Chapter 9

General discussion and future perspectives
CONCLUSIONS

The aim of this thesis was to study the pathophysiology of increased nuchal translucency (NT). This thesis examined the role of lymphatic endothelial development and neural crest cells in increased NT. Furthermore, genetic mechanisms were studied to provide more insight into the high incidence of cardiac defects in fetuses with increased NT. As increased NT is associated with abnormal first-trimester ductus venosus flow velocity waveforms, we also investigated whether a ductus venosus sphincter or local morphological changes in the ductus venosus could explain the altered ductus venosus flow velocity waveforms.

This thesis shows the involvement of abnormal lymphatic endothelial development and neural crest cells in increased NT. We have analyzed genes that contribute to embryonic cardiac and lymphatic development. Loss of function of these genes causes both cardiac and lymphatic abnormalities and nuchal edema, the morphological equivalent of increased NT. We have demonstrated the normal embryonic development of the ductus venosus and the absence of a sphincter. Our results indicate that alterations in ductus venosus flow velocity waveforms in human embryos are not caused by local morphological changes, regardless of chromosomal abnormalities, nuchal edema, cardiac defects or lymphatic anomalies. Our findings implicate that abnormal lymphatic endothelial development is the most important factor in the development of increased NT. Neural crest cell abnormalities also contribute to abnormal lymphatic development. The hemodynamic alterations in the ductus venosus reflect intracardiac pressure changes, but the exact role of hemodynamic alterations in the development of increased NT has remained unclear.

INCREASED NT

Almost 25 years ago the association between an increased fetal NT and chromosomal abnormalities was first reported. Nuchal thickness measurement has been implemented as part of the first-trimester screening for chromosomal abnormalities. Besides chromosomal anomalies, an increased NT has been reported to be associated with a variety of structural malformations and genetic syndromes, of which cardiac defects are the most common. On the other hand, the majority of euploid fetuses with increased NT have a favourable outcome. Despite the large-scale use of NT measurement, the developmental background of increased NT has not been elucidated. Based on the broad spectrum of chromosomal, structural and genetic abnormalities found in fetuses with increased NT, as well as the healthy outcome in the majority of the euploid fetuses, a single origin is not likely. It seems probable that different factors in a developmental process or different processes in early development could be disturbed, in which increased NT represents the common phenotype.
In this thesis we have identified genes involved in both abnormal cardiac and lymphatic development, that show expression in the endothelium. Alterations in these genes and their genetic pathways result in nuchal edema. This shared genetic origin of both cardiac and lymphatic defects, in which lymphatic and cardiac endothelium play important roles, offers an explanation why increased NT has a strong relationship with cardiac defects, opposed to other fetal anomalies that are mainly reported as case series. The association between nuchal edema and abnormal jugular lymphatic sac (JLS) formation explains an increased risk for cardiac defects in fetuses with increased NT. The identified genes are involved in various genetic pathways and have multiple genetic interactions.

Recent studies on the origin of increased NT have suggested that a disturbance or delay in lymphatic endothelial development causes increased NT. Embryonic lymphatic development starts in the cardinal veins with reprogramming of blood endothelial cells towards lymphatic endothelial cells. Lymphatic endothelial cells bud and migrate from the cardinal veins as single cells or clusters and subsequently form the JLS. The JLS normally remodel into lymph nodes after 10 weeks of human gestation. The ingrowth of the right thoracic duct into the left JLS completes the development of the lymphatic system, thereby forming the main drainage site of lymphatic fluid into the venous systemic circulation. Previous studies demonstrated enlarged JLS in both euploid and aneuploid fetuses with increased NT. A delay or disturbance in lymphatic development could explain both the temporary and local fluid accumulation in the nuchal region. The formation of the JLS occurs in close proximity of cranial nerves, which are derived from neural crest cells. Neural crest cells are pluripotent cells that contribute to different structures and tissues, such as the pharyngeal cartilage, cranial nerves, dermis of the head and neck, cardiac outflow tract and the pharyngeal arch arteries. In Chapter 3, we identified neural crest cells in the JLS. This demonstrates the involvement of neural crest cells in jugular lymphatic development. This is line with a study by Miyabara et al. who suggested the involvement of neural crest cells in lymphatic anomalies and cardiac defects. Whether neural crest cells first differentiate into blood endothelial cells and then differentiate into lymphatic endothelial cells, or directly into lymphatic endothelial cells, is not answered yet.

This thesis identifies retinoic acid as a connecting factor between the nervous and lymphatic system. Retinoic acid signalling occurs in lymphatic endothelial cells in the JLS and is required for the formation of the JLS. It is synthesized in nerves and is essential for neural crest cell migration and differentiation. Inhibiting retinoic acid signalling, which causes a disturbance in neural crest cell migration and differentiation, results in fewer lymphatic endothelial cells, an abnormal size of the JLS and nuchal edema. This is in line with studies by Choi et al. and Marino et al. who reported on smaller numbers of lymphatic endothelial cells and smaller lymphatic structures upon a lack of retinoic acid. Bowles et al. reported that an excess of retinoic acid results in more lymphatic endothelial cells. Disturbances in neural crest cell migration and differentiation thus cause anomalous jugular lymphatic development and nuchal edema. Nerves and (lymphatic) endothelium share expression of multiple developmental genes, such as Vegfc and the receptors Neuropilin (Nrp)-1 and Nrp-2. Embryonic endothelium also influences and signals to the neural crest. We speculate on the closely coordinated development of the neural and lymphatic system. These new insights into the closely associated development of the neuronal and lymphatic system and the involvement of retinoic acid need further exploration. The precise contribution of neural crest cells to lymphatic development could be quantified using flow cytometry techniques in a mouse model in which neural crest cells and its descendants are fluorescently labelled. Another question that remains is what is the source of retinoic acid. Because nerves adjacent to the JLS highly express Raldh2, which is essential for the synthesis of retinoic acid, we speculate that these cranial nerves synthesize retinoic acid.

Abnormal neural crest cell migration and differentiation can result in cardiac defects and craniofacial anomalies and skeletal abnormalities. This could explain a part of the spectrum of fetal anomalies associated with increased NT, such as pharyngeal arch artery anomalies and cardiac outflow tract defects. Increased NT is, however, not related to a specific type of cardiac defect. Experimental animal studies have shown that neural crest cells also contribute to the formation of the myocardium and signaling to the epicardium, or contribute directly to the formation of the heart. Future genetic and morphological studies are needed to explore a possible causal relationship.

Historically, increased NT was believed to be caused by cardiac failure. This believe was based on the observation that cardiac defects are the most common malformation in euploid fetuses with increased NT and that a strong relationship exists between increase in NT and the risk of a cardiac defect in euploid fetuses. It was suggested that cardiac failure, secondary to a cardiac defect, induces increased NT. The cardiac failure was thought to be reflected by alterations in ductus venous flow velocity waveforms, which is a frequent finding in fetuses with increased NT and cardiac defects. In this thesis we showed that nuchal edema developed independent of the presence of a cardiac defect in various euploid mutant mouse models. The presence of nuchal edema was consistent with abnormal JLS development. This emphasized the role of abnormal embryonic lymphatic development in the etiology of increased NT in euploid fetuses. The methodology of our experiments, however, hampered us to relate the cardiac defects to cardiac dysfunction, because we were unable to measure intracardiac flow velocities in the mouse embryos that were alive. In order to directly contradict the hypothesis of cardiac failure underlying increased NT, first-trimester cardiac function should be assessed to relate cardiac defects to cardiac function. The reproducibility and accuracy of ultrasound techniques like Speckle-Tracking Echocardiography or Tissue Doppler Imaging currently impede large-scale studies on this subject, especially because fetal hearts at this early gestational age are very small with a relatively fast heart rate.

Two prior studies measured intracardiac flow velocities in first-trimester fetuses and found no significant difference in cardiac function, independent of increased NT, cardiac defects or
anomalies. A previous study\(^6\) of the ductus venosus in trisomy 16 mouse embryos, a model for human trisomy 21, showed nuchal edema and thickening of the ductus venosus endothelium\(^6\). It was suggested that an alteration in local (endothelial) morphology could explain abnormal ductus venosus flow velocity waveforms in fetuses with increased NT. Another explanation for changed ductus venosus flow velocity waveforms is the presence of a sphincter at the ductus venosus inlet, which is described in the first morphological reports on the ductus venosus\(^2\)-\(^4\). In the studies of this thesis, neither thickening of the ductus venosus endothelium or a ductus venosus sphincter were observed. This is in accordance with more recent morphological studies by Mavrides et al.\(^6\) and by Ailamazyan et al.\(^6\). This is also supported by previous experimental studies in fetal sheep that have reported on the effect of ductus venosus relaxation upon nitric oxide and hypoxemia\(^6\) and ductus venosus constriction upon administration of endothelin\(^6\). High blood viscosity and low umbilical vein pressure could also cause an increased blood flow through the ductus venosus\(^6\). Interestingly, these studies all report on relaxation or constriction of the total length of the ductus venosus and do not show a specific sphincter area.

A comparable morphology of the ductus venosus was found in various mutant mouse models with nuchal edema, abnormal lymphatic development and cardiac defects. Local morphological alterations thus can not explain changes in ductus venosus flow velocity waveforms. Similar results were demonstrated in first-trimester human fetuses with abnormal ductus venosus flow velocity waveforms, aneuploidy, cardiac defects and increased NT. Again, the lack of a ductus venosus sphincter was confirmed. We state that ductus venosus flow velocity waveforms are not caused by a local morphological alteration, but most likely reflect intracardiac pressure. Alterations in cardiac pre- or afterload may have a significant effect on ductus venosus flow velocity waveforms. An increase in cardiac compliance and cardiac output coincides with the disappearance of NT\(^5\)-\(^7\). Because peripheral vascular resistance and placental resistance simultaneously decrease, cardiac afterload considerably drops\(^7\). The JLS then remodel into lymph nodes and the lymphatic vessels connect to the venous circulation at 14 weeks of human gestation. The (excess of) lymphatic fluid is then drained to the venous system\(^2\)-\(^3\). Previous studies in first-trimester trisomy 21 fetuses demonstrated an abnormal cardiac function, specifically a diastolic dysfunction, compared to fetuses with normal or increased NT, independent of cardiac anatomy\(^4\)-\(^5\). Tricuspid regurgitation at 11-14 weeks of gestation, which may be caused by small alterations in hemodynamics, disappears simultaneously with the vanishing of the NT\(^7\)-\(^8\) and is strongly associated with chromosomal abnormalities, in particular with trisomy 21\(^7\)-\(^8\). We speculate that hemodynamic changes in the late first to second trimester of pregnancy can explain alterations in ductus venosus flow velocity waveforms as well as the temporary nature of increased NT. Whether hemodynamic alterations solely – without a delay or disorder in jugular lymphatic development – is sufficient to induce increased NT is unknown. Hemodynamic alterations can also induce cardiac defects\(^6\). This provides a second interesting link to explain the strong relationship between cardiac defects and increased NT.

Our study design has several limitations. Ideally, a longitudinal study in which a large number of euploid and aneuploid human fetuses with and without increased NT should be examined using modern ultrasound techniques, such as Tissue Doppler imaging and Speckle-Tracking Echocardiography, to assess the myocardial function, followed by postmortem examination of the heart and nuchal region. This study design is never possible, because only in a small proportion of the pregnancies of fetuses with increased NT a termination of pregnancy will be initiated or fetal demise occurs. In the majority of the fetuses with increased NT with less severe malformations, the pregnancy continues and postmortem examination can not be performed. Consequently, only the severe end of the spectrum of fetal anomalies can be studied by postmortem examination. Instead, with the development of high-resolution ultrasound probes, a longitudinal study in various mutant mouse models, ideally including real intra-cardiac pressure measurements, ultrasound assessment of cardiac function, NT and ductus venosus flow velocity waveforms, followed by morphological examination could be performed.

**INCREASED NT AND THE DUCTUS VENOSUS**

If abnormal endothelial development is an important denominator in the etiology of increased NT, the question rises if this relates to abnormal ductus venosus flow velocity waveforms in fetuses with increased NT and such different anomalies. A previous study\(^6\) of the ductus venosus in trisomy 16 mouse embryos, a model for human trisomy 21, showed nuchal edema and thickening of the ductus venosus endothelium\(^6\). It was suggested that an alteration in local (endothelial) morphology could explain abnormal ductus venosus flow velocity waveforms in fetuses with increased NT. Another explanation for changed ductus venosus flow velocity waveforms is the presence of a sphincter at the ductus venosus inlet, which is described in the first morphological reports on the ductus venosus\(^2\)-\(^4\). In the studies of this thesis, neither thickening of the ductus venosus endothelium or a ductus venosus sphincter were observed. This is in accordance with more recent morphological studies by Mavrides et al.\(^6\) and by Ailamazyan et al.\(^6\). This is also supported by previous experimental studies in fetal sheep that have reported on the effect of ductus venosus relaxation upon nitric oxide and hypoxemia\(^6\) and ductus venosus constriction upon administration of endothelin\(^6\). High blood viscosity and low umbilical vein pressure could also cause an increased blood flow through the ductus venosus\(^6\). Interestingly, these studies all report on relaxation or constriction of the total length of the ductus venosus and do not show a specific sphincter area.

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With the discovery of fetal cell-free DNA in pregnant women, the field of prenatal medicine has recently encountered new non-invasive techniques. The Non Invasive Prenatal Test (NIPT) is considered superior to the combined test in screening for aneuploidy, at least in a high-risk population. The NIPT may replace the combined test in the first-trimester screening for aneuploidy in the near future. An increased NT will, however, still be observed in prenatal care. An increased NT is clinically relevant as this is an abnormal finding, which requires specialized care. Although numerous case studies have reported on the association of increased NT with various structural abnormalities, such as diaphragmatic hernia, craniofacial anomalies, exomphalos, body stalk anomaly and with multiple genetic syndromes, including fetal akinesia deformation sequence, 22q11.2 deletion syndrome, Noonan syndrome and rare skeletal dysplasias, the association between increased NT and this wide spectrum of fetal anomalies has never been proven by a large prospective cohort study, including a proper comparison of prevalence of these anomalies to a control population with normal NT. In euploid fetuses, an increased NT should be considered as a nonspecific sign of (temporary) abnormal embryonic development. The future role of increased NT, including its role as part of a first-tier screening for aneuploidy, is uncertain.

In conclusion, increased NT most likely origins from a disturbance in lymphatic endothelial development. Abnormal neural crest cell migration and differentiation closely interacts with lymphatic endothelial development and is involved in the pathophysiology of increased NT. Hemodynamic alterations can explain alterations in ductus venosus flow velocity waveforms and may contribute to the developmental background of increased NT.

The challenge now lies in integrating the various causes of increased NT and identify the specific underlying mechanisms to elucidate the pathophysiology of increased NT. Future studies should focus on the interactions between (lymphatic) endothelial differentiation, neural crest cell development and hemodynamic influences in large datasets of human fetuses with increased NT. A first step would be to analyze neural crest cell migration in human fetuses with and without increased NT, including its relationship with jugular lymphatic development. Furthermore, due to rapid advances in genetic testing, such as the application of high-resolution whole-genome screening in prenatal medicine, an increasing amount of genetic abnormalities will be discovered in fetuses with increased NT. Examination of these genetic mutations in mouse models can be performed to explore the different pathways leading to increased NT. Improved knowledge of the various mechanisms involved in the pathophysiology of increased NT will improve the clinicians ability to counsel parents on their fetus with an increased NT.
REFERENCES


