SAPIENZA
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The role of miRNA expression in the pathogenesis of early stages of Retinopathy of Prematurity: Pilot Study

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Ci sono cose che non si possono fermare:
la volontà ereditata, i sogni della gente,
lo scorrere del tempo.
Finchè le persone avranno sete di libertà,
queste cose dureranno per sempre
Gol D Roger

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1.Retinopathy of Prematurity (ROP)

1.1 Background

Retinopathy of prematurity (ROP) is a multifactorial disease primarily affecting premature infants with incomplete or abnormal retinal vascularization. It arises when normal retinal blood vessel growth (vasculogenesis) is disrupted, leading to abnormal new vessel proliferation (angiogenesis)[1].This abnormal proliferation occurs at the border between the vascularized and avascular retina, resulting in vessels growing into the vitreous cavity. Without timely intervention, ROP can progress to severe outcomes, including a dense fibrovascular plaque behind the lens and complete tractional retinal detachment. Historically known as retrolental fibroplasia, ROP is one of the leading causes of childhood blindness globally. Prematurity and low birth weight are the major risk factors for ROP. Premature birth, defined as delivery before 37 weeks of gestation, becomes particularly associated with retinal disease in infants born before 32 weeks[2]. The disease has a substantial global impact, particularly in resource-limited settings where advances in neonatal care have increased the survival rates of preterm infants, leading to a corresponding rise in ROP incidence. According to the National Institutes of Health, approximately 1,100 to 1,500 infants in the U.S. require treatment for ROP each year, with 400 to 600 of them likely to experience severe visual impairment.[3]

1.2 Pathogenesis

ROP occurs in two distinct phases, driven primarily by fluctuations in oxygen levels in the developing retina.[4]

1. **Phase 1 (Hyperoxic phase):** From birth to about 31 weeks' gestational age, the retina experiences relative hyperoxia compared to the in-utero environment. This increased oxygen suppresses the production of vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1), two key molecules required for normal retinal vascular development. As a result, retinal blood vessel growth ceases, leaving parts of the retina avascular.
2. **Phase 2 (Hypoxic phase):** After 31 to 34 weeks' gestational age, the metabolic demands of the retina increase, leading to relative hypoxia in the avascular areas. This hypoxia stimulates overproduction of VEGF, causing disorganized vascular proliferation. These abnormal vessels are fragile, prone to hemorrhage, and contribute to tractional forces on the retina, which may lead to retinal detachment.

The pathophysiology of ROP is closely linked to oxygen management in neonatal intensive care. Historical evidence from the 1950s identified excess oxygen supplementation as a contributing factor to severe ROP, leading to significant changes in neonatal care practices. Despite advancements, optimal oxygen regulation remains critical to mitigating the risk of severe ROP

1.3 Classification

1.3.1 Classification

ROP is classified based on the International Classification of Retinopathy of Prematurity (ICROP), which was first introduced in 1984 and revised in 2005 and 2021[5–7]. These revisions reflect evolving understanding of ROP pathophysiology, advances in neonatal care, and improved diagnostic techniques. The classification system encompasses four key features:

1. Location (Zones) (Fig1.):

   - Zone I: The posterior retina within a circle centered on the optic nerve and extending to twice the distance between the optic nerve and the fovea. This region represents the most immature and high-risk area for ROP development.

   - Zone II: Extends from the edge of Zone I to the nasal ora serrata, encompassing the majority of the retinal periphery.

   - Zone III: The outermost crescent of the retina, which represents the most peripheral area. For ROP to be classified as Zone III, the nasal vessels must reach the ora serrata, and no lesions should be present in the two nasal-most clock hours.

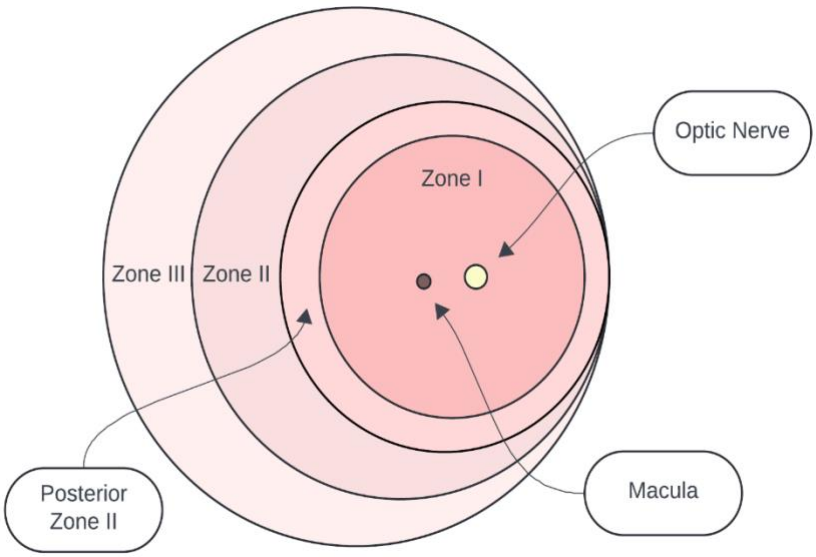


Fig. 1

Schematic representation of the retinal zones used to classify the location of retinopathy of prematurity (ROP) according to the International Classification of Retinopathy of Prematurity (ICROP). The zones are defined as follows: Zone I represents the posterior retina within a circle centered on the optic nerve; Zone II extends from the edge of Zone I to the nasal ora serrata; and Zone III forms the outermost crescent of the retina.

2. Extent of Disease: Measured in clock-hours (from 1 to 12), indicating how much of the retina is affected. This classification provides a standardized way to document the spread of the disease.

3. Severity (Stages):

    - Stage 0: Immature retinal vasculature without pathologic changes.

    - Stage 1: A thin white demarcation line separates vascularized from avascular retina. The line lies flat within the retinal plane and may be associated with abnormal vessel branching.

    - Stage 2: The demarcation line evolves into a "ridge," characterized by width, height, and volume. The ridge may appear white to pink and can include small tufts of neovascular tissue (commonly referred to as "popcorn"), which do not meet the criteria for Stage 3. (Fig.2)

    - Stage 3: Extraretinal neovascular proliferation extends from the ridge into the vitreous, contributing to a ragged appearance. This stage often correlates with significant risk for disease progression. (Fig 3)

- Stage 4: Partial retinal detachment, subdivided into:
    - Stage 4A: Retinal detachment sparing the macula. (Fig.4)
    - Stage 4B: Retinal detachment involving the macula.
- Stage 5: Complete retinal detachment, further classified into funnel configurations (open-open, open-closed, closed-open, and closed-closed) and subcategories such as Stage 5A (optic disc visible), 5B (optic disc obscured by fibrovascular tissue), and 5C (accompanied by anterior segment abnormalities).

4. Plus Disease: Defined by dilation and tortuosity of the posterior retinal vessels within Zone I, often accompanied by other advanced disease signs, such as iris vascular engorgement and vitreous haze. A milder form, known as "preplus disease," represents retinal vascular dilationand tortuosity that is abnormal, but insufficient for plus disease. Importantly, these terms represent a spectrum of retinal vascular abnormalities.

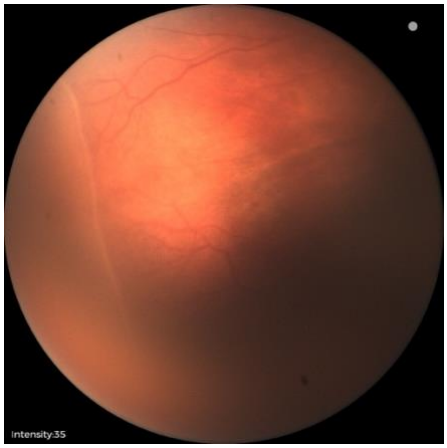


Fig.2

Retinography of a ROP Stage 2 with ridge characterized by width, height, and volume. Note the so-called popcorn lesions posterior to the ridge

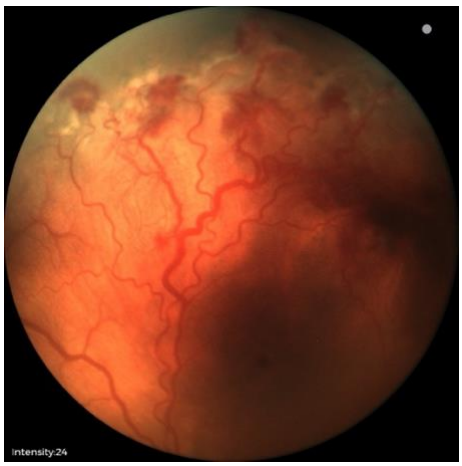


Fig 3

Retinography of a ROP stage 3 with extraretinal neovascular proliferation extends from the ridge into the vitreous,

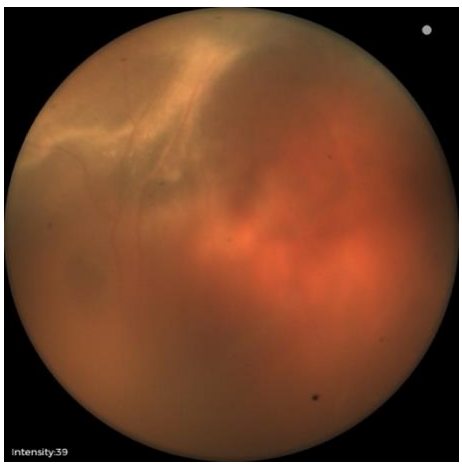


Fig4.

Retinography of a ROP stage 4A with partial retinal detachment, sparing the macula

1.3.2 Recent Additions to the Classification: The 2021 ICROP update introduced several refinements:

- Posterior Zone II: A new subcategory that designates a region two disc diameters beyond the boundary of Zone I, associated with more concerning disease compared to peripheral Zone II.

- Notch: Describes cases where ROP lesions extend 1-2 clock hours into a more posterior zone. For example, "Zone I secondary to notch" denotes lesions predominantly in Zone II but with posterior extension into Zone I.

- Aggressive Retinopathy of Prematurity (A-ROP): Formerly termed "aggressive posterior ROP," A-ROP is characterized by rapid progression, pathologic neovascularization, and severe plus disease without clear staging progression. This phenotype can now occur in larger preterm infants and beyond the posterior retina, especially in resource-limited regions. (Fig.5)

1.3.3 Regression and Reactivation:

Regression refers to disease involution, which may be complete or incomplete, often leaving persistent avascular retina (PAR) susceptible to late complications like retinal thinning and detachment.

Reactivation involves recurrence of acute-phase features, often seen after anti-VEGF treatment, and may progress to advanced stages, including retinal detachment.

Clinical Implications: These refinements emphasize the need for precise documentation and early recognition of ROP progression. For example, distinguishing posterior Zone II disease or identifying notches may prompt earlier intervention. Furthermore, understanding the continuous spectrum of preplus and plus disease guides monitoring and treatment decisions. Enhanced imaging techniques, such as fluorescein angiography and optical coherence tomography (OCT), provide additional tools for assessing vascular abnormalities and retinal detachment configurations.

By integrating these updates, the ICROP aims to standardize ROP classification globally, facilitating research, treatment protocols, and long-term outcome studies.

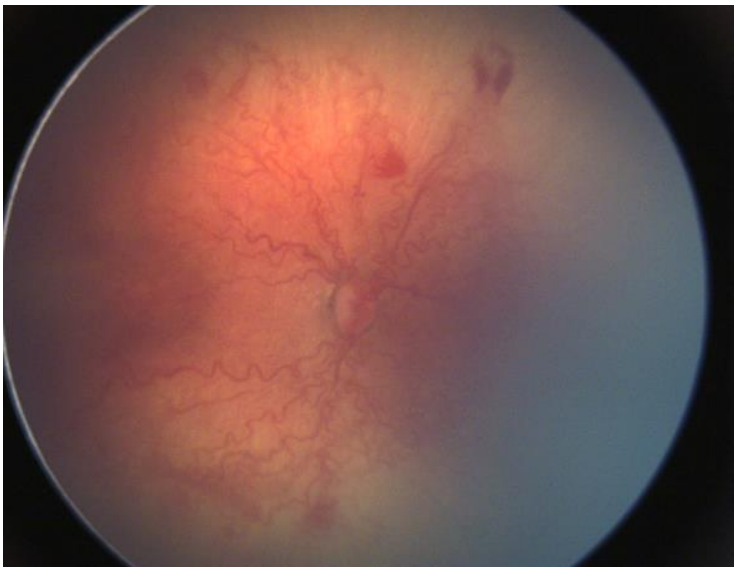


Fig 5. *Retinography of a premature infant with aggressive retinopathy of prematurity (A-ROP), demonstrating marked vascular tortuosity characteristic of plus disease. A-ROP, as defined in the third edition of the International Classification of ROP (ICROP3), includes rapidly progressing forms such as aggressive posterior ROP (AP-ROP), which can bypass the typical staged progression of the disease.*

1.4 Treatment

The management of ROP has evolved significantly since the late 20th century. The landmark **Cryotherapy for Retinopathy of Prematurity (CRYO-ROP) study** in the 1980s demonstrated that ablating the avascular peripheral retina could reduce the risk of severe visual impairment in eyes with threshold ROP.[8] However, nearly one-quarter of treated eyes still experienced unfavorable outcomes, especially in cases involving posterior disease.

In the 1990s, laser photocoagulation replaced cryotherapy as the preferred treatment. Laser therapy, which uses an indirect ophthalmoscope to deliver precise burns to the peripheral retina, remains the standard care for severe ROP. The **Early Treatment for Retinopathy of Prematurity (ETROP) study** further refined treatment guidelines, recommending earlier intervention for high-risk pre-threshold ROP (type 1 ROP)[9]. Type 1 ROP, which includes Zone I disease with plus disease or Stage 3 ROP, should be treated immediately, while type 2 ROP should be monitored closely.

Over the past decade, anti-vascular endothelial growth factor (VEGF) agents have emerged as a promising alternative or adjunct to laser therapy for ROP. VEGF plays a critical role in pathological neovascularization seen in ROP, and anti-VEGF agents have been widely used in adult ophthalmic diseases characterized by neovascularization. Their application in ROP offers a targeted approach to reducing abnormal blood vessel proliferation without the extensive tissue damage caused by laser therapy.

The landmark **BEAT-ROP trial** was the first large-scale study to evaluate the efficacy of anti-VEGF therapy in ROP, specifically investigating intravitreal bevacizumab.[10] The study found that bevacizumab significantly reduced the recurrence of ROP in zone I disease compared to laser treatment. However, concerns were raised about the potential long-term systemic effects of bevacizumab, as VEGF is critical for normal neurodevelopment in infants. Additionally, the prolonged suppression of VEGF levels in the systemic circulation following bevacizumab treatment may increase the risk of adverse developmental outcomes.

The **RAINBOW trial**, a randomized, open-label, multi-center study conducted across 26 countries, compared the efficacy of two doses of intravitreal ranibizumab (0.1 mg and 0.2 mg) with laser therapy for ROP. Ranibizumab, a smaller anti-VEGF molecule, has a shorter half-life in the systemic circulation than bevacizumab, potentially reducing the risk of systemic VEGF suppression. The RAINBOW trial demonstrated that ranibizumab 0.2 mg was associated with higher rates of treatment success compared to laser therapy, with fewer unfavorable structural outcomes and no detectable suppression of systemic VEGF levels. Importantly, the study also reported a lower incidence of high myopia following ranibizumab treatment compared to laser [11].

Despite the short-term benefits of anti-VEGF therapy in reducing the recurrence of ROP and minimizing unfavorable structural outcomes, several questions remain regarding its long-term safety and efficacy. The **RAINBOW Extension Study**, which followed infants treated in the original trial up to 5 years of age, confirmed that ranibizumab 0.2 mg continued to offer superior

ocular outcomes compared to laser therapy, with fewer cases of high myopia and no new ocular complications. Visual acuity outcomes were comparable between the ranibizumab and laser groups, though ranibizumab-treated infants had slightly better vision-related quality of life.

The long-term safety profile of anti-VEGF agents remains an area of concern, particularly regarding neurodevelopmental outcomes. Recent systematic reviews and meta-analyses have suggested an association between bevacizumab and adverse neurodevelopmental outcomes, although these findings may be confounded by the preferential use of anti-VEGF agents in sicker, more immature infants. In contrast, the RAINBOW Extension Study found no significant differences in neurodevelopmental outcomes, growth, or systemic health between infants treated with ranibizumab and those treated with laser therapy, further supporting the safety of ranibizumab[12,13].

Anti-VEGF agents offer several advantages over laser therapy, including shorter administration times, reduced need for specialized equipment and anesthesia, and potentially fewer visual field defects. However, anti-VEGF therapy also carries the risk of disease recurrence, which can occur later than with laser therapy. Infants treated with anti-VEGF agents require longer and more frequent follow-up to monitor for late recurrences and to assess full retinal vascularization.

Intravitreal ranibizumab, now licensed in Europe for the treatment of ROP, appears to be a viable alternative to laser therapy, particularly in infants with posterior disease or those at high risk for unfavorable visual outcomes. However, clinicians must weigh the benefits of improved ocular outcomes

and reduced myopia against the need for long-term follow-up and the potential for unknown systemic risks.

Further research is needed to determine the optimal dosing, long-term systemic safety, and the role of anti-VEGF agents in combination with laser therapy. Ongoing studies, such as the **RAINBOW Extension Study** and similar trials, will continue to provide valuable insights into the long-term outcomes of anti-VEGF therapy in ROP.

2. Development of Retinal Vascularization

2.1 Introduction

Retinal vascularization is a complex and highly regulated process crucial for providing oxygen and nutrients to the inner retina, which is essential for the development and function of retinal neurons[14]. The retina is one of the most metabolically active tissues in the body, making the proper formation of its vasculature vital for normal vision. Retinal vascular development has been studied extensively using animal models, particularly the mouse model, which mirrors many aspects of human retinal vasculature development[15]. These studies have greatly improved our understanding of the signaling pathways and cellular interactions that govern this process, both in normal conditions and in diseases such as retinopathy of prematurity (ROP) and oxygen-induced retinopathy (OIR).

2.2 Anatomy of Retinal Vascularization

The retina contains two distinct vascular systems: the retinal vasculature, which supplies the inner retina, and the choroidal vasculature, which nourishes the outer retina, including the photoreceptors[16]. During retinal vascularization, the inner retina is penetrated by capillaries forming a

laminar meshwork, while the outer retina remains avascular. The development of the retinal vasculature proceeds in a highly organized manner from the optic nerve head outward, forming distinct vascular plexuses: the superficial, intermediate, and deep plexuses.[17,18]

- Superficial plexus: The first plexus forms at the inner surface of the retina, where vessels emerge from the optic nerve head and spread radially across the nerve fiber layer.

- Intermediate and deep plexuses: Angiogenic sprouts from the superficial plexus invade deeper retinal layers, creating secondary networks within the inner retina.

In humans, this process begins in utero, while in mice it occurs postnatally, providing an accessible model to study the intricate details of retinal vascular development.

2.3 Stages of Retinal Vascular Development

2.3.1 Hyaloid Vasculature Formation and Regression

Retinal vascular development begins with the formation of the hyaloid vasculature, a temporary vascular system that nourishes the developing eye in the embryonic stage[19]. Originating from the hyaloid artery, this network

supplies the retina and the posterior lens. However, as the retina matures, the hyaloid vessels regress, and retinal vessels begin to grow from the optic nerve head into the retina.

The regression of the hyaloid system, which is critical for normal eye development, begins around 13 weeks of gestation in humans and is usually complete by the time of birth. Dysregulation of hyaloid vessel regression can lead to persistent fetal vasculature, a condition associated with visual impairment.[20]

2.3.2 Formation of the Retinal Vascular Plexuses

The primary retinal vascular plexus forms by angiogenesis, the sprouting of new vessels from pre-existing ones. This process starts at the optic nerve head and spreads centrifugally toward the retinal periphery. By around 36 weeks of gestation, the primary vascular network reaches the nasal periphery, and by 40 weeks, the temporal periphery of the retina is fully vascularized[21].

After the formation of the superficial plexus, angiogenic sprouts invade deeper retinal layers, establishing two additional layers of capillaries—the intermediate and deep plexuses. These networks grow outward in a pattern like the superficial plexus but penetrate deeper into the retina. By birth, the retinal vasculature is typically complete in full-term infants.

2.4 Molecular Mechanisms in Retinal Angiogenesis

2.4.1 VEGF Signaling

Vascular Endothelial Growth Factor (VEGF) plays a pivotal role in retinal vascular development. Retinal astrocytes, which precede the development of blood vessels, secrete VEGF in response to physiological hypoxia. VEGF stimulates endothelial cell proliferation, migration, and survival, guiding the growth of vessels toward the hypoxic, avascular retina[22–24].

VEGF is tightly regulated to ensure the proper formation of the retinal vasculature. Disruption in VEGF signaling, as seen in oxygen-induced retinopathy (OIR) models, leads to either excessive vessel growth (neovascularization) or vessel regression, both of which contribute to diseases such as ROP[25].

2.4.2 PDGF and Retinal Astrocytes

Platelet-Derived Growth Factor (PDGF) is another key signaling molecule in retinal vascularization. Retinal astrocytes, which express PDGF receptor alpha (PDGFRA), migrate across the inner retinal surface in response to PDGF produced by retinal ganglion cells (RGCs)[26–28]. As these astrocytes spread, they form a template that guides the developing blood vessels. Astrocytes play a dual role in retinal vascularization: they secrete VEGF to stimulate vessel growth and provide a physical substrate for endothelial cell

migration[29,30]. The loss of retinal astrocytes severely impairs vascular development, leading to abnormal or incomplete retinal vascularization.

2.5 Neural-Vascular Interactions in Retinal Development

The retinal vasculature is highly dependent on neural signals, particularly from retinal ganglion cells (RGCs). RGCs regulate vessel growth by sensing hypoxia and responding with the production of VEGF. Through this mechanism, RGCs help match the metabolic needs of the developing retina with the supply of oxygen and nutrients from the growing vascular network[31].

RGCs also produce anti-angiogenic factors, such as semaphorin 3E (SEMA3E), which help restrict the growth of blood vessels to the appropriate layers of the retina, ensuring the proper lamination of the vascular plexuses. Dysregulation of this neural-vascular communication can result in misdirected vessel growth, leading to retinal vascular diseases.

2.6 Sprouting Angiogenesis and Tip/Stalk Cell Dynamics

The process of retinal vascularization is driven by sprouting angiogenesis, wherein specialized endothelial cells at the tip of the sprout guide the direction of vessel growth. These tip cells are highly responsive to VEGF gradients and extend filopodia to explore the surrounding environment[31]. In contrast, stalk cells proliferate and form the vessel lumen, supporting the extension of the sprout.

Tip and stalk cell behaviors are regulated by the Notch signaling pathway. The Notch ligand, Delta-like 4 (DLL4), expressed by tip cells, activates Notch in neighboring endothelial cells, suppressing their tip cell phenotype and promoting stalk cell behavior[32]. This dynamic competition between tip and stalk cells ensures the orderly extension of new vessels.

2.7 Maturation and Remodeling of the Retinal Vasculature

Once the retinal vascular network is established, it undergoes extensive remodeling to form a mature, hierarchical structure. Vessel pruning occurs through the selective regression of redundant or non-perfused vessels, a process driven by endothelial cell migration and apoptosis. This remodeling is influenced by hemodynamic factors, such as blood flow and shear stress, which stabilize perfused vessels and promote the regression of those without flow[33].

Vessel maturation is further supported by the recruitment of mural cells, including pericytes and smooth muscle cells, which provide structural stability to the vessels. [34]Pericyte recruitment is mediated by PDGF signaling, and their presence is essential for the long-term stability and function of the retinal vasculature.

2.8 Conclusion

The development of the retinal vasculature is a highly orchestrated process involving the coordinated actions of multiple signaling pathways, cell types, and environmental cues. VEGF, PDGF, Notch, and other molecular pathways play crucial roles in guiding the formation of blood vessels, while retinal astrocytes and neurons contribute essential signals that shape the vascular network.

Understanding the mechanisms of retinal vascularization not only provides insights into normal retinal development but also highlights potential therapeutic targets for diseases characterized by abnormal retinal vessel growth, such as retinopathy of prematurity. Advances in research using animal models like OIR continue to inform potential interventions to modulate retinal angiogenesis and prevent vision-threatening complications.

3.MicroRNA

3.1 What is miRNA

MicroRNAs (miRNAs) are small, single-stranded, non-coding RNA molecules typically 18-26 nucleotides in length[35]. These molecules play a crucial role in the post-transcriptional regulation of gene expression by binding to complementary sequences in the 3' untranslated regions (UTRs) of target messenger RNAs (mRNAs). These binding leads either to the degradation of the target mRNA or the inhibition of its translation, thus controlling the expression of various proteins involved in cellular processes like apoptosis, differentiation, immune regulation, and angiogenesis[36].
In the context of Retinopathy of Prematurity (ROP), a disease characterized by abnormal retinal neovascularization in premature infants, miRNAs regulate several key angiogenic pathways, contributing to both the normal and pathological development of retinal vasculature. Their ability to fine-tune these processes makes miRNAs central to understanding the molecular mechanisms of ROP and highlights their potential as diagnostic biomarkers and therapeutic targets.

3.2 Role of miRNA in Angiogenesis and Retinopathy of Prematurity

Angiogenesis is the process through which new blood vessels form from pre-existing vasculature, and it plays a critical role in retinal development[36,37]. However, in conditions like ROP, pathological angiogenesis occurs, leading to the abnormal proliferation of blood vessels and subsequent retinal damage.

Several miRNAs have been identified as key regulators of angiogenesis, influencing the balance between pro-angiogenic and anti-angiogenic signals. Studies on animals' model of ROP have identified several key miRNAs involved in ROP pathogenesis:

- miR-210: miR-210 is hypoxia-induced and plays a significant role in the cellular response to low oxygen levels. In the retina, its downregulation is associated with ROP progression, as it acts as a counter-regulator in hypoxic stress, reducing new vascular proliferation[38–41].
- miR-21: Known for its role in promoting cell survival and vascular proliferation, miR-21 is linked to the fibrovascularization process seen in later stages of ROP. Its overexpression contributes to abnormal vessel growth[41–43].
- miR-146a: This miRNA is involved in the inflammatory response and has been shown to modulate endothelial cell activity. It inhibits angiogenesis through its interaction with nuclear factor kappa B (NF-

kB), and its downregulation in ROP contributes to unchecked vascular remodeling[41,44–46].

- miR-221 and miR-222: These miRNAs inhibit endothelial cell proliferation and migration by targeting angiogenesis-related pathways. Their dysregulation in ROP may exacerbate abnormal neovascularization[47].
- miR-23a and miR-200b-3p: These miRNAs were found to be upregulated in hyperoxia-induced rat models of ROP. miR-23a may protect retinal cells under oxidative stress, while miR-200b-3p is implicated in modulating VEGF-A expression, a critical driver of angiogenesis[48–50].
- miR-27b-3p and miR-214-3p: These miRNAs are downregulated in ROP, leading to increased expression of pro-angiogenic factors such as VEGF-B and VEGF-C, thus contributing to pathological neovascularization[47,51].
- miR-143 and miR-126: These miRNAs are directly involved in retinal neovascularization, with roles in both promoting and inhibiting new vessel formation under different conditions[52].

The balance of these miRNAs determines whether angiogenesis is appropriately regulated or leads to pathological outcomes like those seen in ROP.

Recent bioinformatics analyses have also revealed the significant roles of miR-128-3p and miR-9-5p in ROP. These miRNAs were consistently identified as differentially expressed across multiple studies, with miR-128-3p upregulated and miR-9-5p downregulated in ROP.

- miR-128-3p: Associated with angiogenesis and cell migration, miR-128-3p has been linked to the regulation of pathways such as PI3K-Akt and TGF-β signaling, both of which are critical for vascular development and retinal neovascularization in ROP[53].
- miR-9-5p: Primarily known for its role in neural development, miR-9-5p has also been implicated in retinal vascular disorders. Its downregulation in ROP suggests a potential neurovascular role in regulating abnormal retinal angiogenesis[54].

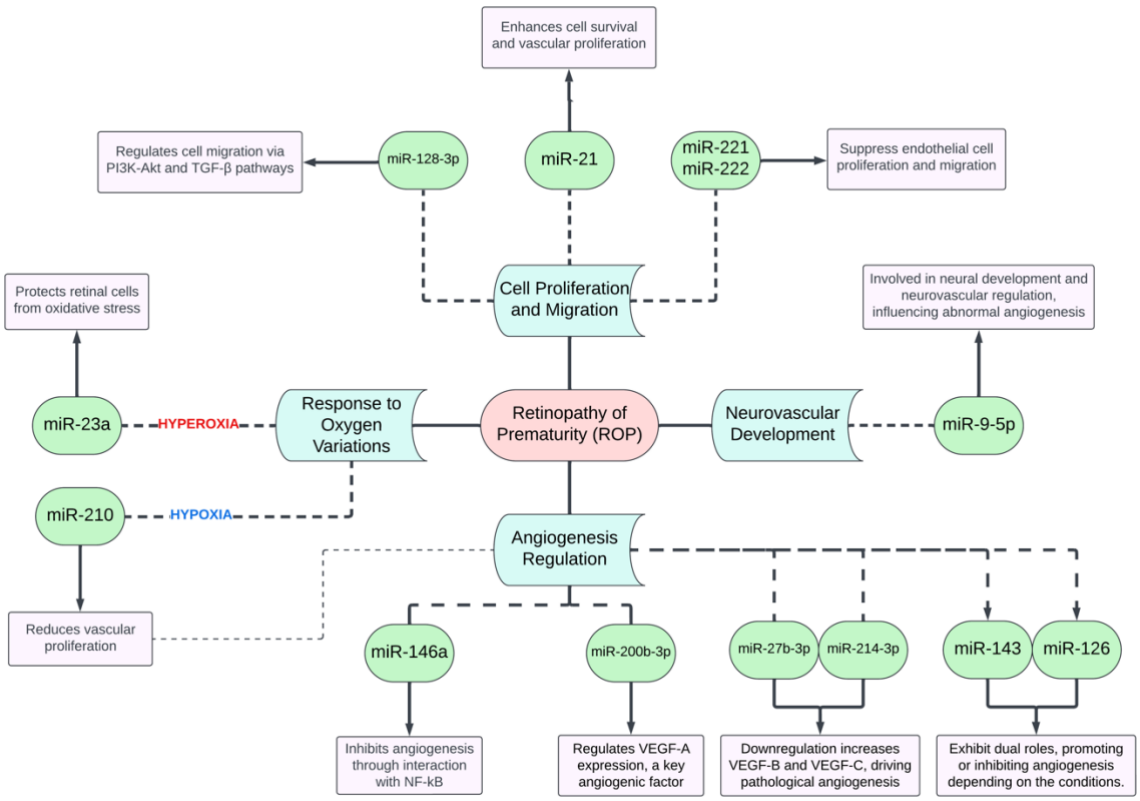


Figure 3. Schematic representation of the roles of key miRNAs in retinopathy of prematurity (ROP).

3.3 Extraction and Analysis of miRNA in ROP

The analysis of miRNA expression in ROP involves the precise extraction and quantification of miRNAs from biological samples, such as plasma, serum, or retinal tissue. Accurate extraction techniques and sensitive laboratory methods are essential for identifying the role of miRNAs in ROP.

3.3.1 Extraction Techniques for miRNA in ROP

The extraction of miRNA from biological samples in ROP studies typically employs either column-based or organic extraction methods:

- Column-Based Extraction: Commercial kits, such as the miRNeasy Serum/Plasma Kit (Qiagen), are commonly used for isolating miRNAs from serum or plasma. These kits rely on the use of silica-based spin columns that bind miRNA molecules selectively, allowing for the removal of contaminants like proteins and larger RNA molecules. This technique is advantageous for its high purity and reproducibility.
- Phenol-Chloroform Extraction: This traditional method involves using a phenol-chloroform mixture to separate miRNAs from other cellular components. The aqueous phase, containing the miRNA, is then purified and concentrated. This method is cost-effective but requires meticulous handling to avoid contamination.

Once miRNA is extracted, it can be analyzed for differential expression in ROP versus control samples[55,56].

3.3.2 Laboratory Analysis of miRNA in ROP

Several methods can be used to quantify and analyze miRNA expression, each with its strengths and limitations. In ROP studies, the most commonly used techniques are:

- Quantitative Real-Time PCR (qRT-PCR): This is the gold standard for miRNA quantification. After miRNA extraction, reverse transcription is used to convert miRNAs into complementary DNA (cDNA), which is then amplified using specific primers. qRT-PCR allows for the precise quantification of miRNAs, providing fold-change data relative to controls. In ROP studies, the SybrGreen dye is commonly used in combination with specific miRNA primers for amplification and detection.
- Microarray Analysis: This technique allows for the simultaneous measurement of multiple miRNAs. In microarray experiments, labeled miRNAs are hybridized to probes on a chip that contains sequences complementary to known miRNAs. This high-throughput method is useful for screening large numbers of miRNAs but is less precise than qRT-PCR for individual miRNA quantification.
- Next-Generation Sequencing (NGS): NGS offers a comprehensive approach for miRNA profiling. It allows for the identification and quantification of both known and novel miRNAs, making it ideal for exploratory studies in ROP. However, NGS is more expensive and time-consuming than other methods[57,58].

3.4 Diagnostic and Therapeutic Potential of miRNA in ROP

The identification of differentially expressed miRNAs in ROP has opened up new avenues for both diagnosis and treatment. miRNAs such as miR-210, miR-23a, and miR-128-3p hold potential as biomarkers for identifying infants at risk for severe ROP. Early detection through miRNA profiling could enable timely interventions to prevent disease progression[59].

In addition to their diagnostic value, miRNAs are promising therapeutic targets. Modulating the expression of specific miRNAs involved in angiogenesis, such as inhibiting pro-angiogenic miRNAs like miR-21 or restoring anti-angiogenic miRNAs like miR-214, could help control abnormal blood vessel growth in the retina.

3.5 Challenges in miRNA Research for ROP

While miRNAs hold great promise in the diagnosis and treatment of ROP, several challenges remain:

- Complexity of miRNA Networks: miRNAs often regulate multiple genes, and individual genes can be targeted by several miRNAs. Understanding these complex interactions is crucial for developing effective miRNA-based therapies.
- Technical Limitations: miRNA extraction, quantification, and analysis require highly sensitive techniques and standardized protocols to ensure reproducibility across studies.
- Differences Between Animal Models and Humans: Although rodent models provide valuable insights into ROP, the physiological differences between species necessitate further genetic and experimental studies in humans to validate the findings and ensure their clinical applicability.

4 Thesis

4.1 Introduction

Retinopathy of prematurity (ROP) remains a leading cause of preventable vision loss among preterm infants, particularly those born at lower gestational ages and with reduced birth weights[2,3]. Despite advances in neonatal care, the pathophysiology of ROP, driven primarily by abnormal retinal vascular development under variable oxygenation levels, remains complex and partially understood. However, numerous angiogenic agents, such as Vascular Endothelial Growth Factor (VEGF), insulin-like growth factor (IGF-I), Angiotensin Converting Enzyme (ACE), Fibroblast growth Factor(bFGF), Tumor Necrosis Factor (TNF-a), and Nitric Oxide (NO), are also crucial in the disease's progression[57].In recent years, microRNAs (miRNAs) have emerged as critical regulators of gene expression, because these 20-22 nucleotide non-coding RNAs participate in numerous cellular processes by regulating gene expression at the post-transcriptional level. Through this mechanism, they influence cell differentiation, apoptosis, immune responses, and angiogenesis[60].

Highly expressed in endothelial cells, miRNAs may help balance inhibitory and activating angiogenic factors, contributing to the regulation of vascular integrity and the angiogenic process. Prior studies in rat models and limited human studies have identified specific miRNAs, such as miR-210, miR-21,

miR-27b, miR-214, and miR-128a, as potential markers of ROP progression and disease severity[41,49,61–63].

This pilot study investigates the expression patterns of selected miRNAs in a cohort of infants with early stage and untreated ROP, aiming to characterize miRNA involvement in the premature stages of the disease. By focusing on preterm infants who did not require therapeutic intervention, this study provides insights into the role of miRNAs in subclinical ROP cases, offering a foundation for future research on potential biomarkers and early diagnostic targets. To the best of our knowledge, this is the first study conducted on humans with the mildest stages of ROP, where sample collection occurs at birth, prior to the diagnosis itself.

4.2 Materials and Methods

4.2.1 Study Population

This study was conducted prospectively in the University Hospital of Policlinico Umberto I and included all infants born before 32 weeks of gestation and weighing less than 1500 grams, during January 2023 and September 2024. ROP screening was done following the Academy of Ophthalmology guidelines and evaluated according to the ICROP criteria[5,64]. All patients had blood samples drawn from a central catheter at birth to avoid hemolysis and minimize sample degradation. Two milliliters of l blood were collected in EDTA-containing tubes as an anticoagulant. Samples were immediately placed on ice and transported to the laboratory within 10 minutes. An initial centrifugation at 2000 × g for 10 minutes separated the plasma from cellular components. The plasma was then transferred to a clean microtube and centrifuged a second time at 2000 × g for 5 minutes to remove any remaining cellular debris. The resulting plasma was aliquoted and stored at -80 °C until RNA isolation. ROP cases were selected based on a diagnosis and maximum stages 2 of ROP were included and ROP that not requiring laser or anti-VEGF therapy. Controls were preterm infants meeting the same gestational and weight criteria but without ROP signs. Infants with congenital heart diseases or other congenital anomalies were excluded from this study. Gender, gestational age, birth weight, delivery pattern, morbidity, Apgar score, Hematocrit, Platelets, Leukocytes, Hemoglobin, Erythrocytes, MCH (Mean Corpuscular

Hemoglobin), MCV (Mean Corpuscular Volume), RDW (Red Cell Distribution Width), MCHC (Mean Corpuscular Hemoglobin Concentration). Informed consent was obtained from both the parents, and the study was approved by the local ethics committee, according to the Declaration of Helsinki.

Based on key findings in previous studies of ROP in rat models and the two available human studies, five miRNAs—miR-210, miR-21, miR-27b, miR-214, and miR-128a—were selected for analysis. We employed the miRNeasy Serum/Plasma Kit (Qiagen, Catalog No. 217184) along with the miRNeasy Serum/Plasma Spike-In Control (Qiagen, Catalog No. 219610) to measure this six MiRNA. RNA extraction and reverse transcription were performed, followed by quantitative PCR (qPCR) to quantify miRNA expression levels and using the TaqMan MicroRNA Assays INV SM (Life Technologies Italia fil. Life Technologies Europe B, Catalog. N. 4427975). PCR cycles were repeated for 45 times.

Fold regulation, fold change and significance were calculated online by using the gene globe data analysis center website. (at www.qiagen.com) Fold change is the ratio of gene expression in the sample to the controls. Fold regulation is the expression of fold change results in a biologically plausible way.

4.2.2Statistical Analysis

The study was designed as a pilot due to the lack of similar research in the literature.

Data analysis was performed using SPSS software (version 27). The normality of continuous variables was assessed using the Shapiro–Wilk test, with results presented as mean ± standard deviation (SD). Differences between groups were analysed using the unpaired t-test for parametric data, and the Mann–Whitney test for nonparametric data. Bivariate relationships were examined through the Pearson correlation coefficient for parametric data, and Spearman's rank correlation for nonparametric data. Categorical variables were reported as counts and percentages, and Fisher's exact test was applied for comparisons.

4.3 Results

We collected samples from 20 patients; subsequently, we excluded 3 patients due to insufficient sample volume, 4 patients due to haemolyzed samples, and one sample was excluded as the patient had deceased. Finally, we included 12 samples in the analysis, 5 from patients with ROP and 7 as controls. Mean gestational age was 27,51±1,30 in the ROP patients and 30,14±1,56 in the controls.

Birth Weight was 958,00±201,04 g and 1363,71±107,935 respectively for ROP and controls. Both the values were significantly different (P-value<0,05). No difference was found for the gender distribution. Mean days of oxygen treatment presented a low significant difference (P-value <0.048) with following parameters 9,4 ± 3,71 for ROP patients and 5±3,05 for the control group. However, no difference was found in the blood parameters between the two groups. Table 1 presents the characteristics of the subjects.

Significant differences were observed in the selected miRNA expression levels between ROP cases and controls, with miR-210, miR-21, and miR-214 notably lower in ROP cases, while miR-128a was significantly higher (Table2). miR-210 levels correlated positively with oxygen exposure, suggesting a potential link between miRNA expression and prolonged oxygen exposure. Interestingly the presence of ROP corelated positively with all the miRNA evaluated and the BW and GA.

Table 1 Characteristic of the study and control group

Characteristics	Total	ROP	Controls	p-value
	n. 12	n. 5	n. 7	
Sex (female), n %	3 (25%)	2 (40%)	1 (14,28%)	**0.222**
Gestational age at birth (weeks), mean±SD	29,49±1.94	27,51±1,3	30,14±1,56	0,03*
Birth weight (grams), mean±SD	1194±254,35	958±201,04	1363,71±107,93	0,01*
HCT	39,855±8,55	48,61±9,67	40,64±3,82	0,20
Plt	250583,33±106764,361	244428,57±85398,78	259200±142294,41	0,63
WBC	7010,13±8327,75	6310,13±4327,56	8110,13±5367,43	0,76
HB	14,958±2,10	13,48±0,81	16,01±2,13	0,34
RBC	4,14±0,71	3,79±0,27	4,39±0,84	0,75
MCV	109,32±2,52	107,92±7,35	110,31±5,93	0,43
MCH	36±2,51	35,72±2,2	36,2±2,87	0,75
MCHC	33,29±1,91	33,04±0,86	32,47±2,48	0,87
RDW	17,75±1,13	16,45±1,79	18,76±1,22	0,34
Day OT	6,93±3,43	9,4±3,71	5±3,04	0,048*

Note: p-values<0.05 are given in bold italic entries

* Unpaired t-test

† Fischer exact test

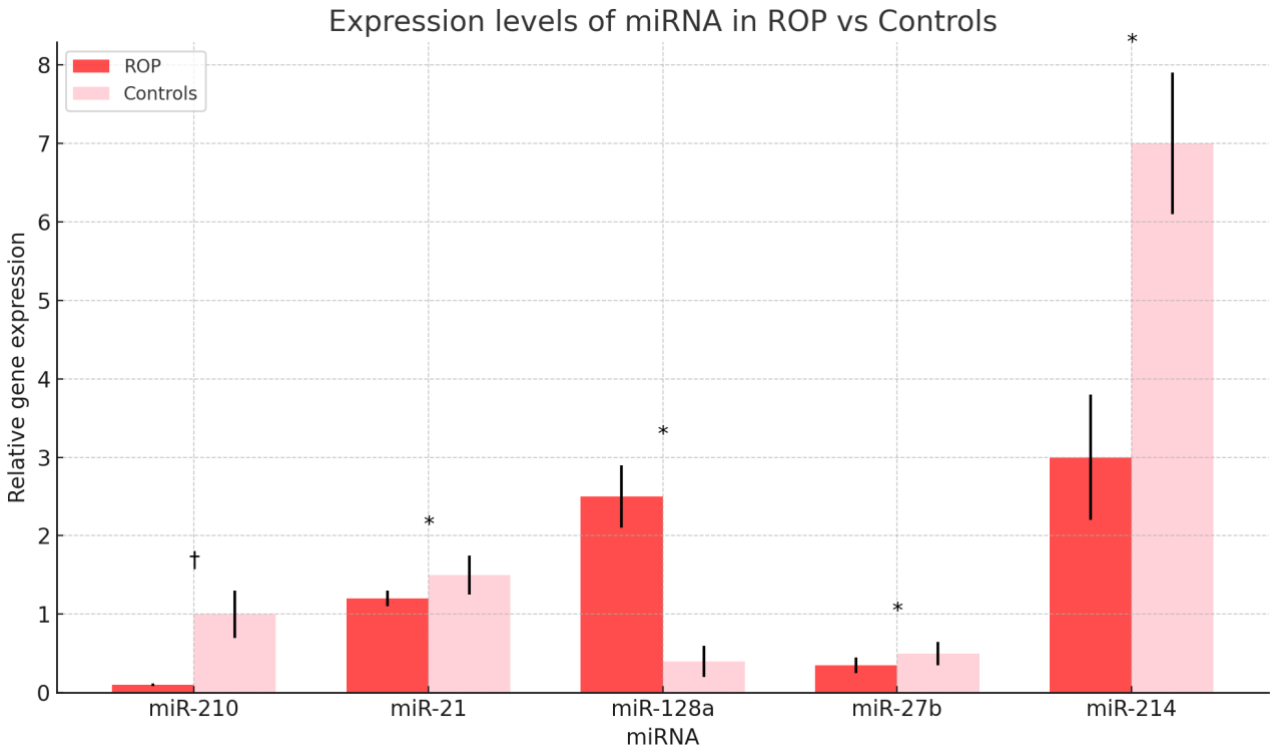
Table 2 MiRNA Values

miRNA	ROP	Controls	p-value
	n. 5	n. 7	
miR-210	0,07±0,06	0,9±0,41	0,005†
miR-21	1,011±0,05	1,41±0,21	0,03*
miR-128a	2,14±0,37	0,38±0,35	0,03*
miR-27b	0,29±0,85	0,45±0,09	0,01*
miR-214	2,66±0,73	7,45±0,85	0,03*

Note: p-values<0.05 are given in bold italic entries

* Unpaired T-test

† Mann-Whitney test



The bar chart illustrates the relative expression levels of five different microRNAs (miRNAs) — miR-210, miR-21, miR-128a, miR-27b, and miR-214 — in two distinct groups: Retinopathy of Prematurity (ROP) patients and healthy controls. The data is represented as mean ± standard deviation for both groups

4.4 Discussion

This pilot study represents the first clinical investigation evaluating specific miRNA expressions in early-stage ROP (maximum stage 2) in untreated patients, focusing on miRNAs that previous research has suggested may play a role in ROP pathogenesis. Our findings suggest a distinct expression pattern of selected miRNAs in patients with early ROP compared to controls, with notable differences in the levels of miR-210, miR-21, miR-128a, miR-214, and miR-27b. These results offer preliminary insights into the molecular alterations associated with early ROP and present potential avenues for early diagnosis and risk assessment.

We observed that miR-210, miR-21, miR-214, and miR-27b were significantly downregulated in the ROP group, while miR-128a showed an upregulation compared to the control group. The downregulation of miR-210 is noteworthy as it has previously been identified as a hypoxia-responsive miRNA in conditions such as diabetic retinopathy, hypoxic-ischemic encephalopathy, and cerebral ischemia. MiR-210 is known to increase during hypoxic states, suggesting a protective response to hypoxic insult. However, in early ROP cases, the decreased miR-210 expression could indicate a shift from a hypoxic response to a proangiogenic phase, as the retina begins to develop abnormally due to oxygen fluctuations typical in premature infants. This pattern might reflect a mechanism in early ROP where miR-210 fails to mount a sufficient protective response against hypoxia, potentially leading to uncontrolled vascular proliferation. Furthermore, our findings revealed a correlation between miR-210 levels and oxygen exposure duration,

supporting its role as a marker of prolonged hypoxic insult and highlighting the need for further investigation into miR-210's role in ROP[38–40].
Interestingly, another study in humans observed a reduction of miR-210 in the early stages of ROP, followed by an increase in later stages, which could make it a promising biomarker for the various phases of ROP. Mir210, which could make it a promising biomarker for the various phases of ROP[41].
Similarly, miR-21 has been linked to proliferative retinopathies and various cancer types, where it acts as a pro-fibrotic and anti-apoptotic factor. In ROP, the decrease in miR-21 may suggest an insufficient protective response to apoptosis and fibrovascular proliferation in early-stage disease. Previous studies have shown that miR-21 increases in the proliferative phase of diabetic retinopathy, a condition with some pathophysiological similarities to ROP[41–43]. Our observation of reduced miR-21 levels in early ROP could indicate that, in these initial stages, miR-21's role as a mediator of fibro-vascularization is not fully activated, potentially correlating with the lack of significant fibrovascular proliferation seen clinically in mild ROP cases.
The upregulation of miR-128a in the ROP group presents an interesting contrast, as this miRNA has been implicated in pathways related to cell differentiation and immune regulation[53,65]. The increase in miR-128a expression in our ROP patients may reflect an early adaptive response to hypoxic conditions or oxidative stress, possibly affecting retinal cell survival and differentiation in a way that contributes to early disease pathogenesis. This finding suggests that miR-128a could be explored as a potential biomarker for early ROP detection and risk assessment in preterm infants, as

its elevation may indicate an underlying vulnerability to abnormal retinal development.

MiR-214 and miR-27b, both found to be significantly downregulated in our ROP cases, are known to target angiogenic pathways, including those involving VEGF. The downregulation of miR-214, previously shown to decrease VEGF levels in ischemic conditions, suggests that in early ROP, the reduced expression of miR-214 may contribute to a lack of adequate inhibition of VEGF-driven angiogenesis, setting the stage for abnormal vascular growth[49,52,60]. Similarly, miR-27b's role in suppressing angiogenesis through VEGF inhibition suggests that its downregulation may facilitate unrestrained angiogenic activity, even in the absence of severe hypoxia, which is characteristic of early-stage ROP[49,51].

Although this study has provided valuable insights into miRNA expression profiles in early, untreated ROP, it has limitations. Primarily, the small sample size restricts the generalizability of the findings and introduces a risk of type I and type II errors. The study's pilot nature was necessary due to the novel focus on early-stage, untreated ROP, but larger cohorts are required to validate these preliminary findings. Additionally, the decision to limit our miRNA selection to five key candidates based on previous studies may have omitted other miRNAs with potential roles in ROP pathogenesis. However, this targeted approach allowed us to explore specific hypotheses regarding the involvement of these miRNAs in ROP while balancing the limitations of a small sample size and resource constraints.

Furthermore, while our study suggests potential diagnostic and prognostic roles for miRNAs in ROP, clinical applications remain distant. Early-stage

miRNA expression profiling in ROP may help identify at-risk infants who could benefit from closer monitoring, but extensive validation is needed before implementing miRNA-based screening or therapeutic interventions. Additionally, environmental and genetic factors influencing miRNA expression, such as gestational age and birth weight, must be accounted for in future studies, as these factors could confound miRNA levels and their association with ROP risk.

In conclusion, our study highlights the differential expression of selected miRNAs in early-stage, untreated ROP, providing initial evidence that miR-210, miR-21, miR-214, miR-128a, and miR-27b may play roles in the pathophysiology of ROP. These findings lay the groundwork for future studies to evaluate miRNA-based biomarkers in ROP and investigate the therapeutic potential of targeting miRNA pathways in early disease intervention.

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