase-induced retinal damage and the complimentary or contradictory actions of the two arginase isoforms need to be better understanded.[340] Potential systemic adverse effects on immune responses and endothelial cell function should also be studied.[108]

6. Hypoxia-inducible factor

All responses to hypoxia in cells share HIF as a common denominator.[66] HIF is a transcription factor and a heterodimeric complex composed of two subunits: an oxygen-dependent subunit HIF-1α (or its analogs HIF-2α and HIF-3α) and a constitutively expressed nuclear subunit HIF-1β (1, 2)[97,394] – also denominated as aryl hydrocarbon receptor nuclear translocator.[122] In normoxic conditions, HIF-1α is hydroxylated by prolyl hydroxylase domain (PHD) in the cytosol. PHDs belong to a small family of proteins. In humans, there are 3forms (PHD1, PHD2, PHD3) with their own specialized activity.[253] The hydroxylation of HIF-1α by PHD provides a binding signal for Von Hippel-Lindau (VHL) tumor suppressor protein,[45] thus enabling ubiquitination by the cullin-2 E3 ligase complex ($CRL2^{VHL}$ E3)[203] and degradation by the 26S proteasome.[45]

Since PHD uses oxygen and iron as cofactors, a state of hypoxia inhibits the enzymatic activities of PHD, allowing the stabilization of HIF-1α.[203] As a result, the HIF-1α level increases and binds to HIF1-β in the nucleus to activate the angiogenic mechanisms which help cells adjust to hypoxia.[97] In addition, the Krebs cycle activity is linked to oxidative phosphorylation which is both impacted by hypoxia.[187] Inhibition of succinate dehydrogenase by hypoxia results in the accumulation of succinate, which is exported to the cytosol, binds to and activates its receptor, GPR91, and leads to inhibition of PHD and activation of HIF expression.[264] Another Krebs cycle intermediate, fumarate, also accumulates during hypoxia and inhibits PHD activity leading to an increase in HIF-α.[128]

Consequently, in hypoxia, HIF-1α hydroxylation is inhibited and it escapes from VHL binding and 26S proteasome-dependent degradation.[203] This results in the stabilization of HIF-1α and subsequent translocation into the nucleus.[384] The protein dimerizes with constitutively expressed HIF-1β to form the HIF-1 complex which binds to E-box-like hypoxia response elements (HREs) in the promoter region of hypoxia-inducible genes.[177] The HIF-1 complex enables the transcription of hundreds of genes, including those involved in angiogenesis, such as VEGF, EPO, platelet-derived growth factor (PDGF), angiopoietin (Ang)-1, and angiotensin-converting enzyme 1; glucose/iron metabolism; stem cell maintenance; and cell survival and proliferation. This process helps the cells

adapt to low oxygen conditions.[171,303,406,425] Synnestvedt and coworkers found that adenosine, another angiogenic factor, is also controlled by HIF-1α.[368] HIF-1 and HIF-2 regulate many common transcriptional targets, but some genes are not co-regulated. For example, the expression of EPO and certain genes linked to iron metabolism are controlled by HIF-2 while HIF-1 controls anaerobic glycolysis.[128]

There is also evidence that non-hypoxic stimuli induce HIF. Under normoxic conditions, various growth factors and cytokines such as IGF-1 and transforming growth factor β may stabilize HIF-1α via specific kinase pathways [PI3K or mitogen-activated protein kinase (MAPK)].[220] ROS can also increase the transcriptional activity of HIF-1, even in normoxia.[53] The same is reported for angiotensin-converting enzyme and angiotensin II receptors.[230]

As described above, relative hypoxia, such as that seen during fetal development, inhibits PHD activity, increasing levels of HIF-1α.[282] This plays a crucial role in retinal vascular development, both directly increasing the transcription of major angiogenic factors[402] and indirectly increasing the apoptotic effects caused by the HIF-1–responsive gene *RTP801*.[37] Preterm infants are exposed to a high oxygen tension after birth,[94] often including supplemental oxygen therapy. Hyperoxia suppresses HIF-1α levels and leads to a reduction in VEGF expression and induction of retinal capillary obliteration.[13,349] Therefore, suppression of HIF-1α by hyperoxia plays a pivotal role in the onset and progression of the first phase of ROP, while its upregulation by tissue hypoxia is crucial for the second phase.[13]

Several studies have demonstrated that HIF stabilization during hyperoxia (hypoxiamimesis) may prevent retinal vessel loss and subsequently, the second phase of ROP. Sears et al demonstrated that PHD inhibitors stabilize HIF in mice and maintain conditions to stimulate physiological retinal vascular development in phase 1 ROP in OIR models.[328] Hoppe and coworkers tested 7 small molecule HIF PHD inhibitors and reported that at least 2 of them administered systemically during the hyperoxic phase prevent vaso-obliteration and subsequent pathologic angiogenesis in OIR mice.[163] One of these molecules is roxadustat, a carbonyl glycine approved in many countries for the treatment of anemia associated with chronic kidney disease.[329] The other, AR0, is a benzolamide constructed by modeling the oxoglutarate binding site of PHD2. Both molecules targeted PHD2 better than PHD1 or 3.[163] The researchers observed that retinal expression of PHD2 became dominant at postnatal day 8, and suggested that the inhibition of PHD at this point should be maximal to obtain the best protection.[163] They also demonstrated that roxadustat can prevent OIR in two ways: directly in the retina, by stabilizing HIF and up-regulating enzymes for aerobic glycolysis, or indirectly in the liver, by stabilizing HIF-1 and stimulating the secretion of angiogenic hepatokines.[164] These findings lead the authors to suggest a clinical trial with intermittent use of low doses of PHD inhibitor in preterm infants, starting one week after birth and continuing to 30 weeks postmenstrual age, to allow oxygen supplementation without impairing normal retinovascular growth.[163] Another interesting consideration is that iron and oxygen-dependent PHDs are erythropoiesis regulators.[128] PHD inhibitors, such as roxadustat, are used to treat anemia, a risk factor associated with ROP. We think that this effect of HIF-PHD inhibitors should also be investigated in the prevention of ROP.

In response to hypoxia, HIF-1 mediates metabolic reprogramming for cellular adaptation, increasing flux through glycolysis and decreasing glycolytic carbon entry into the tricarboxylic acid cycle.[200,344] HIF also induces serine synthesis and metabolism, increasing mitochondrial NADPH production.[344] One-carbon metabolism is folic acid and methylenetetrahydrofolate redutase dependent.[416] Singh and coworkers found that serine/one-carbon metabolism are dependent on hepatic HIF-1 and mediate HIF protection against hyperoxia-induced retinal vessel in OIR.[344] This study suggests that pharmacological stabilization of HIF can induce aerobic glycolysis and control serine metabolism, resulting in a protected phenotype in mice.[344,378]

Other studies suggest that HIF inhibition during phase 2 OIR may be an ideal strategy, as it seems to be directed towards the pathological action of proangiogenic factors (mainly VEGF-A), while maintaining the physiological role of these factors, essential in protecting the retina.[217]

In one mouse OIR model, the peak levels of expression of HIF-1α and HIF-2α were reached after two hours of exposure to hypoxia.[262] Jiang and coworkers demonstrated that inhibition of HIF-1α suppresses the production of pro-angiogenic factors that cause the neovascular phase (phase 2).[180] Studies have demonstrated the role of HIF in the pathogenesis of ROP (Table 1).

Miwa and coworkers studied two HIF inhibitors with different mechanisms: topotecan, which suppresses the accumulation of HIF-1α protein but not mRNA expression and, doxorubicin, which inhibits HIF-αs by preventing its binding to the hypoxia response element[258] (Table 1). Shoda and coworkers screened marine products and found six species of fish with HIF inhibitory effects.[339] Between them, *Decapterus tabl* components suppressed retinal neovascularization in a mouse model of OIR, however, the mode of action and effector compounds have not been clarified.[217,339] Usui-Ouchi et al demonstrated that intravitreal injection of peptides derived from the intrinsically disordered protein CITED2, an endogenous negative feedback regulator of HIF-1α prevented ROP in a mouse model of OIR.[383] It was also reported that the combination of this peptide inhibitor of HIF with a reduced concentration of the anti-VEGF aflibercept causes suppression of neovascularization and stimulates the revascularization of the ischemic retina.[383]

Apurinic/apyrimidinic endonuclease 1/reduction-oxidation factor 1 (APE1/Ref-1) is a multifunctional protein that has a role of redox-transcription activator and of endonuclease.[138,221] APE1/Ref-1 is expressed during retinal development and in retinal and choroidal endothelial cells, retinal pigment epithelial cells, and pericytes.[287] APE1/Ref-1 regulates transcription factors that are involved in retinal neovascular diseases, including HIF-1α, STAT3, and NF-κB.[138] Low oxygen levels and APE1/Ref-1 redox activity induce HIF-1α expression.[138] The inhibition of APE1/Ref-1 as a new option for the treatment of retinal neovascular diseases, including ROP, is addressed by another review.[138]

Phase II and III clinical trials of PHD inhibitors for anemia of chronic kidney disease and of HIF inhibitors for the treatment of cancer are summarized in the reviews by Haase

Table 1 – Results of studies investigating molecules or pathways involved in the pathogenesis of retinopathy of prematurity and related pharmacological agents.

Molecule/ Pathway	Pharmacological agent	Intervention	Tested in	Effect	Adverse effect	Reference
HIF-1	Dimethyloxalylglycine (PHD inhibitor) (Phase 1)	Intraperitoneal injection	Mouse OIR model	Prevents loss of vessels, vascular tortuosity, and tufts formation		328
	Roxadustat or AR0 (PHD inhibitors) (Phase 1)	Intraperitoneal injection	Mouse OIR model	Prevent vaso-obliteration and subsequent pathologic angiogenesis		163
	Topotecan or doxorubicin (HIF inhibitors) (Phase 2)	Intraperitoneal injection	Mouse OIR model	Both prevent pathological but not physiological retinal neovascularization. In addition, topetecan protects the visual function		258
	2-azahypoxanthine (HIF inhibitor) (Phase 2)	Oral administration	Mouse OIR model	Suppress VEGF upregulation and retinal neovascularization		218
HIF-1 + VEGF	HIF-1α siRNA (Phase 2)	Subretinal injection	Mouse OIR model	Neovascular tufts and neovascular nuclei were decreased		180
	CITED2 (HIF-1α inhibitor)	Subretinal injection	Mouse OIR model	Inhibits vaso-obliteration and pathological angiogenesis		383
	HIF-1α siRNA and VEGF siRNA (Phase 2)	Subretinal injection	Mouse OIR model	Result in maximum effects in suppression of VEGF in vitro and in vivo		180
	CITED2 (HIF-1α inhibitor) +Aflibercept (Phase 2)	Intravitreal injection	Mouse OIR model	Suppress neovascularization and stimulate ischemic retinal revascularization		383
VEGF	VEGF (Phase 1)	Intraocular injection	Rat OIR model	Prevention of endothelial cell apoptosis and rescue of retinal vasculature		7
	Anti-VEGF (Phase 2)	Intravitreal injection	Rat OIR model	Reduction of intravitreal neovascularization area	Reduction in weight gain	247
	Bevacizumab (Phase 2)	Intravitreal injection	Clinical Study	Effective for treatment of zone I ROP (compared to laser treatment) but not for zone 2	Persistent avascular retina and recurrent intravitreal neovascularization; serum levels suppressed for 2 months	167,256,413
	Bevacizumab (Low dose) (Phase 2)	Intravitreal Injection	Clinical Study	The lowest effective dose of bevacizumab may be 0,004mg	Ocular results (strabismus, high myopia, nystagmus, retinal detachment) in 1 year identical to those of studies with higher doses	74,393
	Ranibizumab (Phase 2)	Intravitreal injection	Clinical Study	Effective in the treatment of ROP	Reduction in serum VEGF levels for less than 4 weeks	157
		Intravitreal injection	Clinical Study	Ranibizumab at a dose of 0.12 mg and 0.20 mg was shown to be safe and effective at 1 and 2 years of follow-up	Recurrences were frequent	357

(*continued on next page*)

Table 1 (*continued*)

Molecule/ Pathway	Pharmacological agent	Intervention	Tested in	Effect	Adverse effect	Reference
	Ranibizumab versus laser therapy (Phase 2)	Intravitreal Injection	Clinical Study (Clinical trial: NCT02375971)	Ranibizumab 0.2 mg may be superior to laser therapy and has an acceptable safety profile of 24 weeks	Less myopia with ranibizumab than laser therapy. No adverse advents reported with ranibizumab.	243,359
	Aflibercept (Phase 2)	Intravitreal injection	Clinical Study	Serum IGF-1 levels did not change significantly	Serum VEGF levels were suppressed for at least 8 weeks. Aflibercept in systemic circulation after 4 weeks	118
	Aflibercept versus laser therapy (Phase 2)	Intravitreal Injection	Clinical Study	Aflibercept is effective in the treatment of ROP	Aflibercept requires more additional treatments than laser photocoagulation	90
	Aflibercept versus Ranibizumab (Phase 2)	Intravitreal Injection	Clinical Study	Both are effective in the treatment of ROP	Lower rate of ROP recurrence with aflibercept than with ranibizumab	364
	Aflibercept versus Bevacizumab (Phase 2)	Intravitreal injection	Clinical Study		Higher rate of ROP recurrence with bevacizumab than with aflibercept. Serum VEGF significantly reduced for 3 months	170
		Intravitreal injection	Clinical Study	Regression rate significantly higher with aflibercept compared with bevacizumab.	Recurrence rate significantly higher with aflibercept compared with bevacizumab.	312
	Aflibercept versus Bevacizumab versus Ranibizumab (Phase 2)	Intravitreal Injection	Clinical Study	Aflibercept, bevacizumab and ranibizumab are effective for the treatment of ROP	Recurrence is more frequent and earlier with ranibizumab. Bevacizumab is associated with myopic shift	366
	VEGFA shRNA (Phase 2)	Subretinal injection	Rat OIR model	Reduction of VEGF expression; Inhibited intravitreal neovascularization without affecting physiological retinal vascular development or puppy weight gain		395
	VEGF164 shRNA (Phase 2)	Subretinal injection	Rat OIR model	Maintained long-term inhibition of intravitreal neovascularization, limited cell death, and preserved the outer nuclear layer compared with shRNA to VEGFA		181

(*continued on next page*)

Table 1 (*continued*)

Molecule/ Pathway	Pharmacological agent	Intervention	Tested in	Effect	Adverse effect	Reference
β- adrenergic receptors	Propanolol (Phase 2)	Subcutaneous injection	Mouse OIR model	Markedly reduced neovascularization; reduced upregulated VEGF		313
		Oral (0.25 or 0.5 mg/kg/6 hours)	Clinical Study (Clinical trial: NCT01079715)	Effective in reducing the progression of ROP	Serious adverse effects (hypotension, bradycardia) associated with sepsis, anesthetic induction or tracheal stimulation were observed in 5 of 26 infants	101
		Topical administration	Clinical Study (Clinical trials NCT02504944; NCT02014454)	Propanolol 0.2% dose but not 0.1% dose reduces ROP progression		102,103
IGF-1	IGFBP3 (Phase 1 and 2)	Intraperitoneal injection	Mouse OIR model	Protects against the oxygen-induced retinal vessel loss, increases vessel regrowth, and decrease retinal neovascularization		233
	IGF-1	Intraperitoneal injection	Mouse OIR model	Early administration (prior to exposure to hyperoxia) reduce the risk of OIR		386
	Fresh-frozen plasma as a source of IGF-1/IGFBP3 (Phase 1)	Transfusion	Clinical Study	Increases serum IGF-1 and IGFBP3 levels		131
	IGF-1/IGFBP3 (Phase 1 and 2)	Intravenous	Clinical Study	Well tolerated, safe, and efficient in increasing serum IGF-1 levels		234
		Intravenous	Clinical Study (Phase II clinical trial, NCT01096784)	Does not affect the development of ROP	13.1% emerging adverse effects possibly related to study drug	224
EPO and derivates	EPO for preventing red blood cell transfusion	Intravenous/ subcutaneous	A systematic review of 2 clinical studies		Risk factor for ROP (any grade) and a trend for ROP stage > 3 with early EPO treatment	4
	EPO for treatment of anemia	Intravenous/ subcutaneous	Clinical Study	Does not influence markedly the incidence and severity of ROP		330
	EPO (ROP phase 1)	Intravenous/ subcutaneous	Clinical Study (Clinical trials: NCT02036073, NCT03919500)	Effective for type 2 ROP in infant boys or preterm infants with gestational age greater than 28 weeks and birth weight greater than 1500g		365
	Darbepoetin for preventing red blood cell transfusion	Subcutaneous	Clinical Study (small sample size)	Does not influence markedly the incidence and severity of ROP		278

Abbreviations: EPO = erythropoietin; HIF = Hypoxia-inducible factor; IGF-1 = insulin-like growth factor-1; IGFBP3 = insulin-like growth factor-binding protein 3; PHD = prolyl hydroxylase domain; ROP = retinopathy of prematurity; shRNA = short-hairpin RNA; siRNA = small interfering RNA; VEGF = vascular endothelial growth factor.

and coworkers and Fallah and coworkers.[97,129] Although to date, no clinical trials of HIF inhibitors or PHD inhibitors for retinopathies have been performed.[337]

The studies mentioned above provide molecular mechanisms to support the use of HIF PHD inhibitors during hyperoxia or of HIF inhibitors during the second phase of ROP in severely preterm infants at risk of ROP.[163,164] Some of these molecules have passed phase 2 and 3 trials and, once approved for adults, may be eligible for clinical trials in infants, as safety concerns may be lessened using an agent known to be proven safe in adults.[164]

7. Growth factors

7.1. *Vascular endothelial growth factor*

VEGF is a family of glycoproteins composed of 5 structurally similar factors: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PLGF.[34,204,292] Although the term VEGF is a designation for the VEGF family of polypeptides, it generally refers to VEGF-A, the prototype growth factor, originally identified as vascular permeability factor. VEGF-A is the main focus of angiogenesis research.[34,141,204] Members of the VEGF family are homodimeric polypeptides, although natural heterodimers of VEGF-A and PLGF have also been described.[204] The VEGF superfamily and its receptors play a key role in physiological and pathological angiogenesis and vascular permeability.[192,272]

The gene encoding VEGF-A yields alternatively spliced products,[34] termed VEGF isoforms, which have different, sometimes opposite, functions.[275] In humans, these include the relatively abundant VEGF121, VEGF165, VEGF189, and VEGF206, as well as other less abundant forms.[292] The proangiogenic isoforms are designated as VEGF-Axxx, while antiangiogenic isoforms are VEGF-Axxxb (where xxx represents the number of amino acids in the mature form of the protein).[244] The pro- or anti-angiogenic tissue effect depends on the isoform balance. The isoform balance is controlled by mRNA splicing that is influenced by several factors, including HIF-1, IGF-1, and cytokines.[275] VEGF-A165, the most common isoform of VEGF-A found in the human eye, appears to be the most relevant for pathological ocular neovascularization and represents the primary therapeutic target.[244] VEGF-A165b, the best-studied VEGF-Axxxb isoform, has a protective effect for neuronal, epithelial, and endothelial cells.[241] Although VEGF-A165b binds to VEGF receptor (VEGFR) 2, it does not activate it.[411] Konopatskaya and coworkers showed that the administration of VEGF-A165b is a potent inhibitor of retinal neovascularization.[209]

VEGFR-1 and VEGFR-2 (KDR/Flk-1 in mice), together with structurally related receptors, Flt-3/Flk-2 and VEGFR-3/Flt-4, are tyrosine kinase receptors.[290,292] VEGFR-1 and -2 are mainly involved in angiogenesis,[419] while Flt-3 and Flt-4 are involved in hematopoiesis and lymphangiogenesis[292]. VEGFR expression is increased by hypoxia and potentiated by VEGF.[309] VEGFR-2 is the main receptor inducing the proliferation, migration, differentiation, and maturation of endothelial cells, as well as vascular permeability.[334] It preferably uses the phospholipase C-protein kinase C-MAPK pathway for signaling.[334] The *VEGFR-1* gene encodes two isoforms: a full-length transmembrane VEGFR-1 (also known as Flt-1) and a soluble VEGFR-1 (sFLT-1).[192] Flt1 has a higher affinity for VEGF-A, even in soluble form, but has a tyrosine kinase activity approximately 10 times less than VEGFR-2.[370] Therefore, VEGFR-1 can negatively modulate VEGFR-2 activity.[290] The normal placenta has a high expression of sFLT-1,[334] an endogenous VEGF inhibitor, and its potential clinical application as an angiogenesis inhibitor has received considerable attention.[70,245,345]

Neuropilin 1 and neuropilin 2 are non-tyrosine kinase receptors that, in endothelial cells, serve as receptors for semaphorins class 3 (Sema 3), and co-receptors for the VEGF family members.[276] VEGF signaling via neuropilin 1 stimulates endothelial cell migration and adhesion.[276] Studies have shown that neuropilin 1 interacts with VEGFR-1, significantly reducing its binding affinity for VEGF165[116] and considerably enhancing VEGF165 binding to VEGFR-2.[353]

Different cells have been shown to synthesize VEGF-A in the retina, including retinal ganglion cells,[395] astrocytes,[225] Müller cells,[225] retinal pigment epithelium (RPE) cells,[34,272] glial cells,[244] vascular endothelial cells, and pericytes.[272] In pathological intravitreal neovascularization, the VEGF-A signal was located in many of the same cells, specifically Müller cells,[20] astrocytes,[402] and perhaps retinal ganglion cells.[395] VEGFR-2 is primarily found on vascular endothelial cells.[399] The main role of the VEGF/VEGFR-2 system is therefore to signal cascade pathways involved in the proliferation, migration and survival of endothelial cells, leading to tubulogenesis, and later, vessel formation.[244,399]

VEGF has a fundamental role in both phases of ROP.[306] In experimental models, levels of VEGF decrease within 6 hours under normoxic or hyperoxic conditions leading to a decrease in the angiogenic signaling and allowing vaso-obliteration caused by apoptosis of vascular endothelial cells.[7,335] The exogenous administration of VEGF may prevent this effect of hyperoxia in the first phase of ROP.[7] As the retina becomes hypoxic in phase 2 of ROP, VEGF levels rise within 6–12 hours and affect endothelial cells in a paracrine fashion.[295] These endothelial cells increase their expression of high affinity VEGFR-2, specific for proliferative neovascularization.[295]

Pieh and coworkers suggested the involvement of VEGF and VEGFR-2 in ROP development,[294] and Budd and coworkers showed in a rat model of OIR that intravitreal neovascularization is reduced by a VEGFR-2 inhibitor.[41] McColm and coworkers found that both VEGFR-2 and VEGF164, an analog to human VEGF165, are increased secondary to repeated oxygen fluctuations, a factor associated with ROP,[248] and Hartnett demonstrated that they may have roles in phases 1 and 2 of OIR and the early and vascular phases of human ROP.[140] Additionally, results from Hartnett and coworkers supported the hypothesis that overactivation of VEGFR-2 disorders dividing endothelial cells, permitting them to grow outside the plane of the retina in a pattern comparable to intravitreal neovascularization.[139]

The VEGF-VEGFR system is a prime target for antiangiogenic treatment.[334] A standard procedure in clinical practice for the treatment of intravitreal neovascularization is inhibition of VEGF-A by anti-VEGF monoclonal antibodies; however, it is associated with RPE degeneration and may impact the viability of photoreceptors, choriocapillaris, and the

signaling of Müller cells.[11] Different anti-VEGF molecules have been studied for the treatment of ROP (Table 1).

The first anti-VEGF drug to be reported for ROP therapy was bevacizumab.[256] It was approved for cancer therapy but is used off-label for eye diseases.[403] Although it has shown an advantage over laser therapy for ROP stage 3 with plus disease in zone I but not in zone II, recurrence of ROP is not uncommon.[167,255,256] In addition, bevacizumab has been associated with several serious adverse outcomes in clinical studies.[15,96] These results have caused setbacks in the development of bevacizumab for ROP treatment.[78]

The randomized, multicenter RAINBOW trial showed intravitreal ranibizumab (0.2 mg/eye) to be superior to laser therapy, with a 24-week safety profile and fewer unfavorable ocular outcomes.[243,359] These findings led to the approval of ranibizumab for the treatment of ROP in the European Union in 2019.[216] The randomized multicenter CARE-ROP trial that compared 0.12 mg with 0.20 mg ranibizumab also found a safety profile on functional eye outcomes and neurodevelopment although ROP reactivation was a challenge during the follow-up phase.[357]

Wu and coworkers reported that intravitreal therapy with bevacizumab or ranibizumab for infants with ROP resulted in a significant decrease in serum VEGF up to 8 weeks.[414] Serum VEGF levels were less affected after treatment with ranibizumab than with bevacizumab in patients with ROP type 1.[414]

Aflibercept, the most potent inhibitor of pathological angiogenesis among anti-VEGF agents, binds not only to VEGF-A but also to VEGF-B and PLGF.[285,364] Ekinci and coworkers confirmed the efficacy of intravitreal aflibercept in the treatment of ROP, although it required more treatments than laser photocoagulation during follow-up visits.[90] Two randomized phase 3 clinical trials, FIREFLYE (NCT04004208) and BUTTERFLYE (NCT04101721) are underway to compare intravitreal injection of aflibercept with laser therapy for ROP treatment.

As VEGF is necessary for the normal development of many organs, the potential systemic toxicity of anti-VEGF is a concern.[12,48,371,413] Clinical studies seek to find a lower effective dose of bevacizumab that can reduce the risk of impaired neurological development or harmful effects on other organs.[74,393] A recent study by Wallace and coworkers indicated positive retinal structural results with low-dose bevacizumab for ROP treatment, but many patients required additional treatment.[392]

Another angiogenic factor, secretogranin III, has been described as selectively inducing pathological but not physiological angiogenesis.[78] Secretogranin III-targeted therapies are expected to selectively block angiogenesis in diseased but not healthy vessels, while VEGF binds to and drives angiogenesis in diseased and healthy vessels.[77] Experimental evidence on the potential advantages of secretogranin III as a target for the treatment of ROP is addressed in another review.[78]

7.2. *Erythropoietin*

The glycoprotein hormone EPO is an oxygen-regulated growth factor controlled by HIF-2[128] which, in addition to its role in regulating the formation of red blood cells in hematopoiesis,[244] has other important neuroprotective,[155,280] antiapoptotic,[398] antioxidative,[398] and angiogenic[195] functions, and possibly contributes to the maintenance of the blood-retinal barrier.[155] EPO is therefore a pleiotropic growth factor that protects and stimulates the growth of many different cells, including endothelial cells.[78]

EPO participates in physiological and pathological angiogenesis in the retina[422] and exerts its actions through the EPO receptor.[155] Additional EPO receptors have been described that may mediate the tissue protective function of EPO in non-hematopoietic tissue, particularly in the nervous system.[280] Three possible neuroprotective EPO receptors are: a heteroreceptor consisting of the EPO receptor and common β receptor, the Ephrin B4 receptor, and the human orphan cytokine receptor-like factor 3.[38,280,291] Su et al suggested that the common β receptor may act as an integrator of eNOS activation mediated by EPO signaling in endothelial cells[362]; however, other studies do not support a functional interaction between the EPO receptor and the common β receptor.[64]

There is particular interest in EPO derivatives to reduce transfusion requirements for preterm infants[278] and as a neuroprotective agent to stimulate cognitive development[355]; however, a recent randomized trial (PENUT, NCT01378273) found contradictory results with regards to its role in cognitive development.[189] In addition, some studies have found that EPO treatment for anemia of prematurity was associated with an increased risk of severe ROP, although other studies have not confirmed this association (Table 1).

High concentrations of endogenous EPO in preterm infants are associated with high blood concentrations of inflammation-related proteins[404] and with various morbidities, including ROP and respiratory problems[160]; however, this association may depend on the postnatal day or ROP phase. Lundgren and coworkers studied serum EPO levels and anemia as risk factors for severe ROP requiring treatment and concluded that, among infants born before 28 weeks of gestational age, anemia during the first week of life was a significant risk factor but not high levels of EPO.[235] In clinical studies by Holm and coworkers the high systemic concentrations of EPO around postnatal day 14, before ROP-associated neovascularization, were consistent with the possibility that EPO plays a direct role in stimulating angiogenesis.[159,160]

Like VEGF, EPO is an important angiogenic factor and its production is induced by HIF.[141] Morita and coworkers provide evidence that HIF deficient mice under OIR have reduced levels of EPO and no ROP.[260]

A small interfering RNA targeting EPO markedly decreased ROP in OIR mice.[59] EPO may have different functions depending on the phase of ROP development.[60] Concentrations of EPO are low during the vaso-obliterative phase and may contribute to a stop in angiogenesis.[60] Administration of exogenous EPO during this phase may stabilize blood vessels.[142] Conversely, during the vasoproliferative phase of ROP, high EPO levels may increase pathological neovascularization.[142]

The OIR model provided evidence that VEGFA may activate VEGFR2, which subsequently phosphorylates the EPO receptor forming an interaction with the phosphorylated EPO receptor to activate the JAK/STAT pathway in endothelial cells inducing the proliferation of these cells.[422] It is possible that the EPO receptor, activated by EPO or VEGFA, enhances VEGFR2 signaling and mediates the pathological angiogenesis seen

in phase 2 ROP.[422] In normal physiological angiogenesis, the concentration of VEGFA is not likely to be sufficiently high enough to activate the EPO receptor or cause its interaction with VEGFR2.[422] Lastly, it is possible that certain forms of EPO preferentially bind to neuroprotective EPO receptors and may have a protective role for ROP.[141]

7.3. *Insulin-like growth factor-1*

IGF-1 is a polypeptide protein hormone supplied by the placenta and amniotic fluid, and is essential for fetal development, including healthy retinal angiogenesis.[150,214,229] Plasma levels of IGF-1 increase with gestational age, mainly during the third trimester of pregnancy,[214] and suffer a sudden decrease after preterm birth due to the lack of maternal-fetal interaction.[349] Inflammation may further reduce the limited production of IGF-1 in preterm infants.[132]

Low serum IGF-1 levels in preterm infants have been associated with poor postnatal growth,[296] poor brain growth,[133] prematurity-related morbidities,[149] and delays in retinal blood vessels growth.[149,232] A persistent reduction in circulating IGF-1 in preterm infants is strongly correlated with the development of ROP.[46,147,176] Patients with a genetic defect in the production of IGF-1 have reduced retinal vascularity that is not restored after administration of VEGF alone.[148]

IGF-1 binds to the IGF-1 receptor. In an OIR mice model, Kondo and coworkers found that the IGF-1 receptor knockout mice showed less retinal neovascularization compared to a control group.[208] This result also provided evidence that IGF-1 signaling in vascular endothelium plays a key role in retinal neovascularization.[208]

Cakir and coworkers found that in extremely preterm infants, early postnatal hyperglycemia was associated with reduced levels of IGF1 and increased severity of ROP.[46] They also found in a mouse model of hyperglycemia-associated retinopathy that reduced insulin signaling led to a decrease in hepatic IGF-1 production and an increase in neovascularization.[46] IGF-1 administration promoted retinal revascularization and reduced retinal pathological neovascularization.[46] These findings support early IGF-1 supplementation as a potential treatment to decrease the risk of ROP.

Insulin-like growth factor-binding protein 3 (IGFBP3), the major IGF-1-binding protein in serum, is decreased in preterm infants,[232] possibly contributing to the depletion of retinal blood vessels.[293] Lofqvist and coworkers observed that higher serum levels of IGFBP3 correlated with less severe ROP in preterm infants.[233] Clinical studies have been conducted to address the merits of treatment with IGF-1 and IGFBP3 at an early stage to prevent vaso-obliteration in preterm infants (Table 1).

In one phase 2 randomized clinical trial (NCT01096784) the administration of recombinant human IGF-1/IGFBP-3 complex decreased the development of severe bronchopulmonary dysplasia, but did not impact the development of ROP.[224] The reason for the lack of effect on ROP prevention is unclear, but may be related to a need for dosage optimization.[224] The results of this study encourage the continuous evaluation of recombinant human IGF-1/IGFBP-3 complex in preterm infants for the prevention of diseases related to prematurity. An ongoing phase 2b randomized clinical trial (NCT03253263) with a larger number of patients will bring additional data that could help elucidate this issue.[378]

Although VEGF is increased by HIF-1 under hypoxia, IGF-1 is necessary for angiogenesis to occur. Hellstrom et al demonstrated that low levels of IGF-1 prevented VEGF-induced activation of Akt, a kinase critical to the survival of endothelial cells.[152] Therefore, IGF-1 acts as a permissive factor for the growth of VEGF-dependent vascular endothelial cells.[154] It is assumed that if IGF-1 levels are insufficient at birth, VEGF cannot activate Akt and the retina stays avascular,[306] as observed during the first phase of ROP.[152] This leads to increased apoptosis despite normal VEGF levels.[152] Consequently, the retina becomes hypoxic, leading to the accumulation of VEGF in the vitreous[152,225] until IGF-1 levels reach a limit. At this point neovascularization is triggered, beginning the second phase of ROP.[152,306] Apoptosis of endothelial cells is then blocked and neoangiogenesis is promoted, presumably by activating Akt signaling.[152] Vascular proliferation is also regulated by the IGF-1 receptor via the p44/ 42 MAPK pathway that increases VEGF activity.[152,347] Therefore, inhibition of IGF-1 and VEGF can prevent neovascularization in phase 2 of ROP.[152,225] Hellstrom and coworkers also found that low serum IGF-1 levels are a biomarker which can be used to determine risk of ROP in preterm infants weeks before it manifests.[152] As such, IGF-1 was initially included in a clinical algorithm to predict the risk of developing severe ROP.[388]

Recently, Jenssen and coworkers reported that low levels of IGF-1 are associated with early thrombocytopenia, and this in turn is related to the subsequent development of severe ROP in preterm infants.[175] The investigators hypothesize that IGF-1 delivery by platelets is the mechanism linking thrombocytopenia with severe ROP development.[175] This hypothesis requires confirmation by further laboratory and clinical studies.[175] In addition, Cakir and coworkers demonstrated in an OIR mice model that platelet transfusion was associated with decreased retinal VEGF-A and reduced neovascularization.[47] These data suggest that the release of platelet-derived factors has an antiangiogenic action on endothelial cells,[47] and support the hypothesis that, in preterm infants, maintenance of platelet levels, and therefore platelet-derived growth factors, including IGF-1 and VEGF-A, may reduce the risk of ROP development.[147]

IGF-1 plays an important role in cell cycle progression and reversal of endothelial cells apoptosis via the NOS-NO signaling pathway[238] and IGFBP3 regulates its actions.[183] Kielczewski and coworkers showed that IGFBP-3 improves ischemic retinal vascular repair by increasing the expression of eNOS and the generation of NO, an essential signaling molecule that promotes angiogenesis and regulates vascular remodeling.[198] Therefore, the interaction of IGFBP-3 is another potential line of treatment that warrants further exploration.

7.4. *Retinal pigment epithelium and pigment epithelium-derived factor*

The main producers of trophic factors in the eye are the RPE and Müller cells.[206] The RPE is a monolayer of tight cells located between the neurosensory retina and the vascularized choroid.[29] It performs essential functions for the survival of photoreceptors, such as phagocytosis and renewal of shed

photoreceptor outer segments, and secretes important factors for the homeostasis of the external retina.[29,246] The RPE prevents the choroidal vasculature from invading the subretinal space and the outer retina,[184] has a key role in both preserving the blood-retinal barrier and protecting the retina from oxidative and toxic damage,[206] and plays an important role in the pathophysiology of the retina.[11,29] Formed by polarized cells, the RPE secretes components in a directional way.[11,29]

One of the most relevant molecules secreted by the RPE is the *pigment epithelium-derived factor (PEDF)*, an extracellular glycoprotein that is a member of the serine protease inhibitors family (serpin family).[245] With neurogenic and neuroprotective properties, PEDF is one of the most potent anti-angiogenic and anti-inflammatory factors.[11,184,251] Michelis and coworkers demonstrated that PEDF protected photoreceptors from apoptosis, promoting its survival and differentiation.[251] The effects of PEDF on photoreceptors are like those exerted by docosahexaenoic acid (DHA).[251,363] This similarity results from the phospholipase A activity of the PEDF receptor, whose PEDF binding increases the hydrolysis of phospholipids, resulting in the release of fatty acids, including DHA.[251,363] The protective role of DHA on photoreceptors is discussed in more depth in section *12. Long-chain polyunsaturated fatty acids*.

In another study, the transfer of the SERPINF1 gene via viral vectors and the exogenous administration of PEDF protein were found to be valuable in defending photoreceptors against death and degeneration caused by environmental and/or genetic factors.[299] The balance between VEGF and PEDF plays the largest role in maintaining the external retina avascular. This is essential to preserve a suitable environment for photoreceptors and neurons in the retina.[184] Experimental studies have demonstrated PEDF's therapeutic potential for the treatment of retinal angiogenic diseases.[9,184]

7.5. *Other growth factors*

PLGF, a homologous factor of VEGF-A primarly expressed in the placenta but also produced for various cell types[17,56] acts as a pro-angiogenic factor for retinal endothelial cells[5] and plays a role in recruiting immune cells.[428] There are four PLGF isoforms, encoded by the human *PLGF* gene. While all isoforms bind to VEGFR-1, PLGF-2 and 4 also bind to neuropilins 1 and 2.[76] VEGFR-1 signaling activated by PLGF is distinct from the signaling mediated by VEGF-A because different tyrosine residues are phosphorylated on the receptor, leading to target-specific downstream regulation.[76]

In preeclampsia, a serious pregnancy complication often associated with intrauterine growth restriction and preterm birth, there is an increase in plasma levels of soluble antiangiogenic VEGFR-1 (sFLT-1) in relation to the proangiogenics PLGF and VEGF. The circulating sFLT-1/PLGF ratio may serve as a predictor of early preeclampsia.[307]

Although PLGF is associated with pathological rather than physiological angiogenesis, its precise contribution to this process is less understood than that of VEGF.[385] Its expression can be induced by VEGF and hyperglycemia,[182,426] but unlike other members of the VEGF family, PLGF is down-regulated during hypoxia and has an anti-apoptotic effect during hyperoxia,[335,343] likely playing an important role in the aberrant neovascular phase of ROP.[343] Luttun and coworkers showed that PLGF deficiency reduced pathological vascular leakage in a mouse model of OIR.[236]

Even though aflibercept, primarily an anti-VEGF-A, also inhibits PLGF, there are currently no approved therapies for retinopathies that exclusively target PLGF.[76] In fact, the benefits of specific PLGF inhibition in humans with neovascular diseases of the retina remain controversial.[272] Clinical studies and further research are needed to better elucidate the underlying molecular and cellular mechanisms that determine whether PLGF inhibition may have advantages over VEGF inhibition and, in clinical practice, whether or not they can be co-administered.[385]

Angiopoietins are growth factors that modulate physiological and pathological neovascularization particularly in association with VEGF.[326] Ang-1 and Ang-2 are the most evaluated angiopoietins in preclinical studies.[99] The action of Ang-3 and Ang-4 are less described.[99] In humans, only the expression of Ang-1, Ang-2, and Ang-4 is reported.[318] Ang-1, Ang-2, and Ang-4 bind to the Tie2 receptor.[197] Ang-1 and Ang-4 are known agonists of the Tie-2 receptor and are bound to Tie2 in the physiologic state.[197] After physiologic activation, Tie2 is phosphorylated, leading to the activation of the cellular pathways AKT and ERK, which are involved in the reduction of angiogenesis and vascular permeability and favor vascular stability.[197] Furthermore, Tie-2 activation induces eNOS expression, leading to an improvement in endothelial cell function and the expression of survivins, molecules that trigger endothelial cell survival.[99] There is also an inhibition of the inflammatory cascade through the down-regulation of the NF-κB transcription factor pathway.[99] In pathophysiologic states, Ang-2 is highly secreted and acts as a competitive inhibitor of Ang-1 and Ang-4 binding. Ang-2 functions as a negative regulator, leading to dephosphorylation of Tie2.[197] Therefore, the Ang-1/Tie2 signaling pathway plays an important role in maintaining vascular integrity and in the later stages of vascular remodeling[297] while Ang-2 initiates vascular proliferation[14] and is upregulated by hypoxia and VEGF.[277]

An increase in the levels of Ang-1 and Ang-2 in the vitreous of eyes with severe ROP has been documented. Furthermore, the concentration of Ang-2 was found to be significantly higher, indicating that the balance between them may be important for ROP pathophysiology.[326] Additional studies in animal models of OIR demonstrate that combined inhibition of Tie2 and VEGF signaling may be more effective in suppressing intravitreal neovascularization in ischemic retinopathies, in particular ROP, than VEGF inhibition alone.[179,227,369]

Several new agents targeting the angiopoietin/Tie pathway have promising results in phase II and III clinical trials for addressing diabetic macular edema, wet age-related macular degeneration and retinal vein occlusions.[99,197] One such biological agent, faricimab– a bispecific antibody administered by intravitreal injection– acts as an inhibitor of both VEGF and Ang-2.[99,197] Phase III clinical trials support its safety, tolerability, efficacy, and potential to reduce treatment burden of wet age-related macular degeneration and diabetic macular edema.[99,196,197] The findings led to its recent approval in the USA and Japan for the treatment of these retinal vascular diseases and it is under review in the European Union.[338] Another new agent, razuprotafib (AKB-9778), is a small molecule administered via subcutaneous injections.[99] Razuprotafib acts

by selectively inhibiting vascular endothelial protein tyrosine phosphatase and causing Tie-2 phosphorylation and activation, regardless of the presence of Ang-1 or Ang-2.[99,197] In a phase IIa clinical trial, razuprotafib showed good tolerability and an optimal safety profile.[49] The researchers also found that combination therapy of razuprotafib with ranibizumab was more effective for the treatment of diabetic macular edema than ranibizumab alone.[49,99] Therefore, there is evidence from both preclinical studies and initial phase III clinical trials demonstrating that dual inhibition of the VEGF and Ang/Tie pathways can be superior to anti-VEGF therapy alone in treating retinal vascular diseases.[99]

While the studies mentioned above have encouraging results, to-date, there have been no clinical trials with these drugs for ROP. Additional research is needed, including Ang/Tie modulation as a possible target for gene therapy.[99]

Granulocyte colony-stimulating factor (G-CSF) is a growth factor that stimulates hematopoietic progenitor cells. It is extensively used in neutropenic patients,[354] including preterm infants[219]. Several studies report that G-CSF induces anti-inflammatory, anti-apoptotic, and neuroprotective effects.[143,207] Sato et al measured the vitreous levels of twenty-seven types of cytokines in a retrospective study and found levels of six cytokines, including G-CSF, significantly increased in eyes with ROP compared to the control group.[325] In an animal OIR study, G-CSF reduced vascular obliteration and neovascularization by increasing the levels of IGF-1.[205] Shima and coworkers reported that systemic therapy with G-CSF can protect inner retinal layers and retinal ganglion cells from ischemia/reperfusion injury, and these effects may be associated with AKT activation.[336] In a retrospective study with neutropenic neonates, Bhola and coworkers found a tendency to decrease the incidence of ROP requiring treatment in preterm infants who received GCS-F[35]; however, the differences were not statistically significant.[35]

Findings from systematic reviews and clinical trials show that recombinant GCS-F is well tolerated and safe at all gestational ages.[6,52,342] In addition, the biosimilar GCS-F filgrastim was shown to be as safe as the original drug in children.[42] G-CSF may have a potential role in preventing ROP; however, further studies are needed to establish a benefit from its administration in the initial phase of ROP.[244]

Neurotrophins are a family of closely related proteins, originally identified as growth factors for the survival, development, and function of neurons, and later discovered to also play a role in the immune and reproductive systems.[319] These neuroprotective factors activate a specific tropomyosin-related kinase receptor and a common receptor.[79] Some of them, such as nerve growth factor and brain-derived neurotrophic factor (BDNF), play an important role in the process of angiogenesis.[319] Sood and coworkers reported lower serum concentrations of neurotrophin 4 and BDNF during the first 3 weeks of life in preterm infants who developed severe ROP.[356] Rao and coworkers found that BDNF concentrations on P60 were lower in preterm infants who developed ROP.[310]

New treatment modalities using neuroprotective factors have targeted diseases caused primarily by retinal ganglion cell degeneration, such as glaucoma, as well as other ophthalmic diseases, such as macular degeneration, ischemic optic neuropathy, retinitis pigmentosa, and cystoid macular edema.[115] Phase I and II clinical trials of these therapies are summarized in the review by Fudalej and coworkers.[115]

The *PDGF* family comprises four polypeptide chains with structural similarities to the VEGF family and includes five dimeric isoforms: PDGF-AA, -AB, -BB, -CC, and -DD.[111] PDGFs are important mitogens for many types of cells, mainly of mesenchymal origin, and play an important role in angiogenesis and wound healing.[33,407] Accumulated data suggest the important roles of PDGF-CC and PDFG-DD in pathological angiogenesis.[166,211] Wågsäter and coworkers found that PDGF-CC up-regulates matrix metalloproteinase-2 and -9 expression and induces monocyte migration.[391] Inhibition of PDGF-CC or PDFG-DD in animal models suppressed both choroidal and retinal neovascularization.[166,211]

In a study of preterm infants born at a gestational age of less than 28 weeks, Hellgren and coworkers found that severe ROP is significantly associated with low platelet counts and lower serum levels of PDGF-BB, VEGF-A and BDNF at the postmenstrual age of 32 weeks.[147] These data suggest that factors released from platelets may be involved in the regulation of retinal angiogenesis after extreme preterm birth.[147]

Fibroblast growth factors (FGF) are a family of cell signaling proteins,[174] including bFGF (also FGF2) which, like other FGF family members, are a strong mitogen of endothelial, neuronal, and smooth muscle progenitor cells, expressed in the retina.[98] While some studies have shown that bFGF is not necessary for normal or pathological retinal vascularization,[281] other studies attribute it with vascular[174] and retinal development,[315] as well as retinal neuroprotective effects.[186] Sato and coworkers documented increased bFGF expression in the vitreous of preterm infants who have undergone vitrectomy for stage 4 ROP.[325] Fang and coworkers found increased expression of bFGF during the resolution of surface vasculopathy in an OIR mouse model, indicating its possible role in maintaining neuroretinal function in OIR/ROP.[98]

FGF21, a new member of the FGF family, is an important regulator of lipid and glucose metabolism.[114,378] FGF21 activates Nrf2, which in turn regulates the expression of antioxidant enzymes and plays a critical role in the retina's defense against oxidative stress.[114,267] Fu and coworkers demonstrated in a mouse model of OIR that FGF21 reduces pathological retinal vasoproliferation and stimulates physiological retinal vascularization.[112] The inhibitory effects were mediated by adiponectin and appeared to be independent of VEGF-A.[112] Furthermore, Fu and coworkers found that in mice with hereditary retinal degeneration, FGF21 enables the preservation of retinal neuronal responses.[113] The effectiveness of FGF21 against pathological vascular proliferation could make it the next generation standard of care for patients with ROP.[112] Yet, further exploration of the underlying mechanisms is necessary.[112]

A compensatory mechanism between vascular growth factors in ROP has been described. In this, the inhibition of VEGF expression leads to an up-regulation of other angiogenic factors such as bFGF and Ang-1, while the opposite occurs when VEGF is up-regulated in endothelial cells under hypoxic conditions.[415] This phenomenon could partially explain why the inhibition of a single growth factor cannot effectively prevent the recurrence of neovascularization in ROP, and why a combined strategy may be more effective.[415]

8. Semaphorins

The same families of attractive and repulsive molecular cues, including Sema 3 – secreted glycoproteins expressed in the retina – are responsible for the conduction of both blood vessels and nerves throughout the body.[51,84] Most Sema 3, except Sema 3E, bind to neuropilin family receptors to transduce their signals and posteriorly associate with plexin family receptors to form functional receptors.[377] Sema 3E uses Plexin D1 as its main binding receptor.[377]

In an OIR model, Rivera and coworkers proposed that areas of retinal ischemia cause the microglia to prematurely produce interleukin 1 (IL-1) β which sustains microglia activation and leads to microvascular damage by releasing Sema 3A from the adjacent retinal ganglion cells.[314] Although hypoxic neurons initially secrete VEGF, as they become more severely ischemic, the production of Sema 3A becomes predominant.[188] Sema 3A exerts opposing effects on vessels, increases apoptosis of endothelial cells and repels neovessels of the avascular neural retinal towards the vitreous, preventing revascularization of the hypoxic retinal tissue.[188] In contrast, normal vascular regeneration is increased by the IL-1 receptor antagonist or by silencing Sema 3A expression. This preserves the microvascular bed and decreases the subsequent pathological pre-retinal neovascularization seen in ischemic retinopathies.[314]

Through an OIR model, Fukushima and coworkers showed that the binding of Sema 3E to its Plexin D1 receptor activates a signaling pathway that leads to the normalization of angiogenic directionality in ischemic retinopathy and in developing retinas.[117] The increased expression of Plexin D1 in extraretinal vessels prevents disoriented VEGF-induced projections of the endothelial filopodia.[117] Yang et al found a similar result with the intravitreal administration of recombinant Sema 3C in an OIR model.[421] More recently, Noueihed and coworkers discovered in a model of ischemic retinopathy that mesenchymal stromal cells stimulate retinal vascular repair of the ischemic retina through modulation of Sema 3E and IL-17A.[274]

Chen and coworkers found that Sema 3G, which is only expressed in retinal endothelial cells in mice, plays an important role in promoting both regression of pathological neovascularization and healthy vascular formation in blood vessel remodeling.[58] There is evidence that under hypoxic conditions Sema 3G transcription is directly regulated by HIF-2 α.[58] Sema 3G increased the stability of β-catenin in vascular endothelium via neuropilin 2/Plexin D1 receptor – coordinating the interplay between β-catenin and vascular endothelial cadherin.[58] Supplementation with Sema 3G has been shown to have a protective effect on blood vessel remodeling.[58]

The involvement of other semaphorin family classes such as Sema 6A and Sema 4D in ischemic retinal vasculopathies has also been documented.[401,412] Sema 6A appears to play a role in a neuronal stress response, reducing the resistance of ischemic neurons to reparative angiogenesis.[401] Wei and coworkers reported that, in a HIF-1α-dependent mechanism, Nrf2 expression is increased in the ischemic retina and promotes vascularization towards the avascular zone by suppressing the expression of Sema 6A.[401] It is postulated that Nrf2 suppresses the antiangiogenic effect of Sema 6A and reprograms angiogenesis in the ischemic neuroretina.[401] Nrf2 activity can be modulated pharmacologically, suggesting a therapeutic potential for ischemic retinopathies.[401] Additionally, experimental studies by Nakamura and coworkers indicated that RS9, an activator of Nrf2, may be a candidate for the treatment of retinal diseases characterized by pathological vascularization and hyperpermeability.[268]

Wu and coworkers observed an increase in Sema 4D expression in an OIR model and in a model of diabetic retinopathy. Results of the OIR model showed that the presence of Sema 4D/Plexin B1 led to endothelial cell dysfunction, while inhibition of Sema 4D/Plexin B1 prevented endothelial cell dysfunction.[412] A humanized monoclonal antibody, VX15/2503, specifically inhibits Sema 4D by binding to its receptors.[104,412] Two phase 1 clinical trials with VX15/2503, one targeting advanced solid tumors (NCT01313065)[288,412] and the other targeting multiple sclerosis (NCT01764737),[212,412] were successfully completed. Both studies found that VX15/2503 given intravenously at various doses was shown to be well tolerated and safe. Future research should address whether Sema 4D/Plexin B1 inhibition may be useful for the treatment of retinal vascular diseases such as ROP.[412]

Like semaphorins, the ephrin/ephrin receptor system also functions as guidance cues for neurons and the visual pathway during the embryonic period. This influences cell migration and differentiation during developmental processes.[125,250] Membrane-bound ephrin receptors are the biggest subgroup of tyrosine kinase receptors.[125] As noted in a review by Medori, there is a gradient of selected ephrins and their receptors in the retina and visual pathways, creating a map for neurons.[250] In addition, Kozulin and coworkers demonstrated that ephrin A1 to A4 and their A6 receptors play an important role in retinal vascular development.[210] A review by Kaczmarek and coworkers focuses on the role of ephrins and ephrin receptors in retinal diseases.[190] Studies of particular interest to ROP research are those that demonstrate that the blocking of selected ephrine receptors inhibits pathological angiogenesis without affecting the normal development of blood vessels.[190]

In conclusion, the findings of the studies mentioned above support the targeting of semaphorins in vascular regeneration therapy, particularly by directing neovascularization towards the ischemic retina.

9. Succinate, its receptor GPR91, and adenosine

Neurons, endothelial cells and the macroglia (astrocytes and Müller cells) are anatomically and functionally interconnected to orchestrate physiologic and pathologic retinal vascularization.[323] In humans at approximately 15 weeks of gestation, astrocytes emerge from the optic nerve head and establish a network that delineates the path of vascular growth.[302,322]

During episodes of ischemic hypoxia, cells respond to an imbalance of energy and signal to restore vascular supply.[323] In addition to their traditional roles, a physiological function of the Krebs cycle intermediates, of which succinate is the best example, is to serve as signaling factors, which respond to the compromised energy state and lead to an increase in retinal

vascularization.[265] Similarly, metabolic purine products (ATP, ADP, AMP, and adenosine) accumulate during hypoxia and activate purinergic receptors that can contribute to neovascularization.[254,403] As succinate and adenosine levels increase in hypoxic conditions, they activate their cognate G protein-coupled receptor (GPCR), and reestablish adequate blood flow in tissues.[187] In the short term, this occurs through vasorelaxation, while in the long term it is a result of the stimulating effect that GPCR activation has on angiogenesis.[187] Therefore, in response to hypoxia, there is initially rapid vasodilation and accumulation of Krebs cycle intermediates (mainly succinate) and adenosine, followed by stabilization of HIF-1α, alteration of the redox state and activation of survival factors. The primary goal of this process is to restore oxygen delivery and protect the retina.[429] Several studies have shown that a hypoxic microenvironment leads to the accumulation of succinate in macrophages, resulting in stabilization of HIF-1α and the expression of the proinflammatory cytokine IL-1β.[372,405] This induces a ''Warburg effect", a process of metabolic reorganization in which mitochondrial oxidative phosphorylation decreases while aerobic glycolysis increases.[372,405] Unlike succinate, α-ketoglutarate promotes the enzymatic activity of PHD and depletes HIF-1α.[239,405] Thus, α-ketoglutarate and succinate have opposite effects on HIF-1 α -IL-1β signaling.[405]

In an OIR model, Zhou and coworkers observed a considerable increase of IL-1β in the RPE and choroid in the early stages of retinopathy. This increase was correlated with choroidal involution, subsequent progressive loss of photoreceptors and RPE, and visual degradation.[429] Early IL-1β receptor blockade protected the choroid, reduced subretinal hypoxia, and preserved photoreceptors and RPE, resulting in better visual function in OIR animals treated with IL-1β receptor antagonist.[429] These findings suggest that inhibition of IL-1β may constitute a new therapeutic potential, through the prevention of choroidal involution and retinal degeneration.[429]

The signaling system of the succinate receptor (GRPR91) is not only essential for neural and retinal development, but also for its regulatory role in many vital processes that involve pathophysiological mechanisms.[228] In fact, in an OIR rat model, the contribution of succinate and adenosine to the proliferative phase of ROP was established by suppressing the expression or activity of their GPCR. Knockdown of GPR91 disrupts normal retinal vascular development and decreases aberrant intravitreal neovascularization in the OIR model.[323] Similarly, the A2B adenosine receptor antagonism markedly reduces pre-retinal neovascularization.[254]

GPR91 is abundantly expressed in both highly vascularized tissues[145] as well as retinal ganglion cells[226] and behaves as an early sensor for metabolic demands. Sapieha and coworkers provided evidence that GPR91 controls the expression of several important angiogenic factors, such as VEGF, Ang-1, and Ang-2, and suppresses antiangiogenic thrombospondin-1.[323] Studies by Li and coworkers in a rat OIR model suggest that in hypoxia the GPR91-ERK1/2-C/EBP β (c-Fos, HIF-1α) signaling pathway plays a key role in regulating VEGF transcription in retinal ganglion cells.[226]

Succinate signaling operates before HIF-1 stabilization and is therefore an antecedent sensor for metabolic demand and hypoxic stress.[187] The metabolic changes induced by hypoxia in neurons correspond to an attempt to restore the vascular supply.[187]

GPR91 is considered a valuable target in developing molecular interventions.[228] Blocking GPR91 may be an option to prevent secretion of excessive growth factors and to reduce intravitreal neovascularization[168,323]; however, a better understanding of the mechanisms involving the binding of GPR91 to various agonists and antagonists and the relationship between activity and structure is needed to identify compounds that may pave the way for preclinical investigation.[228]

10. Heparan sulfate proteoglycans and heparanase

Heparan sulfate proteoglycans are ubiquitous macromolecules present at the cell surface. As either transmembrane or membrane-anchored proteins, they are important components of the extracellular matrix.[169] Heparan sulfate proteoglycans are composed of a core protein covalently attached to one or more chains of glycosaminoglycan polysaccharide heparan sulfate.[390,417]

Heparan sulfate anionic glycosaminoglycan chains bind to extracellular matrix and cell surface proteins, providing the scaffolding for matrix organization and cell-cell or cell-matrix interactions.[22] As components of the plasma membrane and the basement membrane, heparan sulfate proteoglycans play more than a structural role[22] influencing cell proliferation, differentiation, migration, and shape.[22,324,373]

Heparanase is an endo-β-glucuronidase that cleaves heparan sulfate polysaccharide chains at the cell surface and in the extracellular matrix.[390] An important step in the neovascularization process involves changing the integrity of the extracellular matrix and the subendothelial basement membrane.[169] At sites of injury or inflammation, heparanase degrades heparan sulfate proteoglycans from the basement membrane, allowing extravasation of immune cells to non-vascular spaces[22] and release of heparan sulfate-linked angiogenic growth factors, including bFGF and VEGF, as well as heparan sulfate-fragments, thereby promoting cell proliferation and angiogenesis.[169]

Jie Hu and coworkers demonstrated that heparanase and VEGF are upregulated in hypoxia-induced retinal neovascularization and that heparanase inhibition by phosphomannopentaose sulfate (PI-88 or muparfostat), results in down-regulation of VEGF expression and formation of fewer new blood vessels, suggesting a role for heparanase in the regulation of VEGF expression.[169]

El Asrar and coworkers found an important increase in heparanase expression in vitreous samples from patients with proliferative diabetic retinopathy compared to nondiabetic controls.[3] Heparanase levels positively correlated with VEGF and syndecan-1 levels, suggesting a link between them in the progression of proliferative disease.[3]

Knowledge gathered to date shows that heparanase may be a possible new therapeutic target for retinal diseases characterized by hypoxia-ischemia, such as ROP.[169] The search for molecules that inhibit heparanase has been increasing due to their importance in clinical practice; however, despite enormous efforts, programs to develop heparanase inhibitors

have brought few into clinical trials.[283] They are synthetic or semi-synthetic oligosaccharides and polysaccharides, such as muparfostat (PI-88), pixatimod (PG545), necuparanib (M-402), and roneparstat (SST0001), mainly evaluated in cancer therapy and for inflammatory pathologies.[283] In general, these molecules were well tolerated and side effects were limited, but they all have some limitations related to their nature and origin that can make their standardization difficult. In addition, they all need to be administered parenterally.[283]

Other approaches have been used to achieve pharmacological inhibition of heparanase, including inhibitors based on nucleic acids, and monoclonal antibodies.[283] The search for synthetic small molecules heparanase inhibitors has gained momentum in recent years and remains an option of interest due to their more favorable pharmacokinetic profiles and possibility of oral administration.[259] Some of them were developed and evaluated in pre-clinical stages.[283] The use of high-throughput quantitative screening systems is expected to help accelerate the development of clinically applicable small molecule inhibitors.[259]

11. Metalloproteinases

Metalloproteinases (MMPs) constitute a family of endopeptidases that hydrolyze components of the extracellular matrix.[68] They play a central role in different biological processes, such as remodeling of normal tissue, wound healing, inflammatory responses, embryogenesis, and angiogenesis.[389] Secretion of MMP-2 and MMP-9 by the RPE increases when stimulated by the angiogenic molecules VEGF, fibronectin, and tumor necrosis factor-alpha.[158]

Inflammatory cytokines, growth factors, and ROS control the activity of MMPs, including at the beginning of their transcription.[68] Inversely, MMPs can be inactivated by the tissue inhibitors of MMPs (TIMPs) family of proteins.[68] This family is composed of four proteins (TIMP-1 to 4) with an endogenous regulatory action and present in the extracellular matrix. They inhibit MMPs and members of the “a” disintegrin and metalloproteinase (ADAM) family.[44]

Das and coworkers provided evidence that MMP-2 and MMP-9 levels were significantly increased in association with phase 2 of retinopathy in an OIR model and that systemic inhibition of MMPs led to a significant decrease in neovascularization.[80] These results suggest that pharmacological intervention in the MMPs pathway may constitute an alternative approach in the treatment of proliferative retinopathy.[80]

In an OIR animal model, Di and coworkers reported that an increased retinal expression of MMP-9 upregulates the expression of VEGF, promoting retinal neovascularization.[85] They also demonstrated that intravitreal injection of TIMP-1 markedly reduced retinal expression of MMP-9 and VEGF, and retinal neovascularization. These results support TIMP-1 as a potential target to prevent and treat ROP.[85]

The ADAM family of enzymes is also involved in the degradation of extracellular matrix components.[427] Several subtypes of the ADAM family have been implicated in the pathogenesis of ROP. Weskamp and coworkers showed that pathological neovascularization is reduced in the ADAM17 knockout mice without affecting normal vascular development.[408] Guaiquil and coworkers found that Adam8_/_, Adam9_/_ mice and mice lacking Adam10 in endothelial cells were partially protected from plus disease, suggesting that ADAM 8, 9, and 10 may be targets for the treatment of plus disease.[124] In an animal study, Gutsaeva and coworkers showed that ADAM17 contributes to retinal ischemia-reperfusion-induced inflammation, oxidative stress, and neurovascular cell injury and that regulation of ADAM17 activity can prevent these consequences of retinal ischemia.[126] The TIMP-3 protein, a known physiological inhibitor of ADAM17,[242] decreased the formation of neovascular tufts in a mouse model of OIR, suggesting its potential therapeutic application.[156]

Interest in the development of synthetic inhibitors of MMPs arose more than three decades ago, but suffered a setback as a result of several failed clinical trials.[222] This was largely due to the low individual selectivity for different MMPs because of the high structural homology within this family and, consequently, a high risk of side effects and toxicity.[43] Additionally, most clinical trials were performed using compounds with poor metabolic profile and low bioavailability.[222] An alternative approach may be to use TIMPs designed with limited inhibitory specificities.[44]

The current challenge of developing new molecular entities with selectivity between specific MMPs has led to the emergence of new non-hydroxamate compounds, and a renewed interest in MMPs as therapeutic targets.[222] A review by Lenci and coworkers describes patents published between January 2014, and June 2020, that are related to new MMPs inhibitors with clinical application in several areas.[222] These patents concern compounds that target only a few MMPs, more specifically, MMP-2, -9, -12, -13, -14 (Membrane-type 1-MMP) and -17 (Membrane-type 4-MMP).[222] To date, doxycycline, a broad MMP inhibitor, is the only MMP inhibitor compound approved by the US Food and Drug Administration for therapy of disorders related to elevated MMP activity.[43]

12. Catecholamines

Catecholamines (epinephrine, noradrenaline, and dopamine) play an important role as neurotransmitters in the central and peripheral nervous system and, in addition are hormones in the endocrine system.[286] The targets of catecholamines, especially epinephrine and noradrenaline, are adrenoceptors.[286] The adrenoceptor family is subdivided into three subfamilies: alpha1 (α1), alpha2 (α2), and beta (β).[316] Each of these subfamilies consists of three subtypes (α1A, α1B, and α1D; α2A, α2B, and α2C; β1, β2, and β3).[316] Ristori and coworkers demonstrated that hypoxia causes catecholaminergic overstimulation, activating β-adrenoreceptors.[313] Different studies have shown the effects on the expression of angiogenic factors mediated by β-adrenergic receptors.[375,420] In addition, β-adrenoceptor stimulation also induces proliferation and migration of retinal endothelial cells.[360]

Much of the information on the contribution of β-adrenoreceptors to hypoxia-induced neovascularization emerged from the confirmation that infantile hemangiomas are effectively reduced by treatment with propranolol, a β-1 and β-2 adrenergic receptor antagonist.[215] Subsequently, several studies showed that the action of propranolol re-

sults mainly from the blockade of β2-adrenoreceptors.[178] In a mouse model of OIR, the administration of propranolol during the hypoxic phase prevented the upregulation of HIF-1α and its proangiogenic cascade, reducing retinal neovascularization.[313] This suggests that blocking the β-adrenergic system may contribute to uncoupling hypoxia from vascularization, indirectly modulating oxygen-induced vascularization.[100] Other studies demonstrated that propranolol is promising in the treatment of ROP (Table 1). Fillipi and coworkers showed that oral propranolol was effective in reducing severe ROP, but there were serious adverse side effects.[101] Clinical trials showed that, at the 0.2% dose, propranolol in topical eye drops was well tolerated and effective in preventing progression of ROP to advanced stages, but not at the 0.1% dose.[102,103]

In addition to its antiangiogenic activity in hypoxic stages, propranolol may also have a neuroprotective effect.[100] Despite the promising results of preclinical and clinical trials with propranolol in ROP, randomized clinical trials with larger samples and with adequate dose ranges and routes of administration are needed to draw definitive conclusions.[23,100] That said, the ideal timing of administration is clearer, as the protective effects of propranolol appear to exist only when administered in the proliferative phase and not prophylactically, during the avascular phase.[100]

13. Long-chain polyunsaturated fatty acids

PUFAs such as DHA and AA are essential structural constituents of neuronal and endothelial cells and are indispensable for the physiological functions of the retina, especially with regards to photoreceptors.[106] DHA, eicosapentaenoic acid and alpha-linolenic acid are ω-3 PUFAs, and AA is an ω-6 PUFA.[78] During the third trimester of pregnancy a considerable transfer of long-chain ω-3 and ω-6 PUFAs from the mother to the fetus occurs.[72,51] Because of premature birth, preterm infants lack this maternal supply of essential PUFAs.[266] Connor and coworkers demonstrated in a mouse model of ROP that dietary ω-3-PUFAs decrease the avascular area of the retina, resulting in an increase in the growth of blood vessels after injury, and a reduction in the hypoxic stimulus for neovascularization.[69] The protective effect of ω-3 PUFAs and their bioactive metabolites was mediated, in part, by suppression of tumor necrosis factor-alpha.[69]

DHA protects mitochondrial functionality, promotes differentiation, and prevents apoptosis of photoreceptors.[251] To repair infant DHA, two strategies have been used: intravenous administration of lipid emulsions containing fish oil and enteral supplementation with unicellular oils or fish oil.[378] Results of reviews comparing early supplementation with fish oil versus lipid emulsions without fish oil have yielded inconsistent results.[387,193] As fish oil is rich in DHA and eicosapentaenoic acid, but proportionally low in omega-6 AA, its use in preterm supplementation reduces circulating AA, and this aspect can be potentially negative.[81] A meta-analysis by Qawasmi and coworkers revealed that supplementation of infant formulas with long-chain PUFAs improves visual acuity up to the age of 12 months.[305] Pawlik et al found that parenteral ω-3 supplementation reduces the risk of severe ROP in very low birth weight preterm infants.[289] In a recent clinical trial (NCT03201588), infants born with a gestational age of less than 28 weeks received a daily enteral intake of an oil providing AA (100 mg/kg) and DHA (50 mg/kg) from the third day after birth until 40 weeks of postmenstrual age. Results showed a significant reduction in severe ROP and no significant adverse effects.[151] Higher levels of DHA were only effective in protecting from severe ROP in infants with minimal levels of AA.[153] In another clinical trial (NCT02683317), enteral DHA supplementation (75 mg/kg/day) for fourteen days in preterm infants significantly reduced the risk of stage 3 ROP.[32]

Further studies should seek to identify the ideal fatty acid composition that may contribute to the prevention of ROP and other pathologies associated with prematurity.[153] In addition to evidence of mechanisms involving lipidomics in the pathogenesis of ROP, investigations are also underway on the contribution of proteomics and metabolomics. Detailed clinical and experimental evidence on these mechanisms and on potential therapeutic targets related to their modulation is discussed in another review.[378]

14. Conclusion

Interruption of the angiogenic phase of retinal vascular development by preterm birth leads to ROP, which occurs in 2 phases. Phase 1 is characterized by an early stage of retinal microvascular degeneration and an arrest in the progressive vascularization of the peripheral retina and is associated with reduced levels of VEGF and IGF-1. This is followed by Phase 2, in which retinal ischemia increases HIF levels and induces the transcription of angiogenic factors (VEGF, EPO) and IGF-1, causing neovascularization, which in severe cases can lead to retinal detachment and permanent visual loss. The primary objective of this review was to summarize research on the various molecular mechanisms of ROP and identify potential targets for future studies, specifically related to possible treatment modalities.

In addition to low gestational age and birth weight, exposure to oxygen is the other primary risk factor associated with ROP. Recent results from randomized clinical trials suggest that an oxygen saturation range of 90–95% in the first few weeks of life appears to be the safest level for preterm infants. Another important emerging notion is that rigorous management of oxygen saturation, geared towards avoiding oxygen fluctuations and periods of intermittent hypoxia, helps to reduce the risk of ROP. Additional clinical trials are still needed to optimize oxygen therapy.

Oxidative and nitrosative stress is involved in the early obliterative phase of ROP and induces HIF-1α, VEGF and the JAK/STAT pathway, while also mediating inflammation and angiogenesis. Additional evidence is needed to fully support the safety and efficacy of broad inhibition of ROS generation as a therapy for ROP. Future therapies may target ROS-activated downstream pathological effectors. In addition, more research is needed to assess the potential for regulating NO production by eNOS in preventing ROP.

Oxidative stress increases the activity of arginase, which competes with NOS for the common substrate, L-arginine, causing NOS uncoupling and further exacerbating oxidative stress and inflammation. Upregulation of arginase 2 is associ-

ated with ischemic retinal neurovascular degeneration, while arginase 1 – a marker of anti-inflammatory M2 macrophages – reduces oxidative stress and inflammation. Further studies are needed to evaluate arginase as a therapeutic target for oxidative stress-related retinopathies. The development of specific inhibitors of arginase isoforms and methods for cell-specific targeting may facilitate progress in this area.

HIF mediates the adaptive responses of cells to hypoxia. In the first phase of ROP, HIF stabilization during hyperoxia (hypoxiamimesis) may prevent retinal vessel loss. During the second phase, inhibition of HIF may target the pathological action of proangiogenic factors.

Given the important role of VEGF in the pathological angiogenesis of ROP and the experience with anti-VEGF agents for other retinal neovascular pathologies, there is an interest in the use of anti-VEGF for the treatment of ROP. However, there is great concern about the safety of anti-VEGF therapy in ROP because VEGF is essential for physiological angiogenesis and development of the retina and other organs. Currently, the standard treatment for stage 3 ROP with plus disease (especially that located in zone II) is laser photocoagulation. Although, it destroys parts of the retina and may have potential adverse ocular side effects.

Other growth factors of interest in the pathogenesis and treatment of ROP are:

- EPO – an oxygen-regulated growth factor controlled by HIF-2 and an important angiogenic factor. In phase 1 ROP, low serum levels of EPO may contribute to the interruption of angiogenesis, while in phase 2, high levels of EPO may increase pathological neovascularization.
- IGF-1 – a polypeptide hormone required for VEGF-stimulated angiogenesis to occur. Decreased levels of serum IGF-1 and IGFBP3 in preterm infants increase the risk of ROP. Clinical studies are being conducted to evaluate treatment with IGF-1 and IGFBP3 to prevent stage 1 ROP.
- RPE – a monolayer of tight cells that aids in preserving the blood-retinal barrier and protecting the retina from oxidative damage.
- PEDF – a member of the serine protease inhibitor family (serpin family) secreted by the RPE with potent antiangiogenic and anti-inflammatory properties.
- PLGF – a homologous factor of VEGF-A, associated with pathological rather than physiological angiogenesis. Aflibercept, primarily an anti-VEGF-A, also inhibits PLGF; however, the advantages of PLGF inhibition for treatment of retinal neovascular diseases remain controversial.
- Ang-2 – a growth factor that modulate physiological and pathological neovascularization, particularly in association with VEGF. It is upregulated by VEGF and hypoxia and stimulates neovascularization. Clinical trials in retinal vascular diseases have shown that combined inhibition of the VEGF and Ang-2 is superior to anti-VEGF therapy alone.

A compensatory mechanism between vascular growth factors in ROP has been described, which may partially explain why inhibition of a single growth factor is not effective in preventing recurrence of neovascularization. A combined strategy may be more effective.

The involvement of semaphorin family classes in ischemic retinal vasculopathies has been demonstrated. Some of them, such as Sema 3A, are vasorepulsive molecules that repel neovessels from the avascular neural retina towards the vitreous, preventing revascularization of the avascular zone. Future research should evaluate semaphorins as targets in vascular regeneration therapy for ROP.

The Krebs cycle intermediates, of which succinate is the best example, are sensors of hypoxic stress, promoting angiogenesis trough activation of its GPR91 receptor. Succinate stabilizes HIF-1α and increases IL-1β expression, contributing to the proliferative phase of ROP. GPR91 is considered a valuable target in developing molecular intervention to reduce intravitreal neovascularization.

Heparanase cleaves heparan sulfate polysaccharide chains at the cell surface and in the extracellular matrix, an important step for neovascularization. The search for heparanase inhibitors is increasing; however, studies to-date have focused on their use in cancer therapy and for inflammatory pathologies. Recently, synthetic small molecules heparanase inhibitors were developed and evaluated in pre-clinical studies. They have favorable pharmacokinetic profiles and can be administered orally. Therefore, heparanase should be considered for additional research as a possible new target in the treatment of ROP.

MMPs hydrolyze the extracellular matrix and play an important role in the neovascularization process. MMP-2 and MMP-9 have been implicated in ROP. MMPs can be inactivated by the TIMPs family of proteins. The ADAM family of enzymes is also involved in the degradation of components of the extracellular matrix. Several subtypes of the ADAM family have been implicated in the pathogenesis of ROP. Developing synthetic inhibitors of MMPs with selectivity has been a challenge. An alternative approach may be the use of TIMPs designed with limited inhibitory specificities.

During the second phase of ROP, the administration of a β adrenergic receptor antagonist, propranolol, prevented the upregulation of HIF-1α and its pro-angiogenic cascade, reducing retinal neovascularization. More prospective randomized clinical trials with longer follow-ups are needed to assess the efficacy, safety, and optimal dose for administration of anti-VEGF agents and propranolol for ROP.

Low plasma levels of essential fatty acids DHA and AA in the postnatal period in preterm infants are correlated with progression of ROP. The benefit of DHA supplementation on ROP appears to be influenced by the levels of AA present in the infant, and also by DHA and AA metabolites.

In conclusion, a better understanding of the intricate molecular mechanisms underlying the pathogenesis of ROP, especially in the early stages, will aid the development of new therapeutic approaches to ROP. Despite the benefits of photocoagulation and anti-VEGF therapy during the proliferative phase, these treatment modalities have limitations. New treatments are needed to promote physiological retinal vascular development, vascular repair and inhibit vasoproliferation by regulating the mediators involved in VEGF, IGF-1, or EPO signaling pathways. Researchers are currently evaluating a series of future treatments aimed at reducing excessive oxidative/nitrosative stress, and understanding progenitor cells,[380] and neurovascular and glial vascular interactions.

As advances in medical technology contribute to increased survival rates in extremely preterm infants, the absolute number of infants at-risk of developing severe ROP rises. Therefore, the need to better understand the mechanisms involved in ROP and to discover novel treatment modalities is more important than ever.

15. Methods of literature search

An extensive bibliographical search was performed in the Pubmed, Medline and Embase databases from 1983 through 2022, using the keywords: "Retinopathy of Prematurity," "Retrolental Fibroplasia," "Retinopathy," "Oxidative Stress," "Nitrative Stress," "Free Radicals," "Arginase," "Nitric Oxide," "Preterm Infant," ""Premature Birth," "Angiogenesis," "Neovascularization," "Vascular endothelial growth factor," "Hypoxia-inducible Factor," "Erythropoietin," "Insulin-like Growth Factor-1," "Nerve Growth factors," "Granulocyte Colony-stimulating Factor,""Pigment Epithelium-derived Factor," "Fibroblast Growth Factors," "Endothelial cells," "Extracellular Matrix," "Heparanase," "Semaphorins," "Succinate," "Adenosine," "Retinal Pigment Epithelium," "Matrix Metalloproteinases," "Prostaglandins," "Angiopoietins," "Polyunsaturated Fatty Acids," and "Signal Pathway." Publications regarding hereditary pathologies of the retina were excluded. Abstracts not peer-reviewed and not in English were excluded. To select articles with thematic that fit the scope of this review, the titles and abstracts of the articles were read. In this first selection, the article was also read in its entirety if considered necessary to assess its importance according to the purpose of the review. When the topic of the article was relevant, the article was read in its entirety. In the second selection, the articles were chosen based on criteria such as relevance of the results for the chosen topic, general assessment of the quality of the study and article. Some articles cited in the reference lists of other selected articles were included. As the pathophysiology of ROP and its signaling pathways is complex, the result was that a significant proportion of articles were considered relevant and included in the review. Publications not yet detected were evaluated and added to the review if they met the inclusion criteria. In the first evaluation of non-English articles, abstracts in English were used. If necessary, the translation of the full text was carried out.

. Key References

Retinopathy of prematurity involves complex signaling pathways, with several research studies contributing to a better understanding of its pathophysiology. We highlight the following five studies on different topics in this area:

1) Becker S, Wang H, Simmons AB, Suwanmanee T, Stoddard G J, Kafri T, Hartnett M E. Targeted Knockdown of Overexpressed VEGFA or VEGF164 in Müller cells maintains retinal function by triggering different signaling mechanisms. Sci Rep. 2018;8(1):2003. https://doi.org/10.1038/s41598-018-20278-4.

   This study provided evidence that reducing intravitreal neovascularization by selective knockdown of VEGFA, and specially VEGF164, in Müller cells may have less harmful effects than non-selective inhibition of VEGFA for all retinal cells. It also showed that different signaling mechanisms are triggered depending on whether VEGFA or VEGF164 is inhibited.

2) Yang Z, Wang H, Jiang Y, Hartnett ME. VEGFA activates erythropoietin receptor and enhances VEGFR2-mediated pathological angiogenesis. Am J Pathol. 2014 Apr;184(4):1230-1239. doi: 10.1016/j.ajpath.2013.12.023. Epub 2014 Mar 12. PMID: 24630601; PMCID: PMC3969997.

   This study showed that p-VEGFA receptor 2 activates the erythropoietin receptor, enhancing endothelial cell proliferation by STAT3 activation, resulting in pathological angiogenesis. It provided important insights for a better understanding of pathological angiogenesis signaling.

3) Rivera JC, Sitaras N, Noueihed B, Hamel D, Madaan A, Zhou T, Honoré JC, Quiniou C, Joyal JS, Hardy P, Sennlaub F, Lubell W, Chemtob S. Microglia and interleukin-1β in ischemic retinopathy elicit microvascular degeneration through neuronal semaphorin-3A. Arterioscler Thromb Vasc Biol. 2013;33(8):1881-91. doi: 10.1161/ATVBAHA.113.301331. Epub 2013 Jun 13. PMID: 23766263.

   Findings of this study suggested that in the early stages of hyperoxia-induced retinopathy, retinal microglia is activated to produce IL-1β that maintains the activation of the microglia and induces microvascular damage by releasing semaphorin 3A from adjacent neurons. It broughts important new insights to the understanding of pathological angiogenesis.

4) Shosha E, Xu Z, Yokota H, Saul A, Rojas M, Caldwell RW, Caldwell RB, Narayanan SP. Arginase 2 promotes neurovascular degeneration during ischemia/reperfusion injury. Cell Death Dis. 2016;7(11):e2483. doi: 10.1038/cddis.2016.295. PMID: 27882947; PMCID: PMC5260867.

   This study demonstrated that after retinal ischemia/reperfusion, neurovascular injury is mediated by increased Arginase 2 expression. According to these results, Arginase 2 can be considered a therapeutic target for the treatment of retinopathy related to oxidative stress and, in particular, retinopathy of prematurity.

5) Hu J, Song X, He YQ, Freeman C, Parish CR, Yuan L, Yu H, Tang S. Heparanase and vascular endothelial growth factor expression is increased in hypoxia-induced retinal neovascularization. Invest Ophthalmol Vis Sci. 2012;53(11):6810-7. doi: 10.1167/iovs.11-9144. PMID: 22956610.

   This study provided evidence that in hypoxia-induced retinal diseases, heparanase is upregulated and promotes the VEGF expression. It revealed the importance of heparanase and extracellular matrix in hypoxia-induced neovascularization and suggests heparanase as a new therapeutic target.

Disclosures

The authors have no conflict of interest to declare.

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