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## The placenta in fetal congenital heart disease

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# CHAPTER 5

## mRNA expression to assess hypoxia and angiogenesis in placental tissue of fetuses with a congenital heart disease

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## ABSTRACT

### Objective

Delayed fetal neurodevelopment, lower birthweight and placenta abnormalities are related to congenital heart defects (CHD). We explored mRNA expression assessment of candidate genes related to fetal hypoxia and angiogenesis in decidual tissues of pregnancies with different types of fetal CHD, classified based on aortic flow and oxygenation.

### Method

In this prospective case-control study, mRNA expression was assessed for eighteen candidate genes related to fetal hypoxia and angiogenesis in decidual (maternal) tissues of fetal simple transposition of the great arteries (TGA) (n=14) and left sided CHD (n=13) and in healthy controls (n=31). Cases with a fetal syndrome/genetic abnormality, termination of pregnancy, intra-uterine fetal demise or multiple pregnancies were excluded.

### Results

There were no significant differences in gene expression of the candidate genes between CHD cases and controls and between cases with fetal simple TGA and cases with fetal left sided CHD. Clustering analysis did not differentiate subgroups within the study population.

### Conclusion

Fetal hypoxia and altered angiogenesis on the level of mRNA expression in decidual tissue were not significantly different between CHD cases and controls and between cases with fetal simple TGA and cases with fetal left sided CHD. We hypothesize that other (angio)genetic and molecular pathways lead to morphological placental alterations in pregnancies with fetal CHD.

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## INTRODUCTION

Congenital heart defects (CHD) occur with an incidence of five to eight per thousand newborns, making them the most common congenital anomalies.<sup>1</sup> As fetal and neonatal care have advanced significantly over the past decades, survival improved and the focus of innovation and research has shifted to improving of long-term outcome and quality of life of CHD patients.<sup>1-3</sup> An important aspect of prenatal counseling for parents now includes the potential for neurodevelopmental delay, given its significant impact on the quality of life.<sup>4-10</sup>

Delay in brain maturation and reduced brain volume have been observed through fetal ultrasound and neonatal MRI in CHD cases, suggesting that altered neurodevelopment may occur as early as in the fetal period.<sup>4,5,11,12</sup> Recent studies investigating fetal brain development in CHD have shown that fetal growth restriction (FGR) and placenta insufficiency, resulting in chronic fetal hypoxia, have a more significant impact on fetal brain growth than aortic flow and oxygenation.<sup>6-9</sup> Reduced oxygenation has been identified in the umbilical vein of CHD cases, further supporting the hypothesis that the placenta plays a critical role in brain development in CHD.<sup>13</sup> Therefore, assessment of placental characteristics in CHD cases is important to further understand this complex interaction.

The placental circulation is essential for fetal development and growth and has a complex structure, in which fetal and maternal compartment interact. Both FGR and pre-eclampsia find their origin in the placenta and are more commonly observed in pregnancies with fetal CHD.<sup>14-23</sup> The interaction between the development of the placenta, CHD, and intrauterine (neuro)development is compelling, as placenta insufficiency is related to impaired brain development in fetal CHD.<sup>6-9,24,25</sup> Furthermore, placental diseases and histological abnormalities are more prevalent in CHD pregnancies, even in the absence of FGR.<sup>23</sup> We hypothesize that altered placental development in CHD, particularly in relation to chronic hypoxia and altered angiogenesis, affects (neuro)development, comparable to the findings in fetuses with FGR without CHD.

Maternal spiral arteries in the decidua begin forming and remodeling in the first trimester of pregnancy, driven by trophoblast invasion and influenced by factors such as oxygen levels, maternal BMI, and angiogenic factors. Decidual cells in the basal plate are formed by proliferation of maternal uterine cells, thereby contributing to the placenta.<sup>26,27</sup> As hypoxia and angiogenesis are interlinked processes that significantly influence the function and development of the placenta, and especially decidual tissue, during pregnancy<sup>28,29</sup>, for this explorative study, we examined mRNA expression profiles in decidual tissue of fetal CHD and assessed molecular changes related to altered placental development. With this technique we determined whether the expression of eighteen candidate genes related to (chronic) hypoxia and/or angiogenesis differs in decidual placenta tissue of fetuses with CHD. Secondly, to evaluate the hemodynamic effects of the heart defects on the placenta, we compared the expression of genes related to

(chronic) hypoxia and angiogenesis in decidual tissue of fetuses with two distinct types of CHD: (1) reduced oxygenation and normal cerebral blood flow, represented by simple transposition of the great arteries (TGA), and (2) left-sided CHD, characterized by reduced aortic flow, including hypoplastic left heart syndrome (HLHS), coarctation of the aorta (CoA), aortic valve stenosis (AoS), and/or aortic arch hypoplasia (AAH).

## METHODS

### Patient selection

In this prospective case-control study, 14 CHD cases with reduced oxygenation (simple TGA), 13 CHD cases with reduced flow (4 HLHS, 4 CoA, 2 AoS, 3 AAH) and 31 healthy controls were identified in the biobank of the Leiden University Medical Center (LUMC) in The Netherlands. In this biobank, clinical data and placental tissue of pregnancies with severe fetal CHD were prospectively collected from April 2020 to June 2022. Multiple pregnancies, pregnancies with a genetic abnormality and/or extracardiac malformations, cases with termination of pregnancy and cases with intra-uterine fetal demise were excluded from the study.

The classification of CHDs into distinct groups, based on theoretical hemodynamics, has been previously described.<sup>7,30,31</sup> This study includes cases from two groups: one with normal cerebral blood flow but reduced oxygenation, including simple TGA cases, and another with reduced cerebral blood flow (including left-sided CHD).

### Data collection

The LUMC biobank consists of all consecutive cases of fetal CHD, registered based on cardiac diagnosis. Included cases followed a standard clinical pathway of ultrasound examinations every four weeks for fetal biometry, Doppler, and neurosonography. The biobank is linked to the PRECOR-registry, which registers all CHD fetal and neonatal cases in CAHAL (Centre for Congenital Heart Diseases Amsterdam Leiden), and stores clinical data and outcomes.

After identification of cases, data on maternal characteristics (age, parity, medical history, smoking, obstetric history), fetal characteristics (gender, intra-uterine growth), and the course of pregnancy (mode of birth, gestational age at time of birth, pregnancy complications) were extracted from PRECOR and additional information was retrieved electronic patient files. Data on placental weight were retrieved from the biobank files.

Placenta samples of the identified cases were extracted from the biobank. The samples were taken from the maternal decidua, central (not peripheral) on the placental plate. Samples had a diameter of around 0.5cm and were taken with sterile gloves and instruments as soon as possible after birth, with a maximum of six hours, and stored in RNA-later in a freezer at -80 °C.

## Gene expression analysis

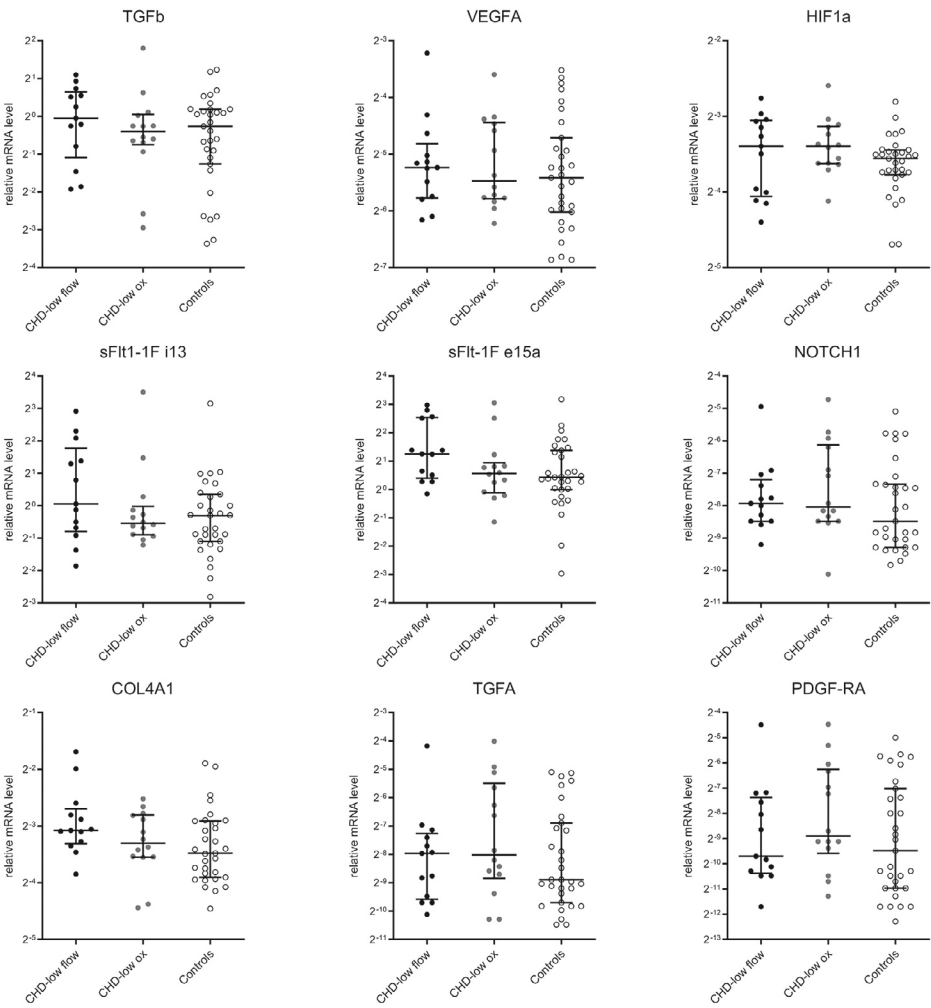
The biopsies were kept on dry ice until the start of the experiment. One quarter of a biopsy was cut off and put directly into 900 µL of Lysis buffer from the RNA kit. Homogenization took place by using a Cordless Pestle Motor with disposable pestles (VWR international B.V.) for 30 seconds. RNA extraction was then performed with NucleoSpin® columns (Macherey-Nagel, Düren, Germany). RNA quantity and integrity were determined on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized from 330 ng of total RNA and real-time quantitative PCR was performed, as described in more detail elsewhere.<sup>32,33</sup> The PCR program consisted of 10 minutes 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

**Table 1: Clinical characteristics of cases and controls studied**

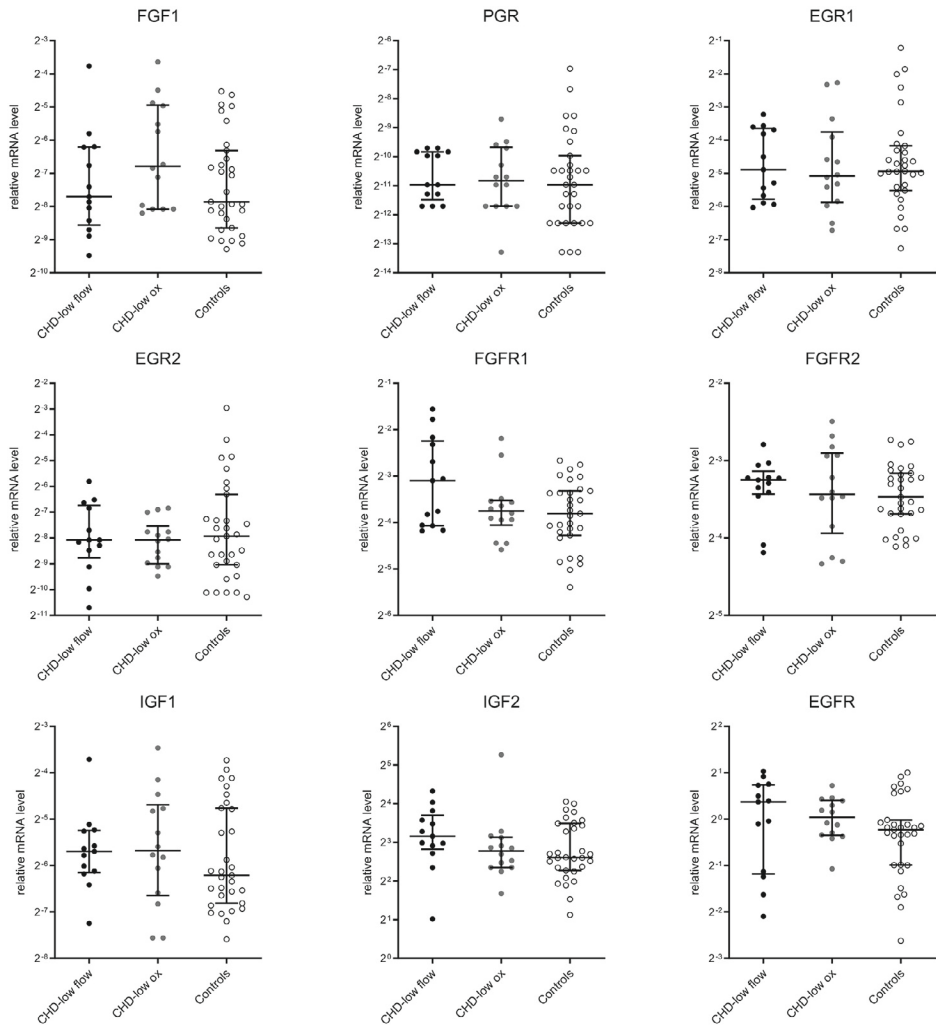
	CHD with reduced oxygenation† n = 14	CHD with reduced flow‡ n = 13	p-value§	Controls n = 31	p-value¶
Maternal age (mean, SD)	32.0 (4.1)	32.6 (4.5)	N.S.	33.3 (4.4)	N.S.
Parity (n,%)			N.S.		N.S.
0	5 (35.7)	7 (53.8)		14 (45.2)	
1	7 (50.0)	4 (30.8)		13 (41.9)	
2	0	2 (15.4)		3 (10.3)	
3	1 (7.1)	0		1 (3.2)	
> 3	1 (7.1)	0		0	
Gestational age at time of delivery (n, %)					
< 37 weeks	1 (7.1)	0	N.S.	0	N.S.
37-41 weeks	13 (92.9)	12 (92.3)		28 (90.3)	
> 41 weeks	0	1 (7.7)		3 (10.7)	
Pregnancy complication in current pregnancy (n, %)					
Pregnancy induced hypertension	0	0	N.A.	0	N.A.
Pre-eclampsia	0	0	N.A.	0	N.A.
Gestational Diabetes	2 (14.3)	0	N.S.	0	N.S.
Birth mode (n, %)			N.S.		N.S.
Vaginal without MRP	11 (78.6)	11 (84.6)		25 (80.6)	
Cesarian section and/or MRP††	3 (21.4)	2 (15.4)		6 (19.4)	
Gender			N.S.		N.S.
Male	11 (78.6)	6 (46.2)		15 (48.4)	
Female	3 (21.4)	7 (53.8)		16 (51.6)	
Birthweight (n, %)					
< 2.3 <sup>th</sup> percentile	0	0	N.A.	0	N.A.
> 97.7 <sup>th</sup> percentile	0	2 (15.4)	N.S.	0	N.S.
Genetic testing (n, %)			<b>&lt;0.001</b>		<b>&lt;0.001</b>
Yes	5 (35.7)	13 (100)		0	
No	9 (64.3)	0		31 (100)	

† simple transposition of the great arteries; ‡ hypoplastic left heart syndrome, coarctation, aortic valve stenosis and/or aortic arch hypoplasia; § p-value CHD with reduced flow vs CHD with reduced oxygenation; ¶ p-value CHD vs controls; †† MRP = manual removal of the placenta









**Figure 1 – Scatterplots showing the medians of RNA expression in decidual tissue per gene in different groups of fetal CHD.** The x-axis represents the type of CHD, the y-axis represents the relative mRNA level, the line indicates the median

Primers for PCR were designed using Primer-Blast, a Basic Local Alignment Search Tool (NIH), and amplicon specificity was checked by a melting curve analysis at the end of each PCR run. Primer sequences are described in Supporting Information Table 1. Five reference genes were measured. Four of these (*HPRT*, *RPL13a*, *B-actin*, *GAPDH*) showing the highest correlation with each other, were further used for accurate gene expression normalization. Eighteen candidate genes related to (chronic) hypoxia and/or angiogenesis (*TGFB1*, *VEFGA*, *HIF1a*, *sFlt-1F i13*, *sFlt-1F e15a*, *NOTCH1*, *COL4A1*, *TGFA*, *PDGFRA*, *FGF1*, *PGR*, *EGR1*, *EGR2*, *FGFR1*, *FGFR2*, *IGF1*, *IGF2*, *EGFR*) were assessed.<sup>23</sup> Expression of the candidate genes and control genes was assessed in the samples (in duplicate). An average (AVR) was calculated for each sample. Per sample, the geometric

mean of the four reference genes was calculated. The delta quantification cycle ( $\Delta Cq$ ) was calculated per gene for each sample. As a validation, histochemistry was performed on five samples, to determine the correct location of the samples. In this samples we found decidual tissue, characterized by differentiated endometrial stromal cells (decidualization) with leukocytes, present in normal decidua (NK cells, T-cells, dendritic cells, and macrophages), including superficial anastomoses of the spiral arteries.

Statistical analyses

Continuous data with normal distributions are presented as means  $\pm$  standard deviation (SD). Categorical data are presented as numbers and percentages (n, %). Independent sample t-tests and Chi-square tests were used where appropriate. An alpha of  $<0.05$  is considered significant. Missing data are described and analyzed. A cluster analysis was performed, using R software (version 3.6.2), to identify subgroups of patients. 280/260 ratios were used to assess the purity of the RNA.

RESULTS

Characteristics of study subjects

Patient characteristics are shown in Table 1, with no significant differences between of cases and controls, and between cases with TGA and cases with left sided CHD. There were no cases with relevant maternal illness, maternal teratogenic medication and maternal smoking. There were no cases with pre-eclampsia (PE), pregnancy induced hypertension (PIH) and/or stillbirth in previous pregnancies. Only prenatal genetic testing differed significantly between the groups.

Table 2: Gross placenta characteristics per type of CHD

	CHD with reduced oxygenation† n = 14	CHD with reduced flow‡ n = 13	p-value§	Controls n = 31	p-value¶
Placenta weight percentile (n, %)					
< 10 <sup>th</sup> percentile	4 (28.6)	5 (38.5)	N.S.	5 (16.1)	N.S.
> 90 <sup>th</sup> percentile	0	2 (6.7)	N.S.	3 (9.7)	N.S.
Umbilical cord insertion (n, %)			N.S.		N.S.
Central/paracentral	14 (100)	12 (92.3)		29 (93.5)	
Marginal/velamentous	0	1 (7.7)		2 (6.5)	

† simple transposition of the great arteries; ‡ hypoplastic left heart syndrome, coarctation, aortic valve stenosis and/or aortic arch hypoplasia; § p-value CHD with reduced flow vs CHD with reduced oxygenation; ¶ p-value CHD vs controls

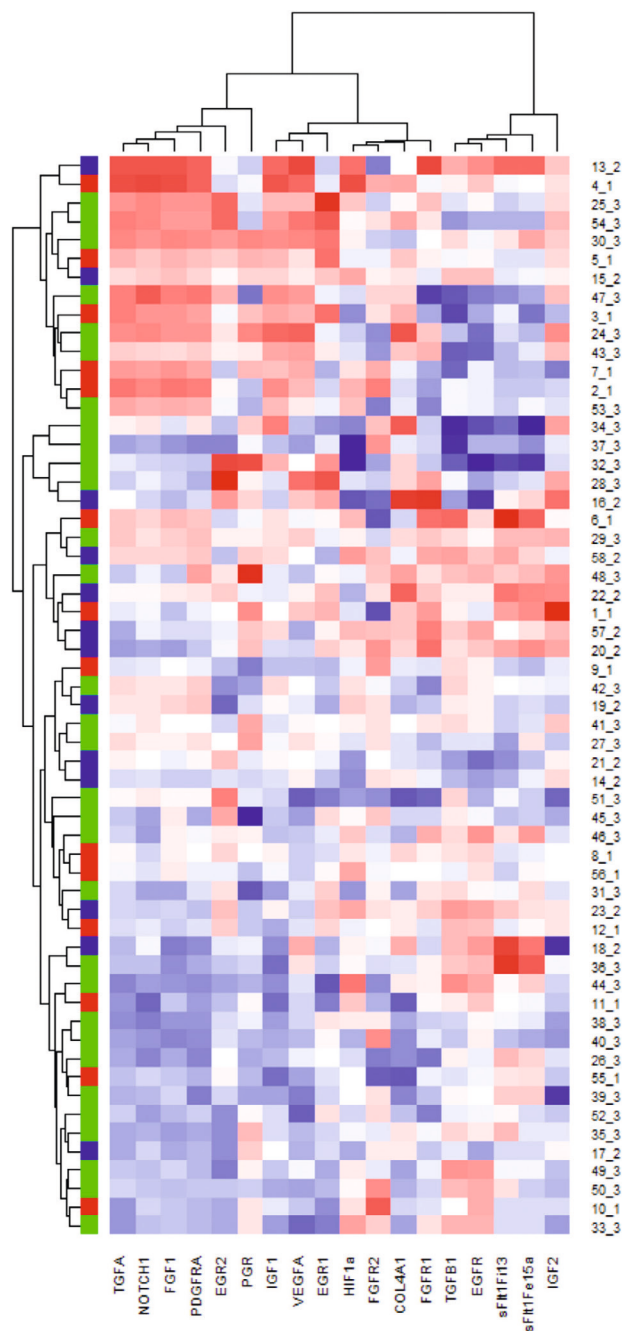
Placenta weight below the 10<sup>th</sup> percentile was more frequently observed in CHD cases (28.6% in CHD cases with TGA, 38.5% in cases with left sided CHD, as compared to 16.1% in controls), without statistical significance (Table 2).

## Gene expression

Scatterplots with mRNA expression per gene are illustrated in Figure 1 and descriptions of the function and clinical associations of the analyzed genes are shown in Table 3. For *HIF1a*, *sFlt-1F i13* and *sFLT-1F e15a*, high levels were associated with adverse pregnancy outcomes. In the other genes, low levels were associated with adverse pregnancy outcomes (Table 3). As presented in Figure 1, no significant differences or trends in medians could be found between the different groups and for all candidate genes. In addition, large variations (scatters) are found.

**Table 3: Description of genes assessed in decidual tissue**

Gene	Function	Clinical associations
TGFB1	Cell growth, cell maturation, apoptosis	Fetal growth, pre-eclampsia
VEGFA	Development of vascular epithelial cells (angiogenesis)	Fetal growth, pre-eclampsia
HIF1a	Response to hypoxia	Fetal growth, fetal hypoxia
sFlt-1F i13	Development of vascular epithelial cells (anti-angiogenic)	Fetal growth, pre-eclampsia
sFlt-1F e15a	Development of vascular epithelial cells (anti-angiogenic)	Fetal growth, pre-eclampsia
NOTCH1	Vascular patterning	Preterm labor, fetal growth, pre-eclampsia
COL4A1	Development of collagen and vascular endothelium	Fetal neurological development, fetal (cerebral) bleeding, fetal growth
TGFA	Cell growth, cell proliferation, cell development	Fetal growth, oral cleft
PDGFRA	Cell growth, cell proliferation	Fetal cardiac development, fetal facial development
FGF1	Embryonic development, cell growth, morphogenesis, tissue repair	Fetal growth, pre-eclampsia
PGR	Immune response, angiogenesis	Preterm labor
EGR1	Embryo implantation, cell differentiation, immune response	Fetal growth, fetal hypoxia
EGR2	Embryo implantation, cell differentiation and migration, immune response	Fetal growth, fetal hypoxia
FGFR1	Development of nerve cells	Neurodevelopment, neurological congenital malformations
FGFR2	Angiogenesis, development of nerve cells	Neurodevelopment, congenital malformations of the musculoskeletal system
IGF1	Tissue growth	Fetal growth, placental development, cardiac development, maternal diabetes
IGF2	Tissue growth	Fetal growth, placental development, cardiac development, maternal diabetes
EGFR	Embryo implantation, cell growth, cell differentiation	Fetal growth, congenital anomalies of the (female) reproductive system



**Figure 2 –Clustering analysis cases based on CHD type and mRNA levels in the decidual samples**  
*Cluster 1 Red* = Group 1 (CHD with reduced oxygenation); *Cluster 2 Blue* = Group 2 (CHD with reduced flow); *Cluster 3 Green* = Group 3 (healthy controls).  
This figure shows the results of a hierarchical cluster analysis with cases grouped into distinct clusters.



There were no significant differences in gene expression of the candidate genes between CHD cases and controls (Table 4a) and between TGA cases and left sided CHD (Table 4b). In addition, *FLT-1/sFLT-1* and *IGF1/IGF2* ratios were plotted and no significant differences were found. A graph of mRNA expression per gene is illustrated in Supporting Information Figure 1, illustrating gene expression in our samples based on the delta quantification cycle ( $\Delta Cq$ ) per candidate gene for all samples combined. In addition, 280/260 ratios were assessed to assess the purity of the RNA, which were all above 1.95. This indicates the used RNA was pure.

A cluster analysis was performed (Figure 2) in which the different groups (indicted by the different colors) did not cluster for the different genes (X-axis) based on the samples (Y-axis). This indicates that with this analysis we could not differentiate subgroups within the study population and thereby not establish a relation between gene expression and the subgroups.

**Table 4a: Messenger RNA levels in decidual tissue of fetal CHD cases vs controls**

	CHD n = 27	Controls n = 31	p-value
TGFB1 (median $\Delta Cq$ , IQR)	1.000 (0.850)	0.865 (0.721)	0.569
VEFGA (median $\Delta Cq$ , IQR)	0.026 (0.023)	0.023 (0.023)	0.378
HIF1a (median $\Delta Cq$ , IQR)	0.095 (0.039)	0.085 (0.019)	0.085
sFlt-1F i13 (median $\Delta Cq$ , IQR)	0.778 (1.914)	0.808 (0.811)	0.221
sFlt-1F e15a (median $\Delta Cq$ , IQR)	1.707 (1.402)	1.348 (1.599)	0.163
NOTCH1 (median $\Delta Cq$ , IQR)	0.004 (0.005)	0.003 (0.005)	0.114
COL4A1 (median $\Delta Cq$ , IQR)	0.116 (0.052)	0.090 (0.066)	0.077
TGFA (median $\Delta Cq$ , IQR)	0.004 (0.009)	0.007 (0.007)	0.272
PDGFRA (median $\Delta Cq$ , IQR)	0.007 (0.006)	0.001 (0.007)	0.315
FGF1 (median $\Delta Cq$ , IQR)	0.007 (0.015)	0.004 (0.010)	0.246
PGR (median $\Delta Cq$ , IQR)	0.001 (0.001)	0.001 (0.001)	0.591
EGR1 (median $\Delta Cq$ , IQR)	0.030 (0.060)	0.033 (0.034)	0.821
EGR2 (median $\Delta Cq$ , IQR)	0.004 (0.006)	0.004 (0.011)	0.680
FGFR1 (median $\Delta Cq$ , IQR)	0.077 (0.095)	0.071 (0.049)	0.178
FGFR2 (median $\Delta Cq$ , IQR)	0.010 (0.033)	0.090 (0.034)	0.404
IGF1 (median $\Delta Cq$ , IQR)	0.019 (0.015)	0.014 (0.028)	0.272
IGF2 (median $\Delta Cq$ , IQR)	7.545 (4.189)	6.103 (6.103)	0.330
EGFR (median $\Delta Cq$ , IQR)	1.110 (0.601)	0.854 (0.483)	0.141

**Table 4b: Messenger RNA levels in decidual tissue in different types of fetal CHD**

	CHD with reduced oxygenation <sup>†</sup> n = 14	CHD with reduced flow <sup>‡</sup> n = 13	p-value
TGFB1 (median $\Delta$ Cq, IQR)	0.759 (0.439)	0.965 (1.094)	0.302
VEFGA (median $\Delta$ Cq, IQR)	0.023 (0.028)	0.027 (0.017)	0.943
HIF1a (median $\Delta$ Cq, IQR)	0.095 (0.033)	0.095 (0.060)	0.793
sFlt-1F i13 (c, SD)	0.685 (0.449)	1.038 (2.845)	0.257
sFlt-1F e15a (median $\Delta$ Cq, IQR)	1.477 (0.990)	2.380 (4.497)	0.094
NOTCH1 (median $\Delta$ Cq, IQR)	0.004 (0.012)	0.004 (0.004)	0.793
COL4A1 (median $\Delta$ Cq, IQR)	0.101 (0.058)	0.118 (0.054)	0.185
TGFA (median $\Delta$ Cq, IQR)	0.004 (0.020)	0.004 (0.005)	0.402
PDGFRA (median $\Delta$ Cq, IQR)	0.002 (0.012)	0.001 (0.005)	0.402
FGF1 (median $\Delta$ Cq, IQR)	0.009 (0.028)	0.005 (0.011)	0.185
PGR (median $\Delta$ Cq, IQR)	0.001 (0.001)	0.001 (0.001)	0.867
EGR1 (median $\Delta$ Cq, IQR)	0.030 (0.057)	0.034 (0.062)	0.830
EGR2 (median $\Delta$ Cq, IQR)	0.004 (0.003)	0.004 (0.007)	0.650
FGFR1 (median $\Delta$ Cq, IQR)	0.074 (0.027)	0.117 (0.152)	0.239
FGFR2 (median $\Delta$ Cq, IQR)	0.092 (0.069)	0.105 (0.021)	0.720
IGF1 (median $\Delta$ Cq, IQR)	0.019 (0.029)	0.019 (0.012)	0.867
IGF2 (median $\Delta$ Cq, IQR)	6.841 (3.680)	8.911 (5.933)	0.085
EGFR (median $\Delta$ Cq, IQR)	1.028 (0.539)	1.294 (1.230)	0.616

<sup>†</sup> simple transposition of the great arteries

<sup>‡</sup> hypoplastic left heart syndrome, coarctation, aortic valve stenosis and/or aortic arch hypoplasia

## DISCUSSION

Placenta-related complications, such as FGR and PE, and histological placental abnormalities occur more frequently in pregnancies with fetal CHD.<sup>23</sup> Delayed fetal neurodevelopment found in CHD may be (partly) explained by reduced fetal growth and changes in placental development, causing fetal hypoxia.<sup>6-9</sup> Therefore, one would expect altered expression of genes related to angiogenesis and hypoxia in decidual tissue of these cases. Our results, however, could not establish a relation between fetal congenital heart disease and altered mRNA expression of genes related to hypoxia and angiogenesis in decidual tissue retrieved after birth. Moreover, we illustrated that mRNA expression assessment is an adequate method to analyze gene expression in decidual tissue.

While our findings did not reveal a direct relationship between fetal CHD and altered gene expression in decidual tissue, the complex interplay between placental development and fetal heart abnormalities suggests that other underlying mechanisms may contribute to

the observed outcomes. The origin of CHD is multifactorial and numerous genes that influence both the development of the placenta and the fetal heart have been identified.<sup>23,34</sup> The placenta-heart axis illustrates the concurrent development of the placenta and the fetal heart and according to this theory, placenta insufficiency and hypoxia in the first trimester may contribute to the development of fetal CHD. This theory is supported by the results of a study by Krala *et al*, in which the impact of early uterine dysfunction on the etiology of, among others, CHD in mice.<sup>35</sup> The other way around, circulatory changes caused by the CHD may cause abnormal development of the placenta. This may cause eventually placenta insufficiency, which contributes to the fetal hypoxia, causing fetal FGR, delayed (neuro)development and adverse pregnancy outcomes.<sup>36</sup> Accordingly, signs of fetal hypoxia and placenta histological abnormalities are more frequently described in placentas of pregnancies with fetal CHD.<sup>23</sup>

In a previous study by Courtney *et al*, transcriptomic and histologic analyses of placental tissues in a small cohort of cases with HLHS (n=16) and TGA (n=8) were performed.<sup>37</sup> In their study, HLHS cases had reduced birthweight and transcriptomic and histologic placental alterations related to reduced cell activity and nutrient transport capability. In TGA cases, normal birthweight and increased cell activity and nutrient transport capability were found. In addition, they found reduced transcriptional levels of the IGF-pathway, which is related to impaired placental development, fetal growth and cardiac development, in HLHS. Strikingly, in TGA cases transcriptional levels of this pathway were elevated. This suggests different compensatory mechanisms between the different forms of CHD, as in HLHS abnormal placental development and FGR was found, while in TGA cases a compensatory mechanism was demonstrated, preventing FGR.<sup>37</sup> In our study, we could not demonstrate altered mRNA expressions of *IGF-1*, *IGF-2* or other candidate genes known to contribute to hypoxia or vascular development/angiogenesis in decidual tissue. These findings, in combination with the results of Courtney *et al*, suggest that placental changes in these cases are caused by hemodynamic effects of the heart defect and compensatory mechanisms, rather than etiological (angio)genetic changes.

Another explanation for the observation that decidual RNA levels were not related to fetal CHD could be the timing of the sampling of the tissue. For the current study, decidual tissue retrieved after birth was used for mRNA expression assessment of the candidate genes, but hypoxia and alterations in angiogenesis may already have been present in the first trimester of pregnancy, especially as maternal vessels in the decidua develop early in the first trimester.<sup>36</sup> In addition, in this study only decidual tissue was assessed, as we know from PE that placenta angiogenesis and hypoxia are important factors influencing the development of the placenta and especially the composition and function of the decidua.<sup>38-45</sup> As the decidual tissue contains maternal vessels rather than fetal vessels and fetal compartments such as villi and trophoblasts.<sup>46</sup> Therefore, changes in genetic expression in fetal vessels, villi and trophoblasts may be masked by

the predominant maternal vessels. Thereby, placental and parenchymal development is dynamic and variable and there is a variability in lesions. It is possible that altered mRNA expression of the candidate genes was present in the first trimester, with a diminished effect during the further course of the pregnancy. Thereby, both the origin of CHD and the development of the decidua and the placenta are multifactorial and influenced by a lot of internal and external factors.<sup>23,34,35</sup> Gene expression of the described candidate genes may be effected by other factors, such as sex differences, maternal BMI and hormones, which could overshadow the effect of the CHD.

In addition, as previously described according to the placenta-heart axis, circulatory changes and hypoxia caused by the CHD affect the development of the placenta and may cause histological changes in placenta tissue (mainly the decidua, corresponding with the maternal side of the placenta) that correspond with placenta insufficiency.<sup>36</sup> Those alterations may have influenced gene expression, however, the effect on RNA expression may be of little significance. Another explanation for our findings is that other genes than the ones assessed here may be related to this phenomenon. Nevertheless and to our knowledge, we assessed relevant genes that are related to hypoxia and altered angiogenesis. The next step could be to perform full RNA sequencing or DNA methylation on placenta tissue of all sides of the placenta (including the fetal part), as these are more elaborate methods that could be performed to identify genes and molecular pathways that have a possible relation with fetal CHD and placental development.<sup>47,48</sup>

## Strengths

We demonstrated that mRNA expression assessment is adequate and suitable for application on decidual tissue, enabling novel opportunities for further research.

By carefully rejecting the hypothesis that altered placentation in fetal CHD is explained or demonstrated by differences in expression of the chosen genes related to (chronic) hypoxia and/or angiogenesis, we have a new insight that brings us a step closer to the understanding of the relation between CHD and altered placental development. With this information, future research can focus on different aspects on placental development and the development of fetal CHD.

## Limitations

The small sample size causes a high variability in our data. Though, the absence of identifiable patterns in our data suggests that the lack of significance is more likely due to the true lack of differential expression of the findings, rather than being attributable to the small sample size. In addition, for this exploratory study we only analyzed samples from the decidua rather than fetal placental compartments such as villi or trophoblasts. As hypoxia can influence both villous maturation and trophoblast development<sup>26,49,50</sup>, placental changes that find their origin in the fetal compartment of the placenta might not be identified in this study.



Not all cases underwent genetic testing. Cases with a genetic diagnosis, either pre- or postnatally tested, were excluded from this study, however, not all cases were tested. In the group of CHD cases with low oxygenation, 35.7% of the cases had genetic testing, all with normal results. This group comprises cases with a simple TGA, a diagnosis that is seldomly related to an underlying genetic disorders<sup>51</sup>. All left sided CHD cases were tested and Whole Exome Sequencing was performed in 9/14 left sided CHD cases (64.3%). All cases included in this study with additional congenital abnormalities and/or dysmorphias after birth were excluded as well. Therefore, we expect that the genetic profile of the vast majority of the included cases is normal.

## CONCLUSION

Though placenta histological abnormalities are more frequently described in placentas of pregnancies with fetal CHD, a relation between fetal CHD and mRNA expression in decidual tissue of genes related to hypoxia and angiogenesis could not be established in this study. This may be explained by the timing and location of the biopsies, the selection of candidate genes or the fact that a different molecular pathway leads to the morphological placental changes in these pregnancies. In future studies, full RNA sequencing or DNA methylation can be used to identify genes and molecular pathways that have a possible relation with fetal CHD and abnormal placentation. In addition, assessment of the fetal compartment of the placenta, including villi and trophoblasts, should be included.

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