Absence of an anatomical origin for altered ductus venosus flow velocity waveforms in first-trimester human fetuses with increased nuchal translucency

N.B. Burger
A. Matias
E. Kok
C.J.M. de Groot
V.M. Christoffels
M.N. Bekker
M.C. Haak

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ABSTRACT

Objective To perform a morphological evaluation of the ductus venosus, heart and jugular lymphatic sac (JLS) in first-trimester human fetuses with normal and abnormal ductus venosus flow velocity waveforms (DV-FVWs) and normal and increased nuchal translucency (NT).

Methods Postmortem examination was performed on fetuses with increased NT or structural malformations with previous NT and DV-FVW measurements. Ductus venosus morphology was examined using markers for endothelium, smooth muscle actin (SMA), nerves and elastic fibers. Fetal hearts were studied by microscopy. The nuchal region was analyzed using markers for lymphatic vessels, endothelium, SMA and nerves.

Results Two trisomy 21 and two trisomy 18 fetuses with increased NT and abnormal DV-FVWs were analyzed. As a control, one euploid anencephalic fetus with normal NT, cardiac anatomy and DV-FVWs was examined. Similar endothelial and SMA expression was observed in the ductus venosus in all fetuses. Nerve and elastic fiber expression were not detected. Three trisomic fetuses showed cardiac defects, one trisomic fetus demonstrated normal cardiac anatomy. The JLS was abnormally enlarged or contained red blood cells in all trisomic fetuses. The control fetus showed a normal JLS.

Conclusions Abnormal DV-FVWs are not justified by alterations in ductus venosus morphology. DV-FVWs most probably reflect intracardiac pressure.

INTRODUCTION

The ductus venosus is a central regulator in the fetal circulation. It directs well-oxygenated blood from the umbilical vein directly to the inferior vena cava and the heart. The pressure gradient in the ductus venosus relates to the different phases of the heart cycle. Ultrasound examination of ductus venosus flow velocity waveforms is used to indirectly assess cardiac function and the fetal hemodynamic condition. Whether the ductus venosus actively regulates blood flow remains uncertain.

The association between abnormal first-trimester ductus venosus flow velocity waveforms, cardiac defects and increased nuchal translucency (NT) is well established. The underlying pathophysiology is, however, unknown. The transient nature of increased NT as well as abnormal first-trimester ductus venosus flow velocity waveforms may imply a common origin of both entities.

Cardiac failure has been suggested to cause both abnormal ductus venosus flow velocity waveforms and increased NT. But a wide variety of cardiac defects are observed in fetuses with increased NT, which mostly do not affect the hemodynamic circulation. Furthermore, not all fetuses with increased NT have a cardiac defect. Fetal signs of cardiac failure are also rarely observed in fetuses with increased NT.

Prior studies demonstrated the involvement of abnormal jugular lymphatic sacs (JLS) in increased NT. Specifically, an abnormal endothelial differentiation of blood endothelial cells towards lymphatic endothelial cells was observed in the JLS. Endothelial signaling plays an important role in embryonic development, such as in the formation of the heart, blood and lymphatic vasculature. A disturbance in endothelial differentiation therefore may also lead to cardiac defects.

A recent review reported on multiple genes, involved in both lymphatic and cardiac abnormalities in mouse embryos with nuchal edema, with an expression pattern in endothelial cells. Given this prominent role of endothelium, we hypothesize that abnormal ductus venosus flow velocity waveforms are caused by altered ductus venosus morphology, such as abnormal endothelium. A study combining ultrasound measurements with subsequent postmortem examinations of the ductus venosus, heart and nuchal region in first-trimester human fetuses has not been performed. The aim of this study was to examine (i) the morphology of the ductus venosus in fetuses with normal and abnormal ductus venosus flow velocity waveforms (ii) the morphology of the JLS in fetuses with normal and increased NT and (iii) the role of endothelium as a possible unifying denominator linking abnormal ductus venosus flow velocity waveforms, cardiac defects and increased NT.
METHODS

Human fetuses
All patients referred to the São João Hospital for tertiary care received ultrasound examinations according to standard protocol. If the parents requested to terminate the pregnancy because of fetal abnormalities at 11-13 weeks of gestation, they were asked to participate in this study. Fetuses were examined prenatally by ultrasound, including assessment of NT thickness, tricuspid regurgitation, ductus venosus flow velocity waveforms and a structural screening for the detection of congenital anomalies. Ultrasound examination was performed between 11-13 weeks of pregnancy by an experienced ultrasonographer (A.M.) using a 5 MHz transabdominal probe (Voluson E8; GE Healthcare).

Nuchal translucency measurement was conducted according to the guidelines of the Fetal Medicine Foundation41. Increased NT was defined as NT above the 95th percentile42. Ductus venosus flow velocity waveforms were obtained according to the technique described by Montenegro et al43. A ventral mid-sagittal plane of the fetal trunk during fetal quiescence was used to measure ductus venosus flow velocity waveforms. Color flow was applied to visualize the venous circulation. The pulsed Doppler sample gate (size 0.5-1 mm) was placed in the ductus venosus. The ductus venosus pattern was easily distinguished from the umbilical vein and inferior vena cava by its characteristic triphasic pattern. The interrogation angle was kept as low as possible and was less than 60 degrees at all times. A minimum of three high-quality flow velocity waveforms was used to establish the measurements for the ductus venosus parameters. Doppler measurements of the ductus venosus involved the lowest forward velocity during atrial contraction in late diastole (A) and the pulsatility index (PI). Ductus venosus flow velocity waveforms were classified as normal or abnormal, depending on the A-wave during atrial contraction in late diastole. A positive A-wave was defined as a normal ductus venosus flow velocity waveforms. An absent or negative A-wave was defined as an abnormal ductus venosus flow velocity waveforms. Chorion villus sampling was performed in all patients. Quantitative-fluorescent polymerase chain reaction (QF-PCR) was applied on all samples to detect aneuploidy. This study was approved by the Medical Ethical Committee of the São João Hospital in Porto, Portugal. All patients received information and gave informed consent. Patients were included from February to December 2014.

Termination of pregnancy was induced using vaginal misoprostol 600 micrograms every 4 hours with a maximum of 3 doses in 24 hours. The fetuses were fixed in 4% paraformaldehyde for 24-48 hours. Next, the fetuses were rinsed in water and stored in ethanol 70% at room temperature. The fetuses were placed in PBS (phosphate buffered saline: 150 mM NaCl, 10 mM NaPi, pH 7.4) at room temperature for the transport to the department of Anatomy, Embryology & Physiology, Academic Medical Center, Amsterdam, the Netherlands.

Postmortem examination of the whole fetus was performed in Amsterdam. Three areas were dissected: (i) the nuchal region; from below the eyes to the clavicles, (ii) the heart-lung region and (iii) the ductus venosus region. The heart-lung region was examined by an experienced (fetal) cardiac patho-morphologist using a dissection microscope. Details on the experimental procedures are presented in the Supplemental Material.

RESULTS

A total of 5 patients with singleton pregnancies at 11-13 weeks of pregnancy were available for this study.

Ultrasound examination
The characteristics of the five fetuses are enlisted in Table 1. Fetus 1 was an anencephalic fetus and was used as a control. One trisomy 18 fetus (fetus 5) showed an enlarged NT of 6.1 mm at the initial ultrasound examination at 11 weeks of gestation. Nuchal thickness measurement was 4.1 mm at the following ultrasound examination at 13 weeks of gestation. A positive A-wave in the ductus venosus was observed in the control fetus as well as in one trisomy 18 fetus (fetus 4). In the other three trisomic fetuses (fetus 2, 3 and 5) a negative ductal A-wave was found (see Table 1).

Ductus venosus morphology
Similar single-layered expression of smooth muscle actin and Pecam1 were identified in the total length of the ductus venosus in the control as well as in all trisomic fetuses on postmortem examination. Ncam1 positive nerve fibers were located near the ductus venosus in the control fetus and all trisomic fetuses (see Figure 1). Ncam1 positive nerve fibers were not observed adjacent to or in the vessel wall of the ductus venosus. LvG elastic fiber expression was absent in the ductus venosus in the control fetus and all trisomic fetuses (see Figure 1). The morphology and expression of all applied markers were comparable in the ductus venosus in the control fetus and all trisomic fetuses (see Figure 1 and Table 2).

Cardiac morphology
The control fetus (fetus 1) and one trisomy 21 fetus (fetus 2) showed normal cardiac anatomy. In the second trisomy 21 fetus (fetus 3) and in two trisomy 18 fetuses (fetus 4 and fetus 5) cardiac abnormalities were identified (see Table 1).

Nuchal morphology and jugular lymphatic sacs
The JLS had a normal size and position44 in the control fetus. The JLS was severely enlarged in two trisomy 21 fetuses and in one trisomy 18 fetus (fetus 4) (see Figure 2). The second trisomy
Table 1. Characteristics of the human fetuses included in this study

<table>
<thead>
<tr>
<th></th>
<th>Fetus 1</th>
<th>Fetus 2</th>
<th>Fetus 3</th>
<th>Fetus 4</th>
<th>Fetus 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultrasound examination:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age at termination (weeks+days)</td>
<td>12+0</td>
<td>13+4</td>
<td>13+4</td>
<td>13+1</td>
<td>13+0</td>
</tr>
<tr>
<td>Crown-rump length* (mm)</td>
<td>56</td>
<td>55,6</td>
<td>57,6</td>
<td>56</td>
<td>46</td>
</tr>
<tr>
<td>Nuchal translucency* (mm)</td>
<td>1,8</td>
<td>5,3</td>
<td>4,5</td>
<td>2,4</td>
<td>6,1</td>
</tr>
<tr>
<td>Nuchal translucency &gt;p95</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Ductus venosus pulsatility index</td>
<td>normal</td>
<td>&gt; p95</td>
<td>&gt; p95</td>
<td>&gt; p95</td>
<td>&gt; p95</td>
</tr>
<tr>
<td>Ductus venosus flow velocity waveform</td>
<td>positive A-wave</td>
<td>negative A-wave</td>
<td>negative A-wave</td>
<td>positive A-wave</td>
<td>negative A-wave</td>
</tr>
<tr>
<td>Structural screening for fetal anomalies</td>
<td>anencephaly</td>
<td>absent nasal bone</td>
<td>-</td>
<td>abnormal nasal bone, omphalocele</td>
<td>-</td>
</tr>
<tr>
<td>Qorion villus sampling:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QF-PCR</td>
<td>euploid</td>
<td>trisomy 21</td>
<td>trisomy 21</td>
<td>trisomy 18</td>
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<td>Postmortem examination:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Postmortem examination</td>
<td>anencephaly</td>
<td>low-set ears, broad nasal bridge, absent nasal bone</td>
<td>low-set ears</td>
<td>low-set ears, abnormal nasal bone, single umbilical artery, unilateral talipes equinovarus, omphalocele containing the liver</td>
<td></td>
</tr>
<tr>
<td>Cardiac examination</td>
<td>normal cardiac anatomy</td>
<td>normal cardiac anatomy</td>
<td>AVSD, ASD type II, slightly overriding hypoplastic aortic arch, hypoplasia (segment B) between left carotid and left subclavian arteries and long hypoplastic isthmus segment</td>
<td>perimembranous subaortic VSD, polyvalvular disease, persistent arterial duct, small aorta and slight tubular hypoplasia of the aortic arch (segment B)</td>
<td>DORV and tetralogy of Fallot with mild (sub) pulmonary stenosis, polyvalvular disease, absent right SVC, persistent left SVC via coronary sinus</td>
</tr>
</tbody>
</table>

* measurement at the initial ultrasound examination

ASD, atrial septal defect; AVSD, atrioventricular septal defect; DORV, double outlet right ventricle; N, no; SVC, superior vena cava; VSD, ventricular septal defect; Y, yes

Figure 1. Immunohistochemical analysis of the ductus venosus in human fetuses with normal and abnormal ductus venosus flow velocity waveforms

Transverse sections of the ductus venosus (a-d). Pecam1, Platelet endothelial cell adhesion molecule-1; SMA, Smooth muscle actin; Ncam1, Neural cell adhesion molecule 1; LvG, Lawson van Gieson. Scale bars represent 300μm.
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Figure 2. Immunohistochemical analysis of the JLS in human fetuses with normal and increased NT

Table 2. Immunohistochemical examination of the ductus venosus

<table>
<thead>
<tr>
<th>Fetus 1: control</th>
<th>ductus venosus</th>
<th>inferior vena cava</th>
<th>umbilical vein</th>
<th>portal vein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pecam1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SMA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LvG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ncam1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fetus 2: trisomy 21</th>
<th>ductus venosus</th>
<th>inferior vena cava</th>
<th>umbilical vein</th>
<th>portal vein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pecam1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SMA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LvG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ncam1</td>
<td>-</td>
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<td>-</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Fetus 3: trisomy 21</th>
<th>ductus venosus</th>
<th>inferior vena cava</th>
<th>umbilical vein</th>
<th>portal vein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pecam1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SMA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LvG</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ncam1</td>
<td>-</td>
<td>-</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Fetus 4: trisomy 18</th>
<th>ductus venosus</th>
<th>inferior vena cava</th>
<th>umbilical vein</th>
<th>portal vein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pecam1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SMA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>LvG</td>
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<tr>
<td>Ncam1</td>
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</table>

<table>
<thead>
<tr>
<th>Fetus 5: trisomy 18</th>
<th>ductus venosus</th>
<th>inferior vena cava</th>
<th>umbilical vein</th>
<th>portal vein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pecam1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SMA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LvG</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>Ncam1</td>
<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
</tbody>
</table>

+ positive staining; - absent staining.
Pecam1, Platelet endothelial cell adhesion molecule 1; SMA, Smooth muscle actin; Ncam1, Neural cell adhesion molecule 1; LvG, Lawson van Gieson

18 fetus (fetus 5) showed a normal size of the JLS, but the JLS contained red blood cells. This was not observed in other trisomic fetuses or the control fetus. In all trisomic fetuses mesenchymal edema was found in the nuchal area. Mesenchymal edema was not observed in the nuchal region in the control fetus (see Figure 2). Details of the immunohistochemical examinations are summarized in Table 3.

DISCUSSION

In this study we examined the morphology of the ductus venosus, the heart and the JLS in aneuploid fetuses and a euploid control fetus with abnormal ductus venosus flow velocity waveforms and increased NT. Our data suggests that ductus venosus morphology is not related to abnormal ductus venosus flow velocity waveforms, aneuploidy, cardiac anatomy and increased NT. We also confirm earlier findings of the lack of a sphincter in the human.
Different hypotheses have been proposed to explain changed ductus venosus flow velocity waveforms. One of the suggested hypotheses is that cardiac failure underlies changed ductus venosus flow velocity waveforms. Ultrasound findings of fetal cardiac decompensation, waveforms. One of the suggested hypotheses is that cardiac failure underlies changed ductus venosus flow velocity waveforms. But similar expression of endothelium was involved in the etiology of increased NT. Our findings are in line with prior morphological studies in euploid and aneuploid mouse embryos and human fetuses with nuchal edema. Furthermore, most cardiac defects do not affect the fetal hemodynamic situation. Accordingly, cardiac failure fails to explain abnormal ductus venosus flow velocity waveforms. Given the important role of endothelium in the development of both cardiac defects and increased NT, we hypothesized that abnormal endothelial development is also involved in changed ductus venosus flow velocity waveforms. But similar expression of endothelium was observed in the ductus venosus in the control fetus and trisomic fetuses. Whether endothelium is the unifying factor to explain the relationship between altered first-trimester ductus venosus flow velocity waveforms, cardiac defects and increased NT cannot be answered in this study and should be explored in future research.

Abnormal ductus venosus flow velocity waveforms cannot be explained by morphological changes or the presence of a sphincter. We hypothesize that abnormal ductus venosus flow velocity waveforms are caused by early to late first-trimester hemodynamic alterations, because of changes in cardiac pre- or afterload, possibly due to an accumulation of fluid in the nuchal area. The fetus has little cardiac diastolic function in the late first trimester when NT develops, because of high placental resistance, rapid growth and an increasing blood volume. This hemodynamic status, together with little preload (because of fluid accumulation in the neck region), could be sufficient to cause temporary impaired cardiac function. In the second and third trimester of pregnancy placental resistance drops and the diastolic function increases. These circulatory adaptations occur simultaneously with the vanishing of NT. Concomitantly, JLS remodel into lymph nodes and the lymphatic vessels connect to the venous system at 14 weeks of human gestation, draining lymphatic fluid into the venous circulation. Nuchal edema could thus be drained by the lymphatic-venous connection, as well as by an improved hemodynamic status. Furthermore, this hypothesis might contribute to the association of increased NT and cardiac defects because hemodynamic alterations can also induce cardiac defects. Altered hemodynamics are thus a mutual denominator in abnormal ductus venosus flow velocity waveforms, cardiac defects and increased NT.

Another mutual factor that may explain anomalies in the ductus venosus, heart and nuchal area is a disturbance in neural crest cell migration and differentiation. Whether neural crest defects are truly involved in ductus venosus pathology was not examined in these fetuses and this question remains unanswered.

Our cases of trisomic fetuses with increased NT show a coincident abnormal development of the JLS and mesenchymal edema. Based on these findings, abnormal JLS formation seems involved in the etiology of increased NT. Our findings are in line with prior morphological studies in euploid and aneuploid mouse and human embryos with nuchal edema. The enlarged JLS showed a diminished expression of lymphatic marker Lyve1 and an increased expression of smooth muscle actin surrounding the JLS in the trisomic fetuses. Smooth muscle cells are normally arranged around blood vessels and around large collecting lymphatic vessels. These findings imply a delay or disturbance in lymphatic development. This is consistent with prior morphological studies in euploid and aneuploid mouse embryos and human fetuses with nuchal edema. Limitations of this study include the impossibility to use healthy fetuses as controls. We have therefore designated an anencephalic fetus (a fetus with normal NT, normal cardiac anatomy, normal ductus venosus flow velocity waveforms and normal QF-PCR) as a control fetus. Only a small number of fetuses could be investigated. Further research with larger numbers of human fetuses with various chromosomal and cardiac defects with and without increased NT in euploid fetuses is warranted.
CONCLUSION

In conclusion, we confirm earlier findings of similar morphology of the ductus venosus in human fetuses, regardless of abnormal ductus venosus flow velocity waveforms, aneuploidy, cardiac defects or increased NT. We propose that abnormal ductus venosus flow velocity waveforms are not caused by local morphological alterations or the presence of a ductus venosus sphincter. We postulate that abnormal ductus venosus flow velocity waveforms reflect a changed hemodynamic status. Furthermore, abnormal jugular lymphatic development is involved in the etiology of increased NT.

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