Placenta 145 (2024) 51–59

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Vascular reactivity is altered in the placentas of fetuses with congenital diaphragmatic hernia

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ARTICLE INFO

Keywords:
Congenital diaphragmatic hernia
Placenta
Vascular reactivity
Artery
Vasodilation
Vasoconstriction
Nitric oxide
Thromboxane

ABSTRACT

Introduction: Infants with congenital diaphragmatic hernia (CDH) often develop pulmonary hypertension but frequently fail to respond to vasodilator therapy, for instance because of an altered pulmonary vasoreactivity. Investigating such alterations *in vivo* is impossible. We hypothesised that these alterations are also present in fetoplacental vessels, since both vasculatures are exposed to the same circulating factors (e.g. endothelin-1) and respond similarly to certain stimuli (e.g. hypoxia). As proof-of-concept, we compared fetoplacental vasoreactivity between healthy and CDH-affected placentas.

Methods: Fetoplacental vascular function of healthy and antenatally diagnosed left-sided CDH fetuses was assessed by wire myography. Placental expression of enzymes and receptors involved in the altered vasoreactive pathways was measured using quantitative PCR.

Results: CDH arteries (n = 6) constricted more strongly to thromboxane A2 agonist U46619 ($p < 0.001$) and dilated less to bradykinin ($p = 0.01$) and nitric oxide (NO)-donor sodium nitroprusside ($p = 0.04$) than healthy arteries (n = 8). Vasodilation to prostacyclin analogue iloprost and adenylate cyclase stimulator forskolin, and vasoconstriction to endothelin-1 were not different between both groups. Angiotensin II did not induce vasoconstriction. Phosphodiesterase inhibitors sildenafil and milrinone did not affect responses to sodium nitroprusside, forskolin, or U46619. The mRNA expression of guanylate cyclase 1 soluble subunit alpha 1 ($p = 0.003$) and protein kinase cyclic guanine monophosphate (cGMP)-dependent 1 ($p = 0.02$) were reduced in CDH versus healthy placentas.

Discussion: The identified changes in the thromboxane and NO-cGMP pathways in the fetoplacental vasculature correspond with currently described alterations in the pulmonary vasculature in CDH. Therefore, fetoplacental arteries may provide an opportunity to predict pulmonary therapeutic responses in infants with CDH.

1. Introduction

Congenital diaphragmatic hernia (CDH) is a birth defect characterised by incomplete closure of the diaphragm. Consequently, abdominal organs enter the thorax and interfere with lung development [1–3]. Almost 70 % of infants with CDH develop severe pulmonary hypertension after birth due to a smaller lung size, as well as an abnormal pulmonary vasculature that is characterised by a reduced cross sectional area, as well as a lower number of arteries in all orders of the vascular tree that harbour enhanced smooth muscle cell deposition and aberrant vasoreactive responses to for example endothelin-1 (ET-1) and nitric oxide (NO) [3–7]. Therefore, these infants frequently fail to respond adequately to vasodilator therapies aiming at reducing pulmonary hypertension [8]. Vasodilator therapies include inhaled nitric oxide (iNO),

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https://doi.org/10.1016/j.placenta.2023.11.015
Received 30 May 2023; Received in revised form 6 November 2023; Accepted 25 November 2023
Available online 2 December 2023

the phosphodiesterase-5 (PDE5) inhibitor sildenafil, the endothelin receptor antagonist bosentan, the phosphodiesterase-3 (PDE3) inhibitor milrinone, and the prostacyclin (PGI_2) analogue iloprost [9].

The altered vasoreactive response in CDH can originate from several alterations in the pulmonary vasculature. Firstly, NO generated by endothelial NO synthase (eNOS) relaxes vascular smooth muscle cells via soluble guanylate cyclase (sGC) and cyclic guanine monophosphate (cGMP), which binds to cGMP-dependent protein kinase. Yet, eNOS expression is decreased in CDH lungs, and PDE5-mediated cGMP degradation is enhanced [10–13]. Interestingly, interference with the NO-cGMP pathway through iNO and sildenafil is often ineffective in reducing CDH-related pulmonary hypertension [8]. Secondly, reduced PGI_2 receptor expression in CDH lungs may impair vasodilation through cyclic adenosine monophosphate (cAMP), which is degraded by PDE3 [5]. This PGI_2-cAMP pathway can be targeted by milrinone and iloprost. Thirdly, the elevated pulmonary vascular resistance in CDH may be attributed to upregulation of the vasoconstrictor ET-1 and its type A (ET_A) and B (ET_B) receptors, which can be antagonised by bosentan [5]. This pathway might also involve angiotensin II upregulation due to increased expression of angiotensin-converting enzyme or elevated levels of thromboxane [14–16].

Until now it has been impossible to study alterations in pulmonary vascular reactivity and predict the response to pulmonary vasodilators *in vivo* for each CDH infant. Both the fetal pulmonary and placental vasculature encompass the fetal circulation and are thus exposed to the same circulating vasoreactive factors, such as ET-1. Moreover, *ex vivo* studies with human tissue have shown that both vascular beds exhibit similar responses to certain stimuli, for instance hypoxia-induced vasoconstriction caused by specific voltage-gated potassium channel signalling systems [6,17–26]. We hypothesise that already-known alterations in the fetal pulmonary vasculature are also present in the fetoplacental vasculature [27,28]. In that case, the fetoplacental vascular reactivity of fetuses with CDH should be altered compared to the fetoplacental vascular reactivity of fetuses without lung disease. In this study, we compared fetoplacental vascular responses between CDH-affected placentas and healthy placentas.

2. Methods

For this study, we collected placentas of healthy ("healthy placentas") and antenatally diagnosed left-sided CDH fetuses ("CDH placentas") immediately after birth at the Erasmus MC, Rotterdam, the Netherlands, in the period from March 2021 until September 2022. The diagnosis of CDH is based on the visualisation of abdominal organs (e.g. stomach, bowels or liver) within the fetal chest with prenatal ultrasound. Controls were placentas from healthy fetuses, who were born through elective caesarean section after an uncomplicated pregnancy. Exclusion criteria were birth before 35 weeks of gestation, the presence of preeclampsia or hypertensive disorders, pre-existing placental abnormalities, viral infections (HIV, hepatitis B, Zika, SARS-CoV-2 in the third trimester), or the need of manual placenta removal. For control placentas we additionally excluded patients with diabetes, fetal growth restriction [29], and major fetal congenital anomalies. Written informed consent was obtained from 22 women (8 healthy, 14 CDH); 7 of the 14 CDH placentas were excluded, either due to manual placenta removal (n = 3), SARS-CoV-2 infection (n = 3), or missing birth of child and placenta (n = 1). We collected the following maternal baseline characteristics: age at birth, medical history and obstetrical history, use of medication, mode of conception, gestational age at delivery, mode of delivery, placental weight, and pregnancy complications. Infant characteristics included birth weight, sex, and survival. The study was approved (MEC-2021-0118, MEC-2017-418, MEC-2016-418) by the local medical ethical committee according to the Dutch medical research with human subjects law.

2.1. Wire-myography experiments

Within 30 min after delivery of the placenta, second-order branches of fetoplacental arteries were identified, dissected, cleaned from surrounding tissue, and stored overnight in Krebs-Henseleit buffer (in mmol/L: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3) aerated with 95 % O_2 and 5 % CO_2 at 4 °C. These vessels were cut into segments of 2 mm length and mounted in 6 ml organ baths (Danish Myograph Technology, Aarhus, Denmark) that were filled with Krebs-Henseleit buffer at 37 °C and were aerated with 95 % O_2 and 5 % CO_2. Tension of the segments was recorded in LabChart acquisition software (AD Instruments). Each segment was normalised to 5.1 kPa tension to approximate *in vivo* conditions as described in previous studies [30–33], and the internal diameter after normalisation was recorded (2808 ± 348 μm in CDH *vs* 2398 ± 358 μm in healthy, $p = 0.05$). After obtaining a stable baseline tension, the maximum contractile response to 100 mmol/l potassium chloride (KCl) was determined in each segment. After washout of the KCl, vasoconstriction or vasodilation was assessed according to one of the following protocols. In addition, we determined the effect of PDE inhibition.

Vasoconstriction was assessed by constructing concentration-response curves using: (1) the thromboxane A2 agonist U46619 (0.1 nmol/l – 1 μmol/l), (2) ET-1 (0.1 nmol/l – 100 nmol/l) pre-incubated with or without an ET_A receptor antagonist (BQ123; 1 μmol/l) or ET_B receptor antagonist (BQ788; 0.1 μmol/l), and (3) angiotensin II (0.1 nmol/l – 10 μmol/l) pre-incubated with or without an angiotensin II type 1 receptor antagonist (irbesartan; 1 μmol/l) or an angiotensin II type 2 receptor antagonist (PD123319; 1 μmol/l).

Vasodilation was assessed after precontraction with 10–50 nmol/l U46619 (the lowest concentration that elicited >75 % contraction of the maximum KCl response). Vasodilator effects were then evaluated using (1) NO-donor sodium nitroprusside (SNP, 0.1 nmol/l – 100 μmol/l), (2) bradykinin (0.1 nmol/l – 3 μmol/l), (3) PGI_2 analogue iloprost (0.1 nmol/l – 1 μmol/l), and (4) adenylate cyclase activator forskolin (1 nmol/l – 30 μmol/l).

PDE inhibition. The effects of the PDE5 inhibitor sildenafil and PDE3 inhibitor milrinone were assessed on NO-mediated vasodilation and adenylate cyclase-mediated vasodilation, respectively, by pre-incubating a part of the vessel segments with sildenafil (1 μmol/l) before the SNP protocol, or with milrinone (10 μmol/l) before the forskolin protocol. The effects of sildenafil and milrinone were also determined on U46619-induced vasoconstriction. All concentration-response curves were constructed after an incubation period of at least 30 min with incubator and compared to a paired control segment of the same placenta without incubator.

2.2. RNA extraction, reverse transcription, and quantitative PCR

Biopsies of the central villous area were taken in almost all placentas that were used for the wire-myography experiments (healthy: 6/8; CDH: 7/7) before dissection of the fetoplacental arteries. These biopsies were snap frozen in liquid nitrogen and stored at −80 °C until further analysis.

For RNA isolation, approximately 30 mg of the biopsy was dissected on dry ice, and thereafter homogenised in RTL lysis Buffer (Bioline, London, UK) with 2 % dithiothreitol (DTT) using a tissue homogeniser (Rotary homogeniser Bio-Gen PRO200, PRO Scientific Inc., Oxford, Connecticut, USA). The RNA was extracted from the homogenate using the ISOLATE II RNA Mini kit (Bioline, London, UK) according to the protocol provided by the manufacturer, and eluted in RNAse free water. RNA concentrations were measured on the NanoDrop ND1000 spectrophotometer (Thermo Fischer Scientific, Cleaveland, OH, USA) and the 260 nm/280 nm ratios were between 1.94 and 2.08, which is considered to represent RNA without pollution. The eluted RNA was stored at −20 °C until complimentary DNA (cDNA) synthesis. The Sensifast cDNA synthesis kit (Bioline, London, UK) was used to obtain cDNA (according to the provided protocol) from 400 ng of each RNA

product using the TProfessional Basic Thermocycler (Biometra GmbH, Göttingen, Germany). The obtained cDNA was diluted (1:10) and stored at −20 °C. Quantitative PCR (qPCR) was performed according to the Sensimix SYBR & Fluorescein kit protocol (Bioline, London, UK) on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc., Puchheim, Germany). The qPCR conditions were: initial denaturation at 95 °C for 8 min and 30 s, followed by 40 cycles comprising 15 s at 95 °C, and 1 min at 60 °C. Directly after, a melt curve (10 s at 95 °C and then 65 °C–95 °C with an increment of 0.5 °C per 5 s) was run for each gene to confirm amplification of a single PCR product. Details of the primer pairs used to perform the qPCR can be found in Supplemental Table 1 [34,35]. Gene expression data were normalised to the geometric mean of three reference genes: pre-MRNA processing factor 38 A, peptidylprolyl isomerase A and β-actin. Based on the expression of the reference genes, one healthy sample was an outlier: Ct > quartile 3 + 1.5 x interquartile range [IQR]. This sample was therefore excluded from further analysis. For all tested genes, the relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.3. Statistical analysis

A sample size calculation was performed based on our previous wire-myography experiments and revealed a minimum sample size of 6 per group with an α level of 0.05 and a statistical power of 80 % [36]. Taking into account the possibility of failed experiments, we aimed for a sample size of 8 placentas per group.

Normality of the data is assessed with the Shapiro-Wilk test. Data are presented as mean ± standard deviation (SD) for normally distributed data or median [IQR] for non-normally distributed data. The Student *t*-test (normally distributed data) or Mann-Whitney *U* test (non-normally distributed data) were used to compare continuous variables. Categorical variables were analysed with a chi-squared test or Fisher exact test. Statistical analyses were performed with R (R Core Team (2020), Vienna, Austria) unless stated otherwise.

Vascular relaxation is expressed as a percentage of the pre-constriction that was created with U46619. Vascular contractile responses are expressed as a percentage of the maximum constriction to 100 mM KCl. Concentration–response curves were statistically analysed with a repeated measures ANOVA in SPSS Statistics (version 28, IBM Corp, Armonk, NY, USA); general linear model–repeated measures (GLM-RM), sphericity was assumed. The differences between vascular relaxation or contraction in the CDH and healthy placentas were compared using a one-way repeated measures ANOVA with the pregnancy condition as between-subjects factor. The effects of antagonists on concentration-response curves were compared to the paired control curve in a two-way repeated measures ANOVA for healthy and CDH experiments separately. Differences in maximum response (E_{max}) between CDH and healthy placentas were analysed using an unpaired *t*-test (normally distributed data) or Mann-Whitney *U* test (non-normally distributed data). The effects of pre-incubators on the E_{max} were analysed using a paired *t*-test (normally distributed data) or a Wilcoxon test (non-normally distributed data), or mixed effects analysis for >2 groups. The averaged concentration-response curves were calculated using sigmoid curve fitting software in GraphPad Prism (version 8, La Jolla, California, USA).

Statistical differences in gene expression between the CDH and healthy placentas were tested using unpaired *t* tests in GraphPad prism. A *p*-value lower than 0.05 was considered statistically significant.

3. Results

Results are depicted for six CDH placentas and eight healthy placentas, as the vessels of one CDH placenta failed to constrict to KCl, precluding further experiments in this sample. Table 1 outlines the clinical characteristics of the mothers and their offspring, as well as the placental characteristics. In the CDH fetuses, the median observed to expected lung-to-head ratio was 39 % [32,33,36–49] and liver position was intra-abdominal in 5 (83 %) fetuses. Out of the eight healthy inclusions, spontaneous vasomotion impaired the analysis of certain vessel segments and led to the exclusion of the following concentration-response curves: one ET-1 curve, two SNP curves with sildenafil, one bradykinin curve, two forskolin curves with milrinone. For the CDH group, n = 6 in all experiments.

Table 1
Clinical data of the study population.

	Healthy (n = 8)	CDH (n = 6)	*p-value*
Maternal age (years)	35 ± 5	32 ± 7	0.32
Nullipara	1 (13 %)	1 (17 %)	1
Caucasian	3 (38 %)	4 (67 %)	0.59
Body mass index (kg/m^2)	23 [21–24]	25 [23–30]	0.20
Highest mean arterial pressure (mmHg)	98 ± 6	95 ± 11	0.50
Spontaneous pregnancy	6 (75 %)	6 (100 %)	0.47
Vaginal delivery	0 (0 %)	6 (100 %)	0.0003
Gestational age at birth (weeks $^{+}$ ^{days})	39^{+2} [39^{+0}-39^{+3}]	38^{+1} [37^{+1}-38^{+2}]	0.017
Male gender	5 (63 %)	5 (83 %)	0.58
Birth weight (g)	3613 ± 496	2861 ± 402	0.009
Birth weight centile	65 ± 33	32 ± 30	0.08
Placental weight (g)	666 [510–739]	497 [464–562]	0.18

Data are expressed as mean ± standard deviation, median [interquartile range], or N (%).

3.1. Vasoconstriction

Absolute maximum contractile responses to KCl were not different between both groups (10.2 ± 3.9 mN in CDH *vs* 11.2 ± 2.9 mN in healthy, $p = 0.57$). Yet, vasoconstriction to U46619 was enhanced in arteries from CDH placentas compared to healthy placentas ($p < 0.001$, Fig. 1A), resulting in an increased maximum constriction (E_{max} 224 ± 32 % *vs* 167 ± 15 %, $p < 0.001$). To study the potential effect of PDE inhibition on vascular contractility, artery segments were also pre-incubated with either sildenafil or milrinone, however, this did not affect constriction to U46619 (Fig. 2A–D). Contractile responses to ET-1 were identical in both groups (Fig. 1B). Pre-incubation of the vessel segments with BQ123 inhibited constriction to ET-1 in healthy placentas ($p = 0.004$) and a similar tendency was observed in CDH placentas ($p = 0.10$), while BQ788 had no effect in both groups (Fig. 3). Angiotensin II did generally not induce vasoconstriction in the fetoplacental arteries; only one healthy and one CDH placenta displayed a modest constriction. Thus, the effects of irbesartan and PD123319 could not be evaluated.

3.2. Vasodilation

Vasodilation to the NO-donor SNP was significantly attenuated in arteries from CDH placentas ($p = 0.04$, Fig. 1C). Pre-incubation of the vessel segments with the PDE5 inhibitor sildenafil did not affect vasodilation by SNP in CDH placentas or healthy placentas (Fig. 2E&F). Bradykinin induced vasodilation in arteries from 4 out of 6 CDH placentas and 6 out of 8 healthy placentas; vasodilation was attenuated in CDH placentas ($p = 0.01$, Fig. 1D). Vasodilation to iloprost and forskolin was not different between CDH and healthy placentas (Fig. 1E&F), nor did pre-incubation with the PDE3 inhibitor milrinone affect vasodilation to forskolin in CDH or healthy placentas (Fig. 2G&H).

3.3. Alterations in placental gene expression

Fig. 4 depicts the gene expression results from placental homogenates. The expression of the guanylate cyclase (GC) 1 soluble subunit alpha 1 (GUCY1A1), and protein kinase cGMP-dependent 1 (PRKG1), was lower in CDH placentas compared to healthy placentas (GUCY1A1: fold change 0.58 [0.46–0.78] *vs* 0.94 [0.86–1.23], $p = 0.003$; PRKG1:

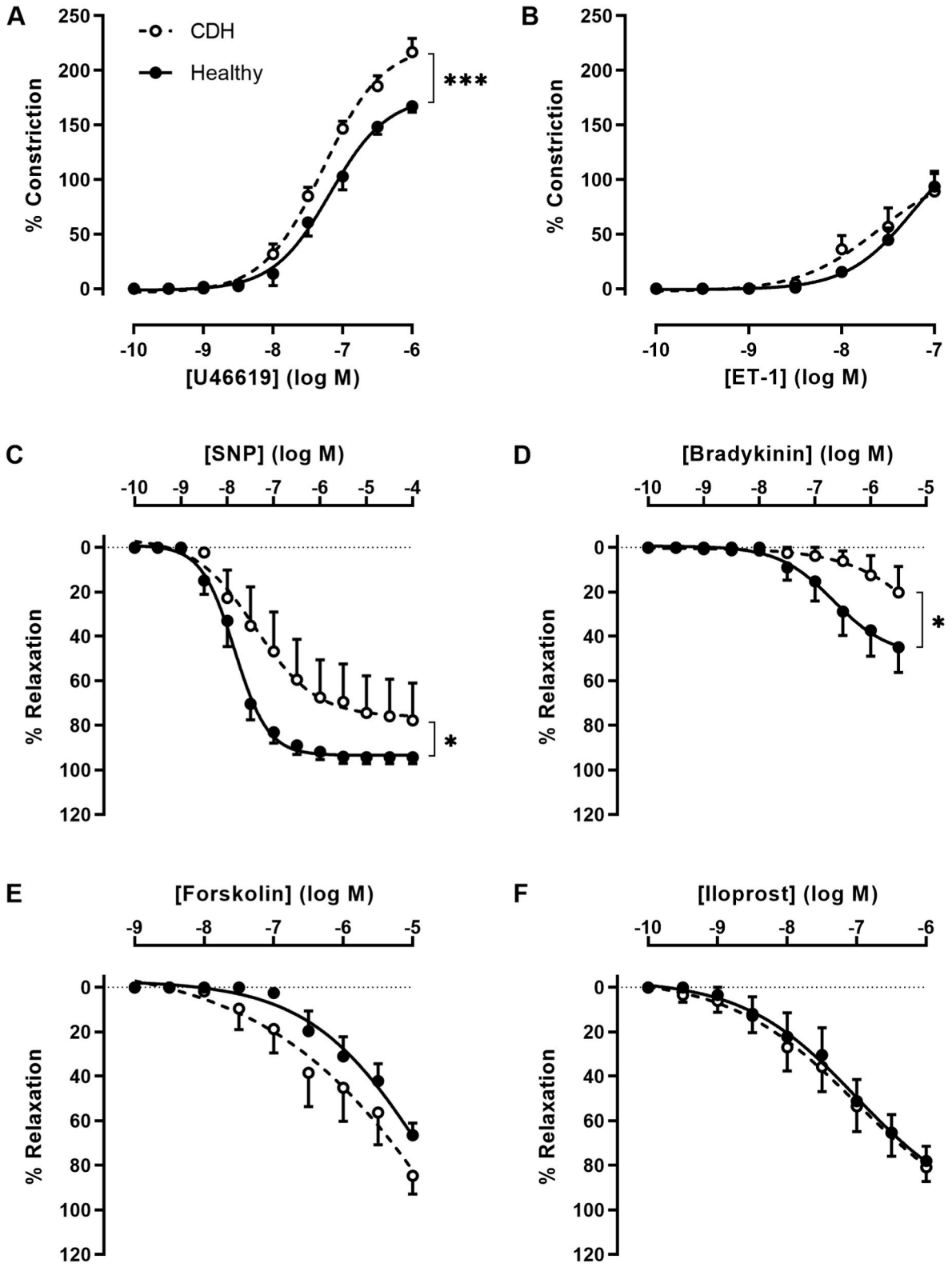

Fig. 1. Comparisons of vascular responses between arteries from congenital diaphragmatic hernia (CDH) and healthy placentas. Concentration-response curves with U46619 (**A**), endothelin-1 (ET-1) (**B**), sodium nitroprusside (SNP) (**C**), bradykinin (**D**), forskolin (**E**), and iloprost (**F**), from wire myography experiments with 8 healthy (filled circles) and 6 CDH placentas (open circles). Constriction (**A&B**) is depicted as percentage of the maximum response to 100 mmol/l potassium chloride, relaxation (**C–F**) as percentage of the precontraction by U46619. CDH curves were compared to control curves using GLM-RM, and depicted as mean ± standard error. $*p < 0.05$, $***p < 0.001$.

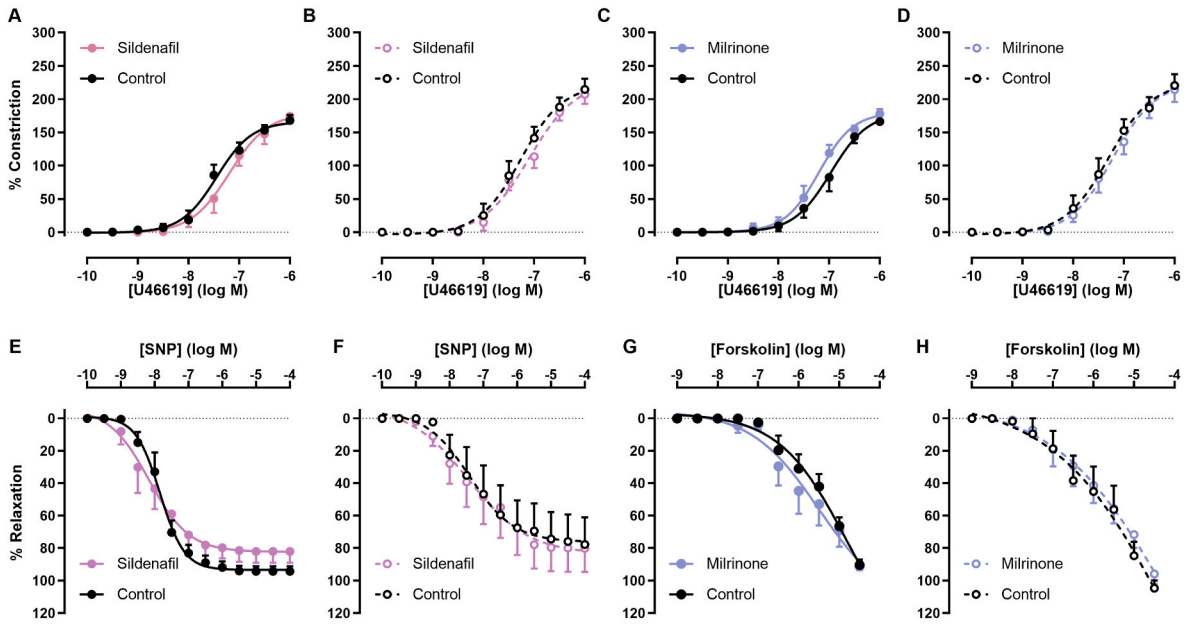

Fig. 2. The effects of sildenafil and milrinone on vasoconstriction and vasodilation in fetoplacental arteries. Concentration-response curves from wire myography experiments with 8 healthy (filled circles, **A,C,E&G**) and 6 congenital diaphragmatic hernia placentas (open circles, **B,D,F&H**). **A-D**) Constriction to U46619 in the absence (Control) or presence of sildenafil or milrinone. **E-F**) Relaxation to sodium nitroprusside (SNP) in the absence (Control) or presence of sildenafil. **G-H**) Relaxation to forskolin in the absence (Control) or presence of milrinone. Due to spontaneous relaxation or severe vasomotion, data were excluded from two healthy SNP curves with sildenafil and two healthy forskolin curves with milrinone. The reponses are depicted as mean ± standard error, for U46619 as percentage of the maximum response to 100 mmol/l potassium chloride, for SNP and forskolin percentage relaxation of precontraction by U46619. The effects of sildenafil and milrinone were statistically tested using GLM-RM.

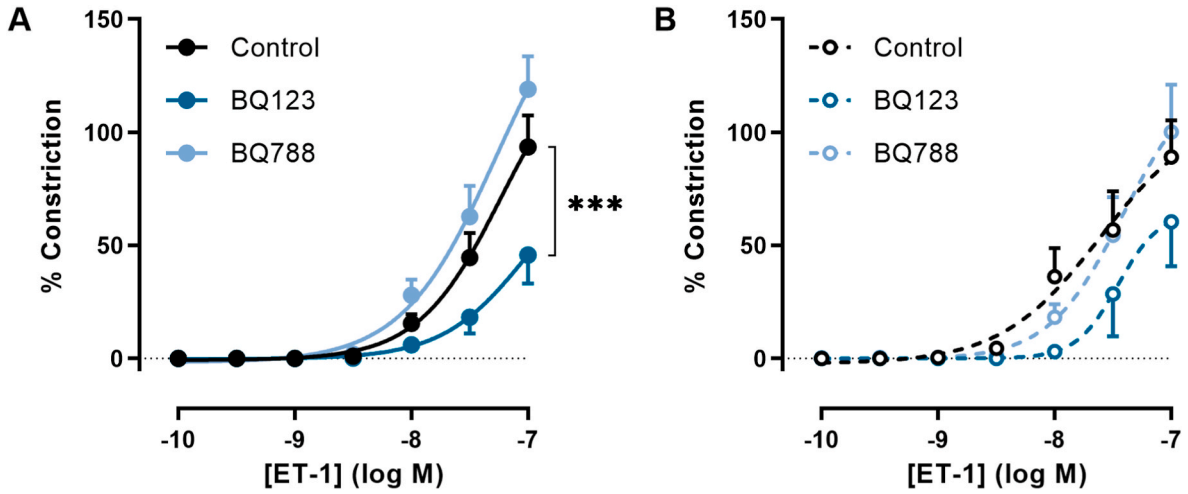

Fig. 3. Vascular constriction to endothelin-1 (ET-1) in fetoplacental arteries. Concentration-response curves with ET-1 from wire myography experiments with 8 healthy (**A**, filled circles) and 6 congenital diaphragmatic hernia placentas (**B**, open circles) in the absence (Control) or presence of BQ123 or BQ788. The effects of BQ123 and BQ788 were statistically tested versus the control using GLM-RM. Data are depicted as mean ± standard error percentage of the maximum response to 100 mmol/l potassium chloride. ***$p < 0.001$.

fold change 0.73 [0.65–0.85] *vs* 1.00 [0.84–1.21], $p = 0.02$). The expression of the thromboxane A2 receptor, bradykinin receptors B1 and B2, as well as GC 1 soluble subunit beta 1, and the enzymes eNOS, prostaglandin E synthase 2 and protein kinase cGMP-dependent 2 were not altered. The Ct value of inducible NOS was >36 in all samples and this gene was therefore considered not expressed.

4. Discussion

This study assessed alterations in arterial fetoplacental vascular reactivity in CDH placentas compared with healthy placentas to explore their potential as proxy for pulmonary vascular aberrancies. These data show that compared to healthy placentas, fetoplacental arteries of CDH fetuses exhibit an enhanced contractile response to the thromboxane A2 receptor agonist U46619 and attenuated vasodilator responses to bradykinin and the NO-donor SNP (Fig. 5). These alterations correspond with the alterations in pulmonary vascular reactivity currently described in infants with CDH as well as ovine and rodent CDH-models [8,10–13,15,16], and thus our data suggest that the fetoplacental vasculature may mirror the pulmonary vasculature.

An imbalanced production of thromboxane and prostacyclin in the pulmonary vessels has been suggested to cause the elevated pulmonary

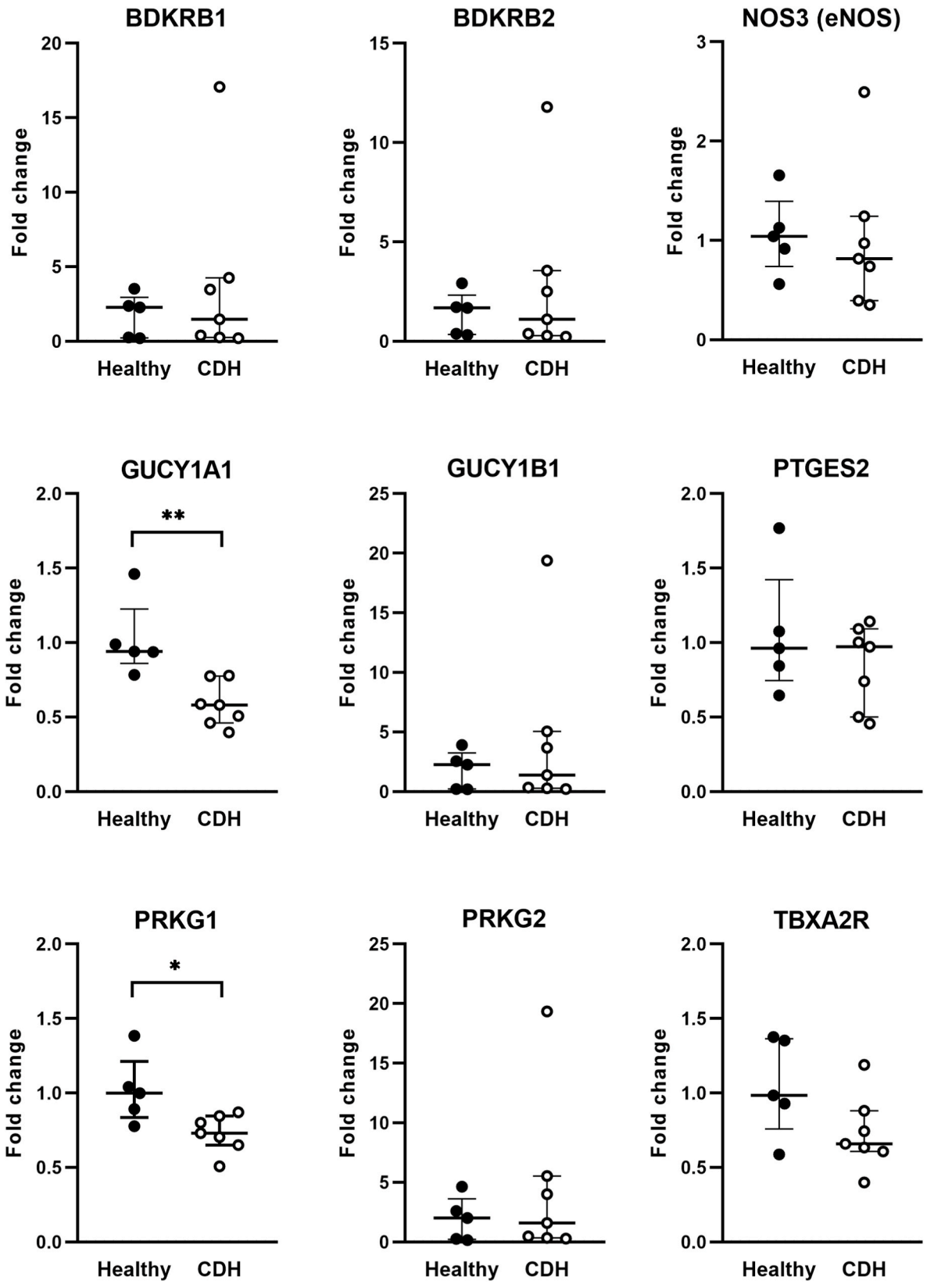

Fig. 4. mRNA expression of genes involved in vasodilation and vasoconstriction in healthy and congenital diaphragmatic hernia (CDH) placentas. Data are depicted as fold change median [IQR]. BDKRB1 indicates bradykinin receptor B1; BDRKB2, bradykinin receptor B2; NOS3 (eNOS), endothelial nitric oxide synthase; GUCY1A1, guanylate cyclase 1 soluble subunit alpha 1; GUCY1B1, guanylate cyclase 1 soluble subunit beta 1; PTGES2, prostaglandin E synthase 2; PRKG1, protein kinase cGMP-dependent 1; PRKG2, protein kinase cGMP-dependent 2; TBXA2R, thromboxane A2 receptor. *$p < 0.05$, **$p < 0.01$.

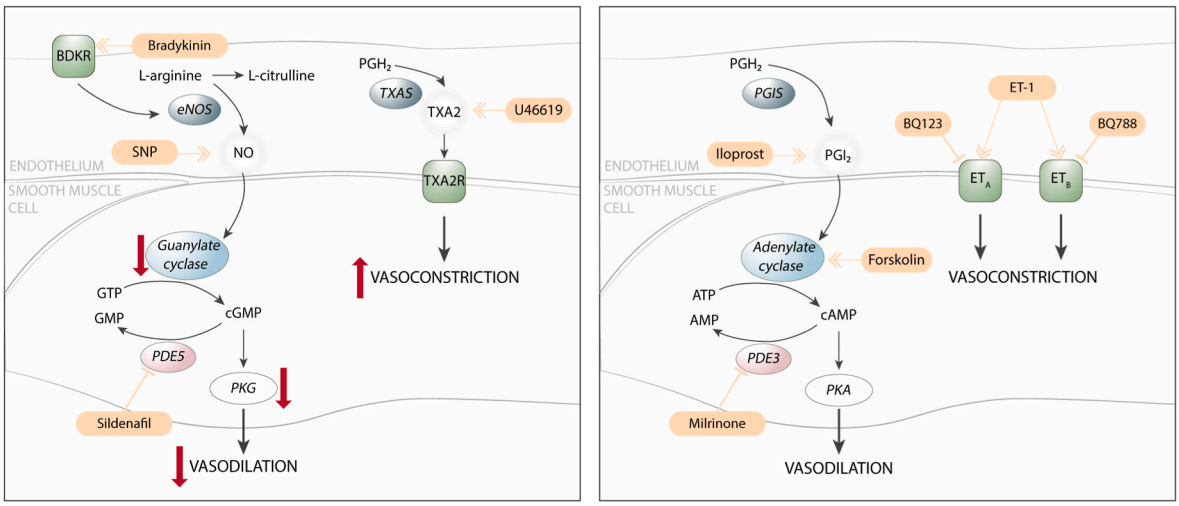

Fig. 5. Schematic representation of vasoreactive pathway alterations in fetoplacental arteries of infants with congenital diaphragmatic hernia (CDH). Alterations in vasoreactive pathways in CDH found in this study are indicated by red arrows in the left panel, specifically: decreased vasodilation to SNP and bradykinin, and enhanced vasoconstriction to U46619. The right panel depicts the vasoreactive pathways in which no alterations were found. (c)AMP indicates (cyclic) adenosine monophosphate; ATP, adenosine triphosphate; BDKR, bradykinin receptor; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; ET_A, endothelin-1 receptor A; ET_B, endothelin-1 receptor B; (c)GMP, (cyclic) guanosine monophosphate; GTP, guanosine triphosphate; NO, nitric oxide; PDE3, phosphodiesterase 3; PDE5, phosphodiesterase 5; PGH_2, prostaglandin H2; PGI_2, prostacyclin; PGIS, prostacyclin synthase; PKA, protein kinase A; PKG, protein kinase G; SNP, sodium nitroprusside; TXA2, thromboxane A2; TXA2R, thromboxane A2 receptor; TXAS, thromboxane synthase.

vascular resistance in CDH infants [15,16]. Our observations of an enhanced vasoconstrictor response to U46619 in CDH placentas indicate that this vascular bed is also increasingly sensitised to thromboxane. A new therapeutic strategy may therefore be to inhibit these vasoconstrictor effects using a thromboxane receptor antagonist. Interestingly, in agreement with this concept, the thromboxane receptor antagonist NTP42, was demonstrated to reduce pulmonary hypertension and pulmonary vascular remodelling in a rat model of pulmonary arterial hypertension [37]. In infants with CDH, administration of tolazoline, an alpha adrenergic receptor antagonist that reduces plasma thromboxane levels, also resulted in reduced pulmonary vascular resistance [16]. On the other hand, it may be possible to reduce the pulmonary vascular resistance by administering a prostaglandin agonist, like iloprost or prostacyclin. Such agonists hold promise for the treatment of neonatal pulmonary hypertension [38–40]. Although the decisions to initiate such therapy are influenced by many factors and differ between centres, prostacyclin is only used in less than 4 % of patients with CDH [41,42]. This is of particular interest as we showed that – unlike the NO-donor SNP – iloprost retained its vasodilator properties in fetoplacental arteries of fetuses with CDH.

Our data confirm that vasodilation through the NO-cGMP pathway is impaired in CDH [13], and may provide an explanation for the impaired responsiveness to iNO that is often observed in postnatal treatment of CDH-induced pulmonary hypertension [8]. Normally, NO has an important physiological role in decreasing the pulmonary vascular resistance at birth [43]. Attenuated responses to both the endothelium-independent NO-donor SNP as well as the endothelium-dependent vasodilator bradykinin (which induces vasodilation by stimulating endothelial NO production), suggest a downstream impairment in vascular smooth muscle cells. Indeed, our data indicate decreased mRNA expression of the sGC subunit GUCY1A1 and PRKG1 in CDH placentas, which could underlie the impaired vasodilation by reducing cGMP formation and lowering intracellular Ca^{2+} concentrations, respectively. The expression of sGC in pulmonary blood vessels is proposed to be stimulated by an enhanced pulmonary blood flow at birth, possibly involving a concurrent developmental switch in sGC expression from smooth muscle cells to endothelial cells [44,45]. We therefore speculate that reduced pulmonary blood flow may contribute to decreased sGC expression and NO responsiveness in CDH. Based on our experimental set-up that did not include flow, it should therefore be noted that an additional contribution of endothelial impairment by shear stress cannot be excluded. Indeed, others have shown decreased pulmonary eNOS activity and NO production in CDH [10,11].

In CDH infants with pulmonary hypertension refractory to iNO, preliminary data suggest that sildenafil administration may reduce pulmonary vascular resistance and improve cardiac output [46]. We are currently awaiting the results from a prematurely terminated randomised controlled trial comparing sildenafil to iNO treatment in CDH-induced pulmonary hypertension [47]. However, both PDE5 inhibition and SNP were less effective in the pulmonary arteries of lambs with CDH compared to controls, despite increased expression of PDE5 in pulmonary arteries of rats with CDH [48], and a therefore larger expected-effect of PDE5 inhibition [13]. The placenta does not seem the best model to study the effectiveness of PDE inhibitors, as we were unable to demonstrate any effects of sildenafil or milrinone, and the effect of sildenafil in the placenta was shown to be modest at most in a previous study [36].

ET-1 receptor antagonists can be used as therapeutics in pulmonary hypertension, and may be of interest in CDH as an earlier study reported an increased expression of ET-1 receptors in the lungs of CDH infants [5]. Our data, however, showed that vasoconstriction to ET-1 was not different in CDH placentas compared to healthy placentas. Thus, most likely the altered expression of ET-1 receptors is limited to the pulmonary vasculature.

5. Limitations

Despite many similarities between the fetoplacental and pulmonary arteries it should be noted that the placenta is not neuronally innervated in contrast to the lungs. This means that the alterations in circulating factors can directly affect placental vascular resistance, but it also means that the fetoplacental vessels will not exhibit the potential contribution of neuronal innervation to pulmonary hypertension. Moreover, for practical reasons we limited our vascular studies to second order arteries, which are characterized by a larger diameter than vessels that are generally considered resistance vessels.

Infants with CDH require intensive care immediately after birth and therefore there is usually a planned birth around 38 weeks gestational

age. In our cohort, all CDH fetuses were vaginally delivered, whereas all controls were delivered through caesarean section. For logistical reasons, this was the best available control group. However, as such, we cannot exclude the effects that induction of labour and mode of labour might have on the placentas. Secondly, infants born after induction of labour had an earlier gestational age and lower birth weight than infants born after caesarean section. As birth percentiles were not different between groups, fetal growth did not affect our results.

Although in this proof-of-concept study, we were able to identify alterations in vasoreactivity of fetoplacental vessels in CDH and relate these to changes in mRNA expression in placental homogenates, it is important for future studies to look at the specific localisation of the altered proteins in the placenta. Moreover, the study was not designed to associate these changes with the individual pulmonary condition of the included infants. Further research in larger cohorts is necessary to determine whether these fetoplacental changes correlate with the alterations in pulmonary vascular reactivity and the postnatal pulmonary treatment responses for each individual CDH infant, thereby taking into account the severity of estimated pulmonary hypoplasia.

6. Conclusions

Our data suggest that the fetoplacental arteries of fetuses with CDH display enhanced vasoconstriction through the thromboxane A2 receptor, as well as attenuated vasodilation through the NO-cGMP pathway, possibly due to reduced expression of guanylate cyclase subunit alpha and protein kinase cGMP-dependent 1. Similarities between our observations and the pulmonary vascular alterations currently described in CDH suggest that fetoplacental arteries may provide a unique opportunity to study and predict pulmonary vascular therapeutic responses in infants who are at increased risk to develop pulmonary vascular diseases. These results also have implications beyond CDH, as other conditions such as fetal growth restriction, are also associated with alterations in fetoplacental vasoreactivity [25] and an increased risk of pulmonary complications [49,50].

Statement of ethics

The research protocol was approved for the CDH subjects (MEC-2021-0118) and exempted from approval for the healthy subjects (MEC-2016-418 and MEC-2017-418) by the local medical ethical committee according to the Dutch medical research with human subjects law.

Funding/support

EJJHO and PLJD are supported by a grant from Sophia Children's Hospital Foundation (SSWO, grant S19-12).

Authors' contribution

EJJH-O, MB, MSH, SHPS, AJE, AHJD, IKMR, and PLJD were all involved in the conception of this paper. EJJH-O, MB, AHJD, IKMR, and PLJD conceptualised and designed the study. EJJH-O, MB, and MSH collected and analysed the data. EJJH-O, MB, MSH, AHJD, IKMR, and PLJD contributed to the data interpretation. EJJH-O, MB, and MSH wrote the manuscript and designed the figures, which were critically reviewed by all authors. All authors contributed to the article and approved the final version of the manuscript.

Data availability statement

The data that support the findings of this study are available from P. L.J. DeKoninck upon reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

Acknowledgements

Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2023.11.015.

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