Title page Early Human Development

Cardiac defects, nuchal edema and abnormal lymphatic development are not associated with morphological changes in the ductus venosus.

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Abstract

Background: In human fetuses with cardiac defects and increased nuchal translucency, abnormal ductus venosus flow velocity waveforms are observed. It is unknown whether abnormal ductus venosus flow velocity waveforms in fetuses with increased nuchal translucency are a reflection of altered cardiac function or are caused by local morphological alterations in the ductus venosus.

Aim: The aim of this study was to investigate if the observed increased nuchal translucency, cardiac defects and abnormal lymphatic development in the examined mouse models are associated with local changes in ductus venosus morphology.

Study design: Mouse embryos with anomalous lymphatic development and nuchal edema (*Ccbe1*$^{-/-}$ embryos), mouse embryos with cardiac defects and nuchal edema (*Fkbp12*$^{-/-}$, *Tbx1*$^{-/-}$, *Chd7*$^{fl/fl}$*;Mesp1Cre, Jarid2*$^{-/-NE+}$ embryos) and mouse embryos with cardiac defects without nuchal edema (*Tbx2*$^{-/-}$*, Fgf10*$^{-/-}$*, Jarid2*$^{-/-NE-}$ embryos) were examined. Embryos were analyzed from embryonic day (E) 11.5 to 15.5 using markers for endothelium, smooth muscle actin, nerve tissue and elastic fibers.

Results: All mutant and wild-type mouse embryos showed similar, positive endothelial and smooth muscle cell expression in the ductus venosus at E11.5-15.5. Nerve marker and elastic fiber expression were not identified in the ductus venosus in all investigated mutant and wild-type embryos. Local morphology and expression of the used markers were similar in the ductus venosus in all examined mutant and wild-type embryos.

Conclusions: Cardiac defects, nuchal edema and abnormal lymphatic development are not associated with morphological changes in the ductus venosus. Ductus venosus flow velocity waveforms most probably reflect intracardiac pressure.

Key words: cardiac defect; ductus venosus; endothelium; increased nuchal translucency; morphology; nuchal edema

Introduction

The ductus venosus is an embryonic shunt located at the level of the liver that connects the umbilical vein and the inferior vena cava [1;2]. The function of the ductus venosus is to transport well-oxygenated blood directly to the heart [1;2]. The narrowest part of the ductus venosus has been suggested to function as an active sphincter to regulate the extent of shunting [1;3;4]. This sphincter mechanism would ensure fetal adaptation to hypoxemia or stress.

The phases of ductus venosus flow velocity waveforms correlate in timing to concurrent phases of the cardiac cycle [5]. Altered ductus venosus flow velocity waveforms may reflect changes in volume and pressure in the cardiac chambers [6]. Ductus venosus flow velocity waveforms are therefore considered to reflect cardiac function [6] and are utilized to assess the fetal hemodynamic performance.

Ultrasound examination of the ductus venosus is increasingly used in daily prenatal obstetrical care [7;8]. Abnormal ductus venosus flow velocity waveforms in the first trimester of pregnancy are related to an increased risk for chromosomal abnormalities, cardiac defects, increased nuchal translucency (NT) and adverse pregnancy outcomes [2;8-16]. The causal mechanism of abnormal first-trimester ductus venosus flow velocity waveforms in fetuses with increased NT is unknown.

Cardiac failure has been suggested to explain altered ductal flow velocity waveforms [2;9;10;14]. But abnormal ductal flow velocity waveforms can not be attributed to a specific cardiac defect that could influence the hemodynamic status [17;18]. Signs of fetal cardiac failure are rarely seen in fetuses with increased NT [19;20] and conflicting evidence on altered intracardiac velocities exists [21;22].

Another theory to clarify abnormal ductus venosus flow velocity waveforms is a local morphological alteration in the ductus venosus. A morphological study of the ductus venosus in embryos with cardiac anomalies and nuchal edema, the morphological equivalent of increased NT, is currently lacking. We tested the hypothesis that nuchal edema, cardiac defects and abnormal lymphatic development are related to local changes in ductus venosus morphology, such as altered endothelial expression or disturbed contributions of smooth muscle cells, and histological construction of the ductus venosus tissue. The morphology of the ductus venosus was examined in three different categories of mutant mouse models; mouse embryos with (i) abnormal lymphatic development and nuchal edema, (ii) cardiac defects with nuchal edema and (iii) cardiac anomalies without nuchal edema.

Material and methods

Embryos

Mouse embryos were analyzed from embryonic day (E) 11.5 to 15.5. These embryonic stages coincide with initial lymphatic developmental processes and the presence of nuchal edema. Cardiovascular development is largely completed at E15.5. These embryonic stages correlate with the timing of the visibility of nuchal edema in human fetuses between 10 and 14 weeks gestational age.

Different knockout and one knockdown mouse models were investigated and compared to wild-type (control) embryos (see Table 1). In the human clinical situation, increased NT is not related to a specific type of cardiac defect, but is associated with a spectrum of cardiac abnormalities. Therefore, multiple different mutant mouse models with lymphatic abnormalities or various cardiac defects with and without the presence of nuchal edema were studied.

First, to examine the ductus venosus in mouse embryos with a lymphatic defect, *Ccbe1*$^{-/-}$ embryos [23] were analyzed. *Ccbe1*$^{-/-}$ embryos display absent lymphatic structures and increased nuchal thickening, as described earlier [23]. Second, to study the ductus venosus in various mouse models with cardiac malformations and nuchal edema, we have analyzed (i) *Tbx1*$^{-/-}$ embryos, showing abnormal development of the cardiac outflow tract, ventricular septal defects and aortic arch anomalies [24;25], (ii) *Jarid2*$^{-/-NE+}$ embryos, displaying ventricular septal defects, non-compaction of the ventricular wall and double outlet right ventricle [26], (iii) *Fkbp12*$^{-/-}$ embryos showing myocardial non-compaction, large ventricular septal defects, hypertrophic trabeculae, deep intertrabecular recesses and thinner left ventricular wall [27] and (iv) *Chd7*$^{fl/fl}$*;Mesp1Cre* embryos, demonstrating ventricular septal defects [28] and a

variety of pharyngeal arch artery defects [29]. Third, to investigate the ductus venosus in diverse mutant mouse models with heart anomalies but without the presence of nuchal edema, we have examined (i) *Tbx2*$^{-/-}$ embryos [30], showing enlarged and dilated ventricles, small endocardial cushions and outflow tract septation defects, such as double outlet right ventricle [31], (ii) *Fgf10*$^{-/-}$ embryos, displaying abnormal direction of the ventricular apex and absent pulmonary arteries and veins [32] and (iii) *Jarid2*$^{-/-NE-}$ embryos [26], showing ventricular septal defects, non-compaction of the ventricular wall and double outlet right ventricle [26]. *Jarid2*$^{-/-}$ embryos showed nuchal edema in some embryos and normal nuchal thickness in other embryos. As a result, *Jarid2*$^{-/-}$ embryos with (*Jarid2*$^{-/-NE+}$) and without (*Jarid2*$^{-/-NE-}$) nuchal edema were examined in two different groups. The number of investigated embryos per mutant mouse model relied on availability. Because of limited availability we could not examine an equal number of the various mutant mouse models (see Table 1).

Guidelines for care and use of mice, approved by the Department of Anatomy, Embryology & Physiology, Academic Medical Center (AMC), Amsterdam, the Netherlands, were followed. Mice were mated overnight and the day of the vaginal plug detection was established as E0.5. Embryos were isolated on E11.5-15.5 and fixed in 4% formaldehyde at 4°C overnight. Subsequently, embryos were dehydrated and the whole embryos were embedded in paraffin. Serial transverse sections of 7µm were made of the ductus venosus area, including all adjacent vessels. Every 5th section was mounted on a slide. The slides were dried at 37°C for at least 24 hours.

Histological staining

Elastic fibers were visualized by Lawson van Gieson (LvG) staining. Deparaffinized slides were rinsed in bidistilled water, stained with Lawson (Klinipath) for 60 min and differentiated shortly in ethanol 96% for ± 10 seconds. Subsequently, slides were rinsed shortly in bidistilled water and stained in Van Gieson's pichrofuchsin (5% fuchsin in 100ml picric acid with 0.25% hydrochloric acid) for ± 6 min. The slides were dehydrated rapidly through a graded series of ethanol and xylene, followed by mounting using Entellan (Merck).

Antibodies

An antibody for smooth muscle actin (SMA, mouse monoclonal antibody clone 1A4 (1:4000), Sigma-Aldrich, St Louis, USA), for nerves (Ncam1 (Neural cell adhesion molecule 1); rabbit polyclonal antibody clone AB5032 (1:1500), Chemicon, Temecula, USA) and for endothelium (Pecam1 (Platelet endothelial cell adhesion molecule-1); goat polyclonal antibody clone SC-1506 (1:2000); Santa Cruz Biotechnology, Santa Cruz, USA) were used.

Immunohistochemistry

The slides were deparaffinated using a xylene to ethanol series. Inhibition of endogenous peroxidase activity was performed by incubating the slides in a solution of 0.3% H_2O_2 in PBS (phosphate buffered saline: 150 mM NaCl, 10 mM NaPi, pH 7.4)/50% ethanol for 30 min. Next, the slides were rinsed twice in PBS for 5 min. In case of Pecam1, the slides were placed in 200ml 1% Antigen Unmasking solution (Vector Laboratories, Burlingame, USA) in a rack and cooked for 5 min at 1000 Watt in a high pressure cooker. The rack was cooled in bidistilled water and once the

pressure cooker was depressurized, the rack was placed on ice for ± 20 min. Next, the slides were rinsed in PBS for 5 min.

All slides (SMA, Ncam1 and Pecam1) were blocked in Tris-sodium buffer (1M Tris, 1.5M NaCl, adjusted to pH 7.4 using HCl) with 0.5% blocking reagent for 30 min. Subsequently, the slides were incubated overnight with the first specific antibody. The next day the slides were rinsed three times in TNT (0.1M Tris-HCl (pH 7.4), 0.15M NaCl, 0.05% Tween-20) for 5 min, followed by 30 min incubation with the second specific antibody in case of SMA and Ncam1 (Envision+ HRP anti-mouse or anti-rabbit, respectively). In case of Pecam1, sections were incubated in biotinylated Donkey-anti-goat IgG (H+L) (Jackson ImmunoResearch Laboratories, #705065147, 1:200) for 30 minutes. The slides were then rinsed three times in TNT, followed by incubation with Streptavidin-horseradish peroxidase (SA-HRP) (Dako, #P0397, 1:100). All slides were rinsed three times with TNT followed by incubation in diaminobenzidine (DAB, Dako kit) for ± 5 min, depending on background staining intensity. The reaction was stopped in bidistilled water. Finally, all slides were counterstained using Mayer's-Hematoxylin for 1 min. Subsequently, slides were rinsed in running tap water for ± 10 min and dehydrated quickly through a graded series of ethanol and xylene. To finish, sections were mounted using Entellan (Merck). All slides were analyzed by microscopy using Leica DFC 320.

Results

Ductus venosus morphology is not changed by altered lymphatic development and nuchal edema (mouse model group I)

A three dimensional view of the ductus venosus and its adjacent vessels is shown in a wild-type E15.5 mouse embryo in Figure 1. *Ccbe1*$^{-/-}$ embryos showed nuchal edema, whereas no nuchal edema was observed in wild-type embryos (see Figure 2). A single layer of smooth muscle actin (SMA) expression was identified in the ventral-caudal part of the ductus venosus in *Ccbe1*$^{-/-}$ and wild-type embryos. The dorsal-cranial part of the ductus venosus was slightly positive for SMA staining in all mutant and wild-type embryos at E15.5. Similarly, single layered SMA expression was identified in other venous vessels, such as in the umbilical and portal vein and in the inferior vena cava. The endothelial surface of the ductus venosus showed equal, positive Pecam1 staining along its entire length in all analyzed mutant embryos and wild-type embryos at E15.5. Ncam1 and LvG staining were absent in the ductus venosus in *Ccbe1*$^{-/-}$ and wild-type embryos at the examined stage (see Table 2 and Figure 2).

Ductus venosus morphology is not affected by cardiac defects and nuchal edema (mouse model group II)

Tbx1$^{-/-}$, *Jarid2*$^{-/-NE+}$, *Fkbp12*$^{-/-}$ and *Chd7*$^{fl/fl}$*;Mesp1Cre* embryos all showed nuchal edema at E11.5-15.5 (see arrows in Figure 3). The ventral-caudal part of the ductus venosus showed similar, positive single-layered SMA expression in *Tbx1*$^{-/-}$, *Jarid2*$^{-/-NE+}$, *Fkbp12*$^{-/-}$ and *Chd7*$^{fl/fl}$*;Mesp1Cre* embryos and their wild-type embryos at E11.5-15.5. Equally, slightly positive SMA staining was found in the dorsal-cranial part of the ductus venosus in all analyzed mutant and wild-type embryos at E11.5-15.5. Single-

layered SMA expression was similarly expressed in other venous vessels. Similarly, positive expression of Pecam1 was observed in the entire ductus venosus in *Tbx1*$^{-/-}$, *Jarid2*$^{-/-\ NE+}$, *Fkbp12*$^{-/-}$ and *Chd7*$^{fl/fl}$*;Mesp1Cre* embryos and their wild-type embryos in all analyzed stages. Ncam1 and LvG expression were absent in the ductus venosus in all examined mutant and wild-type embryos at E11.5-15.5 (see Table 2 and Figure 3).

Ductus venosus morphology is not altered by cardiac malformations without the presence of nuchal edema (mouse model group III)

Nuchal edema was not observed in *Tbx2*$^{-/-}$, *Fgf10*$^{-/-}$ or *Jarid2*$^{-/-NE-}$ embryos at E12.5-14.5 (see Figure 4). The ventral-caudal part of the ductus venosus showed similar, positive, single-layered SMA expression in all examined mutant and wild-type embryos at the investigated stages. Slightly positive SMA expression was identified in the dorsal-cranial part of the ductus venosus in all investigated mutant and wild-type embryos at E12.5-14.5. Expression of single-layered SMA in the ductus venosus was similar in other venous vessels. The entire length of the ductus venosus showed similar and positive staining of the endothelium in *Tbx2*$^{-/-}$, *Fgf10*$^{-/-}$ and *Jarid2*$^{-/-NE-}$ embryos and wild-type embryos at E12.5-14.5. Ncam1 and LvG staining were negative in the ductus venosus in all analyzed mutant and wild-type embryos at the investigated stages (see Table 2 and Figure 4).

Discussion

This is the first study that examined whether nuchal edema, cardiac defects and abnormal lymphatic development are associated with local changes in ductus venosus morphology. We demonstrated a similar expression of single-layered smooth muscle cells and endothelium and the absence of nerves and elastic fibers in the ductus venosus in various mouse models with cardiac and lymphatic defects, irrespective of the presence of nuchal edema. This study shows that the observed cardiac defects, nuchal edema and abnormal lymphatic development are not associated with morphological changes in the ductus venosus in the examined mouse models. Ductus venosus flow velocity waveforms therefore most probably reflect intracardiac pressure.

Prior studies have reported on the strong relationship between abnormal first-trimester ductus venosus flow velocity waveforms, cardiac defects and increased NT [8;10-14]. The pathophysiological mechanism explaining this relationship is unknown. Abnormal endothelial differentiation is a mutual denominator in the development of both cardiac defects [33] and nuchal edema [34-37]. We examined whether a common process, such as an abnormal developmental process in the ductus venosus, is involved in this relationship. But no difference in expression of endothelium or any other used marker was found in the ductus venosus, independent of nuchal edema, lymphatic abnormalities or cardiac anatomy. The underlying cause that can explain the relationship between abnormal ductal flow velocity waveforms, cardiac defects and increased NT thus still awaits further investigation.

Another suggested explanation for changed ductus venosus flow velocity waveforms is cardiac failure [2;9;10;14]. Altered ductus venosus flow velocity waveforms are not

related to a specific type of cardiac defect, neither is a higher pressure in the right ventricle responsible for changed ductus venosus flow velocity waveforms in human fetuses with increased NT [21]. Furthermore, the majority of cardiac malformations are not known to result in overt fetal cardiac failure [17;18]. Typical fetal findings upon cardiac failure, such as pericardial and pleural effusions, edema, ascites and cardiomegaly, are all absent. Abnormal ductal flow velocity waveforms have also been described in fetuses with increased NT as well as with normal NT, without a cardiac defect [19;38]. Cardiac failure due to a cardiac defect is therefore not responsible for altered flow velocity waveforms in the ductus venosus.

We believe first-trimester hemodynamic alterations can explain changed ductal flow patterns. Major differences in cardiovascular function occur in the transition from early to late first-trimester pregnancy. In the first trimester of pregnancy the fetus is relatively impaired in diastolic function because of ventricular stiffness [39], increased cardiac afterload due to higher placental resistance [40] and fetal renal function has not yet developed to balance a possible hypervolemia [10]. A small disturbance in diastolic function may easily result in a hemodynamic imbalance in this phase of pregnancy. Diastolic function distinctly improves in the second and third trimester [10], peripheral vascular resistance decreases, cardiac compliance and output increase [41] and placental resistance reduces, which causes a fall in cardiac afterload [40]. These cardiac adaptations from early to late first-trimester pregnancy occur concomitantly with the disappearance of NT and match the time range of our analyses. Nuchal translucency is transient in nature and normally disappears around 14 weeks of human gestation [42]. A prior study has shown that increased NT is related to altered blood flow in the jugular vein and the ductus venosus [43]. It is possible that the local nuchal

edema is – in addition to the connection of the lymphatic system to the venous circulation – also drained through the lymphatic system by a correction of hemodynamic imbalance at the transition from early to late first-trimester pregnancy. Altered flow velocities in the jugular and ductal vein may also be explained by the fact that the accumulated fluid in the neck region might affect blood viscosity, which results in hemodynamic changes. Abnormal ductus venosus flow velocity waveforms are therefore possibly a secondary phenomenon to increased NT [20].

Altered first-trimester ductus venosus flow velocity waveforms are also associated with aneuploid fetuses with increased NT [9;22;44;45]. Prior studies in first-trimester trisomy 21 fetuses reported on abnormal cardiac function, specifically diastolic dysfunction, compared to fetuses with normal or increased NT, regardless of cardiac anatomy [22;46]. It was suggested that cardiac dysfunction could be attributed to hypervolemia [22]. Thus, these findings also direct toward the involvement of first-trimester hemodynamic alterations in the origin of abnormal ductal flow patterns in aneuploid fetuses.

The existence of a sphincter at the ductus venosus inlet remains contested [1;3;4;47-49]. Again, we did not observe multiple smooth muscle cell layers or elastic fibers – features that are required to establish a contraction in a blood vessel – in the ductus venosus. The ductus venosus thus does not possess characteristics to function as a sphincter. Prior morphological studies in mouse and human embryos [48;49] showed similar results. Our findings are also in line with previous experimental studies in fetal sheep [50-53], reporting on relaxation or constriction of the total length of the ductus venosus, instead of a sphincter region.

We have examined a great diversity of different mouse models in this study. Although we did not observe different staining results in the mouse models as a group, it can not be excluded that if more embryos from a more uniform population were examined, some more subtle differences in for example SMA expression could be detected in the ductus venosus.

Doppler assessment of the ductus venosus could not be performed in this study. The hypothesis of abnormal ductal patterns due to changed hemodynamics could therefore not be tested. Ultrasound examination of ductus venosus flow velocity waveforms could not be performed in the examined mouse models, because we were unable to study alive mouse embryos. Yet, an independent association between abnormal ductus venosus blood patterns and cardiac malformations has been demonstrated [10;12;13].

In order to translate our findings in mouse models to the human situation further research on ductus venosus morphology in euploid and aneuploid human fetuses with increased NT is required. Preferentially, ductus venosus ultrasound assessment in human fetuses followed by morphological examination of the nuchal area, heart and ductus venosus is needed.

In conclusion, this study shows that cardiac defects, nuchal edema and abnormal lymphatic development are not associated with morphological changes in the ductus venosus. Ductus venosus flow velocity waveforms therefore most probably reflect intracardiac pressure.

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Conflict of interest statement

None declared

328 Reference List

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Figure legends

Legend Figure 1: Three-dimensional view of the ductus venosus and its adjacent vessels. The black box represents the area that is shown in Figures 2-4.

Legend Figure 2: Immunohistochemical analysis of the ductus venosus in mouse embryos with a lymphatic defect and nuchal edema (mouse model group I).

Phenotype of *Ccbe1*$^{+/+}$ control (a) and *Ccbe1*$^{-/-}$ (b) mouse embryos at E15.5. Note the nuchal edema in the *Ccbe1*$^{-/-}$ embryo (see arrow). Transverse sections of the ductus venosus (c-j). Pecam1, Platelet endothelial cell adhesion molecule-1; SMA, smooth muscle actin; Ncam1, Neural cell adhesion molecule 1; LvG, Lawson van Gieson. Scale bars represents 50 µm.

Legend Figure 3: Immunohistochemical analysis of the ductus venosus in mouse embryos with a cardiac defect and nuchal edema (mouse model group II).

Phenotype of *Tbx1*$^{+/+}$ control (a), *Tbx1*$^{-/-}$ (b), *Fkbp12*$^{+/+}$ control (c), *Fkbp12*$^{-/-}$ (d), *Jarid2*$^{+/+}$ control (e), *Jarid2*$^{-/-NE+}$ (f), *Chd7*$^{+/+}$*;Mesp1Cre* control (g) and *Chd7*$^{fl/fl}$*;Mesp1Cre* (h) embryos. Note the nuchal edema in all mutant embryos (see arrows). Transverse sections of the ductus venosus (i-oo). Pecam1, Platelet endothelial cell adhesion molecule-1; SMA, smooth muscle actin; Ncam1, Neural cell adhesion molecule 1; LvG, Lawson van Gieson. Scale bars represent 50 µm.

Legend Figure 4: Immunohistochemical analysis of the ductus venosus in mouse embryos with a cardiac defect without nuchal edema (mouse model group III).

Phenotype of *Tbx2*$^{+/+}$ control (a), *Tbx2*$^{-/-}$ (b), *Fgf10*$^{+/+}$ control (c), *Fgf10*$^{-/-}$ (d), *Jarid2*$^{+/+}$ control (e) and *Jarid2*$^{-/-NE-}$ (f) embryos. Note the absence of nuchal edema in all mutant embryos. Transverse sections of the ductus venosus (g-bb). Pecam1, Platelet endothelial cell adhesion molecule-1; SMA, smooth muscle actin; Ncam1, Neural cell adhesion molecule 1; LvG, Lawson van Gieson. Scale bars represents 50 µm.

509 **Table legend**

510 **Legend Table 2:**

511 + positive staining; - absent staining; * positive staining at ventral-caudal part

512 Pecam1, Platelet endothelial cell adhesion molecule-1; SMA, Smooth Muscle Actin;

513 Ncam1, Neural cell adhesion molecule 1; LvG, Lawson van Gieson; IVC, inferior vena

514 cava

515 **Table 1: Number of mouse embryos examined per embryonic day**

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		Embryonic day	Number of mouse embryos
Mouse embryos with a lymphatic defect *(mouse model group I)*			
	Ccbe1$^{+/+}$	15.5	4
	Ccbe1$^{-/-}$	15.5	4
Mouse embryos with a cardiac defect with nuchal edema *(mouse model group II)*			
	Tbx1$^{+/+}$	14.5	2
	Tbx1$^{-/-}$	14.5	3
	Fkbp12$^{+/+}$	11.5-13.5	5
	Fkbp12$^{-/-}$	11.5-13.5	5
	Jarid2$^{+/+}$	14.0	1
	Jarid2$^{-/-\ NE+}$	14.0-14.5	4
	Chd7$^{+/+}$	15.5	2
	Chd7$^{-/-}$	15.5	2
Mouse embryos with a cardiac defect without nuchal edema *(mouse model group III)*			
	Tbx2$^{+/+}$	12.5	3
	Tbx2$^{-/-}$	12.5	3
	Fgf10$^{+/+}$	13.5	2
	Fgf10$^{-/-}$	13.5	6
	Jarid2$^{+/+}$	14.5	1
	Jarid2$^{-/-\ NE-}$	14.5	5

517 **Table 2: Staining results of the ductus venosus in mouse embryos with lymphatic defects and nuchal edema, cardiac defects**

518 **and nuchal edema and cardiac defects without nuchal edema**

Immunohistochemical and histological staining results in mouse embryos with:

	Lymphatic defects and nuchal edema				Cardiac defects and nuchal edema					Cardiac defects without nuchal edema				
Ccbe1-/-	**Pecam1**	**SMA**	**LvG**	**Ncam1**	***Tbx1-/-***	**Pecam1**	**SMA**	**LvG**	**Ncam1**	***Tbx2-/-***	**Pecam1**	**SMA**	**LvG**	**Ncam1**
ductus venosus	+	+*	-	-	ductus venosus	+	+*	-	-	ductus venosus	+	+*	-	-
pre-hepatic IVC	+	+	-	-	pre-hepatic IVC	+	+	-	-	pre-hepatic IVC	+	+	-	-
post-hepatic IVC	+	+	-	-	post-hepatic IVC	+	+	-	-	post-hepatic IVC	+	+	-	-
umbilical vein	+	+	-	-	umbilical vein	+	+	-	-	umbilical vein	+	+	-	-
portal vein	+	+	-	-	portal vein	+	+	-	-	portal vein	+	+	-	-
***Ccbe1*+/+ control**	**Pecam1**	**SMA**	**LvG**	**Ncam1**	***Tbx1*+/+ control**	**Pecam1**	**SMA**	**LvG**	**Ncam1**	***Tbx2*+/+ control**	**Pecam1**	**SMA**	**LvG**	**Ncam1**
ductus venosus	+	+*	-	-	ductus venosus	+	+*	-	-	ductus venosus	+	+*	-	-
pre-hepatic IVC	+	+	-	-	pre-hepatic IVC	+	+	-	-	pre-hepatic IVC	+	+	-	-
post-hepatic IVC	+	+	-	-	post-hepatic IVC	+	+	-	-	post-hepatic IVC	+	+	-	-
umbilical vein	+	+	-	-	umbilical vein	+	+	-	-	umbilical vein	+	+	-	-
portal vein	+	+	-	-	portal vein	+	+	-	-	portal vein	+	+	-	-

*Jarid2-/-*NE+	Pecam1	SMA	LvG	Ncam1	*Fgf10-/-*	Pecam1	SMA	LvG	Ncam1
ductus venosus	+	+*	-	-	ductus venosus	+	+*	-	-
pre-hepatic IVC	+	+	-	-	pre-hepatic IVC	+	+	-	-
post-hepatic IVC	+	+	-	-	post-hepatic IVC	+	+	-	-
umbilical vein	+	+	-	-	umbilical vein	+	+	-	-
portal vein	+	+	-	-	portal vein	+	+	-	-

Jarid2+/+ control	Pecam1	SMA	LvG	Ncam1	*Fgf10*+/+ control	Pecam1	SMA	LvG	Ncam1
ductus venosus	+	+*	-	-	ductus venosus	+	+*	-	-
pre-hepatic IVC	+	+	-	-	pre-hepatic IVC	+	+	-	-
post-hepatic IVC	+	+	-	-	post-hepatic IVC	+	+	-	-
umbilical vein	+	+	-	-	umbilical vein	+	+	-	-
portal vein	+	+	-	-	portal vein	+	+	-	-

Fkbp12-/-	Pecam1	SMA	LvG	Ncam1	*Jarid2-/-*NE-	Pecam1	SMA	LvG	Ncam1
ductus venosus	+	+*	-	-	ductus venosus	+	+*	-	-
pre-hepatic IVC	+	+	-	-	pre-hepatic IVC	+	+	-	-
post-hepatic IVC	+	+	-	-	post-hepatic IVC	+	+	-	-
umbilical vein	+	+	-	-	umbilical vein	+	+	-	-
portal vein	+	+	-	-	portal vein	+	+	-	-

Fkbp12+/+ control	Pecam1	SMA	LvG	Ncam1
ductus venosus	+	+*	-	-
pre-hepatic IVC	+	+	-	-
post-hepatic IVC	+	+	-	-
umbilical vein	+	+	-	-
portal vein	+	+	-	-

Jarid2+/+ control	Pecam1	SMA	LvG	Ncam1
ductus venosus	+	+*	-	-
pre-hepatic IVC	+	+	-	-
post-hepatic IVC	+	+	-	-
umbilical vein	+	+	-	-
portal vein	+	+	-	-

Chd7$^{fl/fl}$;*Mesp1Cre*	Pecam1	SMA	LvG	Ncam1
ductus venosus	+	+*	-	-
pre-hepatic IVC	+	+	-	-
post-hepatic IVC	+	+	-	-
umbilical vein	+	+	-	-
portal vein	+	+	-	-

Chd7$^{+/+}$;*Mesp1Cre* control	Pecam1	SMA	LvG	Ncam1
ductus venosus	+	+*	-	-
pre-hepatic IVC	+	+	-	-

post-hepatic IVC	**+**	**+**	**-**	**-**
umbilical vein	**+**	**+**	**-**	**-**
portal vein	**+**	**+**	**-**	**-**

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