

## REVIEW ARTICLE OPEN ACCESS

# $\beta_3$ -Adrenoceptor Agonism to Mimic the Biological Effects of Intrauterine Hypoxia: Taking Great Strides Toward a Pharmacological Artificial Placenta

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## ABSTRACT

At different stages of life, from embryonic to postnatal, varying oxygen concentrations modulate cellular gene expression by enhancing or repressing hypoxia-inducible transcription factors. During embryonic/fetal life, these genes encode proteins involved in adapting to a low-oxygen environment, including the induction of specific enzymes related to glycolytic metabolism, erythropoiesis, angiogenesis, and vasculogenesis. However, oxygen concentrations fluctuate during intrauterine life, enabling the induction of tissue-specific differentiation processes. Fetal well-being is thus closely linked to the physiological benefits of a dynamically hypoxic environment. Premature birth entails the precocious exposure of the immature fetus to a more oxygen-rich environment compared to the womb. As a result, preterm newborns face a condition of relative hyperoxia, which alters the postnatal development of organs and contributes to prematurity-related diseases. However, until recently, the molecular mechanism by which high oxygen tension alters normal fetal differentiation remained unclear. In this review, we discuss the research trajectory followed by our research group, which suggests that early exposure to a relatively hyperoxic environment may impair preterm neonates due to reduced expression of the  $\beta_3$ -adrenoceptor. Additionally, we explore how these impairments could be prevented through the pharmacological stimulation of the remaining  $\beta_3$ -adrenoceptors. Recent preclinical studies demonstrate that pharmacological stimulation of the  $\beta_3$ -adrenoceptor can decouple exposure to hyperoxia from its harmful effects, offering a glimpse of the possibility to recreating the conditions typical of intrauterine life, even after premature birth.

**Abbreviations:** ATP, adenine triphosphate; BE, base excess; BPD, bronchopulmonary dysplasia; CNS, central nervous system; CTG, cardiocography; Hb, hemoglobin;  $\text{HCO}_3^-$ , bicarbonate; HIE, hypoxic-ischemic encephalopathy; HIFs, hypoxia-inducible transcription factors; IUFD, intrauterine fetal death; IUGR, intrauterine growth restriction; NEC, necrotizing enterocolitis; NIRS, near-infrared spectroscopy;  $\text{O}_2$ , oxygen; OXPHOS, oxidative phosphorylation;  $\text{pCO}_2$ , partial pressure of carbon dioxide;  $\text{pO}_2$ , partial pressure of oxygen; PVL, periventricular leukomalacia; ROP, retinopathy of prematurity; VEGF, vascular endothelial growth factor;  $\beta$ -ARs,  $\beta$ -adrenoceptors.

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## 1 | Introduction

Oxygen (O<sub>2</sub>) is closely associated with the origin and evolution of life because aerobic organisms require molecular O<sub>2</sub> to produce chemical energy in the form of adenine triphosphate (ATP), which is essential for survival [1]. In addition to being a substrate for energy production and acting as the primary electron acceptor, O<sub>2</sub> also plays a crucial role as a signaling factor. Indeed, whether O<sub>2</sub> levels are very low, as in the early stages of embryonic life, or high, as after birth, O<sub>2</sub> modulates cellular gene expression by enhancing or repressing hypoxia-inducible transcription factors (HIFs) [2]. Under hypoxic conditions, the HIF-1 $\alpha$  protein accumulates due to the inhibition of its proteasome-mediated degradation, translocates into the nucleus, dimerizes with the HIF-1 $\beta$  protein, and binds to specific DNA sequences called hypoxia-responsive elements, inducing the transcription of hundreds of target genes [3, 4]. These genes encode proteins involved in adapting to a low-O<sub>2</sub> environment, including the induction of enzymes involved in glycolytic metabolism, erythropoiesis, angiogenesis, and vasculogenesis [5]. In addition to HIF activation, low O<sub>2</sub> levels are also known to promote catecholamine release and adrenergic stimulation [6], which in turn induce the activation of pro-angiogenic and pro-inflammatory factors [7].

Through these pathways—some of which have been well studied and others still under investigation—O<sub>2</sub> levels and their fluctuations influence the proliferation and differentiation of various stem/progenitor cell populations, either by preserving their stemness or inducing their tissue-specific differentiation [3]. For instance, high O<sub>2</sub> concentrations promote the differentiation of megakaryocytes into platelets [8]; on the other hand, hypoxia drives the differentiation of specific progenitors into neurons with distinct neurotransmitter phenotypes [9] and human embryonic stem cells toward a cardiomyocyte phenotype [10], as confirmed by the impaired neurogenesis or cardiomyogenesis observed in HIF-1 $\alpha$  knockout models [11, 12]. Therefore, a vast array of biological processes is governed by simple fluctuations in ambient O<sub>2</sub> concentrations.

O<sub>2</sub> fluctuations are critical for the processes of embryogenesis and morphogenesis. Indeed, embryos develop in a low-O<sub>2</sub> environment, often referred as “Everest in utero” [13], which supports stem cell self-renewal and proliferation [3, 14]. However, under low O<sub>2</sub> conditions, cytotrophoblast proliferation establishes connections with the maternal vasculature, leading to an increase in placental O<sub>2</sub> levels [15]. This rise in O<sub>2</sub> levels triggers the differentiation of cytotrophoblast cells into multinucleated syncytiotrophoblasts, which adopt a more invasive phenotype, facilitating the formation of a well-established maternal-fetal circulation [16]. Additionally, hypoxia-induced proangiogenic factors lay the foundation for the embryonic vascular and placental framework, further elevating O<sub>2</sub> levels. The role played by O<sub>2</sub> in further differentiation of the vascular network is well-documented in animal models, where vascular structures are completed after birth when tissues are exposed to higher O<sub>2</sub> pressures. For instance, proper gliovascular structure in rodent brains forms after birth [17, 18], as well as the vascularization of the mouse retina develops postnatally [19, 20].

### 1.1 | Early Exposure to an O<sub>2</sub>-Rich Environment: A Trap for Preterm Infants

This delicate balance of O<sub>2</sub> fluctuations during intrauterine life explains why a series of pathologies arise when premature birth exposes precociously an immature newborn to a relatively hyperoxic environment. A well-documented example is retinopathy of prematurity (ROP), a potentially blinding ocular disorder whose pathogenesis starts and evolves as a function of O<sub>2</sub> levels [21]. The biphasic vascular course of ROP—first ischemic, then proliferative—results from initial hyperoxic exposure followed by hypoxia due to vascular regression triggered by excessive O<sub>2</sub>. Indeed, the *primum movens* of the disease is represented by the premature exposure of a minimally vascularized retina to a relatively hyperoxic environment, which suppresses proangiogenic factors [21]. This event halts or regresses initial vascularization, a phenomenon ophthalmologists describe as “incomplete vascularization” [22]. As a result, the peripheral retina remains avascular until an approximate postmenstrual age of 32 weeks. Persistent ischemia, coupled with the developing retina's increasing metabolic demands, leads to growing hypoxia in the peripheral areas of the retina, farthest from existing vessels. ROP, therefore, reverses, and the progressive hypoxia developing in the outermost areas of the retina triggers the upregulation of a series of proangiogenic factors, including vascular endothelial growth factor (VEGF), leading to disorganized neovascularization and blood-retinal barrier breakdown [23].

However, in preterm newborns, particularly those of very low gestational age, the retina is not the only region with immature vascularity where early exposure to high O<sub>2</sub> levels triggers alterations typical of prematurity. Premature lungs, for example, are vulnerable to high O<sub>2</sub> exposure, which induces alveolar hypoplasia and abnormal vascular organization, which are the typical hallmarks of bronchopulmonary dysplasia (BPD) [24]. Autopsies of infants who die from BPD, in fact, reveal abnormal alveolar microvessels and impaired expression of angiogenic growth factors and their receptors in the lungs [25]. Additionally, very low tracheal VEGF levels within the first week of life are associated with an increased risk of severe BPD, underscoring the strong relationship between impaired lung vascularization and BPD [26]. Hypoxia is also crucial for maintaining physiological homeostasis of the intestine [27], while hyperoxia can lead to intestinal damage, predisposing the newborn to necrotizing enterocolitis (NEC) [28]. Even in NEC, as in many other prematurity-related conditions, reduced intestinal vascularization and resulting ischemia play a key role in pathogenesis [29]. In addition, the central nervous system (CNS) requires a physiologically hypoxic environment rich in HIF-1 to support normal vascular development [30]. Hyperoxia has been demonstrated to damage the immature CNS in neonatal animals [31]. In rat pups, exposure to 80% O<sub>2</sub> results in reduced brain mass and significant microvascular degeneration, which is more pronounced in the cortex [32]. Similarly, neonatal mice exposed to hyperoxia in the early postnatal period develop vascular structural impairments in the cerebral cortex, such as reduced vessel length and fewer branch points [33]. The close relationship between vascular damage and exposure to high O<sub>2</sub> concentrations is further evidenced by human autopsic studies, which show that the vascularity of the white matter between 16

and 32 weeks of gestational age is rudimentary [34]. Therefore, vascular immaturity at birth, combined with vascular regression due to relative hyperoxia exposure, and the reduced viability of preoligodendrocytes exposed to high O<sub>2</sub> levels [35], might explain the development of typical white matter lesions seen in developing brains, such as periventricular leukomalacia (PVL) [36].

## 1.2 | Premature Birth and Oxidative Stress

In preterm newborns, exposure to a hyperoxic environment induces oxidative stress, damaging neonatal tissues [37]. This damage results from the excessive production of mitochondrial reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, singlet O<sub>2</sub>, and hydroxyl radicals, which overwhelm the neutralizing capacity of antioxidant defense systems. Preterm newborns are particularly vulnerable to oxidative stress due to their low antioxidant capacity [38]. The formation of reactive nitrogen species (RNS) from the reaction of ROS with nitric oxide, along with lipid peroxidation—damaging cell membranes—leads to cellular and mitochondrial damage, contributing to systemic disorders [39]. Oxidative stress is widely linked to brain damage in preterm infants [40], as well as respiratory damage [41]. Similar mechanisms occur during ischemia/reperfusion injury, where the restoration of O<sub>2</sub> to ischemic tissues causes further brain damage [42]. This suggests that both prolonged exposure to high O<sub>2</sub> and sudden increases in oxygenation—even when not excessively high—can be harmful.

In addition to hyperoxia, exposure to intermittent [43, 44] or chronic hypoxia [45, 46] also induces oxidative stress in preterm infants. Although counterintuitive, studies have shown increased production of superoxide anion and hydrogen peroxide during hypoxia [45], with ROS production often occurring outside the mitochondria [45]. Thus, hyperoxia, intermittent hypoxia, and chronic hypoxia all contribute to the development of pathologies in preterm newborns via oxidative stress.

Several animal models are used to mimic O<sub>2</sub>-induced damage related to prematurity [47–49]. Among the various models available, newborn rodents exposed to high O<sub>2</sub> concentrations are often used due to their anatomical similarity to preterm neonates [47, 48]. Rodent hyperoxia models typically involve 80% O<sub>2</sub> exposure, leading to a 2.3-fold increase on average O<sub>2</sub> partial pressure compared to controls [50], similar to what occurs during premature birth [51]. These models, which involve exposure only to hyperoxia rather than mixed models where hyperoxia alternates with periods of hypoxia, allow researchers to explore the effects of hyperoxia without ambiguity. The adoption of the hyperoxia-based model is also based on the observation that the abrupt transition from an intrauterine hypoxic environment to extrauterine hyperoxia is the primary event that disrupts normal fetal maturation, serving as the *primum movens* for subsequent hypoxia in the newborn. This is what happens in animal models of BPD, where hyperoxia exposure (at variable concentrations and for variable times) impairs alveolarization, induces fibrosis, and disrupts vascular growth [47, 48]. The same process occurs in the CNS, where hyperoxia leads to vascular regression and, therefore, tissue

hypoxia [52]. Intermittent hypoxia following apneas—common in preterm newborns [43]—may also result from premature exposure to hyperoxia. In the rodent model, in fact, perinatal hyperoxia affects carotid body development, impairing the developing respiratory control system, which may lead to hypoventilation and reduced responses to acute hypoxia [53]. Thus, the abrupt and premature transition from a comfortable hypoxic fetal environment to a hyperoxic postnatal environment likely triggers the damage seen in preterm infants, disrupting their physiological growth and differentiation.

Overall, research demonstrates that embryonic and fetal well-being are closely linked to maintaining a hypoxic environment, essential for proper differentiation and adequate vascularization via HIF-1-mediated production of angiogenic factors like VEGF, basic fibroblast growth factor, platelet-derived growth factor, angiopoietin-1, angiopoietin-2, Tie2, and monocyte chemoattractant protein-1 [54]. However, premature newborns are vulnerable to damage from excessive O<sub>2</sub> exposure, which emphasizes the need for cautious O<sub>2</sub> administration.

However, if, on the one hand, the extremely relevant role played by physiological hypoxia during intrauterine life is evident, it is surprising how little is known about the embryonic/fetal O<sub>2</sub> levels during a healthy pregnancy, their potential fluctuations as gestational age progresses, and the role that these putative fluctuations may have in modulating biological processes of differentiation.

Recent studies on cord blood gas analysis across different gestational ages, from extremely preterm to term infants, revealed that fetal hypoxia is dynamic, allowing us to speculate that the fluctuations of fetal hypoxia may be key to healthy fetal development [55, 56]. Understanding these O<sub>2</sub> trends during pregnancy would enhance our knowledge of mechanisms that ensure fetal well-being. This knowledge will be extraordinarily valuable for researchers who, figuratively speaking, envision bringing the preterm newborn back into the maternal uterus. This ambitious goal could be achieved either through artificial placenta technology [57, 58] or through pharmacological interventions that mimic the biological effects of intrauterine hypoxia [56, 59], which we refer to in this article as a “pharmacological artificial placenta.”

The aim of this review is to describe the pathway that led us to hypothesize that early exposure to a relatively hyperoxic environment may impair preterm neonates due to reduced expression of  $\beta_3$ -Adrenoceptor ( $\beta_3$ -ARs) and that the use of  $\beta_3$ -AR agonist drugs in these newborns may, at least partially, simulate the benefits of the maternal womb.

## 2 | Intrauterine Fluctuations in O<sub>2</sub> Levels

Fecundation and embryo development occur under anaerobic conditions, and the fetus also thrives in a very low-O<sub>2</sub> environment [60]. However, the literature suggests that low O<sub>2</sub> levels play an important role even before sexual fecundation. In fact, both spermatozoa and oocytes live in a physiologically hypoxic environment. On the male side, the testis is naturally an O<sub>2</sub>-deprived organ, where interstitial O<sub>2</sub> tension in different

animals has been reported to be very low, ranging between 11 and 15 mmHg (approximately 2%) [61–63]. Hypoxia affects not only germ cells but also the seminiferous tubules [64], which are poorly vascularized. Therefore, O<sub>2</sub> reaches the lumen only through diffusion, ensuring that luminal pO<sub>2</sub> levels remain very low [65]. On the female side, numerous studies have demonstrated that the O<sub>2</sub> concentration in follicular fluid is low, and oxygenation decreases as the ovarian follicle develops [66]. Moreover, O<sub>2</sub> levels are particularly low throughout the entire course of the oviduct, approximately 5%, although they fluctuate during different phases of the menstrual cycle [67, 68]. Inside the nonpregnant uterus, O<sub>2</sub> levels drop even further, reaching about 2% [69].

The close complementarity between the male and female genital organs during sexual mating suggests that low O<sub>2</sub> levels are essential for successful reproduction. On one hand, such anatomical complementarity facilitates the entry of spermatozoa into the female vagina and cervix. On the other hand, the particular adherence between the two genitals suggests a biological interest in maintaining the integrity of this hypoxic environment, avoiding contaminations from air entry. Thus, this mode of sexual reproduction in mammals can be interpreted as a biological strategy aimed at preserving the *hypoxia chain*, from the testis to the oviduct. In this respect, the importance of low environmental oxygenation in ensuring successful fecundation is highlighted by improved live birth rates of preimplantation embryos cultured under low O<sub>2</sub> tension, compared to those cultured in normoxic conditions [70, 71].

However, if the hermetic seal between males and females is necessary to ensure successful sexual fecundation, this means that even temporary exposure to a more oxygenated environment can represent a risk. Hypoxia, in effect, appears to favor the vitality of the spermatozoa, while their exposure to higher O<sub>2</sub> concentrations can have detrimental effects [72].

In humans, conception occurs when a sperm cell successfully fertilizes an egg cell in the fallopian tube, where the O<sub>2</sub> concentration is approximately 5% [67]. A hypoxic environment remains necessary even after fecundation, as low O<sub>2</sub> levels have been identified as key regulators of the harmonious processes of embryonic and placental development [14, 60]. However, immediately after conception, the environmental O<sub>2</sub> levels change, and this modulation seems to act as a signal that favors specific developmental processes. For instance, when the morula reaches the uterine cavity, the O<sub>2</sub> concentration decreases to around 2% [67, 73, 74]. Interestingly, this transition to a more hypoxic condition is crucial during the first 2–3 weeks of life, allowing the development of mammalian blastocysts. In this environment, the blastocysts form an inner cell mass of embryonic stem cells that are able to proliferate [75, 76] while maintaining their undifferentiated state [77, 78]. In addition to promoting proliferation, increasing hypoxia facilitates the implantation, anchoring, and invasion of the blastocyst into the maternal uterus [15]. Furthermore, exposure to a decreasing O<sub>2</sub> gradient induces a metabolic shift in cytotrophoblast cells from oxidative phosphorylation (OXPHOS) to glycolytic metabolism (the so-called Warburg effect) [79–81]. This metabolic adjustment offers a proliferative advantage, as it stimulates the production of many intermediates in the pentose phosphate

pathway, such as ribose sugars, which are essential for nucleic acid synthesis. It also results in elevated lactic acid levels, which aid in promoting nesting in surrounding tissues [82]. In this respect, the consistent production and extrusion of lactate induce disaggregation of uterine tissues, facilitating trophoblast invasion [83].

The increase in intrauterine hypoxia during the first weeks of life sets the stage for a series of events that provide the foundation for embryonic vascular and placental development, which in turn leads to a further increase in O<sub>2</sub> availability [84]. Hypoxia promotes cytotrophoblast proliferation, migration across the uterine epithelium, aggregation into cell columns, and differentiation into extravillous trophoblasts [85, 86]. These phenomena support cytotrophoblast infiltration into both the uterine interstitium and the lumen of spiral arterioles. This infiltration not only anchors the fetus to the mother but also progressively remodels the spiral arteries. The smooth muscle and elastic lamina of the vessel are replaced by cytotrophoblasts [16], transforming the high-resistance, low-flow original spiral arteries into low-resistance, high-flow vessels. This ensures a gradual increase in O<sub>2</sub> delivery in the following weeks of pregnancy [15].

Up until approximately the 10th week of human gestation, intrauterine O<sub>2</sub> levels remain very low, less than 20 mmHg (approximately 2% O<sub>2</sub>), similar to those found within the nonpregnant uterus [84, 87]. However, starting from this point and continuing through the early weeks of the second trimester of pregnancy, placental development promotes a significant increase in O<sub>2</sub> availability, as evidenced by a threefold rise in placental oxygenation to around 60 mmHg (approximately 8% O<sub>2</sub>) [87]. This increased microenvironmental oxygenation promotes the differentiation of cytotrophoblasts into multinucleated syncytiotrophoblasts [16], which are essential for the secretion of various hormones that maintain pregnancy [88].

The tripling of placental O<sub>2</sub> content enhances O<sub>2</sub> availability to the fetoplacental unit despite several structural factors that tend to reduce the placenta's O<sub>2</sub>-diffusing capacity. The immature structure of placental villi and their rudimentary vascularity, which result in a reduced exchange surface area, along with the considerable diffusion distance between maternal–fetal circulations, limit O<sub>2</sub> transfer from the mother to the fetus. This explains why the partial pressure of O<sub>2</sub> (pO<sub>2</sub>) in umbilical venous blood, which carries O<sub>2</sub> from the placenta to the fetus, is significantly lower than that of both placental [87] and maternal arterial pO<sub>2</sub> [89] blood.

Extrapolations from animal studies and human fetal studies have shown that by the end of the third trimester of pregnancy, the mean pO<sub>2</sub> concentrations in the umbilical vein and umbilical artery are 28 and 19 mmHg, respectively [90]. However, at least in humans, these concentrations do not appear to remain constant throughout the third trimester. Analysis of human umbilical cord arterial and venous blood samples collected via cordocentesis revealed a decrease in fetal pO<sub>2</sub> and O<sub>2</sub> saturation as gestation advances [91–95]. While cordocentesis provides insights into the physiological state over a wide gestational age range before the peripartum period, studies using this method did not account for the final weeks of pregnancy.

Furthermore, these samples were obtained from pregnancies complicated by maternal infections, fetal structural anomalies, or various prenatal pathologies [91–95]. Only recently has the analysis of umbilical cord blood gas samples allowed for a clearer understanding of O<sub>2</sub> level fluctuations during the last trimester. These studies confirmed that fetal pO<sub>2</sub> and O<sub>2</sub> saturation decrease as gestation progresses but only up until the 33rd–34th week, after which a significant increase in O<sub>2</sub> levels is observed [55, 56, 96].

Essentially, intrauterine life shows that although the embryo/fetus exists in a hypoxic environment, O<sub>2</sub> levels fluctuate. These fluctuations likely serve to modulate seemingly contradictory processes, such as the expansion of stemness and differentiation.

Understanding that O<sub>2</sub> levels vary according to gestational age has important implications for research on artificial placenta technology, which is currently under development and being tested on animals [57]. This knowledge of the physiological fluctuations in O<sub>2</sub> levels during intrauterine life should indicate the oxygenation targets be achieved with extracorporeal membrane oxygenation, which must vary based on the week of gestation to support normal physiological growth and maturation of the fetus through artificial placental technology. Similarly, efforts to mimic the biological effects of increasing intrauterine hypoxia through pharmacologic stimulation [59] will need to consider that fetal hypoxemia increases only until to the 33rd–34th week.

### 3 | Methods for Assessing Fetal O<sub>2</sub> Levels

A pathological decrease in the O<sub>2</sub> supply from the placenta to the fetus can lead to severe outcomes such as intrauterine fetal death (IUFD) or hypoxic-ischemic encephalopathy (HIE). As a result, over the years, various methods have been developed to measure O<sub>2</sub> levels in fetal blood, with the goal of detecting pathological hypoxia early and thereby preventing IUFD and HIE [97].

The most commonly used method for monitoring fetal oxygenation is electronic fetal heart rate monitoring (EFM), or cardiotocography (CTG), which track fetal heart rate to assess fetal well-being or detect signs of intrapartum hypoxia [97]. However, the predictive value of CTG for detecting intrapartum fetal hypoxia is only about 30%, and it has a high false-positive rate of 60% [98]. Therefore, despite clear guidelines for interpreting CTG tracings, the reliability of this technology is limited, providing operators with modest accuracy and, more importantly, only indirect and nonquantitative information about the oxygenation status [98]. Near-infrared spectroscopy (NIRS) has been investigated as a method for evaluating placental function, particularly in cases of fetal growth restriction [99]. While NIRS offers easy and minimally invasive access to oxygenation data, its low spatial resolution limits its usefulness for fetal monitoring [97]. Additionally, the photoacoustic (PA) technique, which uses multiple light wavelengths to provide real-time information on blood O<sub>2</sub> saturation, has recently been proposed as a promising novel method for fetal oxygenation monitoring. The sensitivity of this technique for detecting tissue hypoxia is comparable to that of NIRS, but PA offers more accurate information due to its higher spatial resolution [97].

Another proposed system for measuring fetal oxygenation during labor is fetal pulse oximetry. Initially, transvaginal fetal pulse oximetry was evaluated. This method consists of placing a device through the birth canal after the rupture of the uterine membrane. The device is positioned on the fetal head to measure O<sub>2</sub> saturation [100]. Subsequent studies have sought less invasive but equally reliable methods. Recently, transabdominal fetal pulse oximetry has been developed, utilizing near-infrared light sources and photodetectors placed on the mother's abdomen to measure fetal O<sub>2</sub> saturation in a fully noninvasive manner [101].

In modern obstetric practice, several additional tests have been developed to increase the detection rate of hypoxia. One of these is the analysis of the fetal ECG using ST trait analysis (STAN) [102]. Another method involves assessing fetal scalp blood lactate levels, collected through a small incision in the fetal scalp during labor using a capillary tube [103]. However, this method has been widely questioned due to numerous contraindications, such as maternal infections and fetal coagulation disorders, as well as potential complications like prolonged bleeding, hematoma, or abscess formation at the incision site [104].

These techniques are primarily designed for monitoring oxygenation levels during labor or in the immediate context of complicated childbirth. Generally, these approaches do not allow for prolonged monitoring and have not been used to reconstruct fetal oxygenation status over time.

Umbilical cord blood gas analysis is considered the most accurate and reliable method for quantifying fetal and placental oxygenation and detecting potential fetal hypoxia. This procedure involves isolating a 20 cm segment of the cord between two clamps. Venous and arterial umbilical cord blood is typically collected using blood gas syringes containing spray-dried calcium-balanced lithium heparin and is promptly analyzed using an automatic blood gas analyzer [105]. This analysis enables the detection of metabolic acidosis by measuring pH, pO<sub>2</sub>, partial pressure of carbon dioxide (pCO<sub>2</sub>), lactates, bicarbonate (HCO<sub>3</sub><sup>-</sup>), and base excess (BE) [106]. Additionally, measuring hemoglobin (Hb) levels and arterial saturation allows the calculation of O<sub>2</sub> content and fetal O<sub>2</sub> extraction (the difference between venous and arterial blood O<sub>2</sub> contents, divided by venous O<sub>2</sub> content). Furthermore, cord blood sampling provides insight into placental and fetal metabolic activity, such as lactate and CO<sub>2</sub> production (the difference between arterial lactate or blood CO<sub>2</sub> and their respective venous contents, respectively, divided by venous content) [55, 56].

Thus, umbilical cord sampling is the most useful test for evaluating fetal oxygenation at birth and for diagnosing perinatal asphyxia with accuracy [106]. Moreover, the availability of a large number of cord blood gas analyses collected at different gestational ages allows for the reconstruction of fetal oxygenation status as pregnancy progresses [55, 56].

### 4 | Pros and Cons of Umbilical Cord Blood Gas Analysis

Umbilical cord blood gas analysis is one of the most widely used methods in developed countries to monitor fetal well-being and

detect potential fetal hypoxia during delivery. This procedure is simple, painless for the newborn, relatively economical, and provides immediate and valuable feedback to labor ward staff. Additionally, umbilical cord blood analysis often serves as crucial evidence in forensic contexts [106]. Numerous studies over the years have consistently shown that cord blood gas analysis is significantly more accurate and reliable than CTG [107].

However, it is common practice to collect blood from only one cord vessel, especially when the fetus is under stress or compromised. It is generally believed that blood samples from a single vessel are more likely to contain venous blood, as the vein is larger and easier to sample than the artery [107].

The mode of delivery can affect cord blood values, as demonstrated by the lower oxygenation levels observed in newborns delivered by cesarean section compared to those born via vaginal delivery [55, 56]. Several factors may explain this difference, with the impact of delivery type on placental flow likely playing a key role. Neonates delivered by cesarean section experience lower umbilical transfusion force, as they are placed on the maternal abdomen at a higher plane than the placenta, which creates a gravity gradient that opposes the flow of blood from the placenta to the fetus [108]. Furthermore, women undergoing cesarean sections are at a higher risk of developing hypotension [109] or reduced uterine contractility due to uterine incision and anesthesia [110, 111]. Similarly, fetal oxygenation of neonates born through vaginal delivery can be influenced by the engagement of the fetus through the birth canal or by reduced blood flow during uterine contractions [112]. Moreover, neonates delivered by assisted vaginal breech delivery often show lower umbilical cord artery blood pH and  $pO_2$ , along with increased  $pCO_2$ , compared to those delivered via cephalic-vaginal delivery [113].

Another factor that could influence the reliability of umbilical blood gas measurements is the timeliness of sampling. Measurements of pH,  $pCO_2$ , and  $pO_2$ , for example, become unreliable if the sample is taken more than 60 min after clamping a double-segment of the umbilical cord or more than 20 min from an unclamped segment [114]. More controversial, instead, is the effect on the analytical gas status of delaying cord clamping for at least 30–60 s after delivery, which is currently recommended by leading international scientific societies [115]. A recent review showed that the available data are inconclusive on this topic [116]. On the one hand, some studies have suggested that delayed cord clamping is associated with minimal decreases in pH and  $HCO_3^-$  and increases in  $pCO_2$ , lactate, and BE [117–119]. On the other hand, two randomized controlled trials indicated that acid–base balance was not significantly altered by delayed cord clamping, suggesting that placental gas exchange remains effective for at least 1 min after birth [120, 121]. The data related to oxygenation are particularly interesting: no study has shown any significant difference in venous  $pO_2$ , even with cord clamping delayed up to 45–180 s [116]. In contrast, arterial  $pO_2$  values in samples collected after delayed cord clamping were approximately 3–4 mmHg higher, suggesting an influence of the newborn's respiratory activity on umbilical arterial oxygenation [120]. However, these findings were not confirmed in a subsequent study by the same authors [121].

## 5 | Fetal Oxygenation From the 23rd to the Term of Gestation

Recently, fetal oxygenation status was studied through the analysis of umbilical cord blood samples, with results published separately for full-term [55, 96] and preterm newborns [56]. The decision to report these results separately was made because the sample collection methods were not homogeneous; samples from full-term newborns are usually collected approximately 60 s after birth (delayed cord clamping), while those from preterm newborns are collected immediately after birth (early cord clamping). The results of these studies demonstrated an apparently biphasic trend in fetal oxygenation, where the level of  $O_2$  reaching the fetus from the placenta appears to progressively decrease starting from the 23rd week of pregnancy (the earliest age at which umbilical cord samples could be analyzed) until approximately the 33rd–34th week. After this point, fetal oxygenation reverses this trend, with the placenta seemingly increasing  $O_2$  transfer, possibly due to physiological aging [55, 56]. However, this biphasic trend would have been more evident if we had reported the data from preterm and full-term newborns together, despite the limitations that such a plot entails.

In this review, we combined data from preterm and term neonates by gathering as much information as available. To the previously published data (from full-term neonates born at the University Hospital of Pisa, Italy, between January 1, 2019, and December 31, 2019 and from preterm neonates born between January 1, 2016, and December 31, 2022) [55, 56], we added data from all umbilical cord blood samples collected from newborns (both full-term and preterm) born between January 1, 2022, and December 31, 2023. To emphasize the difference in sample collection between full-term (delayed cord clamping) and preterm (early cord clamping) newborns, the results in the graphs are represented with different colors.

The results were obtained by analyzing 4649 samples, 755 of which were collected from 1450 preterm newborns born between 2016 and 2023, and 3894 of which were collected from 5197 full-term newborns born between 2019, 2022, and 2023.

Umbilical cord blood samples from pregnancies with intra-uterine growth restriction (IUGR) and/or fetal or maternal intrapartum complications (e.g., operative vaginal deliveries involving forceps or vacuum extractor use, abnormal intrapartum CTG requiring emergency cesarean section, meconium-stained amniotic fluid, placental abruption, cord prolapse, chorioamnionitis, maternal sepsis, convulsions, hemorrhage, uterine rupture, or snapped cord) were excluded from the analysis. Samples suggestive of severe acidosis at birth ( $pH \leq 7.00$  and/or  $BE \leq -12$  mmol/L) [107, 122] or deemed unreliable [123] were also excluded. Umbilical parameters with values  $<$  or  $>$  3 SD from their respective means were individually evaluated and (i) corrected if likely mid-entered, (ii) maintained if considered plausible, or (iii) excluded if considered not plausible [97]. The samples were analyzed using an automatic blood gas analyzer (GEM Premier 4000, Instrumentation Laboratory, Lexington, MA, USA). pH,  $pCO_2$ ,  $pO_2$ ,  $SO_2$ , lactate, and Hb were measured, while  $HCO_3^-$  and BE were calculated from the measured pH and  $pCO_2$ , using the Henderson–Hasselbalch equation:  $pH = 6.1$

+  $\log([\text{HCO}_3^-]/\text{pCO}_2 \times 0.03)$  [124] and the formula described by Siggaard–Anderson:  $(1 - 0.014 \times \text{Hb}) \times (\text{HCO}_3^- - 24.8 + [1.43 \times \text{Hb} + 7.7] \times [\text{pH} - 7.4])$  [95]. The  $\text{O}_2$  content (mL/dL) was calculated using the formula:  $(1.36 \times \text{Hb} [\text{g/dL}] \times \text{SaO}_2 [\%]/100) + (0.0031 \times \text{pO}_2 [\text{mmHg}])$ , where 1.36 is the volume of  $\text{O}_2$  (mL) bound by a gram of Hb, and 0.0031 is the solubility coefficient of  $\text{O}_2$  in blood, representing the volume of  $\text{O}_2$  (mL) dissolved in 100 mL of plasma for each mmHg of  $\text{pO}_2$  partial pressure. Fetal  $\text{O}_2$  extraction was determined as the difference between umbilical venous and arterial blood  $\text{O}_2$  contents, divided by the umbilical venous  $\text{O}_2$  content. Fetal  $\text{CO}_2$  and lactate production were calculated as the difference between arterial blood  $\text{CO}_2$  or lactate and venous contents, divided by the respective venous content.

The demographic and gas analytical parameters for all newborns are shown in Table 1. Cord blood gas analysis of samples obtained from preterm newborns revealed lower oxygenation

(lower venous and arterial  $\text{pO}_2$ , and lower  $\text{O}_2$  content), lower  $\text{CO}_2$  and lactate production, and lower glucose extraction compared to samples from full-term neonates. However, the modes of delivery differed significantly between the two groups, with a predominance of cesarean sections in the preterm newborn group and a similar prevalence of vaginal deliveries in the full-term group. Since the type of delivery has been shown to significantly influence cord blood gas analysis, particularly oxygenation [55], the data were disaggregated based on delivery mode. Even after this adjustment, preterm fetuses still showed lower oxygenation,  $\text{CO}_2$  and lactate production, and glucose extraction, although statistical significance was not always achieved for smaller sample sizes (Table 2).

To assess whether the different parameters of interest (umbilical venous and arterial  $\text{O}_2$  levels, fetal  $\text{O}_2$  extraction, Hb, pH,  $\text{HCO}_3^-$ , BE,  $\text{CO}_2$ , and lactate) varied over the course of pregnancy, gas analysis data were plotted against gestational age, and trends were

**TABLE 1** | Umbilical cord blood gas analysis in preterm versus term neonates.

	<b>Preterm newborns (n = 755)</b>	<b>Term newborns (n = 3894)</b>	<b>p value</b>
GA, days, mean (SD)	235 (23)	278 (8)	< 0.001
Birth weight, g, mean (SD)	2030 (673)	3346 (617)	< 0.001
Male, n (%)	393 (52.1)	2003 (51.4)	0.757
Vaginal delivery, n (%)	211 (27.9)	2814 (72.3)	< 0.001
<b>Umbilical venous cord sampling</b>			
pH, mean (SD)	7.321 (0.08)	7.322 (0.07)	0.596
PaCO <sub>2</sub> , mmHg, mean (SD)	42.5 (9.2)	40.1 (8.5)	< 0.001
PaO <sub>2</sub> , mmHg, mean (SD)	24.3 (8.4)	29.1 (8.5)	< 0.001
Bicarbonate, mmol/L, mean (SD)	21.7 (3.0)	20.7 (2.7)	< 0.001
BE(B), mmol/L, mean (SD)	-4.3 (3.4)	-5.2 (3.0)	< 0.001
Lactate, mmol/L, mean (SD)	2.8 (1.4)	3.8 (1.7)	< 0.001
Hemoglobin, g/dL, mean (SD)	14.9 (2.3)	16.2 (1.5)	< 0.001
SaO <sub>2</sub> , %, mean (SD)	55.5 (22.0)	62.3 (18.5)	< 0.001
<b>Umbilical arterial cord sampling</b>			
pH, mean (SD)	7.268 (0.08)	7.243 (0.08)	< 0.001
PaCO <sub>2</sub> , mmHg, mean (SD)	50.8 (11.4)	53.4 (11.5)	< 0.001
PaO <sub>2</sub> , mmHg, mean (SD)	16.4 (7.3)	20.6 (9.5)	< 0.001
Bicarbonate, mmol/L, mean (SD)	22.9 (3.8)	23.2 (3.1)	0.034
BE(B), mmol/L, mean (SD)	-4.5 (4.0)	-5.3 (3.6)	< 0.001
Lactate, mmol/L, mean (SD)	3.1 (1.6)	4.3 (2.0)	< 0.001
Hemoglobin, g/dL, mean (SD)	14.7 (2.4)	16.3 (1.5)	< 0.001
SaO <sub>2</sub> , %, mean (SD)	34.8 (19.1)	38.5 (21.5)	< 0.001
Fetal venous $\text{O}_2$ content, mL/100 mL (SD)	11.3 (4.6)	13.8 (4.2)	< 0.001
Fetal arterial $\text{O}_2$ content, mL/100 mL (SD)	6.9 (3.9)	8.6 (4.9)	< 0.001
Fetal $\text{O}_2$ extraction, %, mean (SD)	35.1 (36.4)	32.8 (41.0)	0.287
Fetal $\text{CO}_2$ production, %, mean (SD)	22.4 (24.8)	36.3 (31.8)	< 0.001
Fetal lactate production, %, mean (SD)	12.6 (20.6)	16.8 (23.5)	< 0.001
Fetal glucose extraction, %, mean (SD)	14.1 (13.8)	16.2 (14.6)	0.004

Abbreviations: BE = base excess, GA = gestational age, PaCO<sub>2</sub> = partial pressure of carbon dioxide, PaO<sub>2</sub> = partial pressure of oxygen.

**TABLE 2** | Umbilical cord blood gas analysis in all enrolled preterm and term newborns separately analyzed by the type of delivery.

	Vaginal deliveries			Cesarean deliveries		
	Preterm neonates (n = 211)	Term neonates (n = 2814)	p value	Preterm neonates (n = 544)	Term neonates (n = 1080)	p value
GA, days, mean (SD)	233 (28)	279 (8)	< 0.001	236 (22)	275 (8)	< 0.001
Birth weight, g, mean (SD)	2084 (732)	3363 (673)	< 0.001	2009 (649)	3304 (440)	< 0.001
Male, n (%)	118 (55.9)	1439 (51.1)	0.180	275 (50.6)	564 (52.2)	0.525
Umbilical venous cord sampling						
pH, mean (SD)	7.336 (0.08)	7.324 (0.07)	0.026	7.315 (0.08)	7.318 (0.08)	0.006
pO <sub>2</sub> , mmHg, mean (SD)	26.6 (8.9)	30.3 (8.3)	< 0.001	23.4 (8.1)	26.1 (8.2)	< 0.001
Hemoglobin, g/dL, mean (SD)	15.6 (1.9)	16.3 (1.5)	< 0.001	14.6 (2.4)	15.9 (1.7)	< 0.001
SaO <sub>2</sub> , %, mean (SD)	58.8 (21.6)	64.0 (17.8)	< 0.001	54.0 (22.1)	57.2 (19.4)	0.022
pCO <sub>2</sub> , mmHg, mean (SD)	39.6 (9.2)	38.7 (7.9)	0.133	43.7 (8.9)	43.5 (8.4)	0.638
Bicarbonate, mmol/L, mean (SD)	20.9 (2.7)	20.2 (2.6)	< 0.001	21.9 (3.2)	22.1 (2.6)	0.941
BE(B), mmol/L, mean (SD)	-4.6 (3.3)	-5.5 (2.8)	< 0.001	-4.2 (3.4)	-4.1 (3.2)	0.509
Lactate, mmol/L, mean (SD)	3.4 (1.4)	4.0 (1.6)	< 0.001	2.5 (1.3)	2.9 (1.8)	< 0.001
Glucose, mg/dL, mean (SD)	94.7 (22.9)	101.8 (21.8)	< 0.001	72.0 (20.5)	83.2 (21.2)	< 0.001
Umbilical arterial cord sampling						
pH, mean (SD)	7.266 (0.08)	7.241 (0.08)	< 0.001	7.269 (0.08)	7.250 (0.09)	< 0.001
pO <sub>2</sub> , mmHg, mean (SD)	18.8 (8.0)	22.5 (9.2)	< 0.001	15.3 (6.7)	15.8 (8.3)	0.338
Hemoglobin, g/dL, mean (SD)	15.5 (2.1)	16.4 (1.4)	< 0.001	14.3 (2.4)	15.9 (1.6)	< 0.001
SaO <sub>2</sub> , %, mean (SD)	41.1 (20.3)	41.7 (21.3)	0.743	31.8 (17.8)	29.0 (19.3)	0.032
pCO <sub>2</sub> , mmHg, mean (SD)	50.4 (11.1)	52.7 (11.2)	0.006	50.9 (11.5)	55.4 (12.1)	< 0.001
Bicarbonate, mmol/L, mean (SD)	22.5 (3.1)	22.7 (3.0)	0.322	23.1 (4.0)	24.7 (3.0)	< 0.001
BE, mmol/L, mean (SD)	-5.0 (3.8)	-5.7 (3.2)	0.005	-4.3 (4.1)	-4.1 (4.1)	0.206
Lactate, mmol/L, mean (SD)	3.8 (1.6)	4.6 (1.8)	< 0.001	2.8 (1.5)	3.4 (2.0)	< 0.001
Glucose, mg/dL, mean (SD)	80.7 (21.2)	84.9 (22.4)	0.024	62.2 (20.0)	70.9 (21.3)	< 0.001
Fetal venous O <sub>2</sub> content, mL/100 mL (SD)	12.5 (4.6)	14.2 (4.1)	< 0.001	10.7 (4.5)	12.5 (4.3)	< 0.001
Fetal arterial O <sub>2</sub> content, mL/100 mL (SD)	8.6 (4.3)	9.4 (4.8)	0.094	6.1 (3.4)	6.3 (4.2)	0.444
Fetal O <sub>2</sub> extraction, %, mean (SD)	28.3 (33.5)	28.6 (40.8)	0.936	38.4 (37.4)	45.8 (39.2)	0.012
Fetal CO <sub>2</sub> production, %, mean (SD)	31.7 (31.0)	39.0 (33.3)	0.003	18.5 (20.5)	29.2 (26.3)	< 0.001
Fetal lactate production, %, mean (SD)	13.1 (19.9)	16.0 (22.9)	0.095	12.7 (20.2)	19.4 (25.2)	< 0.001
Fetal glucose extraction, %, mean (SD)	14.5 (13.1)	16.4 (15.3)	0.132	13.8 (14.2)	15.5 (12.4)	0.218

Abbreviations: BE = base excess, GA = gestational age, PaCO<sub>2</sub> = partial pressure of carbon dioxide, pO<sub>2</sub> = partial pressure of oxygen.

evaluated using regression models. The best-fitting model (linear or quadratic) was identified for each parameter, with the equation of the statistically significant model displayed at the top of each figure. If the most representative test (with a lower *p*-value) was a linear regression, the parameter was considered to follow a linear trend (either increasing or decreasing) over time. Conversely, if a

quadratic regression was more representative, the parameter exhibited a biphasic trend over time: for an increasing quadratic model, the trend reached a maximum value and then decreased, whereas, for a decreasing quadratic model, the trend reached a minimum value and then increased. These tests helped identify the approximate points where the curve reversed.

To assess whether  $O_2$  tension varied throughout pregnancy, venous and arterial  $pO_2$  data, as well as total venous and arterial  $O_2$  content, were analyzed as a function of gestational age (Figure 1A–D). The values from umbilical venous samples demonstrated a biphasic trend in  $O_2$  delivery from the placenta, as indicated by a decreasing quadratic regression:  $pO_2$  levels progressively decrease until 220–230 days of gestation (approximately the 32nd–34th week of gestation), when  $pO_2$  appears to reach its lower point. Then, starting from the 34th week,  $pO_2$  increases until delivery, as described in a recent study [55]. This biphasic trend is also observed for arterial  $pO_2$ , although it is less pronounced compared to venous  $pO_2$ , total venous and arterial  $O_2$  contents (Figure 1A–D).

The biphasic trend in  $O_2$  content is accompanied by the inverse trend in Hb levels (Figure 1E,F). Venous and arterial Hb levels progressively increase from the 23rd week onward, reaching a plateau around 250–260 days of gestation (approximately the 36th–38th week of gestation). Overall,  $O_2$  extraction does not show significant changes throughout the observed period (Figure 1G).

According to the trend of oxygenation, a biphasic modification in venous and arterial pH, coupled with a mirrored trend in venous  $HCO_3^-$  and BE, is observed as pregnancy progresses (Figure 2).

The trend of umbilical cord  $pCO_2$  appears to be opposite to that of  $pO_2$ . Venous  $pCO_2$  shows a progressive increase, following a biphasic trend: starting from the 33rd to 34th week of pregnancy,  $pCO_2$  decreases (Figure 3A,B). The trend in arterial  $pCO_2$ , however, shows a linear increase from the 23rd week until the end of gestation. Overall,  $CO_2$  production does not seem to change significantly until the 33rd–34th week but increases during the later stages of pregnancy (Figure 3C). Glucose extraction appears stable throughout the course of pregnancy, although it increases significantly in the final weeks of gestation (Figure 3D).

No significant change in lactate levels is observed in either venous or arterial samples during the early weeks of pregnancy, but an increase in lactate levels becomes evident starting from the 33rd to 34th weeks of gestation. Lactate production also appears to increase in the final weeks of pregnancy (Figure 3E–G).

The type of delivery significantly influences the cord blood gas analysis results, with a notable impact on oxygenation (Table 2). Therefore, umbilical cord venous and arterial  $O_2$  contents were analyzed separately according to the type of delivery (Figure S1). However, the results confirm the biphasic trend in the  $O_2$  levels that the fetus receives from the placenta, regardless of the type of delivery; this trend is only attenuated in the arterial values of newborns delivered via cesarean section.

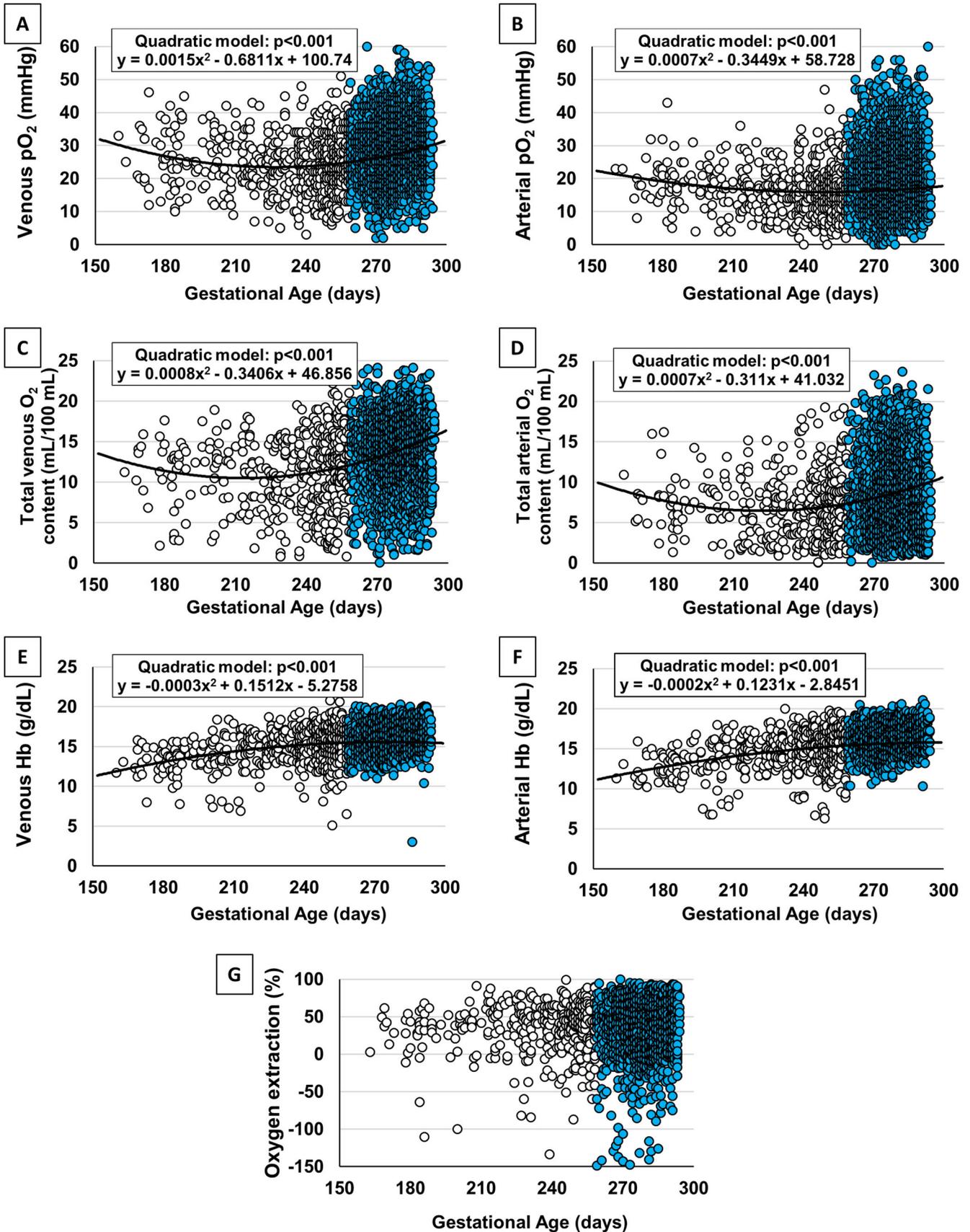
The main criticism regarding the reconstruction of these trends concerns the accuracy of plotting blood gas values obtained from cord blood samples taken immediately at birth in preterm newborns, with samples collected from full-term newborns approximately 60 s after birth. As mentioned above, delayed clamping of 30–60 s does not appear to substantially

alter pH,  $HCO_3^-$ ,  $pCO_2$ , lactate, or BE [117–119], and no effect on umbilical venous  $pO_2$  has ever been observed [116]. However, one study reported that delayed clamping for 2 min increases arterial  $pO_2$  by approximately 4 mmHg, likely as a result of the newborn's first breaths while the umbilical cord remains unclamped [120]. To address this issue, all arterial  $pO_2$  values of full-term newborns were deliberately adjusted by subtracting 4 mmHg. Despite this mathematical adjustment, the results still confirm the previously described trends, although the increase in arterial  $pO_2$  in the late stages of pregnancy is less pronounced (Figure S2). Essentially, the biphasic trend of fetal oxygenation is confirmed even after correcting the bias due to different sampling methods. Oxygen extraction remains unchanged, stable throughout pregnancy (Figure S3).

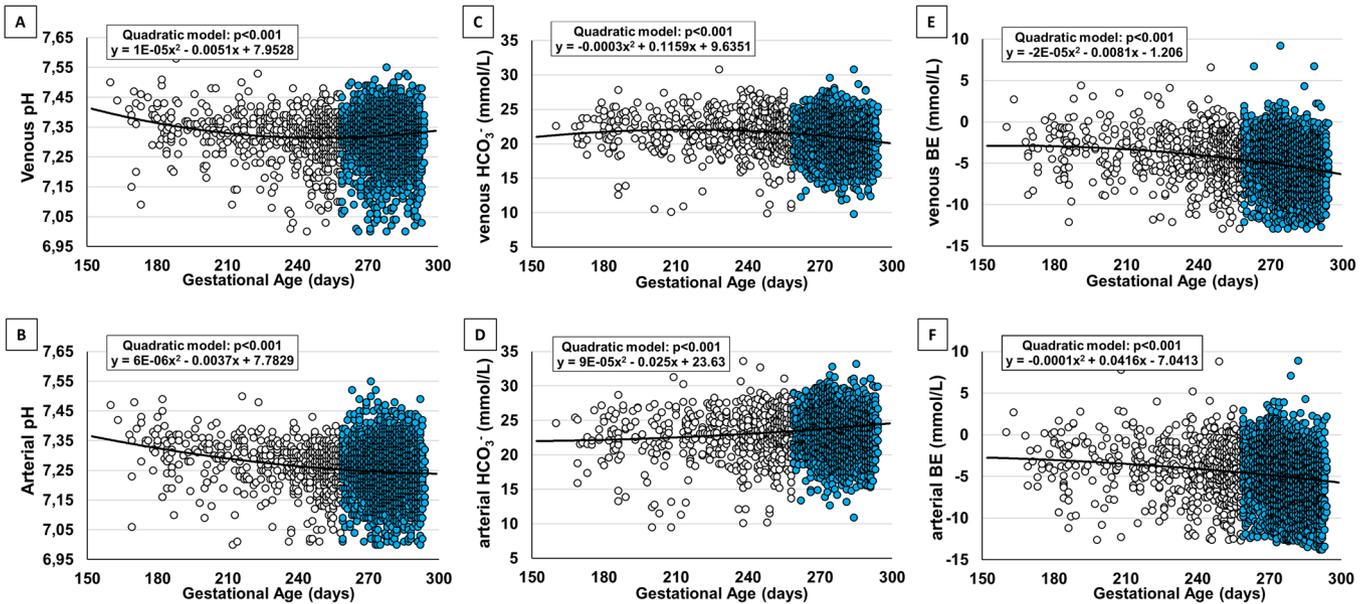
In conclusion, the most innovative aspect of these studies is the demonstration that the levels of  $O_2$  supplied from the placenta to the fetus follow a biphasic trend, with a gradual reduction in oxygenation up to the 33rd–34th week of gestation, followed by an increase until birth. This insight is particularly valuable for researchers aiming to develop artificial placental technologies, who should take into account the physiological fluctuations in oxygenation levels [57, 58]. However, this awareness also prompts researchers to ask what the functional effects of these fluctuations might be.

## 6 | Putative Functional Effects of Fluctuating $O_2$ Levels

During intrauterine life, the embryo and fetus are supplied with varying levels of  $O_2$ : immediately after fecundation, during implantation, and in the first weeks of pregnancy, the embryo lives in a highly hypoxic environment. However, the development of the placenta causes a sharp increase in  $O_2$  levels. Placentation thus represents a major turning point, with a “before” period characterized by significant hypoxia and an “after” period that is decidedly less hypoxic. Despite this early rise in  $O_2$  levels, our data demonstrate that the amount of  $O_2$  reaching the fetus from the middle of the second trimester until the first 4 weeks of the third trimester progressively decreases, reaching a minimum around 32–34 weeks. While the  $O_2$  level in the maternal uterine vessels remains stable throughout pregnancy [125], the progressive reduction in venous umbilical  $pO_2$  suggests that the placenta, as it grows and increases its metabolic activity, progressively extracts more  $O_2$  as pregnancy advances, reducing the amount of  $O_2$  available to the fetus. This interpretation is supported by samples drawn from the intervillous space of the human placenta, where the mean  $O_2$  tension gradually decreases [84, 91, 126], and by evidence that placental  $O_2$  consumption increases over time [60]. Therefore, the placenta, through its increase in  $O_2$  extraction, is responsible for the progressive reduction in fetal oxygenation observed between the second and third trimesters, while the fetus maintains its own  $O_2$  extraction largely unchanged for most of the pregnancy. These findings suggest that the primary function of the placenta is not to ensure maximum  $O_2$  supply to the fetus but rather to act as an anatomical barrier that limits  $O_2$  availability, providing a progressively more



**FIGURE 1** | Umbilical cord oxygenation status. Scatter plots with regression lines representing the umbilical cord oxygenation status (venous pO<sub>2</sub>, A [ $n = 4286$ ]; arterial pO<sub>2</sub>, B [ $n = 4161$ ]; total venous, C [ $n = 2643$ ] and arterial O<sub>2</sub> contents, D [ $n = 2547$ ]); venous Hb, E ( $n = 3034$ ); arterial Hb, F ( $n = 2922$ ); O<sub>2</sub> extraction, G ( $n = 2548$ ) of the whole study population. The equation of the statistically significant model (linear or quadratic) is indicated at the top of each figure. [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]



**FIGURE 2** | Umbilical cord pH, bicarbonate, and BE status. Scatter plots with regression lines representing the umbilical cord pH,  $\text{HCO}_3^-$  concentration, and BE of the whole study population (venous pH, A [ $n = 4544$ ]; arterial pH, B [ $n = 4392$ ]; venous  $\text{HCO}_3^-$ , C [ $n = 3098$ ]; arterial  $\text{HCO}_3^-$ , D [ $n = 2975$ ]; venous BE, E [ $n = 4544$ ]; arterial BE, F [ $n = 4442$ ]). The equation of the statistically significant model (quadratic) is indicated at the top of each figure. [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

hypoxic environment for the fetus, at least until the 33rd–34th week of pregnancy.

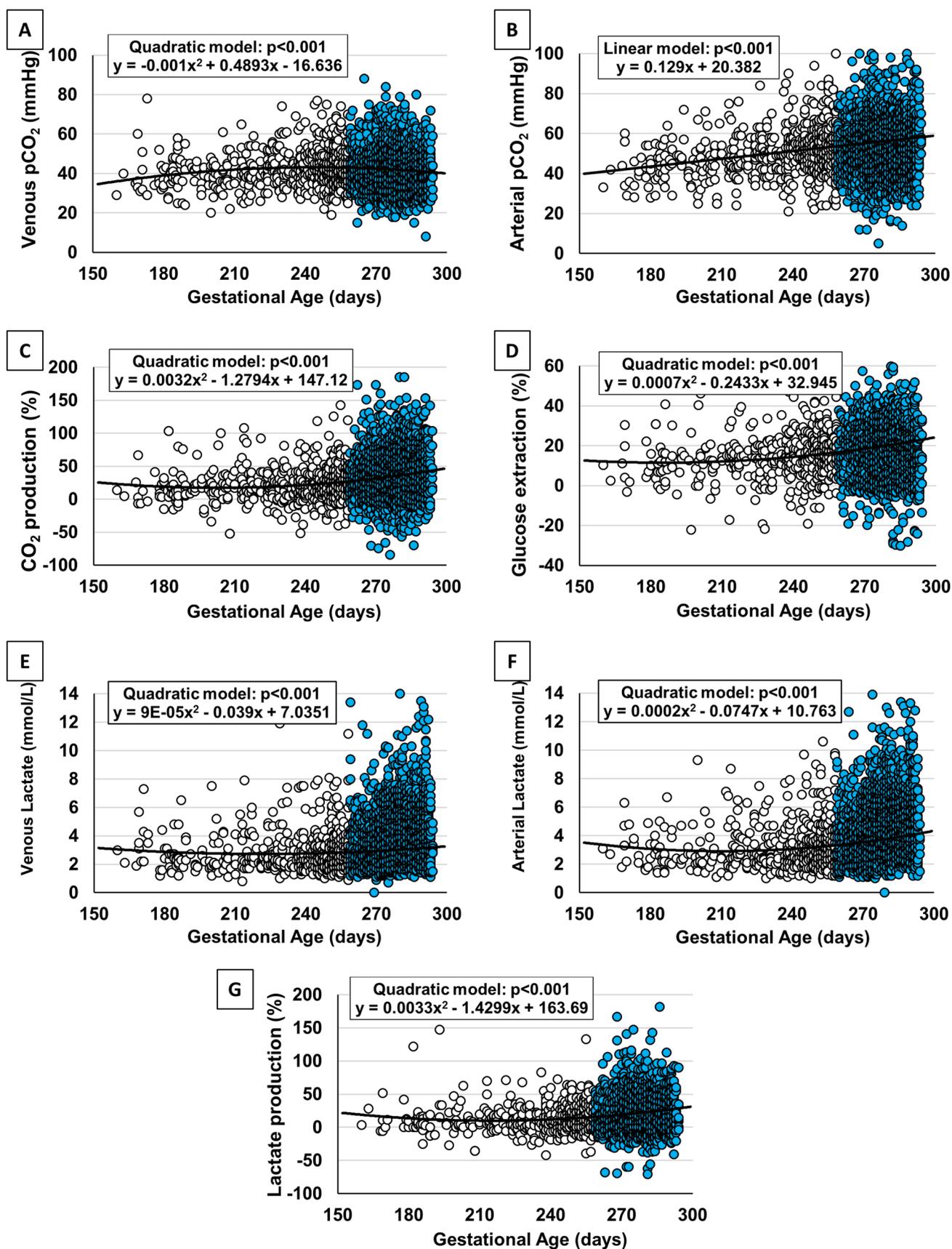
Growing fetal hypoxia until the 33rd–34th week carries several possible consequences:

1. The increasing fetal hypoxia that develops throughout much of pregnancy, until the last 6–8 weeks, upregulates HIF-1 and induces significant tissue-specific stem cell recruitment [127], as an  $\text{O}_2$ -poor environment is essential for preserving naïve stemness potential [128, 129]. The relationship between stemness and  $\text{O}_2$  levels is particularly evident for endothelial precursor cells (EPCs). Cord blood from preterm infants, in fact, shows a gradual increase in EPCs as gestational age progresses; these EPCs are very low at 24–28 weeks and highly expressed at 28–35 weeks [130] or 34 weeks [131]. This trend suggests that EPC expansion is synchronous with increasing hypoxemia, ceasing around the 33rd–34th week, when  $\text{O}_2$  levels start to rise. The close link between hypoxia and stemness is also evident during heart development, which is tightly regulated by HIF-1 expression [10, 132]. Studies in mouse models has shown that hypoxia maintains cardiomyocyte precursors in an undifferentiated state [133, 134], likely allowing them to expand adequately. Similarly, pluripotent stem cells isolated from murine embryoid bodies show impaired differentiation into cardiomyocytes if exposed to hypoxia [135]. Additionally, fetuses made experimentally more hypoxic show an even higher number of immature cardiomyocytes [136], while adult mice gradually exposed to severe systemic hypoxemia show a reactivation of cardiomyocyte mitosis [137].
2. Growing fetal hypoxia, mediated by HIF-1, induces the differentiation of specific tissues in specific anatomic regions [3]. This is particularly evident in the CNS, where

hypoxia regulates various processes, including cell survival, proliferation, catecholaminergic differentiation of isolated neural crest stem cells [138], differentiation of mesencephalic precursor cells into neurons [139], or transition of undifferentiated astrocytes into differentiated astrocytes in the developing retina [140, 141].

3. This hypoxic environment induces a progressive increase in Hb concentration, as its synthesis is stimulated by the activation of the HIF/erythropoietin axis [142, 143]. Despite this increase in Hb, fetal  $\text{O}_2$  content continues to decrease until approximately the 33rd–34th week, confirming this period as a threshold beyond which umbilical venous oxygenation begins to rise.
4. Increasing fetal hypoxia is associated with a progressive increase in cord venous  $\text{pCO}_2$  levels. This finding supports the idea that the placental barrier becomes progressively more effective at separating fetal and maternal circulations over time. As the passage of  $\text{O}_2$  from maternal to fetal circulation is increasingly hindered, the washout of fetal  $\text{CO}_2$  also becomes less efficient. It is possible that these changes in gas exchange are a consequence of placental growth, which physically better separates the two circulations; however, it is also possible that these effects are secondary to the increased metabolic activity of the placenta, which consumes more  $\text{O}_2$  and produces more  $\text{CO}_2$  as it grows [144]. The association of increasing hypoxia and hypercapnia explains the consistent reduction in fetal pH.
5. Despite the increasing hypoxia, fetal metabolism does not appear to be significantly altered, as fetal  $\text{CO}_2$  production and glucose extraction remain largely unchanged.

However, starting from the 33rd to 34th week of gestation, the gas-analytical and metabolic trends described in previous weeks



**FIGURE 3** | Umbilical cord carbon dioxide and lactate status. Scatter plots with regression lines representing umbilical cord carbon dioxide levels (venous pCO<sub>2</sub>, A [ $n = 4529$ ]; arterial pCO<sub>2</sub>, B [ $n = 4367$ ]; CO<sub>2</sub> production, C [ $n = 4315$ ]; glucose extraction (D [ $n = 2775$ ]); venous lactate, E ([ $n = 4119$ ]; arterial lactate, F [ $n = 4051$ ]; fetal lactate production, G [ $n = 3993$ ]). The equation of the statistically significant model (quadratic) is indicated at the top of each figure. [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

abruptly reverse, with fetal oxygenation starting to increase. This gestational age represents the second watershed, beyond which fetal oxygenation rises, likely due to the physiological aging of the placenta, which favors increased diffusion of O<sub>2</sub> [145]. The reduction in the distance between fetal vessels and the trophoblastic membrane, detectable in the last weeks of gestation, facilitates the increase in placental O<sub>2</sub> transport [146]. The concomitance of other events suggests that the reduced barrier efficiency of the placenta in the final stages of pregnancy can be attributed to its aging. Evidence supporting this include the fact that most immunoglobulin G is acquired by the fetus during the last 4 weeks of pregnancy [147], and that the risk of viral vertical transmission, particularly for cytomegalovirus infections [148], increases with advancing gestational age [149]. These observations suggest that, in the last weeks of pregnancy, the placenta's barrier function between maternal and fetal circulation diminishes. However, this apparent reduction in placental performance observed in the last weeks of gestation may actually benefit the fetus, allowing the acquisition of immunocompetence through the transfer of immunoglobulins and the maturation of various functions in utero. In this regard, it is notable that many animals are born immature, with some structures, such as vascular structures, developing only after birth, likely in response to a more oxygenated environment. For example, rodents' glial-vascular interactions are established postnatally [18, 150], and their retinas, avascular at birth, begin to vascularize during the first postnatal week [20]. Rodent puppies, therefore, at birth are exposed in a condition of fragility, as evidenced by the fact that many mammals are born with their eyelids still sealed. On the contrary, human newborns are born more mature, as evidenced by their open eyes at birth and completed retinal vascularization. It is, therefore, likely that in all mammals, developmental maturation is favored by exposure to a more oxygenated environment, but only in some species (including humans), this maturation occurs during intrauterine life, probably due to the increased availability of O<sub>2</sub>, at least from the 33rd to 34th week.

A possible effect of this increased fetal oxygenation in the last weeks of gestation could be to prepare the human fetus for extrauterine life while still in utero, by regulating a series of functions:

1. Increasing fetal O<sub>2</sub> levels in the last weeks of gestation, through the downregulation of HIF-1, induces the differentiation of specific tissues in specific anatomic districts [3]. The surge of O<sub>2</sub> promotes precursor cell differentiation in neural retinal tissue [151], pancreatic cells [152], keratinocytes [153], hepatocyte cell lines [154], and, finally, endothelial cells [155]. The effect of O<sub>2</sub> on the promotion of endothelial cell differentiation allows us to trace the stages of retinal vascularization in the human fetus: from mid-gestation to near-term, the intrauterine environment, which physiologically becomes more hypoxic, promotes the complete development of the astrocytic scaffold and EPC recruitment; during this period, only the EPCs closest to the central retinal artery are affected by an increase in O<sub>2</sub> and differentiate into endothelial cells, ensuring centrifugal vascularization. However, the increased availability of O<sub>2</sub> in the last weeks

of gestation accelerates the complete vascularization of the retina. The example of retinal vascularization is emblematic of the importance of O<sub>2</sub> level fluctuations during intrauterine life: these fluctuations are the driver that ensures the synchronization of the different phases of retinal development [156]. O<sub>2</sub> tension, in addition to other stimuli, is an environmental cue that modulates also cardiomyocyte maturation [157]. Exposure to a more oxygenated environment, in fact, induces postnatal cardiomyocyte cell-cycle arrest, likely through ROS upregulation [158]. Moreover, HIF-1 downregulation by increased O<sub>2</sub> tension has been suggested to promote the maturation of cardiomyocytes, inducing a metabolic switch from anaerobic glycolysis to OXPHOS [133].

2. Rising O<sub>2</sub> levels may favor the switch from fetal to adult Hb. In this regard, recent discoveries suggest that the production of  $\gamma$ -globin and thus the production of fetal Hb (HbF) is regulated by HIF-1 [159], indicating that the increase in O<sub>2</sub> and the subsequent downregulation of HIF-1 may represent a signal for the switch toward adult Hb (HbA) production. Indirect support for this hypothesis comes from the observation that the Hb switch from HbF to HbA begins before birth [160].
3. Increased fetal oxygenation may favor a metabolic shift from a predominantly glycolytic metabolism, which requires minimal O<sub>2</sub>, to a predominantly oxidative metabolism that instead requires greater O<sub>2</sub> supply. The indicators of this metabolic reprogramming are represented by the increase in CO<sub>2</sub> production, glucose extraction, and lactate production in the final weeks of pregnancy.

Cord venous and arterial pCO<sub>2</sub> levels in the last weeks of pregnancy show a peculiar trend: cord venous pCO<sub>2</sub> decreases, and this trend is in line with placental aging, which results in a less efficient barrier effect and therefore greater placental washout of CO<sub>2</sub> [145]. In contrast, arterial pCO<sub>2</sub> continues to increase linearly from the 23rd week until the end of gestation. The persistent rise in arterial pCO<sub>2</sub> even in the last weeks of pregnancy, despite greater placental washout, is likely due to the increased fetal CO<sub>2</sub> production, which follows the fetus's increased metabolic activity. This observation is consistent with the simultaneous increase in glucose extraction and lactate production.

In this context, the rise in metabolism in the final stages of pregnancy suggests a possible metabolic shift toward greater mitochondrial activation. A recent study supports this hypothesis, showing that term human placentas exhibit significantly greater mitochondrial content and mitochondrial respiratory activity compared to first-trimester placentas [161]. The availability of different concentrations of O<sub>2</sub> influences the exploitation of different energy sources: to maintain sufficient ATP production to meet bioenergetic needs, anaerobic glycolysis predominates when O<sub>2</sub> availability is low, while the mitochondrial OXPHOS pathway is favored when O<sub>2</sub> is more abundant [162]. It is well known that HIF-1 reduces mitochondrial function by inhibiting succinate dehydrogenase, that is, chain complex II [163], and, through the intermediation of nitric oxide, by inhibiting mitochondrial cytochrome c oxidase, that is, electron transport chain complex IV [164]. Additionally, HIF-1 reduces

mitochondrial content by decreasing their biogenesis [165] and increased autophagy [166]. Hypoxia not only reduces mitochondrial function but also diverts placental glucose metabolism toward glycolysis. In fact, HIF-1 upregulates the transcription of glucose transporters [167, 168] as well as enzymes of the glycolytic pathway, such as aldolase A, phosphoglycerate kinase 1, enolase, and lactate dehydrogenase-A [169].

Studies on pregnancies with IUGR have confirmed the effects of O<sub>2</sub> levels on metabolic reprogramming and the shift between glycolytic and OXPHOS pathways. Particularly hypoxic placentas, such as those in pregnancies with IUGR, reduce their reliance on O<sub>2</sub>-dependent mitochondrial OXPHOS and preferentially utilize the O<sub>2</sub>-independent glycolytic pathway [170]. Even in this context, the main driver of placental metabolic reprogramming is HIF-1, which is capable of inducing the Pasteur effect [171], that is, the diversion of carbohydrate metabolism from the mitochondrial oxidative pathway to anaerobic glycolysis [172]. Accordingly, the mitochondrial respiratory chain is less active in the placentas of IUGR pregnancies compared to those in uncomplicated pregnancies [173–176]. Some authors interpret this reduced mitochondrial activation as a sign of mitochondrial dysfunction secondary to a non-physiological pregnancy, rather than a compensatory mechanism [176]. However, in placentas from growth restriction pregnancies, ATP production remains unchanged compared to physiological pregnancies [173], suggesting that the switch from mitochondrial OXPHOS to the glycolytic pathway preserves ATP supply while reducing O<sub>2</sub> consumption [172]. Consequently, some genes involved in mitochondrial activity and OXPHOS are downregulated in the placentas of growth-restricted pregnancies [175, 176].

In conclusion, what occurs in the last weeks of an uncomplicated pregnancy appears to be the opposite, a mirror–image scenario to IUGR, equally capable of promoting metabolic reprogramming. In physiological conditions, reprogramming is driven by higher O<sub>2</sub> levels and favors a shift toward predominant OXPHOS metabolism. These final weeks of pregnancy therefore seem to serve as a preparatory period for birth and the transition to a more O<sub>2</sub>-rich environment.

These considerations help researchers address one of the most fascinating and mysterious questions for perinatologists: why is the fetus capable of modulating its metabolic options (prevalent glycolysis vs. prevalent OXPHOS), thereby demonstrating a notable ability to adapt to a wide range of hypoxic conditions, whereas after birth, the newborn loses this capacity to reprogram its metabolism and no longer tolerates life in a hypoxic environment? In fact, even a minimal reduction in neonatal oxygenation after birth—such as lowering the saturation target from 91%–95% to 85%–89%—leads to a marked increase in infant mortality [177, 178]. So, what are the biological “tricks” that allow the fetus this remarkable metabolic versatility, which the newborn irreversibly loses after birth?

Identifying these “tricks,” that is, the biological mechanisms that enable the fetus to thrive in a hypoxic environment, would be a major scientific breakthrough, opening up previously unexplored possibilities. In fact, if even a small remnant of these biological mechanisms, which allow fetal tolerance to hypoxia, was preserved, their pharmacological modulation

might enable the re-emergence of fetal capabilities and increase hypoxia tolerance in newborns. Figuratively, such modulation could be seen as a way to “bring the premature newborn back into the womb.” Essentially, this pharmacological approach could replicate in premature newborns the same beneficial effects they would have experienced had they remained in utero, benefiting from the gradual increase in hypoxia generated by placental growth. For this reason, we have provocatively referred to this hypothetical pharmacological approach as an attempt to create a “pharmacological artificial placenta.”

For several years, our research has been entirely focused on identifying the biological tricks employed by the fetus and placenta. There is substantial evidence suggesting we are on the right path.

## 7 | The Role of $\beta_3$ -ARs in Adaptation to Variable O<sub>2</sub> Concentrations: A Significant Step Toward a Pharmacological Artificial Placenta

### 7.1 | O<sub>2</sub> Dependence of $\beta_3$ -AR Expression

We previously discussed how HIF-1 acts as the primary biological sensor responsible for coupling between low intrauterine O<sub>2</sub> levels with vascular and metabolic adaptive mechanisms. Thus, HIF-1 is undoubtedly one of the most crucial transcription factors that equips the fetus with the specific capabilities needed to thrive in a hypoxic environment. However, even though postnatal exposure to a hypoxic environment also leads to the upregulation of HIF-1, this does not enable the newborn (nor the adult) to tolerate prolonged hypoxia, which is otherwise well tolerated during fetal life. This suggests that, during intrauterine life, other factors or biological mechanisms are essential to fully benefit from the hypoxic environment.

A growing body of research has recently shed new light on the involvement of the adrenergic system in the adaptation to prenatal intrauterine life. Since the late 1990s, the essential role played by the adrenergic system in ensuring embryonic development and fetal survival has become evident, as indicated by the high embryonic lethality observed in mice lacking tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis [179–181]. Adrenoceptors are well-known as key mediators of the sympathetic nervous system. By binding catecholamines (adrenaline and noradrenaline), adrenoceptors regulate the function of organs and tissues, maintaining homeostasis and responding to stress. The nine known adrenoceptor subtypes are divided into three families ( $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ ). Early studies demonstrated that the effects of catecholamines on fetal cardiac reactivity and survival were mediated through  $\beta$ -adrenoceptors ( $\beta$ -ARs), without identifying which specific receptor was most involved [182]. However, later studies identified  $\beta_1$ -AR as the  $\beta$ -AR subtype most responsible for maintaining fetal heart rate in hypoxic conditions [183], while  $\beta_2$ -ARs have been shown to provide growth and differentiation signals in various tissues [184, 185].

The  $\beta_3$ -AR, the last  $\beta$ -AR subtype identified in 1989 [186], is a strong candidate for playing an important and early role in embryonic development, even before the enzymatic system that

produces catecholamines is fully mature, due to its constitutive activity, which allows it to be potentially active even without ligand stimulation [187].  $\beta_3$ -ARs are likely involved in conception, as they are expressed in mammalian oocytes [188, 189] and spermatozoa, where they induce motility [190].  $\beta_3$ -ARs are also expressed in preimplantation embryos [188, 189], in embryos during the early stages of embryogenesis [191], in embryonic tissues and in the placenta [192, 193]. Moreover,  $\beta_3$ -ARs are detected in the mouse uterus [194] and are significantly upregulated in the human pregnant myometrium, where they represent the predominant  $\beta$ -AR subtype and participate in suppressing spontaneous contractions [195, 196]. Because of this, they are considered ideal targets for tocolytic drugs [197]. Finally,  $\beta_3$ -ARs agonism induces vasodilation of the human umbilical artery ring, suggesting that  $\beta_3$ -ARs can regulate fetal–placental circulation [198]. These data suggest a potential role for  $\beta_3$ -ARs in fecundation, embryo implantation, embryo vascularization, and embryonic/fetal well-being. The recent observation that repeated administration of high doses of  $\beta_3$ -ARs antagonists to pregnant animals induced preterm delivery or fetal death [199] further supports the active involvement of  $\beta_3$ -ARs in embryonic/fetal adaptation mechanisms.

The consensus in the literature is unanimous that  $\beta_3$ -ARs in various tissues are upregulated under hypoxic conditions [200–203], and recent evidence suggests direct regulation by HIF-1 [204]. The close relationship between hypoxia and  $\beta_3$ -AR expression is particularly evident in the mouse retina, where both HIF-1 and  $\beta_3$ -ARs are strongly expressed during intra-uterine life. However, their expression rapidly decreases after birth due to exposure to a more oxygenated environment [205]. This observation is consistent with findings in lymphocytes, where  $\beta_3$ -ARs were upregulated under hypoxic conditions but quickly downregulated after re-exposure to  $O_2$  [206]. Therefore,  $\beta_3$ -ARs appear to be particularly sensitive to and modulated by  $O_2$  levels, being upregulated by hypoxia and downregulated by higher  $O_2$  concentrations.

At the same time, a growing body of evidence suggests that  $\beta_3$ -ARs are involved in many biological processes that ensure embryo/fetal well-being and are, unfortunately, reactivated in cancer, allowing both the embryo/fetus and cancer to thrive in an otherwise inhospitable environment [59]. In fact, in addition to their role in vascularization processes induced by hypoxia [207, 208],  $\beta_3$ -ARs, which are intensely expressed in the placenta, promote the development of immune tolerance toward the fetus [209], similarly to how  $\beta_3$ -ARs expressed in cells of the tumor microenvironment promote tolerance toward cancer [206]. Moreover,  $\beta_3$ -ARs seem to participate in the mechanisms by which hypoxia induces the maintenance of stemness traits, as demonstrated by *in vitro* experiments [203] and confirmed by the observation that pharmacological blockade of  $\beta_3$ -ARs induces the differentiation of tumor stem cells [210, 211]. Finally,  $\beta_3$ -ARs are actively involved in hypoxia-induced metabolic reprogramming. In embryonic and cancer stem cells, in fact,  $\beta_3$ -ARs induce a distinctive metabolic rearrangement characterized by increased glucose uptake and accelerated glycolytic metabolism, which, at least partially, replaces mitochondrial OXPHOS [212]. This reprogramming occurs through the  $\beta_3$ -ARs-mediated upregulation of key enzymes involved in glycolytic pathways, such as glucose transporter-1

(Glut-1) and hexokinase 2 (HK-II), as well as transmembrane proteins like monocarboxylate transporter-4 (MCT-4), responsible for increased lactate export. At the same time,  $\beta_3$ -ARs induce the upregulation of uncoupling protein 2 (UCP2), which is responsible for mitochondrial dormancy and the reduction of the OXPHOS pathway [212].

Therefore, the knowledge acquired in recent years suggests that  $\beta_3$ -ARs, significantly upregulated by hypoxia during intra-uterine life, not only manage various functions that ensure embryonic/fetal well-being but also shift energy metabolism toward the glycolytic pathway, which is less dependent on  $O_2$ , rather than the OXPHOS pathway.  $\beta_3$ -ARs thus possess all the characteristics to represent the biological “trick” (or, more likely, one of the biological tricks) that allows the embryo and fetus to thrive in an environment with extremely low  $O_2$  levels.

However, as demonstrated by our findings, in a physiological pregnancy, starting from the 33rd to 34th week of gestation, the availability of  $O_2$  increases, and  $O_2$  levels rise further after birth. It is highly likely that exposure to increasing  $O_2$  concentrations leads to a reduction in  $\beta_3$ -ARs expression, causing the upregulation of glycolytic enzymes to cease and the mitochondria to “awaken.” The combination of these phenomena may result in a metabolic shift from the glycolytic pathway to mitochondrial OXPHOS. From this point onward, due to the virtual disappearance of the  $\beta_3$ -ARs, the dependence on  $O_2$  would become irreversible, and for this reason, the energy needs of the newborn would no longer be met in an  $O_2$ -poor environment.

The involvement of  $\beta_3$ -ARs in numerous processes that ensure fetal well-being (primarily vascularization) and their modulation by  $O_2$  supports the hypothesis that the main damages observable in premature newborns may be caused by the premature downregulation of  $\beta_3$ -ARs following exposure to a hyperoxic environment. Indeed, just as the upregulation of  $\beta_3$ -ARs seems to couple hypoxia with the induction of vascularization [202, 207], the downregulation of  $\beta_3$ -ARs has been hypothesized to be responsible for the coupling between hyperoxia and vascularization arrest [213]. In this regard, it is no coincidence that all pathologies specifically related to prematurity (ROP, BPD, NEC, and PVL) are characterized by an early arrest of vascularization [59].

## 7.2 | $\beta_3$ -AR Agonism to Prevent Hyperoxia Damages

The recognition of the beneficial role played by  $\beta_3$ -ARs during intrauterine life and the possibility that their downregulation after a preterm birth may contribute to neonatal damage opens up promising new scenarios. In fact, if  $\beta_3$ -ARs do not disappear completely after birth, it may theoretically be possible to reactivate some of their functions using currently available drugs that selectively stimulate them. The data available so far suggest that  $\beta_3$ -ARs contribute to fetal well-being as intermediaries of certain biological functions promoted by HIF-1. Thus, pharmacological stimulation of  $\beta_3$ -ARs could maintain these functions even in the absence of hypoxia, HIF-1, or even in a hyperoxic environment. In other words, the pharmacological activation of these receptors could reproduce the effects

normally promoted by hypoxia, even in a high-O<sub>2</sub> environment, thereby counteracting the effects of hyperoxia. Figuratively, this approach could resemble returning the preterm infant to the mother's womb.

Such a perspective is not as far-fetched as it might initially seem. Until a few years ago, inducing Hb production in anemic patients or athletes required moving to the high altitudes to exploit the effect that hypoxia on red blood cells production [214]. However, once it was demonstrated that hypoxia induces red blood cell production through the mediation of erythropoietin [215], its administration became sufficient to treat anemia or enhance athletic performance without the need for altitude [216, 217]. Figuratively, treatment with erythropoietin is equivalent to moving a patient or athlete to the mountains. Similarly, once it is demonstrated that the hypoxic uterine environment promotes fetal well-being through  $\beta_3$ -ARs, it is legitimate to hypothesize that pharmacological stimulation of these receptors in preterm newborns could replicate the effects of the uterine environment. This hypothesis of "returning the preterm infant to the womb" effectively paves the way for a pharmacological artificial placenta.

These considerations explain why our current research focuses on evaluating, in newborn animals, whether  $\beta_3$ -AR agonist administration can mitigate the harmful effects of hyperoxia on various tissues. Initial results have recently shown that pharmacological stimulation of  $\beta_3$ -ARs neutralizes the damage induced by hyperoxia in the colon [218] and ileum [219] of neonatal rats.

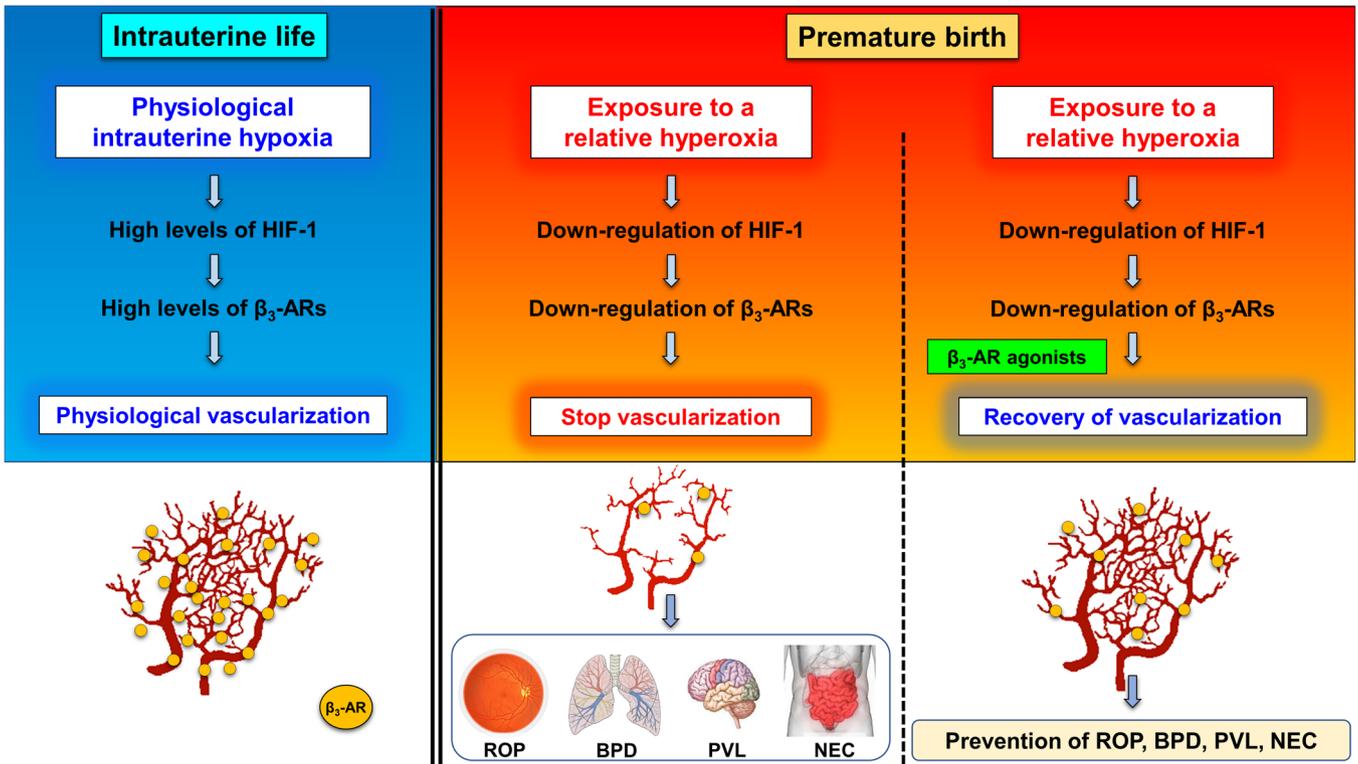
It is well-known that neonatal exposure to hyperoxia alters the structure of the colon mucosa in newborn rats [28, 220, 221] and disrupts the balance of the gut microflora [222] due to the generation of ROS, which compromise the integrity of the intestinal barrier [220, 222]. A first study performed in neonatal rats demonstrated that administration of a  $\beta_3$ -AR agonist prevented the shortening of colonic length caused by hyperoxia and ensured the production of mucins by goblet cells within the proximal colon's lining epithelium [218]. These findings provide the first evidence that targeting  $\beta_3$ -ARs pharmacologically could represent a therapeutic option for diseases characterized by impaired development due to hyperoxia. A second study has recently shown similar protective effects of  $\beta_3$ -AR agonism in the ileum. In this anatomical region, hyperoxia also caused damage, evidenced by increased mucosal thickness [221], disruption of the epithelial barrier through effects on tight junctions, and the dilation of capillaries and lacteals [220]. However, the administration of a  $\beta_3$ -AR agonist preserved the integrity of the gut surface epithelium despite hyperoxic exposure [219]. Notably, the significant reduction in vascularization observed in the ileal submucosa and muscle coat of hyperoxic control rats was prevented by  $\beta_3$ -AR agonist treatment [219]. These findings suggest that  $\beta_3$ -AR agonism can neutralize the effects of hyperoxia, restore conditions similar to those in utero, and pave the way for the prevention of NEC.

Similar results have recently been observed in the retina of neonatal mice. In this case, exposure to a hyperoxic environment typically induces the cessation of vascularization in the central part of the retina [223], which is associated with a

reduced density of retinal astrocytes [224]. This vascular regression, in turn, leads to hypoxic stress in the peripheral retina, triggering subsequent tumultuous and pathological vascularization [223]. A recent study using the oxygen-induced retinopathy (OIR) experimental model demonstrated that treatment with  $\beta_3$ -AR agonists administered during the hypoxic phase promoted physiological vascularization in the central retina, restored blood-retinal barrier integrity, neutralized the effects of hyperoxia, and prevented hypoxic suffering and the induction of pathological peripheral retina vascularization [225]. Although in this third study, the administration of  $\beta_3$ -AR agonists was not synchronous with hyperoxia exposure, also in this experimental setting the activation of  $\beta_3$ -ARs still demonstrated an ability to restore physiological conditions experimentally altered by hyperoxia. These promising results highlight the potential pharmacological prevention of the initial phase of ROP.

Overall, the preliminary data suggest that treatment with  $\beta_3$ -AR agonists neutralizes much of the damage caused by hyperoxia, which is critical in the development of major prematurity-related diseases (Figure 4). The most promising effect appears to be the restoration of normal vascularization, which would otherwise be impaired by hyperoxic exposure. However, the benefits of  $\beta_3$ -AR agonist treatment do not seem to be limited to vascularization. In fact, preliminary data from animal studies suggest that  $\beta_3$ -AR agonism can also reverse certain hyperoxia-induced alterations even in the nervous system. The study conducted on the OIR mouse model showed that the restoration of normal vascularization induced by  $\beta_3$ -AR agonism was accompanied by a recovery in astrocyte density in the central avascular retina. The exact mechanism by which  $\beta_3$ -AR stimulation restores the retinal astrocyte population is not yet clear. However, the induction of specific transcription factors suggests that  $\beta_3$ -AR activation may promote astrocyte migration or maturation [225]. Similarly, a study conducted on neonatal rats exposed to prolonged hyperoxia demonstrated significant effects of  $\beta_3$ -AR agonist treatment on the myenteric neuronal population. Hyperoxia exposure caused a notable increase in neurons expressing choline acetyltransferase and a marked reduction in neurons expressing neuronal nitric oxide synthase (implicated in the production of two opposing neurotransmitters, the excitatory acetylcholine and the inhibitory nitric oxide, respectively). However, these hyperoxia-induced changes in neuronal chemical coding were prevented by  $\beta_3$ -AR agonism [218], restoring conditions typically seen under physiological normoxia.

It is, therefore, likely that the effects of stimulating  $\beta_3$ -ARs during the neonatal period could be even more numerous, warranting further investigation in the future. In particular, we expect that the pharmacological stimulation of  $\beta_3$ -ARs will have significant effects on the metabolic programming of the newborn. Since the activation of  $\beta_3$ -ARs has already been shown to promote the glycolytic pathway at the expense of mitochondrial activity [212], we can anticipate that preterm newborns treated with  $\beta_3$ -AR agonists may experience a metabolic shift toward greater reliance of glycolysis and reduced OXPHOS activity. This shift would likely result in an increased need for glucose, while simultaneously reducing mitochondrial activity. Theoretically, reduced mitochondrial activation should lead to lower



**FIGURE 4** | Putative involvement of  $\beta_3$ -ARs in the pathogenesis of prematurity related diseases.  $\beta_3$ -ARs are actively involved in prenatal vascularization, participating in the coupling between hypoxia and the promotion of vasculogenesis (left). Premature birth involves early exposure to a relatively hyperoxic environment that downregulates HIF-1 and  $\beta_3$ -ARs. If the downregulation of  $\beta_3$ -ARs is responsible for the arrest of vascularization observed in preterm newborns (center), this could be reactivated with pharmacological stimulation of the remaining  $\beta_3$ -ARs (right). Thus, it could be possible to reactivate typically fetal functions promoted by hypoxia (e.g., vascularization) even after birth, even without the need to reduce the  $O_2$  supply, through pharmacological stimulation. This strategy aims to prevent all pathologies related to prematurity that are triggered by an arrest of vascularization. [Color figure can be viewed at [wileyonlinelibrary.com](https://onlinelibrary.wiley.com)]

production of mitochondrial free radicals, decreased dependence on  $O_2$ , and possibly greater tolerance to lower oxygenation levels. All of these conditions could provide theoretical benefits to preterm newborns, but these effects will need to be verified and thoroughly analyzed in future studies.

## 8 | Limitations and Potential Risks

The strategy of reactivating fetal competencies in preterm newborns through the administration of  $\beta_3$ -AR agonist drugs does not aim to completely nullify the effects of early interruption of intrauterine life and premature exposure to a hyperoxic environment. It is evident that the physiological hypoxia experienced by the fetus in the maternal womb regulates, via HIF-1, a myriad of functions, only a portion of which is likely mediated by  $\beta_3$ -ARs. Therefore, our goal is not to replace the extreme complexity of the maternal uterus with drugs, but rather to help preterm newborns maintain at least the biological effects that would have been ensured by the increased expression of  $\beta_3$ -ARs. The objective we reasonably set is to mitigate the consequences of prematurity and limit the damage caused by hyperoxia.

One of the current limitations of this strategy is the lack of studies demonstrating the safety of  $\beta_3$ -AR agonist drugs during the neonatal period, particularly in preterm newborns, although

these drugs have been used in pediatric patients for different indications [226]. At present, there are no reports on the use of  $\beta_3$ -AR agonist drugs in preterm newborns, and thus no specific adverse events have been described. Theoretically, one of the potential risks in preterm infants could be the induction of persistent patent ductus arteriosus. In recent years, our research group has demonstrated the presence of  $\beta_3$ -ARs in endothelial and smooth muscle cells of the murine fetal ductus arteriosus and their involvement in maintaining its patency. Indeed, treating pregnant mice with  $\beta_3$ -AR antagonists led to partial closure of the ductus arteriosus in fetuses [199]. Therefore, it cannot be ruled out that treating preterm newborns with a  $\beta_3$ -AR agonist might contribute to the persistence of a patent ductus arteriosus. Fortunately, evidence showing that  $\beta_3$ -ARs agonism administered to neonatal mice immediately after birth did not prevent the physiological ductus arteriosus closure, raises hope that the role of  $\beta_3$ -ARs in maintaining duct patency is marginal [199].

## 9 | Alternative Strategies

It is reasonable to imagine that treatment with  $\beta_3$ -ARs agonists is not the only possible path to achieving a pharmacological reconstruction of the uterine environment, thus creating a virtual artificial placenta. If the fetus indeed develops in utero through the combined action of HIF-1 and  $\beta_3$ -ARs, pharmacological

upregulation of HIF-1 could be a valid alternative approach to mimic the uterine environment. In this context, prolyl hydroxylase domain (PHD) inhibitors represent a novel class of drugs capable of preventing the hydroxylation and degradation of HIF-1, even in the presence of O<sub>2</sub>. Therefore, these drugs can stabilize and maintain elevated levels of HIF-1 even in the absence of hypoxia. Preclinical studies have suggested that these drugs may be effective in counteracting vascular regression induced by hyperoxia. For instance, the systemic administration of competitive PHD inhibitors, such as dimethyloxalylglycine or Roxadustat, to neonatal animal exposed to hyperoxia prevented retinal vascular regression by increasing levels of HIF-1 $\alpha$  and pro-angiogenic factors such as VEGF and erythropoietin [227, 228]. A number of PHD inhibitors are already in clinical use to mimic the effects of hypoxia for systemic conditions such as anemia [229], acute and chronic kidney disease, or cardiovascular diseases [230]. However, despite the promise of this approach, concerns exist regarding the safety of HIF-1 modulation, especially in immature infants who are still undergoing key developmental processes. The upregulation of a transcription factor like HIF-1, which controls hundreds of genes involved in the regulation of delicate biological functions, may lead to severe adverse effects, such as cancer, thromboembolism, pulmonary hypertension, or hyperkalemia [231].

## 10 | Conclusion

The preclinical results available thus far suggest that pharmacological treatments stimulating  $\beta_3$ -ARs—currently available, inexpensive, and safe for use in pediatric populations—can effectively counteract the effects of early exposure to a highly oxygenated environment on neonatal tissues. The goal of this strategy is to restore in premature newborns the same fetal capabilities that were induced by hypoxia in utero, even in the presence of an O<sub>2</sub>-rich environment. This approach aims to replicate the physiological benefits typically provided by the placenta using a drug, without relying on sophisticated and complex technology. However, further steps are required before advancing to human trials.

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### Conflicts of Interest

The authors declare no conflicts of interest.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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### **Supporting Information**

Additional supporting information can be found online in the Supporting Information section.