Lymphatic development in euploid fetuses with increased nuchal translucency

ABSTRACT

Objective
Increased nuchal translucency in the human fetus is associated with aneuploidy, structural malformations and several syndromes such as Noonan syndrome. In 60-70% of the Noonan syndrome cases, a gene mutation can be demonstrated. Previous research showed that aneuploid fetuses with increased nuchal translucency demonstrate an aberrant lymphatic endothelial differentiation.

Methods
Fetuses with increased nuchal translucency and normal karyotype (n = 7) were compared with euploid controls having a normal nuchal translucency (n = 5). A Noonan syndrome gene mutation was found in three out of seven fetuses with increased nuchal translucency. Endothelial differentiation was evaluated by immunohistochemistry using lymphatic markers (Prox-1, Podoplanin, LYVE-1) and blood vessel markers VEGF-A, NP-1, Shh, vWF and smooth muscle cell marker SMA.

Results
Nuchal edema and enlarged jugular lymphatic sacs were observed in fetuses with increased nuchal translucency, together with abnormal lymphatic endothelial differentiation i.e. the presence of blood vessel characteristics, including high levels of VEGF-A and NP-1 expression. The enlarged jugular lymphatic sacs contained erythrocytes and were surrounded by smooth muscle cells.

Conclusion
This study shows an aberrant lymphatic endothelial differentiation in fetuses with increased nuchal translucency and a normal karyotype (including Noonan syndrome fetuses), as was previously reported before in aneuploid fetuses.
INTRODUCTION

Ultrasonographic measurement of the nuchal translucency (NT), a translucent area in the neck region of the human fetus that can be measured between 10 and 14 weeks of gestation, is a widely used screening method for trisomy 21.1-3 Enlarged NT is associated with a wide range of abnormalities, including chromosomal defects, structural anomalies such as cardiac defects and genetic syndromes.4,5

Recent studies implicate a disturbance in lymphatic development as a likely explanation for the origin of increased NT.6,7 Fetuses with increased NT morphologically show nuchal edema (NE) and a distension of the jugular lymphatic sacs (JLS).6 This also has been described in trisomy 16 mouse embryos8, a mouse model for human trisomy 21.

A recent study of our group demonstrated altered venous-lymphatic endothelial differentiation of the distended JLS in aneuploid mouse embryos and aneuploid human fetuses with NE.7 The lymphatic endothelial cells of the JLS showed a diminished expression of the lymphatic markers Prox-1 and Podoplanin and the abnormal presence of blood vessel markers, including Vascular Endothelial Growth Factor (VEGF)-A and its co-receptor Neuropilin (NP)-1.7 Also, the JLS contained erythrocytes and was surrounded by a layer of smooth muscle cells. Increased Sonic hedgehog (Shh) expression was found in the lymphatic endothelial cells of trisomy 21 fetuses compared with controls.9

So far, no study has been performed to assess the jugular lymphatic system in fetuses with increased NT and a normal karyotype. The goal of the current study was to evaluate the differentiation of the jugular lymphatic system in fetuses with increased NT having a normal karyotype. The lymphatic endothelial differentiation was compared with euploid fetuses having a normal NT (controls). A morphological study was performed using lymphatic endothelial cell-specific markers (LYVE-1, Prox-1, Podoplanin) and blood endothelial cell specific-markers (VEGF-A, NP-1, von Willebrand Factor (vWF), Shh). Smooth muscle actin (SMA) was added as a marker for the presence of smooth muscle cells. FOXC2 and platelet-derived growth factor (PDGF)-B. were used to further investigate the lymphatic endothelial differentiation.

A genetic disorder associated with increased NT and an euploid karyotype is Noonan syndrome.10 This is a disorder characterized by short stature, congenital heart defects, cardiac hypertrophy, a varying degree of intellectual deficit and distinctive facial characteristics.10 In 60-70% of the cases, a gene mutation can be demonstrated.11 In all cases with increased NT included in the present study, genetic and morphological research for the presence of a Noonan syndrome gene mutation was performed. The lymphatic endothelial differentiation was compared between fetuses with a confirmed Noonan syndrome gene mutation, fetuses without a confirmed Noonan syndrome gene mutation and euploid fetuses with normal NT.
**MATERIALS AND METHODS**

**Fetal tissue**

Twelve human fetuses were investigated (controls (euploid fetus with normal NT), n=5; euploid fetuses with increased NT, n=7) (Table 1). The fetuses were prenatally examined by ultrasound and diagnosed with normal or increased NT. Karyotyping of the fetuses was performed, using chorion villus sampling or amniocentesis. In case of a normal karyotype, the Noonan syndrome genes PTPN11 and KRAS gene were evaluated using sequence analysis.

Termination of the pregnancy occurred either by spontaneous abortion (due to cervical insufficiency) or abdominal hysterectomy (for oncological reasons) in case of the control fetuses or by misoprostol induction in the case of increased NT. In case of the fetuses with increased NT, the parents decided to terminate the pregnancy because of the increased NT, demonstrated gene mutation and or / associated ultrasound findings.

The study was approved by the medical ethical committee of the VU University Medical Center. All patients received information and gave written informed consent. A Noonan syndrome gene mutation was found in three of the seven cases with increased NT. One control fetus (gestational age (GA) 20 weeks) was not prenatally examined by ultrasound and no invasive test was performed. However, morphological examination of the fetus revealed no structural defects (including NE) and was therefore considered to have a normal karyotype (Table 1).

Fetal tissue was obtained and fixed in 4% paraformaldehyde. Subsequently, post-mortem evaluation of the whole fetus was carried out. A sequential segmental analysis of the heart was performed by an experienced cardiac morphologist. The neck region was analyzed by microscopic examination of paraffin-embedded transversely serial sections of 8μm. Analysis was performed from eye to clavicle level. In the first trimester fetuses, the lymphatic endothelial differentiation of the JLS was evaluated (controls n = 4, Noonan syndrome with confirmed gene mutation n = 1, increased NT without confirmed Noonan syndrome gene mutation n = 4). In case of the second trimester fetuses (from 17 weeks of gestation onwards), the lymphatic endothelial differentiation of the lymph node tissue was assessed (controls n = 1, Noonan syndrome with confirmed gene mutation n = 2).

**Antibodies and staining procedures**

We used antibodies against LYVE-1 (biotinylated, ReliaTech, Braunschweig, Germany), Prox-1 (ReliaTech), Podoplanin (ReliaTech), NP-1 (Santa Cruz Biotechnology, Santa Cruz Biotechnology, USA), VEGF-A (Santa Cruz Biotechnology), SMA (Sigma-Aldrich, St Louis, USA), FOXC2 (Abcam, Cambridge, UK), PDGF-B (Affinity Bio Reagents, Golden, USA), Shh (Santa Cruz Biotechnology) and vWF (Dako, Glostrup, Denmark).
Sections were deparaffinized and rehydrated. In case of Prox-1, Podoplanin, FOXC2, PDGF-B, Shh and vWF, the sections were incubated for 12 minutes in 0.01M citric buffer of pH6.0 in the microwave at a temperature of 97°C to retrieve epitopes. Inhibition of endogenous peroxidase was performed by incubating the sections for 20 minutes in a solution of 0.3% H2O2. After this, all sections were rinsed twice in PBS and once in PBS/0.05% Tween. Subsequently, they were

Table 1 Characteristics of the fetuses included in the study

<table>
<thead>
<tr>
<th>Gestational age* (wk +days)</th>
<th>CRL* (mm)</th>
<th>Karyotype/ gene mutation</th>
<th>NT (mm)**</th>
<th>Morphological examination</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12+1</td>
<td>49</td>
<td>46,XX</td>
<td>1.2</td>
<td>No defects</td>
</tr>
<tr>
<td>13+3</td>
<td>75</td>
<td>46,XX</td>
<td>1.0</td>
<td>No defects</td>
</tr>
<tr>
<td>13+3</td>
<td>81</td>
<td>46,XX</td>
<td>1.4</td>
<td>No defects</td>
</tr>
<tr>
<td>14+0</td>
<td>84</td>
<td>46,XY</td>
<td>1.2</td>
<td>No defects</td>
</tr>
<tr>
<td>20+3</td>
<td>160</td>
<td>46,XX</td>
<td>ND</td>
<td>No defects</td>
</tr>
<tr>
<td>Increased NT (Noonan syndrome) (n=3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16+0</td>
<td>108</td>
<td>46,XY, PTPN11 gene mutation: c.181G&gt;C,pAsp61Hs</td>
<td>8.2</td>
<td>NE, AVSD, persistent LSCV</td>
</tr>
<tr>
<td>22+2</td>
<td>170</td>
<td>46,XY, KRAS gene mutation: c.173C&gt;T (p.Thr58Ile)</td>
<td>14.0</td>
<td>NE, hypertrophy of atri and ventricles, small pulmonary infundibulum, hypertelorism, low-set ears</td>
</tr>
<tr>
<td>23+5</td>
<td>191</td>
<td>46,XX, PTPN11 gene mutation: Glu139Asp</td>
<td>5.2 (transient)</td>
<td>Hypertrophy of ventricular septum and pulmonary infundibulum, hypertelorism, low-set ears</td>
</tr>
<tr>
<td>Increased NT (without confirmed Noonan syndrome gene mutation) (n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14+4</td>
<td>98</td>
<td>46,XY</td>
<td>6.5</td>
<td>NE, no further defects</td>
</tr>
<tr>
<td>14+4</td>
<td>89</td>
<td>46,XX</td>
<td>6.6</td>
<td>NE, HLHS, polyvalvular disease</td>
</tr>
<tr>
<td>14+5</td>
<td>99</td>
<td>46,XX</td>
<td>7.4</td>
<td>NE, polyvalvular disease</td>
</tr>
<tr>
<td>14+6</td>
<td>94</td>
<td>46,XY</td>
<td>10.0</td>
<td>NE, 3/4th absence of diaphragm, polyvalvular disease, hypertrophic atria and ventricles, hypoplastic right aortic arch and left ductus arteriosus</td>
</tr>
</tbody>
</table>

* Gestational age and crown-rump length at birth. ** Nuchal translucency (NT) measured at the first ultrasound examination. Abbreviations: AVSD, atrioventricular septal defect; ASD, atrial septal defect; ARSA, aberrant right subclavian artery; CRL, crown-rump length; HLHS, hypoplastic left heart syndrome; ND not determined; NE nuchal edema; SCV, left superior caval vein; TOP, termination of pregnancy.
incubated overnight with the primary specific antibody. On the next day, rinsing was performed twice in PBS and once in PBS/0.05% Tween followed by incubation of the sections with the second antibody which differed per staining procedure. No second antibody was required for LYVE-1 as the primary antibody was labeled with biotin. The second antibody for Prox-1, VEGF-A, PDGF-B and vWF was a biotinylated goat-anti-rabbit antibody (Vector Laboratories, Burlinghame, USA). A biotinylated horse-anti-goat antibody (Vector Laboratories) was used for NP-1, FOXC2 and Shh. A horseradish peroxidise-conjugated rabbit-anti-mouse antibody (Dako) was used for SMA. For Podoplanin, this was a biotinylated horse-anti-mouse antibody (Vector Laboratories). Thereafter, rinsing twice in PBS and once in PBS/0.05% Tween took place. The sections, except those following the staining procedures for SMA, were incubated with ABC-reagents (Vector Laboratories) for 40 minutes. All sections were rinsed twice with PBS and once with Tris/maleate pH 7.6, followed by a 10-minute incubation period with 3,3’diaminobenzidin tetrahydrochlorid (DAB; Sigma-Aldrich) for visualization. Finally, all sections were counterstained with haematoxylin (Merck, Darmstadt, Germany), rinsed in tap water, and dehydrated to xylene. The sections were mounted with Entellan (Merck).

RESULTS

The characteristics of the fetuses enrolled in the study are shown in table 1. Morphological examination of the control fetuses revealed no structural abnormalities (n = 5). Two of the three Noonan syndrome fetuses (GA 16+0 weeks, GA 22+2 weeks) showed NE. The Noonan syndrome fetus of GA 23+5 weeks showed increased NT at the first ultrasound examination (5.2mm at GA 12+2 weeks). Repeated ultrasound examination at GA 19+6 weeks showed that the increased NT had disappeared and this fetus showed no NE. A cardiac defect was found in all three Noonan syndrome fetuses. One fetus (GA 16+0 weeks) showed an abnormal heart with a complete atrio-ventricular septal defect and a persistent left superior caval vein. The Noonan syndrome fetus of GA 22+2 weeks showed hypertrophy of the myocardium and a small pulmonary infundibulum. The fetus of GA 23+5 weeks showed an abnormal heart with myocardial hypertrophy of the ventricular septum and pulmonary infundibulum. In addition, low-set ears and hypertelorism were found. No other malformations were demonstrated in these fetuses. All fetuses diagnosed with increased NT and without a confirmed Noonan gene mutation morphologically showed NE (n = 4). A cardiac defect was found in three of the four fetuses. One fetus (GA 14+4 weeks) showed normal cardiac anatomy. No other defects were demonstrated in this fetus. One fetus (GA 14+4 weeks) demonstrated a hypoplastic left ventricle with an atretic aortic and mitral valve and a hypoplastic aortic arch was found. In addition, a dysplastic tricuspid valve and pulmonary valve were detected. One fetus (GA 14+5 weeks) showed a dysplastic tricuspid, pulmonary and mitral valve. No other structural defects were found in these fetuses. The fetus of GA 14+6 weeks demonstrated a dysplastic tricuspid valve and mitral
valve, myocardial hypertrophy of the atria and ventricles, a hypoplastic right aortic arch and left ductus arteriosus. Furthermore, a 3/4th absence of the diaphragm was found. The results of the morphological examinations are described below and are summarized in table 2.

### Table 2 Lymphatic markers of the JLS / lymph nodes of fetuses with increased NT compared with control fetuses

<table>
<thead>
<tr>
<th>LECs of the JLS/lymph nodes</th>
<th>JLS controls (n=4)</th>
<th>JLS increased NT (Noonan syndrome) (n=1)</th>
<th>JLS increased NT (without confirmed Noonan syndrome gene mutation) (n=4)</th>
<th>Lymph node control (n=1)</th>
<th>Lymph node Noonan syndrome (n=2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LYVE-1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Prox-1</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Podoplanin</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NP-1</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Shh</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>vWF</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SMA*</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FOXC2</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PDGF-B</td>
<td>+/-</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
</tr>
</tbody>
</table>

This table summarizes the staining results of immunohistochemical markers in the LECs of the JLS in Noonan syndrome fetus (GA 16 weeks), fetuses with increased NT without a Noonan syndrome gene mutation (GA 14+4-14+6 weeks) compared with the controls with normal nuchal skin (GA 12+1-14 weeks) and summarizes the staining results of immunohistochemical markers in the LECs of the lymph nodes in Noonan syndrome fetuses (GA 22+2-23+5 weeks) compared with control (GA 20+3 weeks) with normal nuchal skin. *SMA = positive staining subendothelially in the LECs of the JLS / lymph nodes. -, absent staining; +/-, slightly positive staining; +, positive staining. JLS, jugular lymphatic sac; LECs, lymphatic endothelial cells; NT, nuchal translucency.

### Lymphatic endothelium

**Control fetuses**

In the control fetuses, the layer of nuchal subcutaneous mesenchyme was thin. The JLS was positioned direct lateral to the jugular vein and carotid artery (Figure 1a). Lymph node tissue was visible within the JLS of the fetuses with GA 12+1, 13+3 and 14+0 weeks, indicating start of reorganization of the JLS into lymph nodes (Figure 1a). In the fetus of GA 20+3 weeks, the JLS were reorganized into lymph nodes (Figure 2a).

The lymphatic endothelial cells of the JLS in the control fetuses between GA 12+1 and 14+0 weeks were positive for the lymphatic markers LYVE-1 (Figure 1d), Prox-1 (Figure 1g) and Podoplanin (data not shown). The lymphatic endothelial cells of all controls showed a slightly positive staining for VEGF-A (Figure 1j) and its co-receptor NP-1 and were negative for vWF and Shh (data
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Figure 1 Immunohistochemical analysis of the JLS
a) Transverse section of the neck of a fetus with normal nuchal skin GA 14 weeks, control) and JLS. LN tissue is visible in the JLS. b) Transverse section of the neck of a Noonan syndrome (NS) fetus with nuchal edema at GA 16 weeks. The JLS is enlarged and erythrocytes are visible within the JLS (arrows). c) Transverse section of the neck of a fetus with increased NT (without a NS gene mutation). The JLS is enlarged and erythrocytes are visible within the JLS (arrows).

Magnifications of the boxed areas of consecutive sections in (a), (b) and (c) show the staining results of the lymphatic endothelium of the JLS with different antibodies. Similar staining of LYVE-1 of the NS fetus (e), the fetus with increased NT (f) and the control fetus (d). Similar Prox-1 expression of the NS fetus (h) and decreased expression of the fetus with increased NT (i) compared to the control fetus (g). Increased VEGF-A staining of both the NS fetus (k) and fetus with increased NT (l) compared to the control fetus (j). Positive SMA staining in the subendothelium of both the NS fetus (n) and fetus with increased NT (o). Negative SMA staining in the control fetus (m). Decreased FOXC2 expression in the NS fetus (q) compared with the control fetus (p). Increased FOXC2 expression in the fetus with increased NT (r) compared to the control (p). Increased PDGF-B staining in both the NS fetus (t) and the fetus with increased NT (u) compared to the control (s). Scale bars (a, b, c) 300 μm; d-u 20 μm.

A, carotid artery; GA, gestational age; LN, lymph node tissue; V, internal jugular vein.
not shown). In all fetuses, FOXC2 expression was observed, whereas the lymphatic endothelial cells stained only slightly positive for PDGF-B (Figure 1p, 1s). No SMA expression was observed subendothelially in the controls with GA between 12+1 and 14+0 weeks (Figure 1m).

The lymphatic endothelial cells within the lymph nodes in the control fetus of GA 20+3 weeks showed a positive LYVE-1 (Figure 2e), Prox-1 (data not shown) and Podoplanin staining (Figure 2i). Staining for VEGF-A was negative (data not shown) and for NP-1 staining was slightly positive (Figure 2g). No Shh and vWF expression was observed (Figure 2k, 2m). A positive FOXC2 and slightly positive PDGF-B staining was observed in the lymphatic endothelial cells of the lymph node of this fetus. SMA expression was observed around the lymph vessels within lymph nodes (data not shown).

**Increased NT (Noonan syndrome)**

The Noonan syndrome fetus of GA 16+0 weeks demonstrated NE and distended JLS with less lymph node tissue compared with the control fetuses. The Noonan syndrome fetus of GA 22+2 weeks also demonstrated NE. The distended JLS of this fetus was only partially reorganized into a lymph node. The size of the lymph node in this case was larger compared with the control fetus of GA 20+3 weeks and had a spongy structure with several dilated lymph vessels visible within the lymph node (Figure 2c-2d). The JLS of the Noonan syndrome fetus of GA 23+5 weeks fetus was fully reorganized into lymph nodes. The size of the lymph node was larger and had a spongy structure with several dilated lymph vessels within the lymph node compared with the control fetus of GA 20+3 weeks (data not shown). The JLS / lymph nodes were positioned lateral to the jugular vein and carotid artery in all three cases (Figure 1b, 2b).

LYVE-1 was expressed in the lymphatic endothelial cells of the JLS in the Noonan syndrome fetus of GA 16+0 weeks and showed a similar staining intensity compared with the control fetuses of GA 12+1-14+0 weeks) (Figure 1d-e). Prox-1 and Podoplanin expression was also similar to the control fetuses (Figure 1g-h and data not shown). VEGF-A and its co-receptor NP-1 showed an increased staining intensity in the lymphatic endothelial cells of the JLS compared with the controls (Figure 1j-k and data not shown). Also, the lymphatic endothelial cells of the JLS were positive for vWF and Shh, in contrast to the controls (data not shown). FOXC2 showed a decreased and PDGF-B an increased expression compared with the control fetuses (Figure 1p, q, s, t). SMA expression was observed subendothelially in the JLS of the Noonan syndrome fetus, implying the presence of smooth muscle cells surrounding the JLS (Figure 1n vs 1m). The JLS of the Noonan syndrome fetus of GA 16+0 weeks and GA 22+2 weeks contained scattered blood cells (arrows Figure 1b-2b).

The lymphatic endothelial cells within the lymph nodes demonstrated normal LYVE-1 positivity in the Noonan syndrome fetuses of GA 22+2 and GA 23+5 weeks and showed a similar staining intensity compared with the control fetus of GA 20+3 weeks (Figure 2e, 2f). Prox-1 (data not shown) and Podoplanin (Figure 2i, 2j) showed a decreased expression in the lymphatic endothelial cells of the lymph vessels within the lymph node of the Noonan fetuses compared with
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Figure 2 Immunohistochemical analysis of lymph node tissue

a) Transverse section of the neck of a fetus with normal nuchal skin GA 20 weeks, control) and LN tissue. b) Transverse section of the neck of a Noonan syndrome (NS) fetus with nuchal edema at GA 22 +2 weeks. The JLS is enlarged and erythrocytes are visible within the JLS (arrows). Magnification of the boxed area demonstrates spongy lymph node tissue with several enlarged lymphatic vessels in the NS fetus (arrow in d) compared to the control (c). (e-l) Magnification of the consecutive sections of the boxed area in (a) and (b) showing the staining results of the lymphatic endothelium with the different antibodies. The asterisks in e-l indicate the investigated lymphatic vessel. A similar LYVE-1 expression (f) compared to (e) and increased expression of NP-1 (h) compared to (g) is seen when comparing the NS fetus to the control fetus. Decreased expression of Podoplanin in the NS fetus (j) compared to the control (i) is demonstrated. Small blood vessels are visible within the lymph nodes in both the NS fetus (l) and control (k), indicated by a positive vWF expression. A positive Shh expression in the NS fetus (n) was found compared to the control (m). Scale bars (a-b) 300 μm; (c-d) 100 μm; (e-l) 20 μm.

A, carotid artery; GA, gestational age; JLS, jugular lymphatic sac; LN, lymph node tissue; V, internal jugular vein.
the control fetus. VEGF-A and its co-receptor NP-1 showed an increased staining intensity in the lymphatic endothelial cells of the lymph nodes compared with the control (data not shown and Figure 2g, 2h). The lymphatic endothelial cells of the lymph nodes were positive for vWF and Shh, in contrast to the control (Figure 2k-n). A clear FOXC2 expression and slight PDGF-B expression was found in the Noonan fetuses which showed a similar expression as compared with the control (data not shown). SMA expression was observed subendothelially of the lymph vessels within the lymph nodes of the Noonan fetuses of GA 22+2 and GA 23+5 weeks, implying the presence of smooth muscle cells (data not shown).

Increased NT (without a Noonan gene mutation)
The four fetuses with increased NT without a confirmed Noonan syndrome gene mutation showed NE and distended JLS with less lymph node tissue compared with the control fetuses (Figure 1a, 1c). The JLS was positioned direct lateral to the jugular vein and carotid artery (Figure 1c). A positive LYVE-1 staining was found and staining intensity was similar compared with the controls (Figure 1d, 1f). The lymphatic endothelial cells of the JLS demonstrated a diminished expression of the lymphatic markers Prox-1 and Podoplanin compared with the control fetuses (Figure 1g,1i and data not shown). VEGF-A (Figure 1j, 1l) and NP-1 protein expression (data not shown) was increased compared with the control fetuses. In contrast to the controls, the lymphatic endothelial cells of the JLS were positive for vWF and Shh (data not shown). A positive FOXC2 staining was observed and staining intensities were similar compared with controls (Figure 1p, 1r). An increased PDGF-B expression was additionally found (Figure 1s, 1u). The JLS were surrounded by a layer of smooth muscle cells (Figure 1o). Furthermore, the JLS of all four fetuses contained blood cells (arrows Figure 1 c).

The JLS of the fetuses with increased NT without a confirmed Noonan gene mutation (GA 14+4-14+6 weeks) were not (yet) reorganized into lymph nodes. Therefore, the lymphatic endothelial cells of lymph vessels within the lymph nodes could not be investigated in these fetuses.

DISCUSSION

In this study we evaluated the nuchal lymphatic endothelial differentiation of seven fetuses with increased NT and a normal karyotype including three fetuses with a confirmed Noonan syndrome gene mutation. The findings in the lymphatic endothelial cells of the fetuses with increased NT were in general comparable with the results in aneuploid fetuses with NE7 showing a decreased expression of the lymphatic specific markers Prox-1 and Podoplanin and an increased expression of the blood vessel specific markers VEGF-A and NP-1 in the fetuses with increased NT compared with controls.

Prox-1, a homeobox transcription factor, is important for the induction of lymphatic development.12 Previous research indicates a disturbed venous-to-lymphatic differentiation of the
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lymphatic endothelial cells in aneuploid fetuses\textsuperscript{7} with Prox-1 suggested to play a central role in this disturbance. It upregulates the expression of the lymphatic marker Podoplanin and suppresses the expression of the blood-endothelial cell specific markers VEGFR-2 and NP-1, which are a receptor and a co-receptor of VEGF-A, respectively.\textsuperscript{13,14} As a result, a decreased Prox-1 expression could result in increased lymphatic endothelial cells specific VEGF-A signalling, which could cause hypervascularization, increased permeability and edema.\textsuperscript{15,16}

In the current study, most fetuses with increased NT (6 out of 7) showed decreased Prox-1 and Podoplanin expression. However, the normal expression patterns of these lymphatic endothelial cell-specific proteins found in the Noonan fetus of GA 16 weeks compared with the controls suggests that Prox-1 independent mechanisms, such as autonomous VEGF-A upregulation could also play a role in the described phenotype. Previous research suggests that an increased VEGF-A expression might be the result of an altered Shh signalling as Shh acts upstream of VEGF-A.\textsuperscript{17,18} This is in accordance with the findings of the current study, showing an upregulated VEGF-A and Shh expression in the fetuses with increased NT. In addition, the positive vWF expression in the lymphatic endothelial cells, which has been described before in trisomy 21 fetuses\textsuperscript{19}, supports the finding of a disturbed lymphatic to blood vessel endothelial differentiation because vWF is normally not expressed in lymphatic endothelial cells.\textsuperscript{20}

Smooth muscle cells surrounded the enlarged JLS in all fetuses with increased NT in contrast to the control fetuses. It has been hypothesized before in trisomy 21 fetuses with increased NT that these smooth muscle cells are associated with a decreased FOXC2 and an increased PDGF-B expression\textsuperscript{9}, which was confirmed in the Noonan fetus of GA 16 weeks. The four fetuses without a gene mutation for Noonan syndrome showed an increased PDGF-B expression but positive expression patterns of FOXC2 in the lymphatic endothelial cells compared with the control fetuses. A possible explanation for the increased PDGF-B expression and subsequent smooth muscle cell upregulation in these cases could be the fact that PDGF-B can be upregulated by VEGF-A.\textsuperscript{21}

In the current research, six of the seven fetuses with increased NT showed a cardiac malformation. Interestingly, abnormal endothelial processes also have been described to play a role in relation to development of cardiovascular defects.\textsuperscript{22} This is also recently suggested in Noonan syndrome associated cardiac defects by Araki et al.\textsuperscript{23}

In summary, we demonstrate enlarged JLS and lymph node tissue with a disturbance in differentiation of the lymphatic endothelial cells in euploid fetuses having increased NT. An upregulated VEGF-A and NP-1 expression are most likely to be involved in this abnormal differentiation which has also been suggested in aneuploid fetuses with increased NT.\textsuperscript{7} With this paper, we show that euploid fetuses with increased NT demonstrate a similar phenotype as aneuploid fetuses with enlarged JLS and aberrant differentiation of the lymphatic endothelial cells. This aberrant differentiation is also found in the lymph node tissue of second trimester fetuses with increased NT.
A wide range of abnormalities is related to increased NT.\textsuperscript{5} Fetuses with increased NT are a heterogeneous group which is also showed in this study. One single cause as an explanation for the pathophysiology of increased NT is therefore not likely. A disturbed lymphatic endothelial differentiation could be the common process related to fetuses with increased NT. We hypothesize that the disturbance in lymphatic differentiation can range from a delayed but physiological development to a more severe disturbance. Previous ultrasound studies showed that in case of euploid fetuses with increased NT and a normal 20 week scan, an uneventful pregnancy outcome can be expected.\textsuperscript{24,25} In the current study, only a small group of euploid fetuses with a severely increased NT and (genetic and / or structural) malformations were assessed which is a limitation of our study. These fetuses probably form the severe end of the spectrum.

Interestingly, Turner syndrome (monosomy X) fetuses also present with a massively increased NT. A recent morphological study of the jugular lymphatic system demonstrated aplasia of the JLS in these fetuses.\textsuperscript{19} This suggests a total lack of lymphatic endothelial differentiation from veins and development towards a JLS in Turner syndrome fetuses. This indicates that increased NT in euploid fetuses and Turner syndrome are most probably caused by different mechanisms (i.e. abnormal lymphatic endothelial cell function vs lack of JLS development).

In conclusion, the present study shows that euploid fetuses with increased NT demonstrate a disturbance in lymphatic endothelial differentiation. These findings are similar to the previous results in aneuploid fetuses with increased NT. The possible relation between lymphatic and cardiac abnormalities needs further research.

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