Increased nuchal translucency

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2021

document version
Publisher's PDF, also known as Version of record

Increased nuchal translucency
From etiology to clinical consequences

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ISBN: 978-94-6361-613-3
Cover design and layout: Optima Grafische Communicatie (www.ogc.nl)
Printing: Optima Grafische Communicatie (www.ogc.nl)

INCREASED NUCHAL TRANSLUCENCY From etiology to clinical consequences

VRIJE UNIVERSITEIT

INCREASED NUCHAL TRANSLUCENCY
From etiology to clinical consequences

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor of Philosophy aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. C.M. van Praag,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de Faculteit der Geneeskunde
op woensdag 15 december 2021 om 9.45 uur
in een bijeenkomst van de universiteit,
De Boelelaan 1105

door
KYRA EVA STUURMAN
geboren te Tilburg

promotoren:	prof. dr. E.J. Meijers-Heijboer
	prof. dr. L. Henneman
copromotor:	dr. M.W. Elting
promotiecommissie:	prof. dr. M.C. Cornel
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Alles van waarde is weerloos

Lucebert, 1974

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Chapter 1

Introduction

In many countries, an increased nuchal translucency (NT) is used as a screening tool for Down syndrome in pregnancy. When the karyotype is normal after follow-up testing, however, the fetus is still at risk for structural anomalies and genetic disorders. The etiology of an increased NT has yet to be unraveled. In addition, with the development and implementation of new prenatal screening techniques and diagnostic testing possibilities, the use of the NT measurement has changed over the past 5-10 years. In this thesis the fundamental questions, the clinical aspects and the current value and future purpose of an increased NT were studied.

INCREASED NUCHAL TRANSLUCENCY

Nuchal translucency (NT) refers to the translucent area in the neck region of the developing fetus and can be visualized by ultrasound scan between 11 and 13+6 weeks of gestation (Figure 1), after which it normally resolves. An increased NT represent nuchal edema and is defined at equal or greater than 3.5 mm (99th percentile) according to the Dutch Society of Obstetrics and Gynecology guidelines (NVOG, 2012) and is prevalent in 1% of all pregnancies (Snijders et al., 1996; Snijders et al., 1998; Lichtenbelt et al., 2015). Some authors, however, even suggest to use a cut-off above 3.0 mm (95th percentile) (Maya et al., 2017; Petersen et al., 2020; Sagi-Dain et al., 2021), but this is still debated. An increased NT raises the suspicion of a chromosomal and/or structural anomaly in the fetus and is therefore an indication for invasive diagnostic follow-up testing. In 1992, Nicolaides et al. (1992b) was the first to describe the use of NT measurement in the first trimester of pregnancy as a marker to identify fetal aneuploidy. Previous studies, mainly case reports, on first trimester nuchal fluid had primarily focused on cystic hygroma, which is a congenital malformation of the lymphatic system with fluid collections usually extending along the entire back region of the fetus with clearly visible septations. There are, however, authors who regard cystic hygroma as an outdated term for a severely increased NT (Molina et al., 2006). Nicolaides et al. (1992b) showed that the risk of chromosomal abnormalities increased with the increase of thickness of the nuchal translucency. The implementation of NT measurement was widely embraced by physicians and pregnant women, due to its usefulness in screening the general population with a non-invasive test and as a result preventing unwanted miscarriages resulting from invasive prenatal diagnostic tests. However, it has to be stated that the measurement of NT alone is nowadays not regarded as an accurate screening test as the detection rate for trisomy 21 is only 70% (Alldred et al., 2017; ACOG, 2020). In the years thereafter Nicolaides' group proposed a new method to improve aneuploidy risk assessment, which combined data on nuchal translucency measurement, maternal age and maternal serum biochemistry (free Beta-HCG and pregnancy associated plasma protein

A (PAPP-A)) (Brizot et al., 1994; Nicolaides et al., 1994). In the Netherlands this is called first trimester combined screening (in Dutch: 'combinatietest') and was first introduced in 2007 as a prenatal screening test for Down syndrome (trisomy 21) (Gezondheidsraad, 2007) in a national screening program. In 2011, Edwards syndrome (trisomy 18) and Patau syndrome (trisomy 13) were added to this screening program. The aim of prenatal screening for fetal aneuploidy is to enable autonomous reproductive choices by pregnant women and their partners and not preventing the birth of children with specific anomalies (Dondorp et al., 2015).

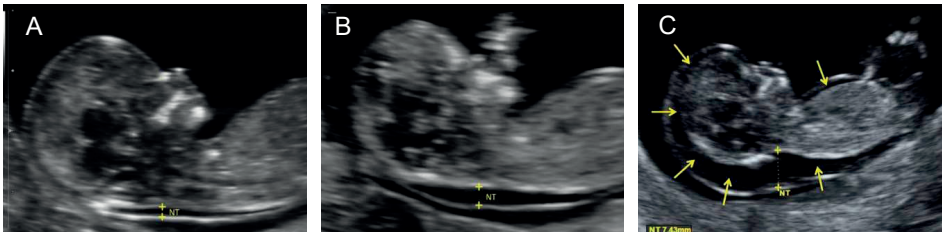

Figure 1 Stages of nuchal edema (A) Normal nuchal translucency. Collection of fluid under the skin at the back of the fetal neck region between 11 and 14 weeks of gestation, measured on ultrasound. (B) Increased nuchal translucency. The collection of fluid exceeds 3.5 mm (defined as cut-off value in the Netherlands) in measurement on ultrasound. (C) Hydrops fetalis. The presence of abnormal fluid collections in fetal soft tissues and serous cavities, at least in two compartments (skin edema, ascites, pleural effusion, pericardial effusion) (Gersak, 2013)

SUGGESTED ETIOLOGY OF AN INCREASED NT

The etiology of an increased NT has not been elucidated and the diversity in which congenital structural abnormalities and genetic syndromes occur in association with increased NT (Nicolaides et al., 1992a; Pandya et al., 1995; Hyett et al., 1997b; Bilardo et al., 1998; Souka et al., 1998; Souka et al., 2001; Senat et al., 2002; Souka et al., 2005; Bilardo et al., 2007; Axt-Fliedner et al., 2009; Bilardo et al., 2010; Ayras et al., 2013), suggests that one single explanation for the development of nuchal edema is not very likely. Three main theories on the development of nuchal edema have been postulated in the past: I) cardiovascular failure (Hyett et al., 1997b), II) alterations in the extracellular matrix (Brand-Saberi et al., 1994a; Brand-Saberi et al., 1994b; von Kaisenberg et al., 1998) and III) lymphatic development abnormalities (van der Putte, 1977; Byrne et al., 1984).

I Cardiovascular failure

Cardiac defects are frequently found in fetuses with an increased NT (Hyett et al., 1995; Hyett et al., 1997a). It was therefore thought that these defects could result in developing cardiovascular failure such as increased end-diastolic right ventricular pressure or ductus venosus flow abnormalities, which subsequently would result in nuchal edema

(Montenegro et al., 1997). However, not all fetuses with an increased NT have cardiac defects and the most frequently diagnosed cardiac defects in fetuses with an increased NT are septal defects (Miyabara et al., 1989; Hyett et al., 1995; Haak et al., 2002a), which are not commonly associated with hemodynamic compromise during fetal life (Haak et al., 2002a). Ductus venosus flow abnormalities are also seen in fetuses with a chromosome aberration who do not show a cardiac defect (Carvalho, 1999) and in fetuses with a normal NT (Oh et al., 2007; Martínez et al., 2010). Additionally, fetuses with an increased NT show a plethora of extra-cardiovascular anomalies (Souka et al., 1998). Therefore an increased NT cannot be explained by hemodynamic failure due to cardiac defects only.

II Extracellular matrix alterations

Extracellular matrix alterations, e.g. irregularly arranged and densely packed collagens, have been found in different aneuploidies, with trisomy 21 fetuses having altered collagen VI suggested to be due to overexpression of the *COL6A1* and *COL6A2* genes on chromosome 21 (Brand-Saberi et al., 1994a; Brand-Saberi et al., 1994b). However, in trisomy 16 mouse embryos, which resemble the human trisomy 21, a similar change in the extracellular matrix was seen, but the *COL6A1* and *COL6A2* genes are not located on the murine chromosome 16 (von Kaisenberg et al., 1998). Moreover, in trisomy 13 and 18, extracellular matrix changes were also seen, albeit slightly different alterations than seen in trisomy 21, therefore not explained by the overexpression of the *COL6A1* and *COL6A2* genes. Also, the altered composition in extracellular matrix morphology has only been found in chromosomal abnormal fetuses (Brand-Saberi et al., 1994a; Brand-Saberi et al., 1994b; von Kaisenberg et al., 1998). The extracellular matrix changes have not been investigated in euploid fetuses with an increased NT. Although Brand-Saberi et al. (1994a; 1994b) and Von Kaisenberg et al. (1998) used only one time-point in early pregnancy for investigating the extracellular matrix, Quarello et al. (2007) confirmed that overexpression of the *COL6A1* and *COL6A2* genes in fetuses with trisomy 21 was present irrespective of the NT increase or gestational age. Subsequently, if expression of *COL6A1* and *COL6A2* and alterations in the extracellular matrix would be causal for the development of increased NT, the transient nature of an (increased) NT is then not explained.

III Abnormal lymphatic development

An altered lymphatic development has been suggested as an important cause of increased NT. In 1902, Sabin first described the development of lymphatic vasculature (Sabin, 1902). The internal jugular veins in the neck form jugular lymphatic sacs (JLS) by budding and fusing. The peripheral lymphatic system is formed by sprouting from these sacs. It is also suggested that small peripheral lymph vessels can arise from angioblast-derived lymphatic endothelial progenitors (Risau, 1995; van de Pavert et al., 2014). The development of the lymphatic system is finalized when the right thoracic duct grows

into the left JLS, which then drains the interstitial fluid from the nuchal region into the systemic circulation. The transient and local nature of an increased NT can therefore be explained by lymphatic maldevelopment, because the lymphatic system undergoes finalization of development at the time the nuchal edema appears (Sabin, 1909). A disturbed differentiation of lymphatic endothelial cells is associated with distension of JLS and nuchal edema (Haak et al., 2002b). Bekker et al. showed in an ultrasound study a significant association between an increased NT and the presence of (enlarged) JLS (Bekker et al., 2005a). Additionally, impaired neural crest cell migration has been suggested as a possible cause for abnormal lymphatic and cardiovascular development (Bekker et al., 2005b). Thus far, however, the underlying mechanism of lymphatic maldevelopment causing an increased NT has not yet been completely elucidated. Therefore, part of this thesis focused on the lymphatic development and possible prior associated neural crest cell migration in mouse embryos to gain more information on the development of an increased NT.

ASSOCIATION OF AN INCREASED NT WITH SEX, ETHNICITY AND FAMILIAL OCCURRENCE

Sex

Extensive research has been performed to evaluate the influence of the sex of the fetus on the risk of abnormal maternal serum markers (de Graaf et al., 2000; Spencer et al., 2000; Yaron et al., 2001). However, the impact of sex on *normal* nuchal translucency has been described in only a small amount of studies. Some of these studies show that, in a general unselected population, male fetuses appear to have a slightly larger NT in the normal range than female fetuses (Lam et al., 2001; Larsen et al., 2002; Cowans et al., 2009; Daouk et al., 2012). Other studies show no difference (Yaron et al., 2001; Prefumo et al., 2003). The impact of sex of the fetus on *increased* NT (NT≥3.5 mm) is to the best of our knowledge been described in only two studies (Timmerman et al., 2009; Ayras et al., 2015a). Timmerman et al. (2009) showed preponderance for male fetuses in the lower range of increased NT (3.0-5.4 mm) but a higher prevalence of female fetuses with NT above 5.5 mm. A possible explanation might be the incidence of Turner syndrome in the female group, which is in general associated with a higher increased NT. Ayras et al. (2015a) also showed that more male fetuses had an increased NT, but female fetuses with increased NT had a thicker NT than male fetuses. The authors, however, had excluded abnormal sex chromosomes beforehand and still found more chromosome anomalies in the female fetuses. Both studies also commented on the outcome of euploid fetuses with increased NT; Timmerman et al. (2009) showed a better prognosis for the male fetuses, but in the Ayras at al. study (2015a) there were no sex differences

in the long term outcome. In the past five years, no studies have been published about sex differences in fetuses with an increased NT. Based on the sparse literature a definite conclusion of sex differences in fetuses with an increased NT cannot be made.

Ethnicity

With regards to ethnicity, differences in NT have been suggested. However, no consensus exists and all studies published to date lack significance (Chen et al., 2002; Spencer et al., 2005; Cowans and Spencer, 2011).

Familial occurrence

Recurrent increased NT has been described in a handful of mostly case reports. In all but one the increased NT was part of a familial chromosomal abnormality such as a microdeletion or autosomal recessive disorder. Only one of these reports showed recurrence of transient nonseptated cystic hygroma at 14 weeks of gestation in 18 sibling pairs out of 4200 pregnancies. These pairs did not show any other fetal anomaly on prenatal ultrasound scan, had normal karyotype and normal outcome (Rotmensch et al., 2004). It suggests a genetic etiology without clinical consequences postpartum.

ASSOCIATION OF AN INCREASED NT WITH CONGENITAL STRUCTURAL DEFECTS AND (GENETIC) SYNDROMES

The added value of prenatal chromosomal microarray (Table 1) to detect submicroscopic deletions or duplications in fetuses with an increased NT has been described extensively (Brisset et al., 2003; Hillman et al., 2013; Lund et al., 2015; Oneda and Rauch, 2017; Yang et al., 2017; Sinajon et al., 2020). A 4-7% diagnostic yield is added to conventional karyotyping in search for structural chromosomal aberrations such as trisomy 21 (Grande et al., 2015), but besides its clear association with chromosomal abnormalities including submicroscopic deletions and duplications, fetuses with an increased NT are also at risk for congenital structural defects such as cardiac defects, renal and abdominal wall anomalies (Nicolaides et al., 1992a; Hyett et al., 1995; Pandya et al., 1995; Souka et al., 1998). These structural anomalies can be part of a (genetic) syndromal disorder. Over the years, many studies about this association between increased NT, congenital malformations and (genetic) syndromes have been published (Bilardo et al., 1998; Souka et al., 1998; Souka et al., 2001; Souka et al., 2005; Ayras et al., 2013). The list of associated syndromes seems endless, but numbers often do not reach significance to proof its association (Souka et al., 1998). Thus far only Noonan syndrome and its associated RASopathies (a clinically defined group of genetic disorders caused by germline pathogenic variants in genes that encode components or regulators of the Ras/MAPK

pathway) have been evaluated systematically and proven to be of clinical significance in relation to increased NT (Achiron et al., 2000; Baldassarre et al., 2011; Croonen et al., 2013; Hakami et al., 2016; Ali et al., 2017; Stuurman et al., 2019).

Studies on the association of increased NT with normal karyotype and intellectual disability and other neurodevelopmental disorders such as autism spectrum disorders, cerebral palsy and epilepsy show that, when the karyotype is normal and ultrasound does not show any abnormalities, there is still an increased risk of intellectual disability and autism spectrum disorders (Brady et al., 1998; Senat et al., 2002; Miltoft et al., 2012; Ayras et al., 2015b; Hellmuth et al., 2017). However, the absolute risk is low (Hellmuth et al., 2017) and does not exceed the background risk in the general population on a congenital anomaly in the unborn child. There seems to be no increased risk on developing epilepsy and cerebral palsy (Hellmuth et al., 2017).

Although innumerable studies on the outcome of fetuses with an increased NT have been published over the last 25 years, more insight on the diverse clinical postpartum phenotype is needed to assume significant associations, especially the association between an increased NT and RASopathies. This latter association is mainly based on case reports and overview studies are lacking.

CLINICAL IMPLICATIONS OF AN INCREASED NT AND THE CHANGING LANDSCAPE OF PRENATAL SCREENING

Since 1994, prenatal invasive diagnostic testing has been offered to the pregnant woman when an increased NT is identified. Depending on the gestational age and preference of the pregnant woman, this is either chorionic villus sampling (from 11 weeks of gestation onwards) or amniocentesis (from 15 weeks of gestation onwards). In the nineties, standard chromosomal karyotyping was used to identify aneuploidy. In the late 2000s, QF-PCR (Table 1) has been introduced in the Netherlands and is currently well established in the prenatal setting. In euploid fetuses with increased NT, QF-PCR is usually followed by prenatal chromosomal microarray, especially when additional structural anomalies are seen. This flow of establishing an increased NT followed by prenatal invasive testing still stands to this day, although due to the introduction of non-invasive prenatal testing (NIPT, testing fetal cell-free DNA in the maternal blood circulation) in 2017, the uptake of official NT measurement in first trimester combined screening has decreased enormously. The percentage of pregnant women in the Netherlands choosing first trimester combined screening before the introduction of NIPT was 37% in 2016 (Liefers 2017; van der Meij et al., 2021). This already relatively low uptake of first trimester

combined screening plummeted to around 3% after the introduction of NIPT, as part of the TRIDENT-2 study, for all pregnant women in April 2017 in the Netherlands as, due to its accuracy, most women preferred to have NIPT (van der Meij et al., 2019; van der Meij et al., 2021). Therefore, the NT measurement does not act as the backbone of first trimester screening anymore and first trimester combined screening will stop to exist altogether in the fall of 2021. Although NIPT is more reliable in predicting Down syndrome, Edwards syndrome and Patau syndrome than the combined test (Kagan et al., 2015), the disappearance of NT measurement resulted in discomfort among some professionals (Tamminga et al., 2015; Martin et al., 2018). A possible explanation for this is the association with structural anomalies and genetic syndromes in the fetus other than trisomy 13, 18 and 21 and the possibility to identify other structural anomalies with the ultrasound scan that is performed to measure the NT. In the Netherlands, only a dating scan and a 20-week ultrasound scan are offered to the pregnant woman as part of standard prenatal ultrasound care. With the disappearance of the NT measurement, the 'extra' possibility of examining the fetus disappeared as well. However, as of September 1st, 2021 a first trimester ultrasound scan ('13-week scan') has been introduced as part of the national prenatal screening program for early detection of (large) structural anomalies. In this scan a NT measurement with a cut-off at 3.5 mm will also be performed, which will likely result in increasing referral numbers for increased NT in the coming years.

Table 1 Genetic terms, abbreviations and explanations

Abbreviation	Genetic term	Explanation
QF-PCR	Quantitative Fluorescence Polymerase Chain Reaction	Laboratory technique used to amplify specific regions of DNA and quantify the amount of DNA present of those regions.
CMA	Chromosomal microarray	Molecular cytogenetic method for analyzing copy number variations relative to ploidy level in the DNA of a test sample compared to a reference sample
CNV	Copy number variation	Section of the genome that is repeated. The number of repeats in the genome varies in the human population. This can be a normal genetic variant without clinical consequence or can be a structural variation like duplication and deletion.

THE STUDY PROJECT OF THIS THESIS

Until this day the etiology and complexity of an increased NT has not been fully elucidated. Therefore, the studies as described in this thesis were performed to 1) gain more insights into the etiology of the increased NT, and 2) collect more clinical data on the association of an increased NT and postnatal outcome. The results may help clinicians' and pregnant women's decision making when facing the complex issues surrounding a fetus with an increased NT.

RESEARCH QUESTIONS

In this thesis the following research questions will be addressed:

1. What are the contributing factors to lymphatic (mal)development and specifically, what is the role of neural crest cell migration in the development of lymphatic endothelial cell differentiation and nuchal edema? (Chapter 2)
2. What is the clinical outcome of fetuses with an increased nuchal translucency?
    a. What is the diagnostic yield of prenatal microarray after an increased NT? (Chapter 3)
    b. Are there specific copy number variations (CNV) contributing to an increased NT, even when there is a normal outcome postpartum? (Chapter 4)
    c. What relatively recently discovered genes are associated with an increased NT? (Chapter 7)
3. Is there an association between an increased NT and diagnosing a RASopathy?
    a. When should a RASopathy be tested and what are the associated prenatal findings other than an increased NT? (Chapter 5 and 6)

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Chapter 2

Involvement of neurons and retinoic acid in lymphatic development: new insights in increased nuchal translucency

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Prenatal Diagnosis, 2014 Dec; 34:1312-9

ABSTRACT

Objective Increased nuchal translucency originates from disturbed lymphatic development. Abnormal neural crest cell (NCC) migration may be involved in lymphatic development. Because both neuronal and lymphatic development share retinoic acid (RA) as a common factor, this study investigated the involvement of NCCs and RA in specific steps in lymphatic endothelial cell (LEC) differentiation and nuchal edema, which is the morphological equivalent of increased nuchal translucency.

Methods Mouse embryos in which all NCCs were fluorescently labeled (*Wnt1-Cre;Rosa26*^{eYfp}), reporter embryos for in vivo RA activity (DR5-luciferase) and embryos with absent (*Raldh2*$^{-/-}$) or in utero inhibition of RA signaling (BMS493) were investigated. Immunofluorescence using markers for blood vessels, lymphatic endothelium and neurons was applied. Flow cytometry was performed to measure specific LEC populations.

Results Cranial nerves were consistently close to the jugular lymph sac (JLS), in which NCCs were identified. In the absence of RA synthesis, enlarged JLS and nuchal edema were observed. Inhibiting RA signaling *in utero* resulted in a significantly higher amount of precursor-LECs at the expense of mature LECs and caused nuchal edema.

Conclusions Neural crest cells are involved in lymphatic development. RA is required for differentiation into mature LECs. Blocking RA signaling in mouse embryos results in abnormal lymphatic development and nuchal edema.

INTRODUCTION

Nuchal translucency (NT) can be visualized by ultrasound between 10 and 14 weeks of human gestation. Increased NT is associated with aneuploidy, such as trisomy 21, trisomy 18 and trisomy 13, structural (cardiovascular) anomalies and various genetic syndromes (Hyett et al., 1997; Snijders et al., 1998; Souka et al., 1998), but also with a healthy outcome of the fetus. Nuchal edema is the morphological equivalent of increased NT and represents mesenchymal edema (Haak et al., 2002). Although the developmental background of increased NT is still poorly understood, the coincident abnormal enlargement and persistence of jugular lymph sacs (JLS) indicates a role for disturbed lymphatic development (Haak et al., 2002; Gittenberger-De Groot et al., 2004; Bekker et al., 2006).

Lymphatic vasculature development in the mammalian embryo starts in the cardinal veins with reprogramming of blood endothelial cells (BECs) in the cardinal veins towards a lymphatic phenotype (Srinivasan et al., 2007). In mice, transcription factors Sox18 and COUP-TFII (Nr2f2) are expressed within the venous endothelium of the cardinal veins in a polarized fashion at embryonic day (E) 8.5 (Francois et al., 2010; Srinivasan et al., 2010). These transcription factors induce expression of homeobox transcription factor Prox1 in this subpopulation of endothelial cells on the anterior cardinal veins around E9.5. Expression of Prox1 initiates lymphatic endothelial cell (LEC) specification and continuation (Wigle and Oliver, 1999; Francois et al., 2008; Srinivasan et al., 2010; Hagerling et al., 2013). Prox1^{+} cells bud and migrate as single cells or clusters (Hagerling et al., 2013) dorsolaterally from the cardinal veins at E10.5 to E12.5 and start to express podoplanin (Francois et al., 2012; Yang et al., 2012), thereby differentiating from precursor Prox1^{+}podoplanin^{-} (pre-)LECs, located in the cardinal vein. Mature Prox1^{+}podoplanin^{+} LECs migrate from the cardinal vein and form JLS at E11.5 in regions where lymphangiogenic growth factor Vegfc is expressed (Karkkainen et al., 2004; Tammela and Alitalo, 2010).

In trisomy 16 mouse embryos, a mouse model for human trisomy 21, abnormal lymphatic development and nuchal edema have been described (Gittenberger-De Groot et al., 2004). In these mouse embryos, aberrant lymphatic development coincided with significantly smaller cranial nerve X and altered positioning of cranial nerves IX, X and XI, located adjacent to the JLS and jugular vein. It was therefore suggested that abnormal neurogenesis disturbed (lymphatic) endothelial differentiation of the JLS and the jugular vein (Bekker et al., 2005). Abnormal neurogenesis is thought to be caused by disrupted migration or differentiation of the cranial nerve progenitor cells, the neural crest cells (NCCs). A subset of NCCs differentiates into cranial NCCs, forming cranial nerves

at E10 to E13 (Muller and O'Rahilly, 2011). The multiple shared developmental genes by endothelium and nerves, such as Vegfc and neuropilin (Nrp) receptors-1 and Nrp-2 (Karkkainen et al., 2004; Tammela and Alitalo, 2010), further support a coordinated development of the neuronal and (lymphatic) vascular system.

Another common factor in nervous and lymphatic vascular development is retinoic acid, the active metabolite of vitamin A. The retinoic acid-converting enzyme retinaldehyde dehydrogenase 2 (Raldh2/Aldh1a2) is responsible for the majority of retinoid synthesis during embryogenesis (Niederreither and Dolle, 2008). Embryos without functional Raldh2 exhibit severe disturbances in embryonic development, including increased endothelial cell proliferation and loss of endothelial cell maturation, leading to disrupted vascular remodeling (Lai et al., 2003). In *Raldh2*$^{-/-}$ embryos, alterations in NCC migration (disturbed axonal outgrowth of the IXth–XIIth cranial nerves) were observed (Niederreither et al., 2003). Moreover, a role for retinoic acid in lymphatic vasculature development was demonstrated in other mouse models in which retinoic acid signaling was disturbed (Lai et al., 2003; Niederreither et al., 2003; van de Pavert and Mebius, 2014). In these models, it was shown that retinoic acid in combination with cAMP increased expression of LEC markers in mouse embryoid bodies and also initiated lymphatic differentiation in BECs of the cardinal vein (Marino et al., 2011). Deletion of the main enzyme involved in degradation of retinoic acid, Cyp26B1, resulted in more LECs (Bowles et al., 2014; van de Pavert and Mebius, 2014). Our study confirmed that retinoic acid is involved in the earliest steps of LEC initiation (Bowles et al., 2014). However, it is not known whether nerves are involved in regulation of LEC differentiation.

Here, we demonstrate that NCCs are involved in lymphatic vascular development. Differentiation of pre-LECs towards mature LECs was induced by retinoic acid, whereas blockade of retinoic acid signaling resulted in abnormal lymphatic development. For the first time, we have induced nuchal edema by blocking retinoic acid signaling. Thus, we propose a close relation between NCCs and the development of the lymphatic vascular system, in which retinoic acid is critical for the differentiation of LECs.

METHODS

Animals

Wnt1-Cre;Rosa26^{eYfp}, *Raldh2*$^{-/-}$ and DR5-luciferase mouse embryos have been described previously (Niederreither et al., 1999; Foster et al., 2008; Svensson et al., 2008). Mice were kept at standard animal housing conditions. Mice were mated overnight, and the day of the vaginal plug detection was noted as E0.5. Embryos were isolated and fixed

in 4% formalin in phosphate buffered saline (PBS) for 30 min and cryoprotected in 15% sucrose in PBS for 2 h. Subsequently, embryos were incubated overnight in 30% sucrose in PBS at 4 °C and embedded in optimum cutting temperature compound (Tissue-Tek, Qiagen, Venlo, The Netherlands). Institutional animal experimentation committees approved all animal experiments.

Modulation of retinoic acid signaling

As described before (van de Pavert et al., 2014), to inhibit retinoic acid signaling, we supplied pregnant C57BL/6 mice with pan-retinoic acid receptor antagonist BMS493 (Tocris Bioscience, Bristol, UK; 5 mg/kg) or vehicle (DMSO) 1 : 10 in nut oil by oral gavages twice a day with intervals of 10 to 12 h. Treatment started at E10.5 and was terminated when mice were sacrificed at E12.5 to E14.5.

Antibodies

The antibodies 8.1.1 (anti-podoplanin), MP33 (anti-CD45) and ER-MP12 (anti-CD31) were affinity purified from hybridoma cell culture supernatants with protein G-Sepharose (Abcam, Cambridge, UK). ER-MP12 (anti-CD31) and MP33 (anti-CD45) were directly labeled with Alexa-Fluor 488 or Alexa-Fluor 555. Biotin conjugated anti-GFP (rabbit polyclonal antibody, GeneTex, Irvine, USA) was visualized using streptavidin-Alexa- Fluor 488 or 647. Anti-Lyve1 was directly labeled to eFluor 660 (monoclonal antibody ALY7 eBioscience, Breda, The Netherlands). Anti-Prox1 (rabbit polyclonal antibody RELIAtech, Richmond, USA), anti-neuronal class III β-tubulin (mouse monoclonal antibody clone TUJ1, Covance, Rotterdam, The Netherlands) and anti-luciferase (rabbit polyclonal antibody ab21176, Abcam, Cambridge, UK or goat polyclonal antibody AB3256, Chemicon, Tamecula, USA) were used as unconjugated primary antibodies. These primary antibodies were conjugated to the appropriate secondary antibody: Alexa-Fluor 488, Alexa- Fluor 546, Alexa-Fluor 647-conjugated anti-rat IgG, anti-mouse IgG, anti-goat IgG, anti-hamster IgG, anti-rabbit IgG (Invitrogen Life Technologies, Breda, The Netherlands).

Immunofluorescence

Fixed and cryoprotected embryos were cryosectioned at 8 µm, and the sections were air dried for at least 2 h. Next, sections were placed in acetone for 10 min, after which they were air dried for 10 min. Sections were blocked in PBS supplemented with 10% (v/v) serum and subsequently stained with primary antibodies for 30 to 45 min and, if the primary antibody was not labeled, followed by a secondary antibody step of 30 min incubation with Alexa-Fluor-labeled conjugate (Invitrogen Life Technologies). All incubations were carried out at room temperature. Slides were rinsed with PBS and covered with Vinol + DAPI. All images were acquired using the TCS SP-2 confocal laser-scanning

microscope (Leica Microsystems, The Netherlands BV). Images were processed in Adobe Photoshop CS3.

Flow cytometry

Embryos from BMS493 treated mice and control embryos were used for flow cytometry (FACS). Cell suspensions were obtained and stained as previously described (van de Pavert et al., 2009; van de Pavert et al., 2014). Dead and hematopoietic cells were excluded by using 7AAD (Invitrogen Life Technologies) and CD45. Fluorescence was measured on the CyAn ADP analyzer, and results were analyzed using Summit V4.3 (Beckman Coulter, Woerden, The Netherlands).

Statistical analysis

Statistical analysis of the FACS was performed using analysis of variance or two-tailed unpaired Student's t-test. A probability value less than 0.05 was considered significant.

RESULTS

JLS development occurs adjacent to nerve fibers

Previously, we have shown a correlation between nerve fibers and the JLS in E12 to E18 mouse embryos (Gittenberger-De Groot et al., 2004; Bekker et al., 2005). To determine the location of nerve fibers in relation to the developing lymphatic system, we analyzed C57BL/6 embryos at E11.5 and E12.5, when lymphatic vasculature development was initiated and formed. In all embryos analyzed, we observed nerve fibers and ganglia adjacent to the JLS (Figure 1A and 1B). More specifically, the JLS was located lateral from the cardinal vein, and the nerve fibers were observed between the lateral side of the cardinal vein and JLS. The nerve fibers surrounded the JLS completely.

Neural crest cells are involved in lymphatic vasculogenesis

Cranial nerve fibers adjacent to the JLS are derived from NCCs (Kulesa and Gammill, 2010). Thus, to analyze the presence of NCCs in the lymphatic vasculature, we examined *Wnt1-Cre;Rosa26*^{eYfp} embryos, in which specifically NCCs and all descendants were fluorescently labeled (Foster et al., 2008). Using immunofluorescence, NCC-derived cells, being YFP^{+}, were observed in the JLS in E13.5 *Wnt1-Cre;Rosa26*^{eYfp} embryos (arrows in Figure 2A and 2B). These data suggest a relation between NCCs and lymphatic vascular development.

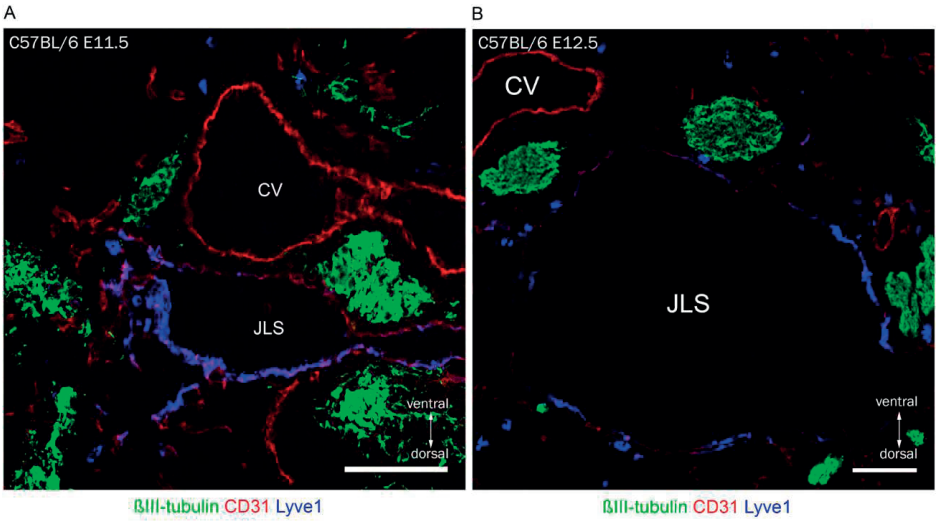

Figure 1 Development of the JLS occurs adjacent to nerve fibers. Representative pictures of transverse sections of the consistent co-localization of nerve fibers, stained for βIII-tubulin (in green), lateral of the cardinal vein (CV, CD31 in red) and surrounding the JLS (Lyve1 in blue) at different stages of embryonic development, E11.5 (n = 4) (A) and E12.5 (n = 2) (B) in C57BL/6 embryos. Bar represents 100 µm. Orientation is shown by the arrow.

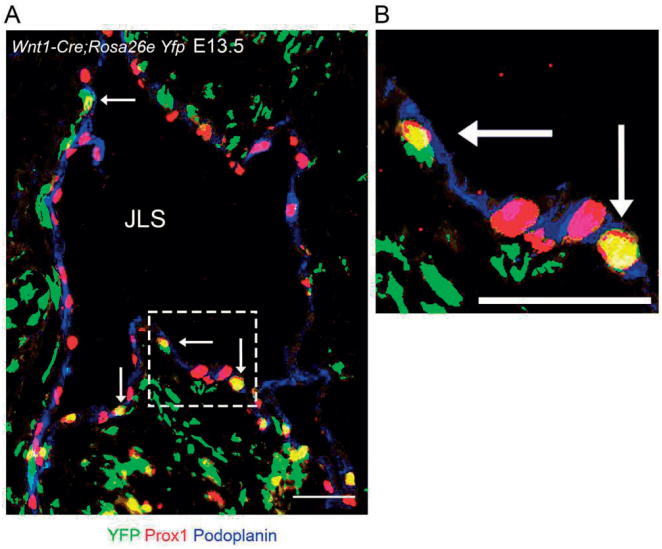

Figure 2 Neural crest cells (NCCs) are involved in lymphatic development. Transverse section of the jugular lymph sac of an E13.5 NCC lineage trace *Wnt1-Cre;Rosa26*^{eYfp} embryo stained for YFP (in green), Prox1 (in red) and podoplanin (in blue). Arrows indicate NCC-derived YFP^{+}Prox1^{+} cells in the JLS. Data are representative of two individual experiments in E13.5 (n = 2) embryos. Bar represents 50 µm (A). Higher magnification of the dotted box is shown in (B).

Retinoic acid signaling occurs within lymphatic cells at the JLS

Retinoic acid is important for differentiation and migration of NCCs, as well as for differentiation of LECs. To visualize which LECs responded to retinoic acid at different developmental stages, DR5-luciferase retinoic acid responsive elements (RARE) reporter embryos, in which luciferase is expressed in response to intracellular retinoic acid signaling, were analyzed at E11.5 to E13.5. At all embryonic stages, Lyve1^{+}Luciferase^{+} cells were observed at specific locations in the JLS (arrows in Figure 3A). High expression of luciferase was observed in neuronal structures adjacent to the JLS (N in Figure 3A) and in LECs nearby nerve fibers (arrowhead in Figure 3A).

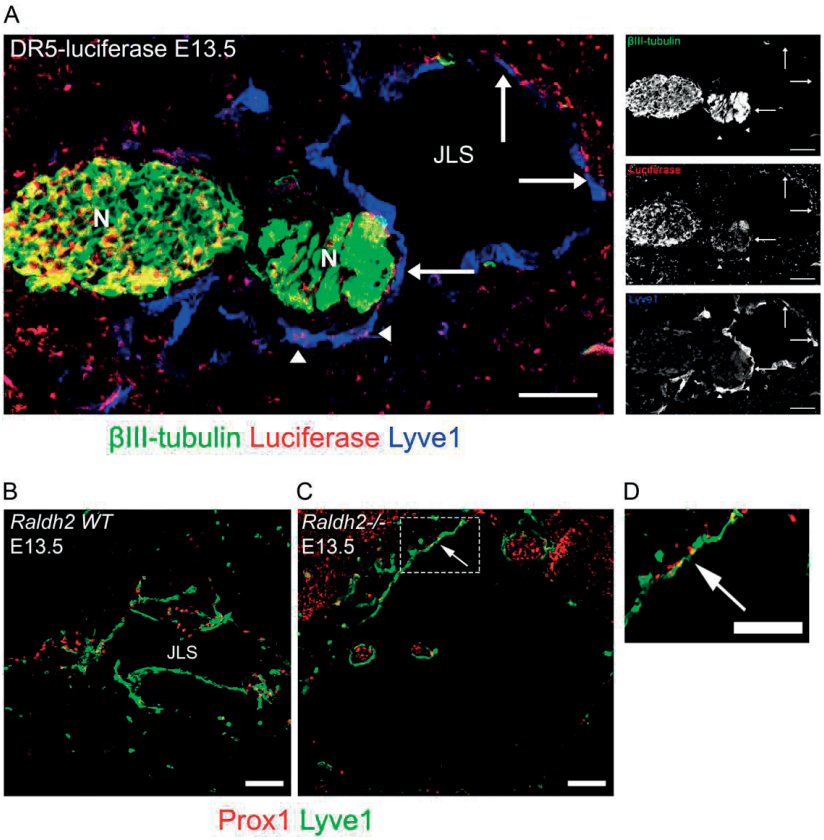

Figure 3 Retinoic acid signaling within LECs is necessary for differentiation. Transverse section of an E13.5 DR5-luciferase embryo stained for βIII-tubulin (in green), luciferase (in red) and Lyve1 (in blue). Arrows indicate expression of luciferase (in red) in LECs in the JLS, arrowhead indicates luciferase (in red) expression dorsal of the JLS and N indicates luciferase (in red) expression in adjacent neuronal structures (βIII-tubulin in green). Data are representative of ten individual experiments in E11.5 (n = 4), E12.5 (n = 4) and E13.5 (n = 2) embryos. Bar represents 50 µm (A). A representative picture of a sagittal section of E13.5 *Raldh2*$^{-/-}$ embryos (n = 2) compared with wild type littermate control embryos stained for Prox1 (in red) and Lyve1 (in green). Arrow indicates a small population of LECs within the lining of the large structure. Bar represents 100 µm (B, C). Higher magnification of the dotted box is shown in (D).

Retinoic acid induces differentiation of (pre-)LECs

To address whether retinoic acid is necessary for proper development of the JLS, we analyzed E10.5 and E13.5 *Raldh2*$^{-/-}$ embryos. We did not identify any lymphatic structures at E10.5 (Figure S1) but observed disturbed neuronal and vascular development as described before (Niederreither et al., 2003). Notable, *Raldh2*$^{-/-}$ embryos exhibited nuchal edema at E13.5. We observed a large structure with an irregular and disorganized lining in E13.5 *Raldh2*$^{-/-}$ embryos, whereas the JLS was observed at a comparable anatomical location in the littermate wild type embryos (Figure 3B and 3C). However, fewer LECs were observed within the lining of this structure and the JLS (Figure 3D), as present in the littermate wild type embryos, could not be detected. Hence, retinoic acid is essential in LEC differentiation.

Disruption of retinoic acid signaling causes aberrant lymphatic vascular development

Having established that retinoic acid is necessary for LEC differentiation and JLS formation, we examined at which developmental stage retinoic acid affects differentiation of (pre-)LECs. We blocked retinoic acid signaling during embryogenesis *in utero* by treating pregnant C57BL/6 mice with BMS493, a pan-retinoic acid receptor antagonist. Nuchal edema was observed in E14.5 embryos from BMS493 treated mice (Figure 4A). In E13.5 embryos from BMS493 treated mice, a JLS containing fewer Prox1 and Lyve1 cells compared with embryos from control treated mice was observed (Figure 4B). In contrast to *Raldh2*$^{-/-}$ embryos, the JLS in embryos from BMS493 treated mice was smaller compared with that of the JLS in control embryos.

Next, we quantified the effect of inhibiting retinoic acid signaling by analyzing the relative amount of cells within three different populations involved in LEC differentiation: BECs ($CD31^+CD45^-Lyve1^-podoplanin^-$), pre-LECs ($CD31^+CD45^-Lyve1^+podoplanin^-$) and mature LECs ($CD31^+CD45^-Lyve1^+podoplanin^+$) (Figure 5A). As podoplanin is expressed on LECs after budding from the cardinal vein,13,14 this marker was used to distinguish pre-LECs from mature LECs. We excluded Lyve1 expressing macrophages by excluding $CD45^+$ events (Figure 5A and 5B).

Inhibiting *in utero* retinoic acid signaling with BMS493 resulted in a relative larger pre-LEC population at the expense of mature LEC population compared with the control treatment in these embryos at E12.5 ($p = 0.02$) and E13.5 ($p = 0.001$) (Figure 5C and 5D). In contrast, in E14.5 embryos from BMS493 treated mice, no significant difference was observed in the pre-LEC versus mature LEC ratio (Figure 5E).

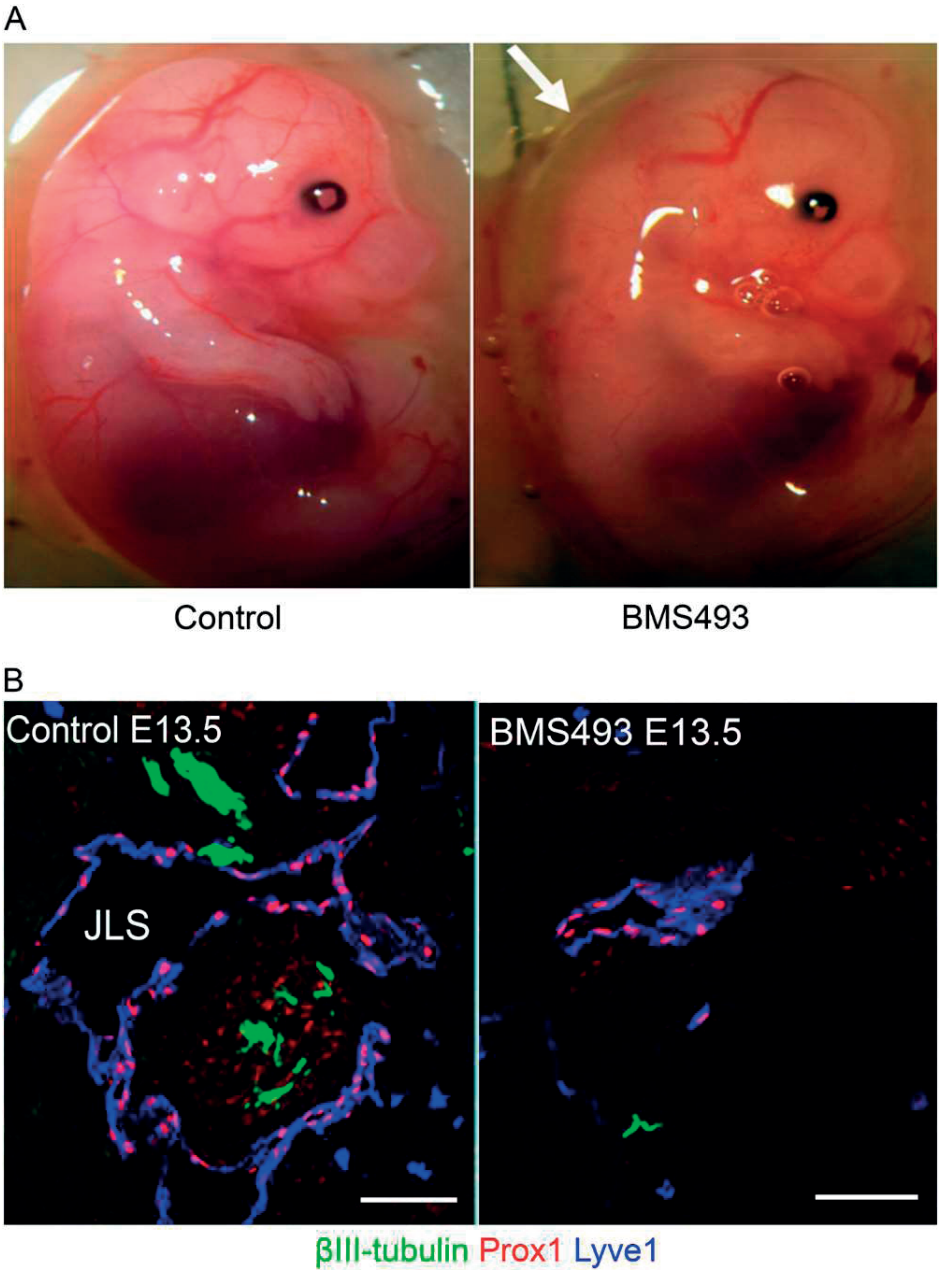

Figure 4 Blocking retinoic acid signaling in utero affects LEC differentiation and induces nuchal edema. Upon treatment of BMS493 pregnant mice, E14.5 embryos showed nuchal edema (arrow in A). Sagittal sections of E13.5 embryos from BMS493 treated mice were stained for βIII-tubulin (in green), Prox1 (in red) and Lyve1 (in blue). Note the small JLS with less Prox1$^+$ and Lyve1$^+$ cells. Difference in βIII-tubulin positive nerve fibers might be due to the previously described effect of retinoic acid on development of the nervous system. Data are representative of three individual experiments in E13.5 (n = 3) embryos. Bar represents 100 μm (B).

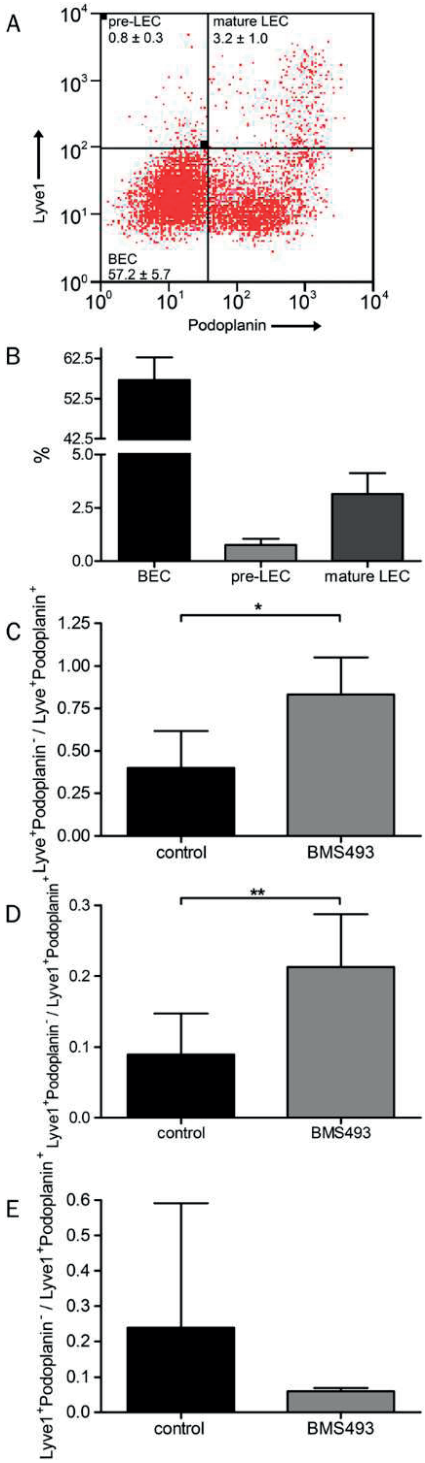

Figure 5 BMS493 blocked pre-LEC differentiation towards mature LEC. Identification of BEC, pre-LEC and mature LEC populations in E13.5 (n = 3) C57BL/6 embryos through FACS analysis. Data are given as mean ± SD. Only CD31+, alive (7AAD-) and nonhematopoietic (CD45-) cells were used in the analysis. Data are representative of three individual experiments (A). Percentages of BECs, pre-LECs and mature LECs present in E13.5 C57BL/6 embryos (n = 3) (B). In the same litters as used for immunofluorescence staining, ratios were measured by FACS of pre-LECs versus mature LECs in E12.5 (C), in E13.5 (D) and in E14.5 € embryos from BMS493 treated mice and control embryos. Embryos from BMS493 treated mice showed a higher pre-LEC versus mature LEC ratio compared with control embryos at E12.5 and E13.5. Asterisk * indicates a significance of p = 0.02, and asterisk ** indicates a significance of p = 0.001. Data are representative of nine individual experiments in E12.5 (n = 3), in E13.5 (n = 3) and in E14.5 (n = 3) embryos from BMS493 treated mice (C–E).

DISCUSSION

This is the first study reporting that NCCs are observed in the JLS. We showed that retinoic acid is essential for the differentiation of pre-LECs into mature LECs and that blocking retinoic acid signaling results in aberrant lymphatic development and nuchal edema. Also, we induced nuchal edema in mouse embryos by disturbing retinoic acid signaling.

We showed that nerve fibers and ganglia are consistently located in close proximity to the initial lymphatic structures. Furthermore, NCCs were identified in the JLS in NCC reporter mouse embryos. NCCs are pluripotent cells that contribute to several structures in the cervical area, such as thymus, pericytes (Zachariah and Cyster, 2010), mesenchymal cells and cranial nerves (reviewed by Sauka-Spengler and Bronner-Fraser (Sauka-Spengler and Bronner-Fraser, 2008)). Also, NCCs can incorporate into walls of blood vessels (Jiang et al., 2000) and are involved in development of pulmonary arteries (Waldo and Kirby, 1993). We are the first to show that NCCs are present in LECs and in the JLS. This finding suggests involvement of NCCs in LEC and JLS formation and accordingly in the formation of nuchal edema. The involvement of NCCs in lymphatic vascular development and in nuchal edema may also explain the variety of fetal malformations associated with increased NT, such as cardiovascular defects (Kirby and Waldo, 1995; Jiang et al., 2000; Huang et al., 2010), craniofacial malformations (Huang et al., 2010) and skeletal anomalies (Van Ho et al., 2011), because these anomalies are all related to disturbances in NCC migration or differentiation.

During initiation of lymphatic vasculature development, the main source of the LECs are the BECs, as was previously shown using several lineage tracing models (Srinivasan et al., 2007). However, because a small portion of BECs originate from NCCs and the BECs subsequently differentiate into LECs, the origin of these LECs might be the NCC. Alternatively, NCCs could differentiate directly into LECs, without ever being a BEC. Further studies into the potential of NCCs to differentiate either directly or indirectly to an LEC are required to gain more insight.

As retinoic acid is a common factor in both differentiation of NCCs (Niederreither et al., 2003) and LECs (Marino et al., 2011; Bowles et al., 2014; van de Pavert and Mebius, 2014), we investigated the role of retinoic acid in the formation of the LEC population. It was previously shown that retinoic acid affected lymphatic endothelial differentiation and lack of retinoic acid resulted in a smaller amount of LECs and smaller lymphatic structures (Marino et al., 2011; Choi et al., 2012). Accordingly, excess of retinoic acid in *Cyp26B1*$^{-/-}$ mouse embryos resulted in more LECs (Bowles et al., 2014). Having es-

tablished the identification of specific stages in BEC towards LEC differentiation, we observed that blocking retinoic acid signaling resulted in fewer mature LECs, whereas the pre-LECs were more abundant at E12.5 and E13.5. This indicated that retinoic acid signaling was needed to specifically allow the final differentiation step towards mature LECs at E12.5 and E13.5 and extends on earlier published data on retinoic acid mediated differentiation (Lai et al., 2003; Niederreither et al., 2003; Van Ho et al., 2011). In E14.5 embryos from BMS493 treated mice, no significant difference was found in the pre-LEC versus mature LEC ratio. This can be explained by the fact that at this stage, lymphatic vessels are formed by proliferation of mature LECs, which probably not depends on retinoic acid and thus results in a larger mature LEC population.

We observed that some LECs responded to retinoic acid, by using the E11.5 to E13.5 DR5 reporter mouse embryos. This seems in contradiction to the E11.5 RARE-lacZ reporter embryos used in a previous study (Bowles et al., 2014). However, the difference in embryonic stage and reporter construct might explain why the RARE activity in a small portion of the JLS was previously not observed. Lack of retinoic acid signaling in the *Raldh2*$^{-/-}$ and embryos obtained from BMS493 treated mice resulted in aberrant LEC differentiation and nuchal edema. Interestingly, it was shown that *Raldh2*$^{-/-}$ embryos exhibited uncontrolled endothelial proliferation (Lai et al., 2003). This would fit our observation of large endothelial sacs, existing of blood and lymphatic endothelial cells, caused by uncontrolled endothelial growth and differentiation. On the contrary, the JLS in BMS493 treated embryos were much smaller. This could be caused by the start of BMS493 treatment at E10.5, which is the moment during which formation of LECs and the JLS was already initiated. This resulted in an arrested small abnormally formed JLS structure after BMS493 supplementation. In contrast, the *Raldh2*$^{-/-}$ embryos have had a retinoic acid deficiency throughout the initiation of lymphatic development, thus resulting in a completely disorganized LEC differentiation and migration.

Although it is now clear that retinoic acid is involved in the differentiation of LECs, the source remains unknown. In prior studies on the formation of lymph nodes (van de Pavert et al., 2009; van de Pavert et al., 2014), we have suggested that nerve fibers located near the lymph node anlagen are the source of retinoic acid. Also, we observed that these nerve fibers are abundantly expressing the enzyme Raldh2, which is essential for the synthesis of retinoic acid. In this study, we observed high retinoic acid signaling activity in the adjacent nerve fibers and ganglia. Therefore, the most likely source of retinoic acid are nerve fibers and ganglia adjacent to the location where LECs bud off from the cardinal vein to further differentiate into mature LECs. Further studies are required to establish that nerves are indeed the source of retinoic acid.

CONCLUSION

We are the first to identify NCCs in the JLS, and for the first time, we have imposed nuchal edema in mouse embryos by inhibiting retinoic acid signaling. Involvement of NCCs can be direct, by differentiation of NCCs into LECs. Also, NCCs could affect lymphatic development indirectly. After NCCs differentiated into nerves, these nerves could act as a source for retinoic acid. Retinoic acid is subsequently needed for proper lymphatic endothelial differentiation and formation of the JLS.

It is remarkable that nerves were observed consistently near the first lymphatic structures. As our previous studies hinted at the role of nerves being the source of retinoic acid, these nerves adjacent to lymphatic structures could also be the source for retinoic acid during initiation of lymphatic endothelial differentiation. Further studies are needed to determine precisely how nerves are involved in establishing the location of the JLS.

The complete pathophysiological background of increased NT in relation to the wide spectrum of associated fetal anomalies is still insufficiently understood. It seems likely that this is based on a complex and multifactorial process, linked to one or more embryonic pathways. We propose that increased NT and its associated fetal malformations originate from a disturbance in a common developmental process in which NCCs and LEC development are prominent factors.

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SUPPLEMENTARY MATERIAL

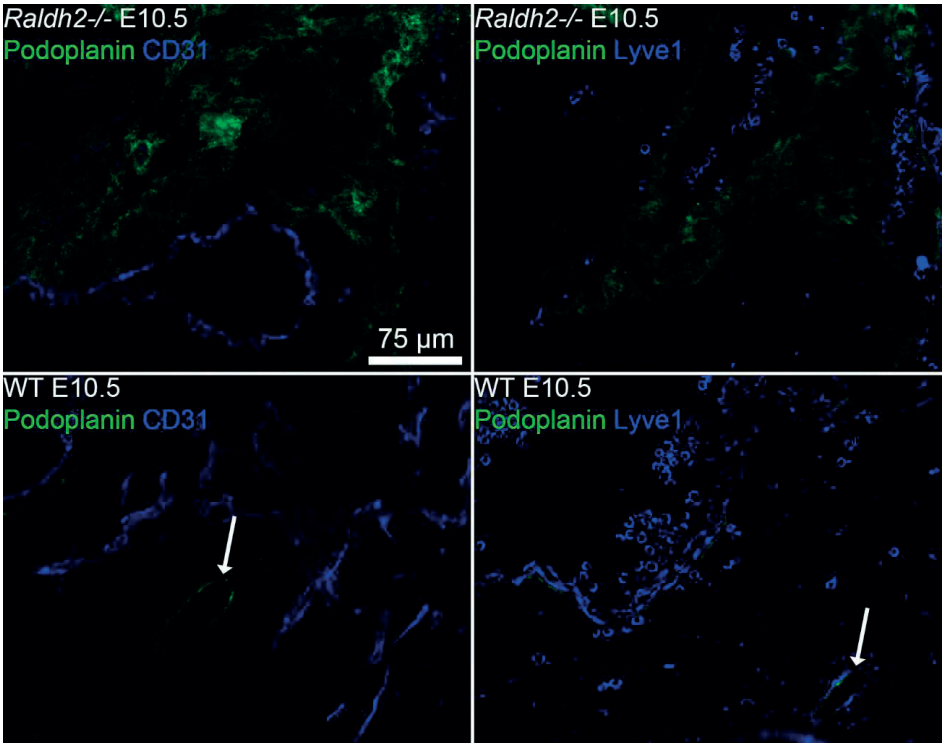

Figure S1 Retinoic acid signaling within LECs is necessary for differentiation. Lymphatic structures were absent in E10.5 *Raldh2*$^{-/-}$ embryos. Podoplanin and Lyve1 positive lymphatic structures were not observed in the region where the JLS would normally form. The arrows in the wild type point to a small JLS. Data are representative of two E10.5 *Raldh2*$^{-/-}$ and two wild type embryos. Bar represents 75 µm.

Chapter 3

Isolated increased nuchal translucency in first trimester ultrasound scan: diagnostic yield of prenatal microarray and outcome of pregnancy

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Accepted in Frontiers in Medicine, section Obstetrics and Gynecology, 2021

ABSTRACT

Background Increased nuchal translucency (NT) is associated with aneuploidy. When the karyotype is normal, fetuses are still at risk for structural anomalies and genetic syndromes. Our study researched the diagnostic yield of prenatal microarray in a cohort of fetuses with isolated increased NT (defined as NT ≥ 3.5 mm) and questioned whether prenatal microarray is a useful tool in determining adverse outcome of the pregnancy.

Materials and methods A prospective study was performed, in which 166 women, pregnant with a fetus with isolated increased NT (ranging from 3.5 to 14.3 mm with a mean of 5.4 mm), were offered karyotyping and subsequent prenatal microarray when karyotype was normal. Additionally, all ongoing pregnancies of fetuses with normal karyotype were followed up with regard to postnatal outcome. The follow up time after birth was maximally four years.

Results 149 of 166 women opted for prenatal testing. 77 fetuses showed normal karyotype(52%). 73 of 77 fetuses with normal karyotype did not show additional anomalies on early ultrasound scan. 40 of 73 fetuses received prenatal microarray of whom 3 fetuses had an abnormal microarray result: 2 pathogenic findings (2/40) and one incidental carrier finding. In 73 fetuses with an isolated increased NT, 21 pregnancies showed abnormal postnatal outcome (21/73, 28.8%), 29 had a normal outcome (29/73, 40%) and 23 were lost to follow-up (23/73, 31.5%). 7 out of 73 live born children showed an adverse outcome (9.6%).

Conclusion Prenatal microarray in fetuses with isolated increased NT had a 5% (2/40) increased diagnostic yield compared to conventional karyotyping. Even with a normal microarray, fetuses with an isolated increased NT had a 28.8% risk of either pregnancy loss or an affected child.

INTRODUCTION

One of the techniques used in prenatal diagnostic testing is chromosomal microarray (array CGH or SNP array). Before prenatal microarray was available, chromosomal karyotyping was the standard technique to perform when soft markers or structural anomalies were seen on fetal ultrasound scan. With the introduction of chromosomal microarray a higher resolution of the genome can be achieved compared to conventional karyotyping and therefore a prenatal microarray is nowadays used as a standard tool if structural anomalies are seen on fetal ultrasound scan. Studies have shown that prenatal microarray for a wide range of abnormal ultrasound findings increases the percentage of genetic abnormalities detected by approximately 5-17% when compared to standard karyotyping (Wapner et al., 2012; Hillman et al., 2013; Oneda and Rauch, 2017). In our hospital we implemented prenatal array CGH in 2011 first in pregnancies in fetuses with an increased nuchal translucency without additional abnormalities on a first trimester ultrasound (dating scan or scan in the context of first trimester combined screening), and who had a normal karyotype with standard karyotyping or Rapid Aneuploidy Detection through Quantitative Fluorescence-Polymerase Chain Reaction (QF-PCR).

Nuchal translucency (NT) is defined by the translucent area in the neck region of the developing fetus, which can be visualized by ultrasound between 11-13+6 weeks of gestation. Isolated increased NT is defined as a NT being the sole anomaly without any other soft markers or structural defects visible on fetal ultrasound. Worldwide, NT measurement was used for first trimester combined screening of trisomies 21, 18 and 13 and monosomy X. An increased nuchal translucency increases the risk of chromosomal aneuploidy (Nicolaides et al., 1992). Overall, approximately half of the fetuses with an increased NT show an abnormal karyotype (Snijders et al., 1998). The fetuses with normal karyotype are, however, still at increased risk for a wide variety of structural defects and genetic abnormalities (Bilardo et al., 1998; Souka et al., 1998; Souka et al., 2001; Souka et al., 2005), such as cardiac defects (Hyett et al., 1997) and Noonan syndrome (Croonen et al., 2013; Stuurman et al., 2019). Some of these abnormalities may be explained by submicroscopic genomic deletions or duplications and therefore can be detected only by prenatal microarray.

Many studies have reported data on the use of prenatal microarray in fetuses with (isolated) increased NT, with the first reports starting as early as 2003 (Brisset et al., 2003) and approximately five English studies in the years 2020 and 2021 (Sinajon et al., 2020; Xue et al., 2020; Zhao et al., 2020; Hui et al., 2021; Sagi-Dain et al., 2021). These studies show a relatively wide range (0-20%) of additional genetic findings in fetuses with increased

NT with regard to prenatal microarray when compared to standard karyotyping. Additionally, isolated increased NT shows a lower diagnostic genetic yield than increased NT with additional structural defects on (second trimester) ultrasound scan (Grande et al., 2015). However, in the last years non-invasive prenatal testing (NIPT) has been introduced and is offered to pregnant women for screening of trisomy 21, 18 and 13 as a first tier test (van der Meij et al., 2019). As a result, first trimester combined screening is only performed in approximately 3% of pregnancies in the Netherlands at the moment (van der Meij et al., 2021). On the other hand, worldwide a first trimester ultrasound scan at 13 weeks of gestation is still appreciated as an important tool for early detection of possible fetal anomalies or genetic disorders (Edwards and Hui, 2018; Kenkhuis et al., 2018). In the Netherlands, from September 1st onwards a first trimester ultrasound scan (the '13 week scan') is offered to all pregnant women free of charge as part of the National Prenatal Screening Program. This scan will screen for growth and structural anomalies and will also include NT measurement. As a result of the implementation of this first trimester ultrasound scan, it is expected it will lead to more referrals to tertiary health centers.

Isolated increased NT on first trimester ultrasound is also associated with an increased risk on adverse outcome of the pregnancy. The rate of adverse pregnancy outcome is strongly correlated with the severity of the increased NT and the presence of additional anomalies on first or second trimester ultrasound scan (Souka et al., 2005; Bilardo et al., 2010).

The aims of this study were twofold: 1) to assess the diagnostic yield of prenatal microarray in a cohort of fetuses with isolated increased NT, and 2) to assess the value of prenatal microarray by determining the outcome of pregnancy in a larger cohort of fetuses with isolated increased NT, including fetuses in whom prenatal microarray was performed.

MATERIALS AND METHODS

Patients

A prospective study was performed in which 166 women who had first trimester combined screening in one of the referral centers and showed an increased NT in the fetus were referred to the Amsterdam UMC, location VUmc for a prenatal invasive procedure. These women were routinely offered conventional karyotyping. Because QF-PCR was introduced at the Amsterdam UMC in 2010, 14 samples received QF-PCR in addition to karyotyping. When a normal karyotype in the fetus was confirmed and no other abnor-

malities on early ultrasound scan (before 16 weeks of gestation) were seen, the patient was offered subsequent additional microarray analysis. Before performing microarray analysis couples were counseled about the testing process, benefits and limitations of testing and possible outcomes. An informed consent was signed and couples received an additional information letter. The local institutional ethics board approved the study.

The exclusion criteria were: pregnancies in which teratogenic medication was used, monochorionic twin pregnancies and women with significant underlying medical conditions.

Increased NT measurement was defined as equal to or greater than 3.5 mm and NT measurement was performed between 11 and 13+6 weeks of gestation, according to the Dutch Society of Obstetrics and Gynecology guidelines (NVOG, 2012). Because NT measurements were performed at referral centers, the gestational age at the time of the measurement was not registered at our center.

Samples

All samples from the dataset used for microarray analysis were received between January 1st 2011 and August 1st 2013 from the department of Obstetrics and Gynecology at Amsterdam UMC, location VUmc. All, except for one, of the samples were chorion villi samples (CVS). One sample was amniotic fluid. This time interval was chosen because in this period the use of prenatal microarray was first introduced and analyzed at our center.

Parental blood samples were simultaneously tested and interpreted to differentiate between potential familial or de novo pathogenic copy number variations (CNVs). In case DNA was isolated from cultured chorionic villi cells and a female fetus was concerned, DNA was tested for maternal contamination. In case parental blood samples were not received, microarray on the fetus was not performed and the patient was excluded from the study.

Postnatal follow up

All ongoing pregnancies with isolated increased NT (with or without prenatal microarray) and normal second trimester ultrasound scan (increased NT had resolved) presented at our hospital between January 2011 and August 2013 were followed up after birth. In the Netherlands, the first check up right after birth is performed by a midwife when an uncomplicated birth has occurred. In case of a more complicated birth (e.g. Caesarean section) a pediatrician will perform the first check. When no problems occur, the children are followed up routinely by a doctor specialized in youth development according

to the national guidelines. With regard to this study, the first author saw the children for the first time between three to six months after birth if earlier problems did not occur. They were checked for dysmorphic features and developmental parameters. One year and four years after the first visit the children were evaluated again. In case of concerns the children were evaluated earlier than the regular intervals. The maximum follow up time after birth was four years.

Cell culture and DNA extraction

Samples were received in the laboratory for cytogenetic studies. Results of conventional karyotyping and QF-PCR were awaited first. In case of a normal result, microarray was subsequently performed on DNA extracted from a part of the sample. Patients were excluded from microarray when there was not enough fetal material to perform a subsequent microarray analysis.

DNA was extracted from uncultured cells using Wizard® (Promega, Madison, WI, USA) and from cultured cells using Qiagen BioRobot® (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. Maternal contamination was tested with fragment analysis using PowerPlex16 (Promega, Madison, WI, USA).

Microarray

Agilent CGH 180K oligo array (Agilent Technologies, Santa Clara, USA; Amadid 023363) was used as array platform and performed according to manufacturer's instructions. The overall median probe spacing of this platform was 13 kb. As reference DNA a commercial reference pool of Kreatech, consisting of healthy men and women, was used (sex-matched experiments).

Data analysis was performed using Nexus Copy Number versions 5.0, 6.1 and 7.0, and interpreted using Cartagenia BENCH 4.0 Feb-2012 (genome build hg18 and hg19).

Standard settings for CNVs in Nexus were applied: threshold for probe median: gain 0.3 and loss -0.3. Minimal probes for a call: 20 per segment. The interpretation of CNVs has been done according the criteria as described previously (Koolen et al., 2009). We analyzed trios to assess whether CNVs were de novo or inherited.

The microarray was considered normal if only benign class 1 or 2 CNVs were detected. The microarray was considered abnormal if a most likely clinically relevant (class 4 and 5) CNV was found. Variants of unknown clinical significance (class 3) were discussed internally before reporting them.

Statistical analyses

The data were analyzed in SPSS V.22. For statistical analysis descriptive statistics were used.

RESULTS

Between January 2011 and August 2013 166 women were seen in our hospital because of increased NT in the fetus. The mean maternal age for all pregnancies with increased NT was 33.8 years (range 21-46 years). The mean thickness of increased NT of all pregnancies was 5.4 mm (range 3.5-14.3 mm). 149 out of 166 women opted for invasive prenatal testing (87%) with standard karyotyping or QF-PCR. 77 of 149 fetuses had normal QF-PCR or karyotype results (52%). The mean NT thickness of these 77 fetuses was 4.8 mm (range 3.5-10.2 mm). The other 72 of 149 fetuses had a chromosome abnormality. 32 fetuses had trisomy 21 (21.5%), 24 fetuses had trisomy 18 (16%), four fetuses had trisomy 13 (2.7%), 11 fetuses had 45,X (7.4%) and one fetus had 47,XXY (0.7%). Four of the 77 fetuses (5.2%) had an increased NT plus additional anomalies on early ultrasound scan at approximately 14 weeks of gestation and were excluded from this study (Fig. 1).

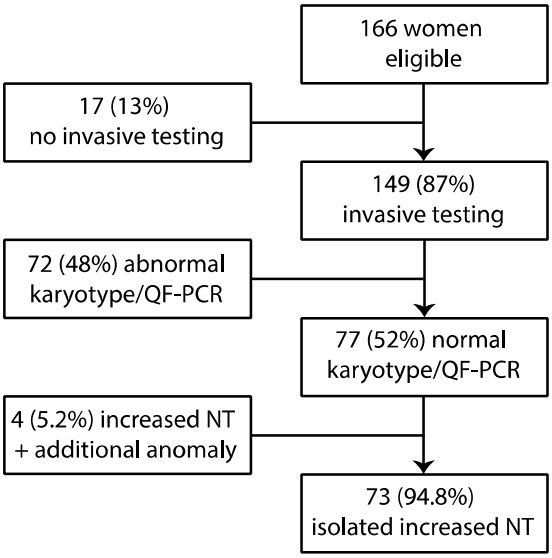

Figure 1 Eligible fetuses with increased NT and normal karyotype/QF-PCR

The remaining 73 fetuses were eligible for microarray. The mean NT thickness was 4.7 mm (range 3.5-10.2 mm) On 40 of 73 fetuses a prenatal microarray (55%) was performed. The mean NT thickness was 4.7 mm (range 3.5-9.3 mm). The remaining 33 fetuses did

not receive a prenatal microarray due to an insufficient amount of DNA, no access to parental samples or microarray declined by the parents as a subsequent test (Fig. 2). The mean NT thickness was 4.7 mm (range 3.5-10.2 mm) (Table 1).

Table 1 Overview of NT measurement in relation to performed prenatal microarray

NT measurement (mm)	Microarray: Yes, normal (n)	Microarray: Yes, abnormal (n)	Microarray: Not performed (n)
3.5-4.4	21	1	21
4.5-5.4	8	0	4
5.5-6.4	7	1	4
≥6.5	2	0	4

NT, nuchal translucency

Diagnostic yield of prenatal microarray

Two of the 40 fetuses (5%) that underwent microarray analysis had a likely pathogenic CNV and one fetus had incidental finding (2.5%). Of the 2 fetuses with pathogenic CNV, one had a 10.9 Mb duplication on chromosome band 10q25.1q26.12. Of note, this fetus had a normal second trimester ultrasound scan; yet the pregnancy was terminated due to the pathogenic finding. The other pathogenic chromosome abnormality was a 8.2 Mb duplication on chromosome 2p25. This pregnancy was terminated before the second trimester ultrasound scan was performed and further FISH analysis showed a paternally inherited unbalanced translocation between chromosome 2 and 22. Both pathogenic CNV findings were not detected with standard karyotyping. In a third fetus, an incidental finding was identified: a 37 kb deletion on chromosome 15q26.1 was detected. In this deletion the *RLBP1*-gene (MIM 180090) is located, which is involved in autosomal recessive diseases of the retina. The deletion was paternally inherited. The fetus did not show any anomalies on second trimester ultrasound scan and a healthy girl was born. We did not identify de novo variants of unknown significance (VUS) in the 40 fetuses that underwent microarray (Table 2, Fig. 2).

Table 2 Overview of identified CNVs by prenatal microarray

Case	NT	Chromosome	Size and type	Categorization
1	5.5	10q25.1-26.12	10.9 Mb duplication	Pathogenic
2	3.9	2p25	8.2 Mb duplication	Pathogenic
3	5.2	15q26.1	37 kb deletion	Incidental finding

CNV; copy number variation

Pregnancy outcome in relation to prenatal microarray

Outcome of pregnancy in fetuses with normal prenatal microarray

Three of the 40 fetuses who underwent prenatal microarray had an abnormal result. Therefore, 37 fetuses had a normal prenatal microarray result. 16 fetuses had a normal outcome (16/37, 43%), 11 were lost to follow up (11/37, 30%) and 10 had an abnormal outcome (10/37, 27%), independent of second trimester ultrasound scan. The fetuses with an abnormal outcome showed the following anomalies: two fetuses died in utero, three developed hydrops (two were terminated and one resulted in intrauterine fetal death), one developed fetal akinesia due to a postnatally through exome sequencing confirmed homozygous *RyR1* (MIM 190901) pathogenic variant and the pregnancy was terminated, one had a severe congenital heart defect (situs ambiguus of the atria with left isomerism, double outlet left ventricle, unbalanced atrioventricular septal defect, hypoplastic right ventricle) on second trimester ultrasound scan and the pregnancy was subsequently terminated. With regard to the fetuses with hydrops the following additional testing was performed: two of three fetuses received DNA analysis for RASopathies and lysosomal testing, which was normal in both. No further testing was carried out. The other fetus did not receive any additional testing due to parental choices. Two pregnancies were terminated in a private clinic at the parents' request due to parental anxiety on a poor outcome and one fetus had a postnatal confirmed congenital disorder of glycosylation type 2m (CDG-2m) (Fig. 2).

Outcome of pregnancy in fetuses without prenatal microarray

In the group of 33 fetuses without prenatal microarray, 12 showed normal postnatal development (12/33, 36%), 12 were lost to follow up (36%) and 9 fetuses showed an abnormal (postnatal) outcome, independent of second trimester ultrasound scan (9/33, 27%). The following anomalies with regard to abnormal outcome were reported: one intrauterine fetal death, one with a congenital heart defect (tricuspid valve atresia) and the pregnancy was subsequently terminated. One pregnancy was terminated at the parents' request due to parental anxiety on a poor outcome. Three children showed developmental delay after birth, without an underlying (genetic) diagnosis at the time of last evaluation. In all three children a postnatal microarray was performed and did not show any pathogenic copy number variations. One of the children with developmental delay also had craniosynostosis. DNA analysis for a specific craniosynostosis syndrome was performed and no pathogenic variants were found in the *FGFR1* (MIM 136350), *FGFR2* (MIM 176943), *FGFR3* (MIM 134934) and *TWIST* (MIM 601622) genes. A VUS was found in the *TCF12* gene (MIM 600480), which was also found in the healthy unaffected mother. One fetus was diagnosed with severe brain anomalies at 25 weeks of gestation and this pregnancy continued till term birth. The baby died several hours after birth

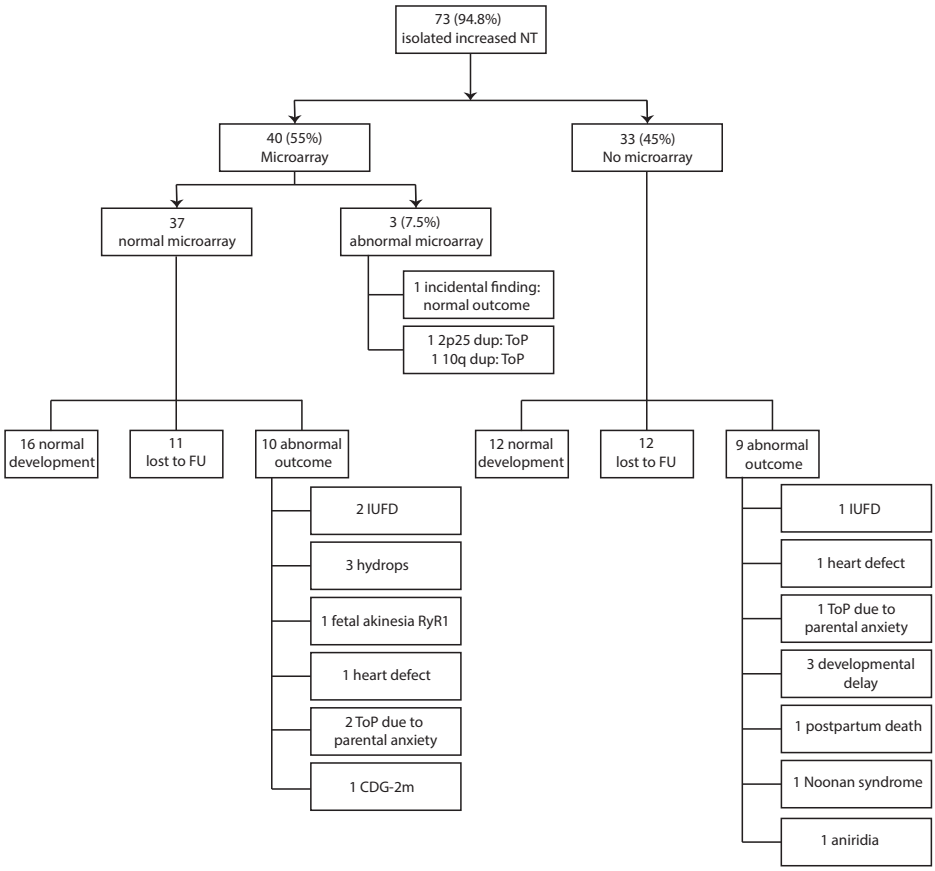

Figure 2 Clinical and genetic outcome in relation to microarray

and additional postnatal chromosome microarray was normal. No other testing had been performed and no underlying (genetic) diagnosis was made at the time. One child had Noonan syndrome with a prenatally confirmed pathogenic *PTPN11* gene variant (MIM 176876). One child had aniridia. DNA analysis performed on a buccal swab sample detected a mosaic *PAX6* gene (MIM 607108) duplication. (Fig. 2)

Both microarray and non-microarray groups taken together, in 73 fetuses with an isolated increased NT, 21 pregnancies had an abnormal postnatal outcome (12 in the microarray group (including the two pathogenic CNVs), 9 in the non-microarray group) (21/73, 28.8%), 29 had a normal outcome (including the fetus with the incidental finding) (29/73, 40%) and 23 were lost to follow up (23/73, 31.5%). Abnormal postnatal outcome was defined as any event that prevented the birth of a healthy normally developed child, such as termination of pregnancy (including due to parental anxiety) or an affected child after birth.

In total seven live born children showed adverse outcome. The overall risk of having an affected child in pregnancies with isolated increased NT in the fetus is therefore 9.6% (7/73).

Pregnancy outcome in relation to second trimester ultrasound scan

Second trimester ultrasound scan in fetuses with prenatal microarray

In the group with 37 fetuses with normal prenatal microarray 19 fetuses had a normal ultrasound scan (19/37, 51%) of which 15 had a normal outcome after birth (15/19, 79%), three were lost to follow up and one had an abnormal outcome (CDG-2m, 5%). Ten fetuses (10/37, 27%) were either lost to follow up in the pregnancy or did not receive a second trimester ultrasound scan due to intrauterine fetal death or termination of pregnancy due to parental anxiety on a poor outcome. Seven fetuses showed anomalies on the second trimester ultrasound scan, which included hydrops development in three fetuses, fetal akinesia in one fetus, fetal growth restriction (FGR) in two fetuses, and a heart defect in one fetus. In addition, choroid plexus cysts (CPC) were observed in an eighth fetus. As a result, the second trimester ultrasound scan was not entirely normal in 8/37 fetuses (22%).Two of the three fetuses with hydrops were subsequently terminated, the other died in utero. For two of three fetuses DNA analysis for RASopathies and lysosomal testing was performed, which was normal in both. No further testing was carried out. The other fetus did not receive any additional testing due to parental choices. One of the fetuses with suspected fetal growth restriction (FGR, in general defined as estimated fetal weight and abdominal circumference <p10 with a significant bending growth curve according to ISUOG Practice Guidelines (Lees et al., 2020)) on second trimester ultrasound scan had actually a normal birth weight of 2700 gram and was healthy and the other was lost to follow up. The pregnancy of the fetus with the heart defect was terminated and the one with choroid plexus cysts was lost to follow up. (Fig. 3)

Second trimester ultrasound scan in fetuses without prenatal microarray

Twenty-one of 33 fetuses without prenatal microarray had a normal second trimester ultrasound scan (21/33, 64%), of which 11 had a normal outcome after birth (11/21, 52%), six were lost to follow up (6/21, 29%) and four had an abnormal outcome (global developmental delay in three, Noonan syndrome in one, 4/21, 19%)). In the three children with neurodevelopmental delay chromosomal microarray was performed when the delay became evident. The results were normal in all three. No specific clinical diagnosis was made in all three children and as a result, no further testing was performed at that time. Seven fetuses (7/33, 18%) were either lost to follow up in the pregnancy or did not get a second trimester ultrasound scan due to termination of pregnancy due

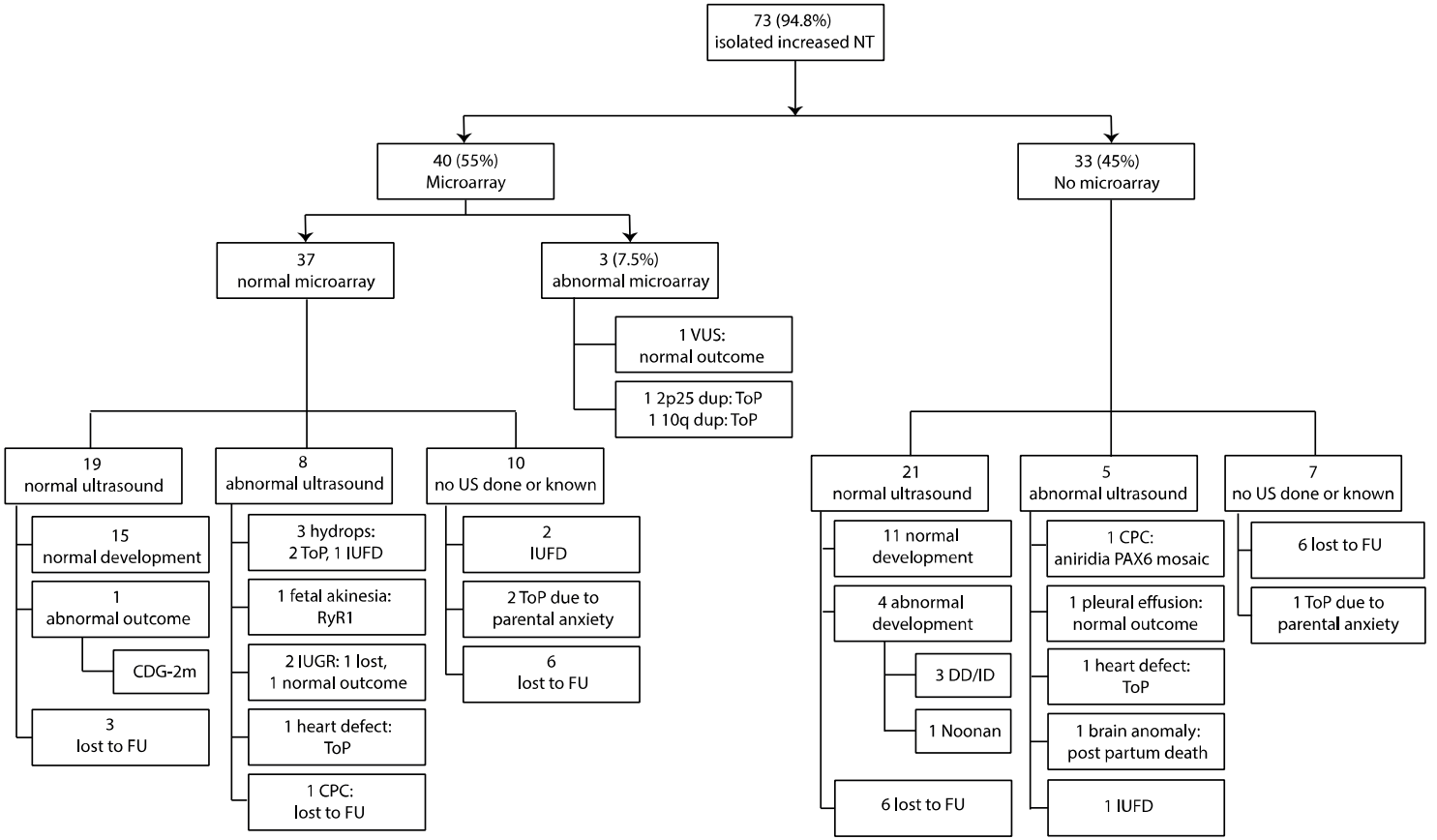

Figure 3 Clinical and genetic outcome in relation to second trimester ultrasound scan

to parental anxiety on a poor outcome. Five fetuses showed anomalies on the second trimester ultrasound scan (5/33, 15%), which included one intrauterine fetal death, one heart defect, one fetus with pleural effusion and echogenic bowel, one fetus with severe brain anomalies and one showed CPC. As mentioned earlier, the pregnancy with the heart defect was terminated. The fetus with pleural effusion and echogenic bowel had a normal outcome, the fetus with the brain anomalies died shortly after birth and the fetus with CPC had postnatal aniridia. (Fig. 3)

Of all 73 fetuses with normal karyotype (without taking microarray into account), 40 fetuses showed a normal second trimester ultrasound scan. Five of the 40 fetuses with a normal second trimester ultrasound scan turned out to have an abnormal postnatal outcome (5/40, 12.5%).

Off note, two fetuses with second trimester ultrasound anomalies (suspected FGR in one and pleural effusion and echogenic bowel in the other) had a normal outcome and were placed in the normal postnatal outcome group in overall conclusion. Additionally, two fetuses with abnormal ultrasound scans (one with suspected FGR and one with CPC) were lost to follow up after birth. Thus, if reported as abnormal outcome –e.g. an anomaly was seen on ultrasound scan- the risk of overall adverse pregnancy outcome increased to 34% (25/73 fetuses).

There was an unusual high percentage of follow up loss in all groups. All pregnant women and live born children who were lost to follow up were first invited to the clinic by regular mail. If no reply came or a no show appeared, the pregnant women or parents in case of live born children were contacted through phone. When no answers came after multiple tries, the patient was placed in the lost to follow up category.

DISCUSSION

The first aim of this study was to gain more insight in the diagnostic use of prenatal microarray in fetuses with isolated increased NT and normal karyotype and/or QF-PCR and to evaluate the outcome of these fetuses. The results show that prenatal microarray increased the diagnostic yield in this group of patients with 5% (2 out of 40 performed microarrays).

More than 30 studies have been published on the diagnostic yield of prenatal microarray in fetuses with an increased NT since Brisset et al. (2003) in 2003 were the first to report on this topic. A few of the earliest studies on prenatal microarray did not detect any

submicroscopic deletions or duplications (Schou et al., 2009; Huang et al., 2014), but the authors used low resolution microarrays. Therefore smaller deletions and duplications might have been missed. Other studies, such as the one from Lund et al. (Lund et al., 2015) reported a much higher diagnostic yield of 12.8% compared to our study. The authors explanation for this higher rate is the use of a high resolution prenatal microarray (50 kb). However, in our lab the same platform was used and we report a much lower diagnostic yield. Although Lund et al. comment on including fetuses with increased NT without other anomalies on the NT-ultrasound scan, it is unclear if a detailed follow up scan at a later gestational age showed abnormalities in these fetuses. If so, this might explain their higher rate of pathogenic findings. In 2015, Grande et al. (2015) reviewed all published papers on fetuses with an increased NT and established an overall 5% diagnostic yield for increased NT as a sole finding and a 7% yield for fetuses with increased NT and associated anomalies. Our percentage of pathogenic findings is therefore in line with most other studies (Grande et al., 2015).

In our cohort two pathogenic CNVs were detected. The first one we identified was a 10.9 Mb duplication on chromosome segment 10q25.1q26.12. Yunis and Sanchez first described the 10q duplication syndrome in 1974 (Yunis and Sanchez, 1974). Pure trisomy 10q24àqter anomalies are characterized by pre- and postnatal growth restriction, severe intellectual disability and structural defects of heart, eyes, kidneys and lower limbs. More distal trisomy anomalies starting from 10q25 have a less severe phenotype and lack major structural anomalies (Klep-de Pater et al., 1979; Taysi et al., 1983; Briscioli et al., 1993; Van Hemel and Eussen, 2000). Our case resulted in a termination of pregnancy, after a second trimester ultrasound scan was performed and no anomalies were identified. In the second case the 8.2 Mb duplication on chromosome 2p25 was shown to be due to unbalanced translocation between chromosome 2 and 22. Because of the large amount of repetitive sequences and the absence of probes for the p-arm on chromosome 22 a small deletion on this chromosome was not picked up with prenatal microarray. However, additional standard karyotyping and FISH showed the unbalanced translocation. The translocation was paternally inherited. 2p25 duplications have not been well defined, but are expected to cause structural anomalies and intellectual disability (Heathcote et al., 1991; Aviram-Goldring et al., 2000; Gruchy et al., 2007; De Rocker et al., 2015). These two pathogenic CNVs could not be picked up with conventional prenatal karyotyping and therefore microarray has an added value to the first-tier tests. Thus, the results from our cohort confirm the added relatively low diagnostic yield of 5% in fetuses with an increased NT. However, it remains debatable whether performing additional prenatal microarray is worthwhile in fetuses with isolated increased NT. In Grande's review (2015) (1695 pregnancies in 17 studies) the same 5% diagnostic yield in fetuses with an isolated increased NT is reported. However, one can easily justify performing a prenatal microar-

ray in fetuses with increased NT as invasive prenatal testing is performed for lower risks such as testing in a pregnancy in which parents have a previous child with a random de novo pathogenic variant. Therefore, prenatal microarray in fetuses with isolated increased NT and normal Rapid Aneuploidy Detection is justified and aligns with the growing trend to offer this test to all patients with (isolated) increased NT and to provide counselling so the patient can make a well-informed decision.

A second aim of our study was to gain insight into the development of children who presented with an isolated increased NT in pregnancy and to what extent prenatal microarray adds value in terms of predicting their outcome. Although we had a relatively high percentage of pregnancies and children who were lost to follow up, we identified an overall percentage of 28.8% in adverse outcome in fetuses with normal karyotype, including terminations of pregnancy due to hydrops, intrauterine fetal demise and structural defects as well as adverse outcome after birth. Specifically, the overall percentage of adverse outcome in all live born children was approximately 9.6%. These percentages of adverse outcome in fetuses with increased NT and normal karyotype and their outcome in live born children are in agreement with several other studies (Souka et al., 2001; Senat et al., 2002; Bilardo et al., 2007). Additionally, the children who were born and presented with developmental problems all had an underlying diagnosis that could not have been picked up with prenatal microarray. Furthermore, the case with Noonan syndrome would not have been detected prenatally with the suggested criteria from Croonen et al. (2013), because the NT in this fetus had resolved and a detailed second trimester ultrasound scan did not identify additional anomalies specific for Noonan syndrome. However, Noonan syndrome would have been diagnosed with the recommendations from Stuurman et al., as every fetus with an isolated increased NT above 5.0 mm is eligible for RASopathy testing (Stuurman et al., 2019).

The percentage of live born children with an adverse outcome was not altered as a result of performing prenatal microarray. Prenatal microarray only shows a relatively low additional diagnostic yield in fetuses with isolated increased NT and the live born children in this study who did not receive a prenatal microarray, but were eligible for microarray postnatal, did not have an abnormal postnatal microarray result . Therefore the question is raised whether prenatal whole exome sequencing would be a suitable additional test in fetuses with an isolated increased NT. The first studies on prenatal whole exome sequencing (WES) suggested a 10-25% increase in diagnostic yield in fetuses with various sonographic abnormalities (Carss et al., 2014; Drury et al., 2015; Hillman et al., 2015). Approximately six studies on prenatal WES have included fetuses with an isolated increased NT and normal microarray results (Drury et al., 2015; Choy et al., 2019; Daum et al., 2019; Lord et al., 2019; Petrovski et al., 2019; Xue et al., 2020). The diagnostic yield

of prenatal WES in these studies vary from 0% to 13% with an average of 5%. In our cohort of fetuses the CDG-2m, *RyR1*-related myopathy, Noonan syndrome and possibly the mosaic *PAX6* duplication might have been detected with prenatal WES. However, whole exome sequencing in fetuses with isolated increased NT warrants a careful approach as there is a substantial risk of unsolicited pathogenic findings that might not be related to the ultrasound findings (e.g. hereditary arrhythmias or hereditary cancer) and can cause anxiety in the future parents. Additionally, a prenatal phenotype is different than a postnatal phenotype and it might be complicated to interpret variant of unknown significance (VUS) for their pathogenicity. In the Netherlands, therefore, these VUS are not reported back during pregnancy at the moment. Resolved edema is a favorable prognostic factor in the outcome (Muller et al., 2004), so the timing of offering prenatal WES can be important as well.

It is interesting to note that only two of the affected children had an abnormal second trimester ultrasound scan. A second trimester ultrasound scan is an important tool in the evaluation process, but may give false reassurance as is seen in our cohort.

Like other studies in the field, our patient group was small as well for the outcome of prenatal microarray as for the outcome of follow up. It also lacks long-term follow up and a control group. Additionally, there was a high percentage of pregnancies and children that were lost to follow up.

In conclusion, prenatal microarray is of small but added value (5%) as a diagnostic test to identify (submicroscopic) chromosomal anomalies in fetuses with an isolated increased NT and should be offered as standard clinical practice. However, even if the result of first trimester microarray was normal, in our study isolated increased NT thickness was still associated with a 28.8% (21/73) risk of pregnancy loss (spontaneous or induced) or an affected child. There remains an increased and not negligible risk for an adverse outcome in live born children that had an isolated increased NT (7/73, 9.6%). In the near future, prenatal WES might be offered more frequently, preceded with genetic counseling.

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Chapter 4

Common copy number variations in fetuses with increased nuchal translucency

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Submitted

ABSTRACT

This study examined whether certain common copy number variations (CNV) on prenatal chromosomal microarray are more present in fetuses with isolated increased nuchal translucency compared to a control group of healthy unrelated adults. Special interest was given to CNVs within or around genes associated with lymphatic development as lymphatic maldevelopment is thought to be one of the causes for the development of nuchal edema. The results show that there is no difference in the frequency of common variance between fetuses with increased NT and healthy unrelated adults, which might explain the relatively limited extra yield of chromosomal microarray. There was also no significant involvement of genes related to lymphatic development either within the shared CNVs or stand-alone.

INTRODUCTION

Increased nuchal translucency (NT) has been an key factor in identifying fetuses with a chromosome abnormality in the first trimester of pregnancy (Nicolaides et al., 1992). However, there is still a large percentage of fetuses with an increased NT that are not explained by pathogenic chromosomal aberrations. These fetuses are still at risk for structural anomalies and genetic syndromes (Hyett et al., 1997; Souka et al., 1998), but the majority has a good postnatal outcome when prenatal chromosomal microarray and second trimester ultrasound scan are normal. Likely, multiple factors such as environmental and epigenetic factors are involved in the development of an increased NT without causing a disruptive phenotype. On cellular level, lymphatic maldevelopment has been proposed as a major key factor in the development of nuchal edema (Haak et al., 2002) and therefore pathogenic variants in genes related to lymphatic development may contribute to the development of nuchal edema.

The aims of this short study were twofold. First, we examined whether certain common copy number variations, especially around genes related to lymphatic development, are more present in fetuses with an isolated increased NT than in the general population without causing a unfavorable outcome postnatal. Second, we used a customized gene panel with genes related to lymphatic development to search for deletions and/or duplications involving these genes to explain these CNVs in fetuses with an increased NT.

MATERIALS AND METHODS

Patients

From our database at the Department of Human Genetics at Amsterdam UMC, location VUmc, all fetuses with an increased NT (defined as ≥ 3.5 mm, according to the Dutch Society of Obstetrics and Gynecology guidelines (NVOG, 2012)) that had received prenatal chromosomal microarray between 1 January 2011 and 1 August 2013 were selected. Previous to prenatal microarray, conventional karyotyping had already shown a normal karyotype in all these fetuses. Prenatal microarray had been performed with informed consent from all parents. In addition, data from healthy unrelated parents who had received a chromosomal microarray due to findings of unknown significance in their live born children were collected. This data was collected in the same time period as the fetuses with increased NT. The fetuses and unrelated parents were divided into two datasets.

Dataset 1 included 40 fetuses with an isolated increased NT (defined as ≥3.5 mm and median thickness of 4.2 mm). Fetuses with increased NT and additional ultrasound anomalies were excluded from the study.

Dataset 2 included 86 healthy adults (51 women and 35 men with average age of 39.8 years each), who received chromosomal microarray subsequent to microarray performed in their live born children. The parents were unrelated to the fetuses with increased NT (dataset 1). The indication for the parental microarray was a variant of unknown significance (VUS) in their live-born child. Dataset 2 was the control group. The local institutional ethics board approved the study.

Samples

The fetal samples, either chorionic villi samples or as amniotic fluid, had been sent in from the Department of Obstetrics and Gynecology from Amsterdam UMC, location VUmc. Parental blood samples for maternal contamination and trio-analysis.

DNA extraction

DNA was extracted from uncultured cells using Wizard® (Promega, Madison, WI, USA) and from cultured cells using Qiagen BioRobot® (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. Maternal contamination was tested with fragment analysis using PowerPlex16 (Promega, Madison, WI, USA).

Chromosomal Microarray

Agilent CGH 180K oligo array (Agilent Technologies, Santa Clara, USA; Amadid 023363) was used as array platform and performed according to manufacturer's instructions. The overall median probe spacing of this platform was 13 kb. As reference DNA a commercial reference pool of Kreatech, consisting of healthy men and women, was used (sex-matched experiments).

Data analysis was performed using Nexus Copy Number versions 5.0, 6.1 and 7.0, and interpreted using Cartagenia BENCH 4.0 Feb-2012 (genome build hg18 and hg19). Standard settings for CNVs in Nexus were applied: threshold for probe median: gain 0.3 and loss -0.3. Minimal probes for a call: 20 per segment. The interpretation of CNVs has been done according the criteria as described previously. We analyzed trios to assess whether CNVs were de novo or inherited. The microarray was considered normal if only benign class 1 or 2 CNVs were detected. The microarray was considered abnormal if a most likely clinically relevant (class 4 and 5) CNV was found. Variants of unknown clinical significance (class 3) were discussed internally before reporting them (Koolen et al., 2009). Frequency plots were created using Matlab, version 3.3.1.(Scheinin et al., 2014).

Gene panel

Genes from the NGS gene panel 'Lymphedema', related to lymphatic development (Table 1) were checked for involvement in possible recurrent CNVs as well as duplications or deletions within these genes. Additionally, CNVs in genes that are related to lymphatic development, but were considered research genes and were excluded from the diagnostic NGS gene panel 'Lymphedema' were checked. We did not run a NGS panel on the fetal material and therefore, point mutations were not analyzed.

Table 1. Genes involved in lymphatic development

Genes known to be involved in lymphatic development (present in a diagnostic gene panel used in our dataset)	Genes possibly involved in lymphatic development (research genes, not included in gene panel)
ABCC9, ALG8, BRAF, CBL, CCBE1, CDK19, FAT4, FLT4, FOXC2, GATA2, GJA1, GJC2, GLA, HGF, HRAS, ITGA9, KIF11, KRAS, MAP2K1, MAP2K2, MET, MPI, NAGA, NRAS, PEPD, PMM2, PTPN11, PTPN14, RAF1, RELN, SHOC2, SOS1, SOX18, SPRED1, TUBGCP6, VEGFC	ANGPT1, ANGPT2, FIGF, VEGFD, LYVE1, PDPN, PROX1, EMILIN1, NTN4, FGF2, VEGFA, FGFR3, NFATC1, CEACAM1, ESM1, TIE1, NRP2, E2F7, E2F8, LMO2, EFNB2, EPHB4, NR2F2, TBX1, ADM, RAMP2, CALCRL, TMEM204, PPFIBP1, GIPC1, RAC1, SPRED1, SPRED2, ITGB1, ACVRL1, ACVR2B, DLL4, NOTCH1, NOTCH2, ELMO1, GJB2, VCAM1

Statistics

The data were analyzed in SPSS V.22. For statistical analysis, an independent t-test was used. A probability value of less than 0.05 was considered significant.

RESULTS

CNVs in fetal samples and healthy controls

In total 3240 CNVs were detected in all samples. In the 40 fetal samples 989 CNVs were detected, with a mean of 24,73 CNVs per sample. The 86 healthy controls contained 2251 CNVs in total, with a mean of 26.17 per control. Using a t-test no difference was detected in the amount of CNVs established in the fetal samples and healthy controls (p-value 0.26). CNVs in both groups were predominantly located around the centromeres.

Pathogenic variants in the fetal samples

Two of the 40 fetuses of dataset 1 (5%) had a clearly pathogenic de novo CNV: a 10.9 Mb duplication on chromosome 10q25.1q26.12 and 8.2 Mb duplication on chromosome 2p25. Both pathogenic findings were not detected on prior prenatal karyotyping. No *de novo* variants of unknown significance in the 40 fetuses were identified.

Overlapping CNVs between fetal samples and healthy adults

All microarrays in dataset 1 and 2 were checked for overlapping CNVs within their own dataset and compared between datasets (Figure 1). There was no single CNV that was present in all fetuses with increased NT or in all adult samples. In addition, there was no common CNV more frequently present in the fetuses compared to the adult samples. We also did not detect any susceptibility loci in either the fetuses with increased NT or the healthy adults.

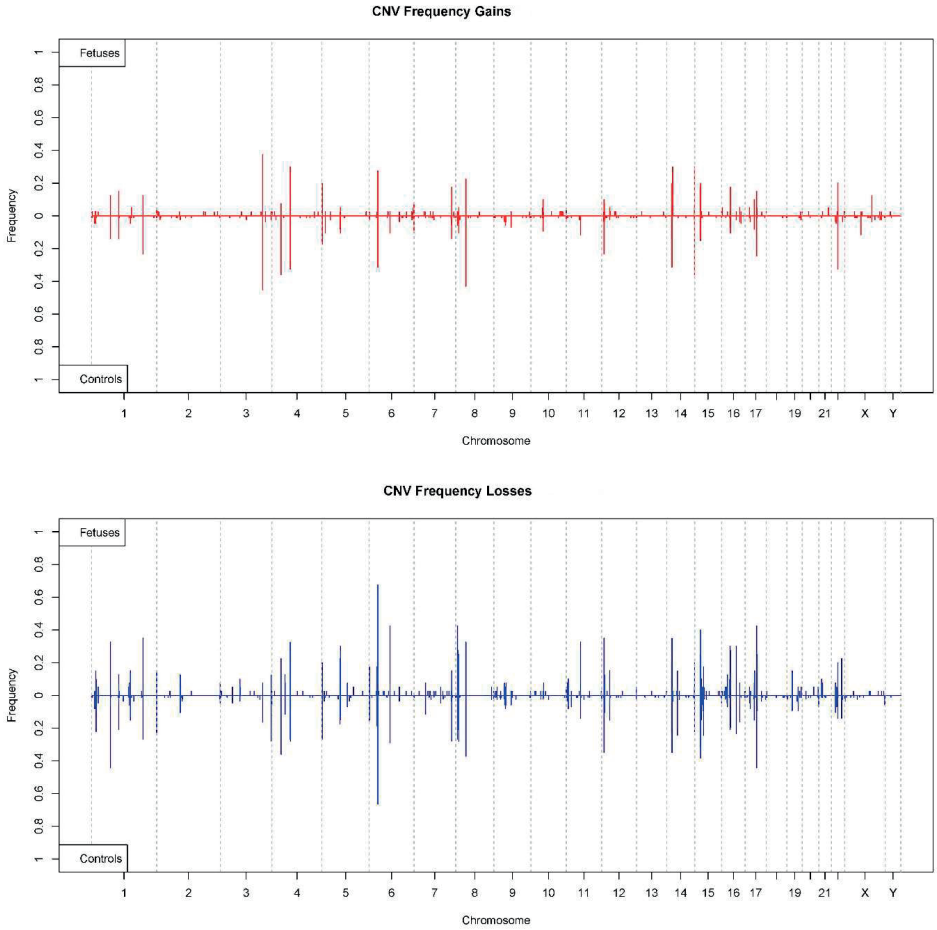

Figure 1 Overview of CNV losses and gains of fetal samples versus adult samples

Involvement of genes related to lymphatic development

There was one fetus with a copy number gain of the *SHOC2* gene (MIM 602775). This gene was located in the pathogenic duplication of chromosome 10q25.1q26.12. In all other fetuses, no involvement of known 'lymphatic' genes was found. One adult sample

showed involvement of a lymphatic gene in one CNV; a deletion of the *GIPC1* gene (MIM 605072) on chromosome 19p13.12. All other 85 adult samples did not show involvement of 'lymphatic' genes in their CNVs.

DISCUSSION

We examined whether fetuses with isolated increased NT showed shared CNVs that might contribute to the lymphatic nature of this condition without causing a clear disruptive phenotype. There were no CNVs more frequently present in fetuses with increased NT compared to the healthy control group. Additionally, there was no significant involvement of genes related to lymphatic development either within the shared CNVs or stand-alone.

Two pathogenic duplications were detected in the fetuses with an isolated increased NT. The pathogenic 10q25.1q26.12 duplication contained the *SHOC2* gene. The *SHOC2* gene is involved in RASopathies and an increased NT is reported to be a prenatal finding in fetuses affected with Noonan syndrome (Stuurman et al., 2019). Usually point mutations or deletions of (part of) the *SHOC2* gene are found to cause Noonan syndrome. Duplications, however, are more difficult to interpret, but it can not be excluded that the *SHOC2* gene was involved in causing the prenatal finding of increased NT in this fetus. In the other fetus there was no involvement of lymphatic genes in its 2p25 duplication. Additionally, these two fetuses did not share any CNV of interest.

The *GIPC1* gene deletion found in a healthy parent was the only aberration found in the healthy controls. This gene is involved in lymphatic development in zebrafish and frogs, but its role in humans is unclear (Hermans et al., 2010). It is also unknown whether this adult had an increased NT him/herself.

Although no difference was seen in common CNVs between both datasets, the prenatal status concerning an increased NT in healthy parents of these fetuses or in the healthy adult control group is unknown.

This study did not perform DNA sequencing of the lymphatic genes. Point mutations could still contribute to the development of a lymphatic phenotype, such as Hennekam syndrome (MIM 235510). However, we followed up on all pregnancies in which an isolated increased NT (personal data) was confirmed and did not find any child with lymphatic concerns. In addition to the two fetuses with isolated enlarged NT, two other fetuses were confirmed to have a genetic diagnosis after birth. One with *RyR1* myopathy

based on a homozygous *RyR1* mutation (MIM 180901) and one fetus with congenital disorder of glycosylation type 2m (CDG-2m) (MIM 300896) based on *SLC35A2* (MIM 314375) *de novo* mosaic mutation. The *SLC35A2* gene is not known to be related to lymphatic development and it is therefore unclear whether this gene is associated with lymphatic development. *RyR1* gene variants have been associated with fetal hydrops, which in general starts out with increased NT (McKie et al., 2014; Meier et al., 2017; Suzumori et al., 2018). Therefore, there may be an association between *RyR1* and lymphatic development. However, an increased NT is found in 1% of all pregnancies (Snijders et al., 1998) and every child has a 3-5% risk of a random inborn error. The likelihood that these simultaneously occur is therefore not unusual.

This study shows there is no indication for a causal role of (common) CNVs for increased NT. It is interesting to speculate that the absence of genes related to lymphatic development in the fetal and adult CNVs results in similar CNVs in these groups and that CNVs therefore only play a minor role in the explanation of an increased NT. This is also shown by the relative limited extra yield of prenatal microarray in fetuses with increased NT (Grande et al., 2015). We hypothesize that an increased NT is more likely caused by transient lymphatic imbalance, which may be caused by point mutations in genes involved lymphatic development, environmental and/or epigenetic factors. Performing whole exome sequencing with CNV analysis might be more useful than microarray alone.

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Chapter 5

Prenatal diagnostic testing of the Noonan syndrome genes in fetuses with abnormal ultrasound findings

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Eur J Hum Genet 2013 Sep;21(9):936-42

ABSTRACT

In recent studies on prenatal testing for Noonan syndrome (NS) in fetuses with an increased nuchal translucency (NT) and a normal karyotype, mutations have been reported in 9–16% of cases. In this study, DNA of 75 fetuses with a normal karyotype and abnormal ultrasound findings was tested in a diagnostic setting for mutations in (a subset of) the four most commonly mutated NS genes. A de novo mutation in either *PTPN11, KRAS* or *RAF1* was detected in 13 fetuses (17.3%). Ultrasound findings were increased NT, distended jugular lymphatic sacs (JLS), hydrothorax, renal anomalies, polyhydramnios, cystic hygroma, cardiac anomalies, hydrops fetalis and ascites. A second group, consisting of anonymized DNA of 60 other fetuses with sonographic abnormalities, was tested for mutations in 10 NS genes. In this group, five possible pathogenic mutations have been identified (in *PTPN11* (n = 2), *RAF1, BRAF* and *MAP2K1* (each n = 1)). We recommend prenatal testing of *PTPN11*, *KRAS* and *RAF1* in pregnancies with an increased NT and at least one of the following additional features: polyhydramnios, hydrops fetalis, renal anomalies, distended JLS, hydrothorax, cardiac anomalies, cystic hygroma and ascites. If possible, mutation analysis of *BRAF* and *MAP2K1* should be considered.

INTRODUCTION

Noonan syndrome (NS) is an autosomal dominant condition with an incidence of 1:1000–2500 live births. It is characterized by characteristic facies, short stature, congenital heart defects (CHD), skeletal abnormalities, cryptorchidism and variable development delay (Allanson, 1987; Sharland et al., 1992; Noonan, 1994). NS is one of the 'RASopathies', a specific class of developmental disorders, caused by germline mutations in genes, encoding proteins of the RAS–mitogen-activated protein kinase (RAS–MAPK) pathway. This pathway has an essential role in the control of the cell cycle, differentiation, growth and cell senescence. Dysregulation has profound developmental consequences. About 50% of known NS cases have a mutation in the *PTPN11* gene (Tartaglia et al., 2001; Tartaglia et al., 2002). Heterozygous gain-of-function mutations in other genes perturbing RAS–MAPK signaling have also been identified in NS patients: *KRAS, SOS1, BRAF, RAF1, MAP2K1, NRAS, SHOC2* and *CBL* (Schubbert et al., 2006; Nava et al., 2007; Pandit et al., 2007; Razzaque et al., 2007; Roberts et al., 2007; Tartaglia et al., 2007; Cirstea et al., 2010; Martinelli et al., 2010). Several of these genes are also involved in Cardio-Facio-Cutaneous (CFC) syndrome and Costello syndrome (Hennekam, 2003; Roberts et al., 2006; Tartaglia et al., 2011). Because of the high variability of clinical symptoms and the genetic heterogeneity establishing a diagnosis of one of these syndromes is often difficult. Patients are most frequently diagnosed postnatally, but also prenatal characteristic findings are described. Costello syndrome is associated with polyhydramnios, fetal overgrowth, a relative macrocephaly and to a lesser extent with nuchal thickening, hydrops, ventriculomegaly, pyelectasia and fetal atrial tachycardia/arrhythmia (Van den Bosch et al., 2002; Lin et al., 2009; Smith et al., 2009).

Prenatal features of NS are increased nuchal translucency (NT), distended jugular lymphatic sacs (JLS), cystic hygroma, hydrops fetalis, pleural effusion, polyhydramnios, CHD and renal abnormalities (Witt et al., 1987; Nisbet et al., 1999; Menashe et al., 2002; Bekker et al., 2007). The first prenatal DNA diagnosis of NS in a fetus with massive cystic hygroma, pleural effusion and ascites showed a mutation in the *PTPN11* gene (Schlüter et al., 2005). Lee et al (Lee et al., 2009) performed a retrospective review of prenatal *PTPN11* testing. The two most common indications for testing in this study were increased NT (44%) and cystic hygroma (48%). *PTPN11* mutations were identified in 9% of fetuses (2 and 16% of fetuses with increased NT and cystic hygroma, respectively). In a prospective DNA diagnostic study on fetuses with a normal karyotype and an increased NT, we previously identified a de novo mutation rate of 15.8% (3/19 fetuses) by parallel sequencing of *PTPN11* and *KRAS* (Houweling et al., 2010).

In the present study, we investigated the DNA of 56 additional fetuses with a normal karyotype and one or more abnormal ultrasound findings for a mutation in one or more of the NS genes in a diagnostic setting. Parallel sequencing of *PTPN11, KRAS, SOS1* and *RAF1* was offered, as these are the most frequent mutated genes in patients diagnosed with NS after birth. The actual number of genes tested in each fetus depended on the amount of DNA available for testing (chorionic villi or amniotic fluid, cultured or uncultured cells). In an attempt to define whether parallel sequencing of these four genes revealed the highest mutation frequency in fetuses with an abnormal ultrasound, we investigated in a second anonymized study the DNA (amplified by whole genome amplification to allow a sufficient amount of DNA) of 60 other fetuses with increased NT, hydrops fetalis and/or CHD and a normal karyotype, for mutations in 10 genes of the RAS–MAPK pathway.

The aim of this study is to provide a protocol for prenatal NS testing, to serve as a useful aid to facilitate parental counseling and targeted DNA testing.

MATERIALS AND METHODS

Patients

The first, diagnostic, study group consisted of 75 fetuses with a normal karyotype and one or more abnormal ultrasound findings. This group contains all fetuses sent to our laboratory from different clinical genetics centers in the Netherlands for a period of 2 years. Part of the positive cases have been described in case reports (Houweling et al., 2010; Bakker et al., 2011). Ultrasound findings considered as indication for prenatal sequencing were increased NT (greater than the 95th percentile (p95)), cystic hygroma, distended JLS, ascites, hydrops fetalis, pleural effusion, polyhydramnios, CHD and renal abnormalities.

From all mutation-negative cases the ultrasound findings provided by the referring physician were marked, and ultrasound findings that were not mentioned were regarded not to be present. From all the mutation-positive cases the applicants were asked to deliver detailed information about gestational age (GA) and corresponding ultrasound findings. Blood of both parents was available for testing of detected mutations and variants.

The second, anonymized, study group consisted of 60 other fetuses with increased NT, hydrops fetalis and/or CHD and a normal karyotype. Medical records of these cases were

reviewed before anonymizing the samples. Because of the anonymous design of the study, parents could not be tested and results were not communicated to the parents.

Sequence analysis

In the diagnostic study group, parallel sequencing of the coding regions and splice sites of *PTPN11, KRAS, SOS1* and *RAF1* was performed. The number of genes tested depended on the amount of DNA available for testing (chorionic villi or amniotic fluid, cultured or uncultured cells) and the GA of the pregnancy. In 15 fetuses only *PTPN11* was tested, 9 fetuses were tested for *PTPN11* and *KRAS*, 11 fetuses were tested for *PTPN11, KRAS,* and *SOS1*. In 40 fetuses all four genes were analyzed. The order of genes tested in case not enough DNA was available (*PTPN11, KRAS, SOS1, RAF1*) is based on the reported mutation frequency in postnatally diagnosed NS cases.

In the anonymized study group, 10 ng of fetal DNA was amplified using the Illustra Genomiphi V2 DNA amplification kit (GE Healthcare, Piscataway, NJ, USA), according to the manufacturer's protocol. The coding regions and splice sites of 10 genes were sequenced: *PTPN11, KRAS, RAF1, SOS1, BRAF, NRAS, SHOC2, MAP2K1, MAP2K2,* and *HRAS*. Mutations were confirmed on the unamplified fetal material. Primers and PCR/sequencing conditions are available upon request.

Interpretation of sequence variants

For all detected variants that were not previously described in literature, an in-silico-based method (Alamut software version 2.0, Interaction Biosoftware, Rouen, France) was used to assess the effect of the mutation. Parameters used in this program include the Grantham score, SIFT and PolyPhen analysis, PhyloP score and analysis of frequency of the mutation in the population.

Statistical analysis

The data were collected in SPSS 16.0. For statistical analysis we used the Student's t-test and the Fischer's Exact test. A statistically significant two-sided threshold was set at $P<0.05$. Because of the small number of fetuses also descriptive analysis was performed.

RESULTS

Diagnostic study group

The diagnostic study group included 75 fetuses with a normal karyotype and abnormal ultrasound findings, described in NS.

Table 1 shows the prenatal ultrasound findings in the total group and classified by the presence or absence of a mutation. The most frequently identified prenatal characteristics are increased NT (n ¼ 50; 66.7%), cystic hygroma (n = 17; 22.7%), cardiac anomalies and hydrops fetalis (each n = 15; 20.0%), distended JLS (n = 12; 16.0%), hydrothorax (n = 9; 12.0%), renal anomalies (n = 7; 9.3%), polyhydramnios (n = 3; 4.0%) and ascites (n = 1; 1.3%). No postnatal information of the mutation-negative cases is present. However, we never received a request for testing of the remaining NS genes once the baby was born, suggesting that no NS-specific features were present. However, it cannot be excluded that part of the pregnancies have been terminated because of the abnormalities seen on the ultrasound.

Table 1 Prenatal findings of 75 fetuses with a normal karyotype

Findings	Total group (n = 75)	Mutation-positive group (n = 13) (%)	Mutation-negative group (n = 62) (%)	P-value
Increased NT (%)	50/75 (66.7)	13/13 (100)	37/62 (59.7)	0.003[a]
Mean NT (mm) at 11–14 weeks	7.3 (3.6–14)	8.0 (4.2–14)	6.5 (3.6–11.9)	0.854[b]
Cystic hygroma	17/75 (22.7)	4/13 (30.8)	13/62 (21.0)	0.475[a]
Distended JLS	12/75 (16.0)	7/13 (53.8)	5/62 (8.1)	0.000[a]
Ascites	1/75 (1.3)	1/13 (7.7)	0/62 (0.0)	0.173[a]
Hydrothorax	9/75 (12.0)	7/13 (53.8)	2/62 (3.2)	0.000[a]
Cardiac anomalies	15/75 (20.0)	5/13 (38.5)	10/62 (16.1)	0.120[a]
Renal anomalies	7/75 (9.3)	6/13 (46.2)	1/62 (1.6)	0.000[a]
Hydrops fetalis	15/75 (20.0)	4/13 (30.8)	11/62 (17.7)	0.279[a]
Polyhydramnion	3/75 (4.0)	3/13 (23.1)	0/62 (0.0)	0.004[a]

JLS, jugular lymphatic sacs; NT, nuchal translucency.
[a]Fisher's Exact test.
[b]Student's *t*-test.

In 13/75 (17.3%) fetuses a mutation was detected. Table 2 shows their pre- and postnatal clinical characteristics. The mutations and clinical features of four of these fetuses have also been described elsewhere; case 1 has been described by Bakker et al (Bakker et al., 2011) and cases 9, 12 and 13 have been described by Houweling et al. (Houweling et al., 2010). Nine fetuses had a de novo mutation in *PTPN11*, three in *RAF1* and one in *KRAS*. Eight of the 12 different mutations detected in this study have been described earlier in postnatally identified NS patients: *PTPN11*; c.174C>G (p.(Asn58Lys)) (Musante et al., 2003), c.182A>G (p.(Asp61Gly)) (Tartaglia et al., 2001), c.184T>G (p.(Tyr62Asp)) (Tartaglia et al., 2002), c.205G>C (p.(Glu69Gln)) (Musante et al., 2003), c.417G>C (p.(Glu139Asp)) and c.854T>C (p.(Phe285Ser)) (Tartaglia et al., 2002), *RAF1* c.770C>T (p.(Ser257Leu)) (Razzaque et al., 2007), and *KRAS* c.173C>T (p.(Thr58Ile)) (Schubbert et al., 2006). One mutation has been previously described in a patient with the clinical diagnosis of LEOPARD syndrome: *PTPN11* (c.1381G>A, p.(Ala461Thr)) (Yoshida et al., 2004). The remaining

Table 2 Prenatal and postnatal findings of 13 mutation-positive fetuses in the diagnostic study group

Prenatal and Postnatal findings	Case 1 (Bakker et al[24])	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
DNA mutation	RAF1 c.770C>T (p.Ser257Leu) *de novo*	PTPN11 c.174C>G (p.Asn58Lys) *de novo*	RAF1 c.770C>T (p.Ser257Leu) *de novo*	RAF1 c.775T>C (p.Ser259Pro) *de novo*	PTPN11 c.1381G>A (p.Ala461Thr) *de novo*	PTPN11 c.182A>G (p.Asp61Gly) *de novo*	PTPN11 c.854T>C (p.Phe285Ser) *de novo*
Prenatal findings							
Increased NT (mm)	4.4	4.2	10.0	7.6	6.5-11.2	Unknown	7.8
Cystic hygroma	No	No	No	Yes	No	Yes	No
Distended JLS	No	No	Yes	No	No	No	Yes (8.5-14 mm)
Ascites	No	No	Yes	No	No	No	No
Hydrothorax	No	Yes (right side)	Yes (both sides)	Yes (both sides)	Yes (left side)	Yes (right side)	No
Hydrops fetalis	No	No	Yes	Yes	Yes	No	No
Polyhydramnios	No	Yes	No	Yes	No	Yes	No
Cardiovascular anomalies	Yes Mild TR, amlalignment VSD, heart axis deviation, RV dysfunction, PE	Yes Ductus venosus agenesis	No	No	No	No	Yes Hypoplastic left heart, retrograde flow aorta
Renal anomalies	Yes Bilateral pyelectasis	No	Yes Bilateral echogenicity	Yes Hydronephrosis	No	Yes Unilateral pyelectasis	No
Facial features	Yes Low-set ears with uplifted earlobes, small nose, sloping forehead, brachycephaly	Yes Mild ptosis					No
Limb anomalies		No		Yes Clubfeet		Yes Syndactyly 4th and 5th finger	No

Table 2 Prenatal and postnatal findings of 13 mutation-positive fetuses in the diagnostic study group (*continued*)

Prenatal and Postnatal findings	Case 1 (Bakker et al[24])	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Indication for testing	Increased NT, facial features	Increased NT, ductus venosus agenesis	Increased NT	Increased NT	Increased NT	Increased NT	Increased NT, distended JLS and hypoplastic left heart
Pregnancy course	TOP GA 22+1	Planned sectio Caesarea at GA 32, deceased suddenly 1 day post partum	TOP GA 17+3	TOP GA 20+2	TOP GA 16+4	TOP GA 23+3	TOP GA 16+1
Postnatal findings							
Facial features	Low-set posteriorly angulated ears, broad nose, brachycephaly	Downslanted palpebral fissures, hypertelorism, low-set posteriorly angulated ears, broad neck, low anterior hairline	Deep nasal bridge, hypertelorism, low-set posteriorly angulated ears, anteverted nares, broad mouth, long philtrum	Low-set ears	Short nose, anteverted nares, low-set posteriorly angulated ears		Low-set ears, retrognathia
Cardiovascular anomalies	Subaortal stenosis, LVH, perimembrous VSD	No		Deep interventricular fissure externally			HLH ASD type II, perimembranous VSD, mitral stenosis, aortic stenosis, hypoplasia aortic arch
Renal anomalies		No				No	
Other features	Redundant nuchal skin, generalized skin edema	Severe hydrops, brachydactyly, virilized genital		Severe hydrops, clubfeet		Hydroma colli	

Table 2 Prenatal and postnatal findings of 13 mutation-positive fetuses in the diagnostic study group

Prenatal and Postnatal findings	Case 8	Case 9 (Houweling et al[27])	Case 10	Case 11	Case 12 (Houweling et al[27])	Case 13 (Houweling et al[27])
DNA mutation	PTPN11 c.227A>T (p.Glu76Val) *de novo*	KRAS c.173C>T (p.Thr58Ile) *de novo*	PTPN11 c.184T>G (p.Tyr62Asp) *de novo*	PTPN11 c.205G>C (p.Glu69Gln) *de novo*	PTPN11 c.417G>C (p.Glu139Asp) *de novo*	PTPN11 c.181G>C (p.Asp61His) *de novo*
Prenatal findings						
Increased NT (mm)	11	14	5.1	8.5	5.2	8.2
Cystic hygroma	Yes	No	No	Yes (6.9 mm)	No	Yes
Distended JLS	No	Yes	Yes (7.1x8.7 mm)	Yes	Yes	Yes
Ascites	No	No	No	No	No	No
Hydrothorax	Yes (left side)	Yes (both sides)	No	No	No	No
Hydrops fetalis	Yes	No	No	No	No	No
Polyhydramnios	No	No	No	No	No	No
Cardiovascular anomalies	No	No	No	Yes Hypoplastic right heart with small arteriae pulmonalis	No	Yes Pericardial effusion, AVSD
Renal anomalies	No	Yes Bilateral pyelectasis (right 9.6 mm, left 7.7 mm)	No	No	No	Yes Bilateral pyelectasis
Facial features	Yes Unclassified because of edema	Low-set ears		Yes Low-set ears	No	
Limb anomalies	No					Yes Short femura (<p5)
Indication for testing	Hydrops fetalis	Increased NT	Increased NT	Increased NT	Increased NT, distended JLS	Increased NT, AVSD, short femura
Pregnancy course	TOP GA 17+4	TOP GA 22+2	TOP	TOP GA 21+5	TOP GA 23+5	TOP GA 16+0

Table 2 Prenatal and postnatal findings of 13 mutation-positive fetuses in the diagnostic study group (*continued*)

Prenatal and Postnatal findings	Case 8	Case 9 (Houweling et al[27])	Case 10	Case 11	Case 12 (Houweling et al[27])	Case 13 (Houweling et al[27])
Postnatal findings						
Facial features	Extremely low-set posteriorly angulated ears, hypertelorism	Hypertelorism, low-set ears			Hypertelorism, low-set left ear	Low-set posteriorly angulated ears, hypertelorism, webbing of the neck
Cardiovascular anomalies	No	Hypertrophy			Hypertrophy interventricular septum	Complete AVSD
Renal anomalies	No	Bilateral pyelectasis				
Other features	Severe skin edema	Nuchal edema			Loose nuchal skin	

AVSD, atrio ventricular septum defect; GA, gestational age; HLH, hypoplastic left heart; JLS, jugular lymph sacs; LVH, left ventricular hypertrophy; NT, nuchal translucency; PE, pericardial effusion; RV, right ventricle; TOP, termination of pregnancy; TR, tricuspid regurgitation; VSD, ventricular septum defect; *increased NT: measured at 11-14 weeks

three mutations had not been described in literature before the detection in this study: *RAF1* (c.775T>C, p.(Ser259Pro)), *PTPN11* (c.181G>C, p.(Asp61His)) and *PTPN11* (c.227A>T, p.(Glu76Val)). These mutations were considered to be pathogenic as the mutations were not present in both the parents. Furthermore, they affect highly conserved amino acids, and in-silico analysis (Alamut software) predicts a pathogenic effect. Additionally, further evidence for pathogenicity of these mutations is given by the fact that the c.181G>C and c.227A>T mutations in *PTPN11* were detected as somatic events in malignancies (Tartaglia et al., 2006). As the Ser259 residue is critical for autoinhibition of *RAF1*, mutations affecting this residue facilitate binding of *RAF1* to GTP-bound *RAS* and its activation (Pandit et al., 2007).

Twelve fetuses with a mutation had an increased NT with a mean NT of 8 mm at GA of 11–14 weeks (range 4.2–14 mm). The NT value of case 6 was not measured at this term. Apart from the increased NT, all mutation-positive fetuses had one or more other sonographic abnormalities. These were, in order of frequency: distended JLS (n = 7; 53.8%), hydrothorax (n = 7; 53.8%), renal anomalies (n = 6; 46.2%), cardiac anomalies (n = 5; 38.5%), cystic hygroma (n = 4; 30.8%), hydrops fetalis (n = 4; 30.8%), polyhydramnios (n = 3; 23.1%) and ascites (n = 1; 7.7%). In three fetuses limb anomalies were described (syndactyly of the 4th and 5th finger, clubfeet and short femura). In five fetuses, facial characteristics such as low-set ears, uplifted earlobes, small nose, sloping forehead and brachycephaly were noted. Twelve of 13 pregnancies were terminated after extensive counseling. One woman had a planned caesarian section at GA 32 weeks, but the neonate deceased suddenly 1 day post partum.

An increased NT, distended JLS, hydrothorax, renal anomalies and polyhydramnios were significantly more common in the mutation- positive group (Table 1). The mean NT at GA 11–14 weeks was 8 mm in the mutation-positive group versus 6.5 mm in the mutation-negative group, which is not statistically significant (P = 0.854). In the mutation-positive group a tendency of increasing NT with advancing GA was observed in the group as a whole (Figure 1). This trend is not seen in the mutation-negative group.

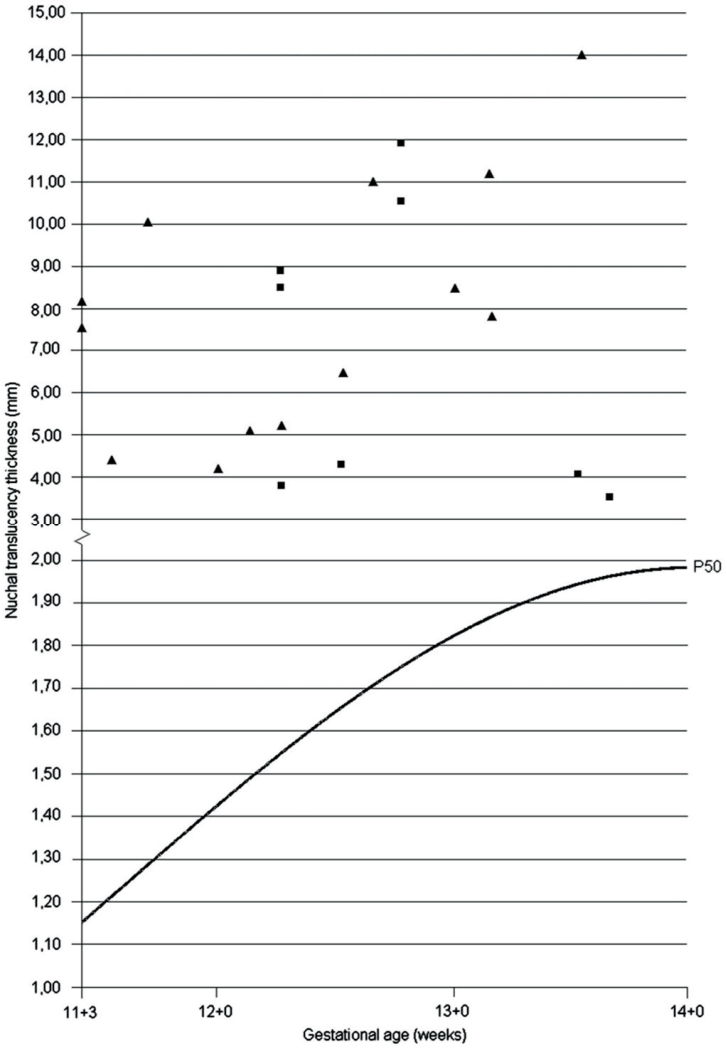

Figure 1 NT values of mutation-positive cases (n = 13) and mutation- negative cases (n = 8) from the diagnostic study group, according to GA. Only those cases for which NT values between 11–14 weeks of gestation were available, are shown. Mutation-positive cases are depicted by gray dots; mutation-negative cases are depicted by black dots.

Anonymized study group

Our second study group consisted of anonymized DNA from 60 fetuses with abnormal prenatal ultrasound findings including increased NT, hydrops fetalis and/or cardiac anomalies, referred to our department for routine chromosomal analysis.

Indications for karyotyping were increased NT in 17/60 fetuses (28.3%), cardiac anomaly in 35/60 (58.3%), hydrops fetalis in 7/60 (11.7%) and in one fetus both a cardiac anomaly

and hydrops fetalis were indications for testing (1.7%). Other characteristic ultrasound findings were cystic hygroma/distended JLS (n = 2; 3.3%), ascites (n = 5; 8.3%), hydrothorax (n = 5; 8.3%), renal anomalies (n = 3; 5.0%) and polyhydramnios (n = 3; 5.0%) (Table 3). All karyotypes were normal.

Table 3 Prenatal findings in 60 anonymous fetuses with a normal karyotype

Prenatal findings	Total group		Mutation-positive group		Mutation-negative group		
	(n = 60)	(%)	(n = 5)	(%)	(n = 55)	(%)	P-value[a]
Indication DNA research							
Increased NT (> 95th centile)	17/60	(28.3)	2/5	(40.0)	15/55	(27.3)	0.616
Hydrops fetalis	8/60	(13.3)	1/5	(20.0)	7/55	(12.7)	0.524
Cadiac anomaly	36/60	(60.0)	2/5	(40.0)	34/55	(61.8)	0.380
Other findings							
Cystic hygroma/distended JLS	2/60	(3.3)	1/5	(20.0)	1/55	(1.8)	0.161
Ascites	5/60	(8.3)	0/5	(0)	5/55	(9.1)	1.000
Hydrothorax	5/60	(8.3)	1/5	(20.0)	4/55	(7.3)	0.363
Renal anomaly	3/60	(5.0)	0/5	(0)	3/55	(5.5)	1.000
Polyhydramnion	3/60	(5.0)	0/5	(0)	3/55	(5.5)	1.000

DNA, deoxyribonucleic acid; JLS, jugular lymph sacs; NT, nuchal translucency
[a]*Fisher's Exact test*

In five fetuses (8.3%) a mutation in one of the 10 NS genes was found. Besides the previously described pathogenic mutation c.854T>C (p.(Phe285Ser)) in *PTPN11* (Tartaglia et al., 2002), four unclassified variants were identified: *PTPN11* (c.430C>T, p.(Pro144Ser)), *RAF1* (c.935T>G, p.(Val312Gly)), *BRAF* (c.1150A>G, p.(Arg384Gly)) and *MAP2K1* (c.1039G>A, p.(Ala347Thr)). Although these variants have not been described before and de novo occurrence could not be tested, we hypothesize that these mutations are likely to be causative as the in-silico analysis predicts a deleterious effect. Although the frequency of these variants has not been tested in healthy controls, the absence of these mutations in postnatally tested cases in our laboratory (B100 for *RAF1*, B150 for *BRAF* and *MAP2K1*, and B1700 for *PTPN11*), and in public databases (NHLBI GO Exome Sequencing project, and dbSNP XML build 135), supports the fact that these changes are not frequently identified polymorphisms. However, in the absence of parental DNA, further evidence on the pathogenicity of these unclassified variants can only be generated by biochemical and/or functional characterization.

To provide further evidence for the involvement of *BRAF* and *MAP2K1* mutations (that were not tested in the diagnostic study group), we anonymized 27 DNA samples (all samples with sufficient DNA left) from that group and sequenced both genes. One

sample contained a mutation in the *MAP2K1* gene (c.383G>A, p.(Gly128Asp)). Because of the anonimization of the samples, no clinical features of the fetus can be described, and the parents could not be tested. The exact mutation has not been described in literature, but is regarded to be pathogenic as a mutation of the same amino acid (p.Gly128Val)) has been reported in a patient with CFC syndrome (Schulz et al., 2008).

No significant differences in clinical characteristics were found between the mutation-positive and negative fetuses in this study group.

DISCUSSION

Analysis of the diagnostic study group consisting of 75 fetuses with a normal karyotype and one or more abnormal ultrasound findings suggestive for NS syndrome revealed a de novo mutation in 13 fetuses in one of the four tested genes known to be related to NS (*PTPN11, KRAS, SOS1* and *RAF1*). This corresponds to a positive test rate of 17.3%. As part of this cohort (19 fetuses sent in by one of the Dutch medical centers) has previously been described by Houweling et al. (2010), we also have calculated the mutation frequency in the undescribed cases. In the additional 56 fetuses, 10 mutations have been identified, which corresponds to a mutation frequency of 17.9%. This slightly higher percentage is probably explained by the fact that in the first 19 cases only two genes have been tested. As no pathogenic mutations have been detected in the *SOS1* gene, analysis of three genes (*PTPN11, KRAS* and *RAF1*) would have revealed the same mutation frequency. The fact that no *SOS1* mutations have been detected, although *SOS1* mutations underlie twice as much NS as do *RAF1* mutations, is likely explained by the fact that the milder *SOS1*-related NS may be difficult to detect in utero with ultrasound. Lee et al. (Lee et al., 2009) analyzed 134 fetuses with one or more sonographic findings suggestive of NS for *PTPN11* mutations only and found a positive test rate of 9%. In our previous study parallel testing of *PTPN11* and *KRAS* in fetuses with a normal karyotype and an increased NT showed a mutation frequency of 15.8% (Houweling et al., 2010). The lower-positive test rate reported by Lee et al. (2009) might be due to the fact that only *PTPN11* was tested.

In the present study, prenatal testing of *PTPN11, KRAS, SOS1* and *RAF1* was offered to parents carrying a fetus with a normal karyotype and ultrasound abnormalities as seen in NS. The actual number of genes tested depended on the amount of DNA available for testing. The prenatal findings of the mutation-positive fetuses found in our diagnostic study group were increased NT, distended JLS, hydro- thorax, renal anomalies, cardiac anomalies, cystic hygroma, hydrops fetalis, polyhydramnios and ascites. An increased

NT, distended JLS, hydrothorax, renal anomalies and polyhydramnios were significant more common in mutation-positive fetuses. However, features in the mutation-negative fetuses that are not specifically mentioned were considered not to be present. Bakker et al (Bakker et al., 2011) reviewed the literature on prenatal findings in NS and mentioned increased NT/cystic hygroma (35.7%), distended JLS (16.7%), hydrothorax (40.5%), renal anomalies (23.8%), cardiac anomalies (38.1%), scalp/skin edema (33%), polyhydramnios (50%) and ascites (14.3%). Baldassarre et al. found most frequently polyhydramnios (38.3%) and increased NT (41%) (Baldassarre et al., 2011). The two most commonly reported indications for prenatal testing reported by Lee et al were increased NT (44%) and cystic hygroma (48%) (Lee et al., 2009). In our study mutation-positive fetuses showed an increased NT and at least one of the following additional features: polyhydramnios, hydrops fetalis, renal anomalies, distended JLS, hydrothorax, cardiac anomalies, cystic hygroma or ascites. Therefore, we recommend these characteristics as important indications for prenatal NS testing. Future studies should reveal whether testing of the NS genes in fetuses with isolated NT is worthwhile.

In the second study group of 60 anonymized fetuses with abnormal prenatal ultrasound findings, we detected a potential-positive test rate of 8.3%. No significant differences were found between mutation- positive and negative fetuses regarding prenatal findings. These two observations are different from the results in the first study group. However, a more detailed analysis of the difference in occurrence of features between the two cohorts is not possible, because the inclusion criteria in the two study groups are not the same. Furthermore, because of the anonymous nature of the second group the description of sonographic abnormalities on the request form might not be complete. The mutation rate could actually by higher than 8.3%, as false negative results cannot be ruled out. Both preferential amplification and allele dropout are known problems accompanying whole genome amplification (Zheng et al., 2011), as all mutations have been verified on the original (unamplified) DNA, false-positive cases have been excluded. The lower-positive test rate can also be explained by the fact that a cardiac anomaly was the most common (60%) indication for testing in this study group. Two out of five fetuses with a mutation had a cardiac anomaly (40%). The findings of both the diagnostic and the anonymized study group support the fact that cardiac anomalies alone will not differentiate between the presence or absence of a mutation in one of the NS genes and should thus not be the main indication for further investigation. Additionally, it is notable that cardiac anomalies are less common in our positive fetuses (38.5% in the diagnostic study group and 40.0% in the anonymized study group) compared with the general NS population (66–87%) (Allanson, 1987; Marino et al., 1999; Bertola et al., 2000; Croonen et al., 2008). Also Menashe et al (Menashe et al., 2002) noted that heart malformations were evident prenatally only in a small group of NS patients (27%), which

could be explained by the fact that the most common heart malformations, pulmonary stenosis and hypertrophic cardiomyopathy, develop during pregnancy or early childhood. This observation suggests that the cardiac anomaly in NS is a cryptic condition in early gestation and has an evolving phenotype in utero and in postnatal life (Achiron et al., 2000).

Interestingly, we only know of an increased NT in two of the mutation-positive fetuses from the anonymized study group. The anonymous nature of this study group did not allow us to discriminate between the possibilities that in the other three mutation-positive fetuses from this group an increased NT was not present but not mentioned, or was not present at all. Therefore, it cannot be ruled out that mutations in the NS genes are found in fetuses with a normal NT.

Although we are aware of the fact that prenatal testing for NS and the possibility to terminate a pregnancy is not common in all countries, based on our findings in the positive fetuses from the first cohort and current literature, we would recommend prenatal testing of NS genes when an increased NT is seen in combination with at least one of the following additional features: polyhydramnios, hydrops fetalis, renal anomalies, distended JLS, hydrothorax, cardiac anomalies, cystic hygroma or ascites. When chromosomal analysis has revealed a normal karyotype, parents should be referred to a clinical geneticist for counseling and an ultrasound examination at 16 weeks. If thereafter genetic testing for NS is indicated, it is advised to collect parental blood simultaneously with the fetal sample, as in case of the identification of a mutation in the fetus, the parental DNA can immediately be tested for de novo occurrence. This saves precious time and limits the uncertainty about pathogenicity of the mutation. The recommended genes for testing are *PTPN11, RAF1* and *KRAS*. If sufficient time and DNA is left, mutation analysis of *BRAF* and *MAP2K1* should be considered. For optimal decision-making by the parents, the results of the tests performed should be disclosed when the option of terminating the pregnancy (in the Netherlands at 24 weeks of gestation) is still open. We recently adapted our DNA diagnostic protocol according to the criteria described in this paper. Although parallel sequencing of these five genes is possible within two weeks on B2 mg of fetal DNA, there is a great need for other detection methods involving less DNA, more tests and a shorter turnaround time, to allow the analysis of more genes and thus to further increase the mutation detection rate. Furthermore, future clinical studies of mutation-negative cases, both prenatally and postnatally, are necessary to better define the inclusion criteria for NS testing.

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Chapter 6

Prenatal ultrasound findings of RASopathies in a cohort of 424 fetuses: update on genetic testing in the NGS era

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J Med Genet. 2019 Oct;56(10):654-661

ABSTRACT

Background this study evaluates 6 years of prenatal rasopathy testing in the Netherlands, updates on previous data and gives recommendations for prenatal rasopathy testing.

Methods 424 fetal samples, sent in for prenatal rasopathy testing in 2011–2016, were collected. Cohort 1 included 231 samples that were sequenced for 1–5 rasopathy genes. Cohort 2 included 193 samples that were analysed with a 14-gene next generation sequencing (NGS) panel. For all mutation-positive samples in both cohorts, the referring physician provided detailed ultrasound findings and postnatal follow-up. For 168 mutation-negative samples in cohort 2, solely clinical information on the requisition form was collected.

Results in total, 40 (likely) pathogenic variants were detected (9.4%). All fetuses showed a variable degree of involvement of prenatal findings: increased nuchal translucency (NT)/cystic hygroma, distended jugular lymph sacs (JLS), hydrops fetalis, polyhydramnios, pleural effusion, ascites, cardiac defects and renal anomalies. An increased NT was the most common finding. Eight fetuses showed solely an increased NT/cystic hygroma, which were all larger than 5.5 mm. Ascites and renal anomalies appeared to be poor predictors of pathogenic outcome.

Conclusion Fetuses with a rasopathy show in general multiple ultrasound findings. The larger the NT and the longer it persists, the more likely it is to find a pathogenic variant. Rasopathy testing is recommended when the fetus shows an isolated increased NT ≥5.0 mm or when NT of ≥3.5 mm and at least one of the following ultrasound anomalies is present: distended JLS, hydrops fetalis, polyhydramnios, pleural effusion, ascites, cardiac defects and renal anomalies.

INTRODUCTION

Noonan syndrome (MIM: 163950) is characterised by postnatal short stature, distinctive facial features, congenital heart defects, variable degree of developmental delay and other structural abnormalities (Noonan, 1968). The incidence is 1 in 1000–2500 live births (Nora et al., 1974; Allanson, 1987; Sharland et al., 1992). Noonan syndrome and phenotypically overlapping syndromes such as Costello syndrome (MIM: 218040) and cardiofaciocutaneous syndrome (MIM: 115150) are part of the so-called rasopathies and are caused by at least 16 genes (*BRAF* (MIM: 164757), *CBL* (MIM: 165360), *HRAS* (MIM: 190020), *KRAS* (MIM: 190070), *LZTR1* (MIM: 600574), *MAP2K1* (MIM: 176872), *MAP2K2* (MIM: 601263), *NRAS* (MIM: 164790), *PPP1CB* (MIM: 600590), *PTPN11* (MIM: 176876), *RAF1* (MIM: 164760), *RIT1* (MIM: 609591), *SHOC2* (MIM: 602775), *SOS1* (MIM: 182530), *SOS2* (MIM: 601247) and *SPRED1* (MIM: 609291)), the *PTPN11* gene being the most prevalent gene (Tartaglia et al., 2001; Tartaglia et al., 2002). Prenatal features of rasopathies have long been documented and can include increased nuchal translucency (NT) and/or cystic hygroma, distended jugular lymph sacs (JLS), hydrops fetalis, polyhydramnios, pleural effusion, ascites, cardiac defects and renal anomalies (Witt et al., 1987; Benacerraf et al., 1989; Nisbet et al., 1999; Baldassarre et al., 2011; Croonen et al., 2013; Bakker et al., 2014). It has been previously estimated that mutations in the rasopathy genes are found in 6.7%–19% of fetuses with increased NT and additional anomalies on ultrasound (Pergament et al., 2011; Croonen et al., 2013). To date, only five studies have systematically evaluated the prenatal phenotype of Noonan syndrome (Achiron et al., 2000; Baldassarre et al., 2011; Croonen et al., 2013; Hakami et al., 2016; Ali et al., 2017).

Croonen et al (Croonen et al., 2013)performed a detailed study of prenatal diagnostic Noonan syndrome testing in fetuses with abnormal ultrasound findings (including enlarged NT above the 95th percentile, cystic hygroma, distended JLS, ascites, hydrops fetalis, pleural effusion, polyhydramnios, congenital heart disease and renal abnormalities) and showed a 17.3% mutation detection rate. The aim of Croonen's study was to provide a protocol for prenatal Noonan syndrome testing (Croonen et al., 2013). However, only 1–4 genes (*KRAS, PTPN11, RAF1,* and *SOS1*) were tested in their diagnostic study group (fetus with normal karyotype and specific abnormal ultrasound findings) and 10 genes (*BRAF, HRAS, KRAS, MAP2K1, MAP2K2, NRAS, PTPN11, RAF1, SHOC2* and *SOS1*) in their anonymised study group (fetus with normal karyotype and increased NT, cystic hygroma, fetal hydrops and/ or cardiac anomalies). Currently, a next generation sequencing (NGS) gene panel of 14 genes is used in the Netherlands in order to detect mutations in both prenatal and postnatal cases suspected of a rasopathy. Recently, a new guideline with regard to prenatal testing of a rasopathy in the presence of an in-

creased NT was implemented in the Netherlands (VKGN, 2017); therefore, we considered it of clinical relevance to update on the previously published data.

Additionally, this study focuses on expanding the knowledge of the prenatal phenotype of a rasopathy. We performed a retrospective study in a consecutive series of 193 fetuses at time of diagnostic testing suspected of a rasopathy and another consecutive series of 231 fetuses in which a prenatal NGS panel of 14 genes involved in rasopathies was performed.

METHODS

Patients

Included in this study is a consecutive series of 424 prenatally sampled fetuses with one or more of the following ultrasound findings: increased NT/cystic hygroma (defined as NT ≥3.5 mm), distended JLS, pleural effusion, ascites, polyhydramnios, cardiac defects and/or renal anomalies and a normal chromosomal microarray result. Cystic hygroma is considered an outdated term and combined with increased NT in this study (Molina et al., 2006). The samples were received from the Clinical Genetics departments of all University Medical Centers in the Netherlands and analysed in the Genome diagnostic laboratory of the Radboud University Medical Center in Nijmegen between January 2011 and December 2016. Of these 424 samples, 231 were received and analysed between January 2011 and September 2014 (cohort 1) and 193 between October 2014 and December 2016 (cohort 2). Samples were sent in as cultured or uncultured amniotic fluid sample, chorionic villi sample or DNA extracted from amniotic fluid or chorionic villi samples. In a few samples, DNA from the umbilical cord or fetal fibroblasts after termination of pregnancy was sent in.

Referring clinical geneticists of the mutation positive samples were asked to provide additional details on the clinical prenatal phenotype and postnatal phenotype if available. The mutation negative samples from cohort 2 were analysed only by the clinical information provided on the requisition form by the referring doctor.

DNA analysis

In cohort 1 (231 samples), 1–5 most commonly described rasopathy genes (*BRAF, KRAS, MAP2K1, PTPN11* and/or *RAF1*) were parallel sequenced. All detected (likely) pathogenic variants or variants of uncertain significance were subsequently tested in parents if parental DNA was available.

Cohort 2, consisting of 193 fetal samples, was tested using the NGS rasopathy gene panel. The coding sequences and splice sites of *A2ML1* (MIM: 610627), *BRAF, CBL, HRAS, KRAS, MAP2K1, MAP2K2, NRAS, PTPN11, RAF1, RIT1, SHOC2, SOS1* and *SPRED1* were sequenced by ion semiconductor sequencing (Ion AmpliSeqTM Noonan panel, ThermoFisher Scientific) combined with Sanger sequencing. All detected (likely) pathogenic variants or variants of uncertain significance were subsequently confirmed by Sanger sequencing and tested in parents if parental samples were available. Five class variant classification system was used: clearly not pathogenic variant (Class 1), unlikely pathogenic variant (Class 2), variant of uncertain clinical significance (Class 3), likely pathogenic variant (Class 4) and clearly pathogenic variant (Class 5) (Bell, 2007).

Statistical analysis

The data were analysed in SPSS V.22. For statistical analysis, a $\chi 2$ test, independent t-test, Shapiro-Wilk test, Mann-Whitney U test, logistic regression and descriptive statistics were used. A probability value of less than 0.05 was considered significant.

RESULTS

Total

Over the 6-year period (2011–2016), 44 out of 424 fetuses were found to have a (likely) pathogenic variant or variant of unknown significance in one of the 14 rasopathy genes (10.4%). Forty variants had either been described before and shown to be pathogenic (Class 5) or were likely pathogenic (Class 4) (9.4%). Four variants were first considered variants of unknown significance (Class 3), but after segregation analysis, they were considered as likely benign variants (Class 2) as they were parentally inherited and the parent did not show a rasopathy phenotype. These variants were found in the *RAF1, RIT1, SOS1* and *HRAS* genes. Twenty-seven of the 40 (likely) pathogenic variants were found in the *PTPN11* gene (67.5%). Five fetuses showed a (likely) pathogenic *RAF1* gene variant (12.5%). Three pathogenic variants were seen in the *RIT1* gene (7.5%). In the *BRAF, HRAS, MAP2K1, SHOC2* and *SOS1* genes, a (likely) pathogenic variant was found in one fetus each (2.5% each gene) (Figure 1). Thirty-three of 40 (82.5%) (likely) pathogenic variants were de novo, two were inherited from an affected mother (5%) and five showed unknown inheritance, because parents were not tested (12.5%).

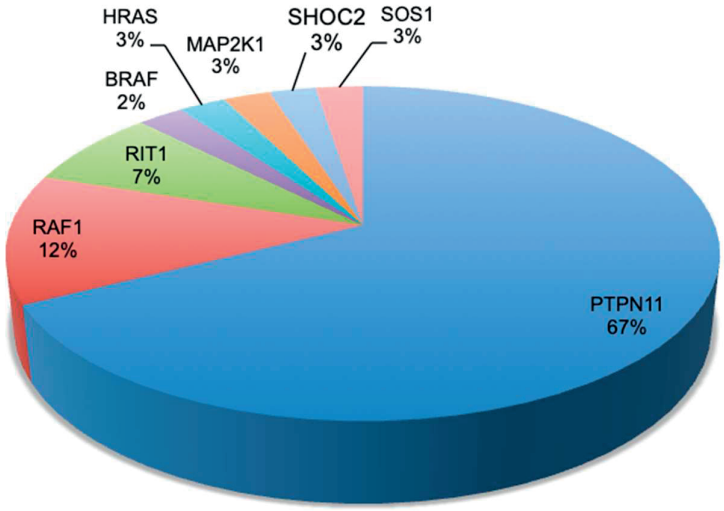

Figure 1 Genes involved in the mutation-positive samples (n=40 (%))

Cohort 1

For cohort 1, in which 1–5 genes were analysed, 15 (likely) pathogenic variants were found in 231 samples (6.5%). Thirteen (likely) pathogenic variants were found in the *PTPN11* gene (5.6%), one pathogenic variant in the *RAF1* gene (0.4%) and one likely pathogenic variant in the *MAP2K1* gene (0.4%) (Table 1).

Table 1 Mutation positive samples

Gene	2011-2016 (n (%))	Cohort 1: 2011-Oct 2014 (1-5 genes) (n (%))	Cohort 2: Oct 2014-2016 (NGS panel) (n (%))
PTPN11	27/424 (6.4)	13/231 (5.6)	14/193 (7.3)
RAF1	5/424 (1.2)	1/231 (0.4)	4/193 (2)
RIT1	3/424 (0.7)	N/A	3/193 (1.6)
SOS1	1/424 (0.2)	N/A	1/193 (0.5)
HRAS	1/424 (0.2)	N/A	1/193 (0.5)
MAP2K1	1/424 (0.2)	1/231 (0.4)	0/193 (0)
BRAF	1/424 (0.2)	0/231 (0)	1/193 (0.5)
SHOC2	1/424 (0.2)	N/A	1/193 (0.5)
KRAS	0/424 (0)	0/231 (0)	0/193 (0)
All other genes in NGS panel	0/424 (0)	N/A	0/193 (0)
Total mutation positive	40/424 (9.4%)	15/231 (6.5%)	25/193 (13%)

Cohort 2

For cohort 2, in which a NGS panel of 14 genes was analysed, 25 (likely) pathogenic variants were found in 193 samples (13%). Of the 25 (likely) pathogenic variants, 14 were found in the *PTPN11* gene (7.3%), four were found in the *RAF1* gene (2%) and three were found in the *RIT1* gene (1.5%). One (likely) pathogenic variant was present in four separate samples in the *SOS1, HRAS, BRAF* and *SHOC2* genes (each gene 0.5%), respectively (Table 1).

Two of the 14 pathogenic variants in the *PTPN11* gene were maternally inherited. Both mothers were suspected of having Noonan syndrome when the fetal DNA sample was sent in for rasopathy testing.

Distribution of NT between negative and positive samples in cohort 2

The distributions of NT in cohort 2 were evaluated comparing the mutation-negative and mutation-positive samples (Table 2, Figures 2 and 3). Tests for normality on samples with measured NT (n=110) showed that mutation-positive samples were normally distributed (mean of 8.46 mm with ±3.7 SD), while mutation-negative samples were non-normal (median of 4.7 mm with ±2.0 IQR) where ~50% of mutation-negative samples had an NT <5 mm. As a result, NT thickness in mutation-positive samples was significantly larger compared with mutation-negative samples ($p<0.001$, $Z=3.858$).

Table 2 Distribution of NT in cohort 2 (mutation-negative versus mutation-positive samples)

NT (mm)	Mutation negative samples (n=168) (%)	Mutation positive samples (n=25) (%)
<3.5	3 (2)	1 (4)
3.5-3.9	25 (15)	2 (8)
4.0-4.9	22 (13)	0 (0)
5.0-5.9	21 (12.5)	1 (4)
6.0-6.9	6 (4)	3 (12)
7.0-7.9	4 (2)	1 (4)
>8.0	10 (6)	11 (44)
Increased, but unknown	21 (12.5)	4 (16)
Nuchal fold at 20 weeks of GA	34 (20)	1 (4)
Not measured	22 (13)	1 (4)

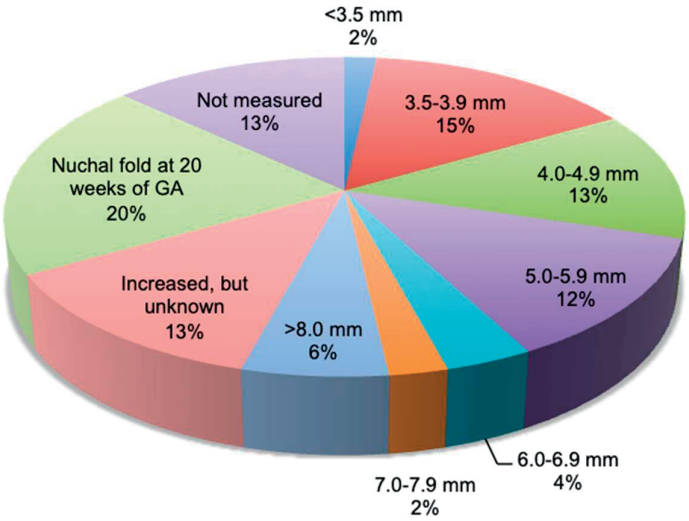

Figure 2 Distribution of nuchal translucency in cohort 2: mutation-negative samples (n=168 (%))

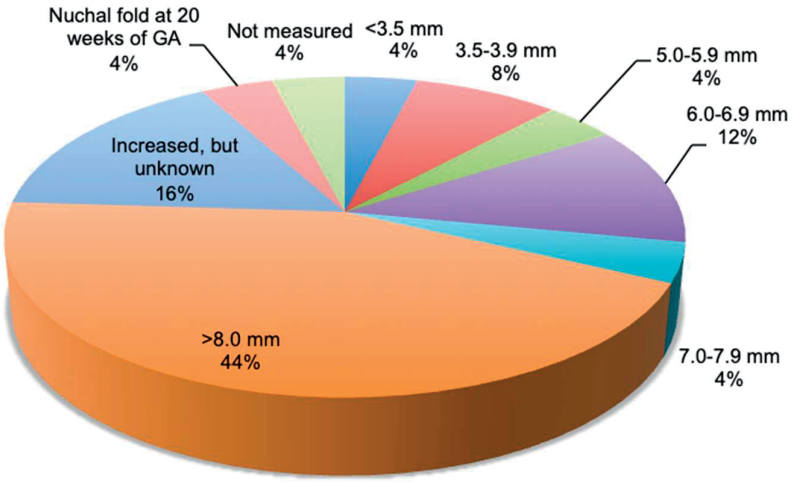

Figure 3 Distribution of nuchal translucency in cohort 2: mutation-positive samples (n=25 (%))

Clinical findings of all samples in cohort 2

The ultrasound findings of all samples in cohort 2 were analysed according to mutation-positive and mutation-negative samples (Table 3). Increased NT/cystic hygroma in the first trimester was the most prevalent finding in all samples (86% of all samples). Although there is a statistically significant difference in the thickness of the NT between the mutation-positive and mutation-negative group, there was no statistic difference in the presence of an increased NT/cystic hygroma between mutation-positive and mutation-negative samples. The ultrasound finding ascites was not statistically more

prevalent in the mutation-positive samples. All other ultrasound findings (JLS, hydrops fetalis, pleural effusion, cardiac anomalies, renal abnormalities and polyhydramnios) were statistically more prevalent in the mutation-positive samples (Table 3).

Table 3 Cohort 2: Prenatal ultrasound findings in fetuses with normal chromosomal microarray and analyzed for rasopathy using Next Generation Sequencing panel

Prenatal findings	Total (n (%))	Mutation positive (n (%))	Mutation negative (n (%))	p-value
Increased NT/cystic hygroma in first trimester*	166/193 (86)	23/25 (92)	143/168 (85)	0.282
Persistent NT	61/193 (32)	16/25 (64)	45/168 (27)	0.000
JLS	24/193 (12)	12/25 (43)	12/168 (7)	0.000
Hydrops fetalis	26/193 (13)	14/25 (56)	12/168 (7)	0.000
Pleural effusion	32/193 (17)	11/25 (44)	21/168 (13)	0.000
Ascites	8/193 (4)	3/25 (12)	5/168 (3)	0.069
Cardiac anomalies	34/193 (18)	12/25 (48)	22/168 (13)	0.000
Renal anomalies	10/193 (5)	4/25 (16)	6/168 (4)	0.027
Polyhydramnios	7/193 (4)	4/25 (16)	3/168 (2)	0.006
Other anomalies	32/193 (17)	9/25 (36)	23/168 (14)	0.009

NT, nuchal translucency; JLS, jugular lymph sacs

Clinical findings in mutation-positive fetuses in cohorts 1 and 2

The 40 pathogenic mutation positive fetuses showed a variable degree of involvement of prenatal findings. Increased NT/cystic hygroma (including persistent NT (defined as NT still visible on ultrasound at 14 weeks of gestation)) was the sole finding in eight fetuses (20.0%). The NTs were between 5.5 and 13 mm for seven samples, whereas one showed edema around the fetus without exact measurement. Five of these fetuses were terminated at 16 and 22 weeks of gestation, respectively, without autopsy, therefore eliminating follow-up, and one resulted in a premature delivery at 17 weeks of gestation. Two fetuses were life born. In this isolated increased NT group, seven fetuses harboured a pathogenic *PTPN11* variant and one a pathogenic *RIT1* variant. Nine fetuses were shown to have an increased NT/cystic hygroma with one other finding (22.5%): three had JLS, two had hydrops, one had polyhydramnios, one had a cardiac defect, one showed bilateral hydrothorax and one had unilateral clubfoot. Seven fetuses harboured a pathogenic PTPN11 variant and two a pathogenic RAF1 variant. The remaining 23 fetuses had at least two or more ultrasound findings (23 out of 40 fetuses, 57.5%) (Table 4). Although ductus venosus anomalies have been reported as being associated with Noonan syndrome, we found evidence in only one of the mutation-positive fetuses in our study (Staboulidou et al., 2011; Demirci et al., 2015; Vigneswaran et al., 2018). This fetus, however, also showed 6 of the specific rasopathy ultrasound features.

All 40 mutation-positive samples were analysed for clinical outcome (Table 4). Twenty-eight pregnancies were terminated before 24 weeks of gestation (70%). Two fetuses were born prematurely and died shortly after birth (at 17 and 27 weeks of gestation, respectively)(5%), two pregnancies ended in intrauterine fetal death (5%) and eight pregnancies ended in a term birth (20%). Of these births, one child died during birth, one shortly after birth and one child a few years after birth. Five children with prenatally confirmed Noonan syndrome are still alive (12.5%). There were no significant differences in clinical findings in mutation positive samples cohort 1 versus cohort 2. It was expected that cohort 2 included fetuses with more ultrasound findings due to proposed new criteria by Croonen et al. (Croonen et al., 2013). However, not all genetic centres in the Netherlands used these new criteria. Additionally, we did not find a correlation between genotype and phenotype. There were a few recurrent mutations and within those mutations a shared phenotype did not appear to be present. Clinical information about the non-pathogenic variants is provided in the Supplementary Table S1.

Table 4 Mutation positive samples (cohort 1 and 2) and the detected ultrasound findings.

Case	Gene	Genomic change	Protein change	NGS panel	De novo	Pathogenicity	NT (mm)	Persistent NT	JLS	Hydrops fetalis	Pleural effusion	Ascites	Cardiac anomalies	Renal anomalies	Polyhydramnios	Other anomalies	Pregnancy outcome
1	PTPN11	c.124A>G	Thr42Ala	+	?	P	edema around fetus at 10 weeks	+	-	-	-	-	-	-	-	-	Live birth, deceased at age 2
2	PTPN11	c.179G>T	Gly60Val	+	+	P	8.6	+	+	+	+	-	pericardial effusion, DORV	-	-	lemon shaped head	ToP
3	PTPN11	c.179G>T	Gly60Val	+	+	P	8.9	+	+	-	-	-	AVSD	-	-	abnormal skull	ToP at 21 weeks
4	PTPN11	c.182A>G	Asp61Gly	+	?	P	edema around fetus at 11 weeks	+	+	+	-	-	AVSD, hypoplastic left heart	pyelectasis	-	-	ToP
5	PTPN11	c.184T>G	Tyr62Asp	-	+	P	6.1	-	-	-	-	-	-	-	mild 34 weeks	-	Live birth
6	PTPN11	c.184T>G	Tyr62Asp	-	+	P	11.0	+	-	-	-	-	-	-	-	-	ToP
7	PTPN11	c.184T>G	Tyr62Asp	+	+	P	9.8	-	-	+	+	-	-	-	-	-	ToP at 16 weeks
8	PTPN11	c.213T>G	Phe71Leu	+	+	LP	increased	+	-	+	-	-	-	-	-	-	ToP at 14 weeks
9	PTPN11	c.214G>T	Ala72Ser	-	+	P	9.3	+	+	-	-	-	hypoplastic right heart, small triscuspid valve	-	-	-	ToP at 23 weeks
10	PTPN11	c.214G>C	Ala72Pro	+	+	LP	8.1	+	-	+	+	+	-	-	-	-	ToP
11	PTPN11	c.215C>T	Ala72Val	-	+	LP	7.0	-	-	-	-	-	-	-	-	-	ToP at 16 weeks
12	PTPN11	c.228G>T	Glu76Asp	-	- mat	P	5.7	-	-	-	-	-	AVSD, hypoplastic left heart	-	-	-	ToP at 21 weeks
13	PTPN11	c.417G>C	Glu139Asp	+	+	P	6.0	+	-	+	+	-	-	-	-	-	ToP at 17 weeks
14	PTPN11	c.767A>G	Gln256Arg	+	?	P	3.8	-	+	-	-	-	-	-	-	-	Live birth
15	PTPN11	c.794G>A	Arg265Gln	-	+	P	6.0	-	-	-	-	-	-	-	-	-	Live birth
16	PTPN11	c.854T>C	Phe285Ser	-	+	P	8.7	+	+	+	+	+	pericardial effusion	mild pyelectasis	-	lemon shaped head	ToP at 23 weeks
17	PTPN11	c.854T>C	Phe285Ser	-	+	P	7.7	+	+	-	+	very mild	-	-	+ 27 weeks	low set ears	Live birth at 27 weeks, postnatal death
18	PTPN11	c.854T>C	Phe285Ser	+	+	P	increased	+	+	-	-	-	PS	-	+	-	ToP
19	PTPN11	c.922A>G	Asn308Asp	-	+	P	5.5	-	-	-	-	-	-	-	-	-	ToP at 22 weeks

Table 4 Mutation positive samples (cohort 1 and 2) and the detected ultrasound findings. (*continued*)

Case	Gene	Genomic change	Protein change	NGS panel	De novo	Pathogenicity	NT (mm)	Persistent NT	JLS	Hydrops fetalis	Pleural effusion	Ascites	Cardiac anomalies	Renal anomalies	Polyhydramnios	Other anomalies	Pregnancy outcome
20	PTPN11	c.922A>G	Asn308Asp	+	+	P	13.0	+	-	-	-	-	-	-	-	-	ToP >20 weeks
21	PTPN11	c.922A>G	Asn308Asp	+	+	P	9.6	+	-	-	-	-	-	-	-	-	Premature delivery at 17 weeks
22	PTPN11	c.1381G>A	Ala461Thr	-	+	P	9.2	-	+	+	+	+	fibrous VSD	horse shoe kidney	-	malrotation bowel, ambiguous genitalia	IUFD at 17 weeks
23	PTPN11	c.1403C>T	Thr468Met	-	+	P	>3.5	-	+	-	-	-	-	-	-	-	ToP at 22 weeks
24	PTPN11	c.1403C>T	Thr468Met	+	- mat	P	8.0	-	+	-	-	-	-	-	-	-	Live birth
25	PTPN11	c.1504T>G	Ser502Ala	+	?	P	13.7	+	+	+	-	-	AVSD	pyelectasis	-	absent ductus venosus, dysmorphic facial features	ToP
26	PTPN11	c.1507G>C	Gly503Arg	+	+	P	3.6	-	-	-	+ bilat	-	-	-	-	-	ToP at 24 weeks
27	PTPN11	c.1530G>C	Gln510His	+	+	P	12.0	+	+	+	+	+	cardiomyopathy, dysplastic mitral valve	pyelectasis	+	brachycephaly, hepatomegaly, hypertelorism, large tongue, non-rotation bowel	IUFD at 31 weeks
28	RAF1	c.770C>T	Ser257Leu	+	+	P	8.2	n/a	-	+	-	-	-	-	-	-	ToP at 12 weeks
29	RAF1	c.770C>T	Ser257Leu	+	+	P	5.5	+	+	-	-	-	VSD	-	-	SUA	ToP at 20 weeks
30	RAF1	c.770C>T	Ser257Leu	+	+	P	6.9	-	-	+	+	-	-	-	-	-	ToP at 19 weeks
31	RAF1	c.778A>C	Thr260Pro	+	+	LP	6.5	+	-	+	+	-	hypertrophic myocardium, PS	-	+	echogenic bowel	Live birth, postnatal death
32	RAF1	c.782C>G	Pro261Arg	+	+	P	8.0	+	-	-	-	-	-	-	-	unilateral clubfoot	ToP at 16 weeks
33	RIT1	c.319A>G	Met107Val	+	+	P	nuchal fold at 19 weeks	?	+	+	+	+	-	pyelectasis	+	-	Died during child birth
34	RIT1	c.319A>G	Met107Val	+	?	P	7.3	+	-	-	-	-	-	-	-	-	ToP
35	RIT1	c.319A>G	Met107Val	+	+	P	18.0	-	-	+	+	+	hypoplastic right ventricle, tricuspid atresia, pulmonary atresia, VSD	-	-	low set ears	ToP at 14 weeks

Table 4 Mutation positive samples (cohort 1 and 2) and the detected ultrasound findings. (*continued*)

Case	Gene	Genomic change	Protein change	NGS panel	De novo	Pathogenicity	NT (mm)	Persistent NT	JLS	Hydrops fetalis	Pleural effusion	Ascites	Cardiac anomalies	Renal anomalies	Polyhydramnios	Other anomalies	Pregnancy outcome
36	MAP2K1	c.383G>A	Gly128Asp	-	+	LP	increased	+	-	+	+	-	-	-	-	-	ToP at 18 weeks
37	SHOC2	c.4A>G	Ser2Gly	+	+	P	nuchal fold at 21 weeks	+	-	+	+	-	cardiomegaly, RV hypertrophy	-	+	ventriculomegaly	live birth at 37 weeks
38	BRAF	c.1391G>T	Gly464Val	+	+	LP	11.8	n/a	+	-	-	-	ToF, abnormal ductus venosus, abnormal a. hepatica	-	-	-	ToP at 13 weeks
39	SOS1	c.806T>G	Met269Arg	+	+	P	edema around fetus	-	+	-	-	-	-	-	-	macrocephaly, SUA	ToP at 18 weeks
40	HRAS	c.38G>A	Gly13Asp	+	+	P	3.4	+	+	+	+	-	VSD	hydronephrosis	+	-	ToP at 24 weeks

NT, nuchal translucency; JLS, jugular lymph sacs; P, pathogenic; LP, likely pathogenic; mat, maternal; ToP, termination of pregnancy; IUFD, intrauterine fetal death; DORV, double outlet right ventricle; AVSD, atrioventricular septum defect; PS, pulmonary stenosis; VSD, ventricular septum defect; RV, right ventricle; SUA, single umbilical artery; bilat, bilateral; n/a, not applicable

DISCUSSION

Recently, a new Dutch guideline was implemented with regard to prenatal testing for a rasopathy. This guideline showed that only a handful of studies were informative for rasopathy testing and it therefore was the motivation for this study. We present the largest cohort of prenatally tested samples for rasopathies. In a previous paper by Croonen et al, testing for a rasopathy in pregnancy was recommended when an enlarged NT is present and at least one of the following features: distended JLS, cystic hygroma, hydrops fetalis, hydrothorax, cardiac anomalies, renal anomalies, polyhydramnios and ascites (Croonen et al., 2013). In this paper, we provide updated recommendations for testing for a rasopathy based on our clinical and genetic findings in a cohort of 424 fetuses.

Mutation detection

In our cohort of 424 fetuses, an overall pathogenic mutation detection rate of 9.4% was found, with a 6.5% mutation detection rate in cohort 1 (testing of 1–5 genes) and 13% in cohort 2 (NGS panel cohort). Croonen et al. found a 17.3% mutation rate in their diagnostic group tested for Noonan syndrome (Croonen et al., 2013). It is unclear what causes the difference in mutation detection rate. The inclusion criteria might play a role, because no isolated enlarged NT was included in their study. However, Croonen et al. included cystic hygroma as a separate entity, whereas in our study, these anomalies were combined. If all 58 isolated increased NT samples with NT <5.0 mm had been excluded in our cohort 2, mutation detection in this cohort would have increased to 18.5% (25/135). Additionally, in cohort 1, 1–5 genes were tested, whereas in cohort 2, 14 genes were tested. This latter presumably leads to a higher mutation detection rate. If in cohort 2 only the five genes from cohort 1 were tested, the percentage of mutation detection in cohort 2 would have been 9.8% compared with 13% with NGS panel. However, not every sample in cohort 1 was tested for five genes. Therefore, the mutation detection percentage in cohort 1 might be an under-representation compared with the same genes tested in cohort 2. The difference in mutation detection between our two cohorts seems to be due to the increased amount of genes tested.

In addition to the Croonen et al. paper, Ali et al. (2017) recently published a cohort of 39 fetuses with enlarged NT and normal karyotype which underwent testing for Noonan syndrome. The authors used two laboratories with a selected panel of 11 and 9 genes, respectively. They found a mutation percentage of approximately 10%. This is approximately the same percentage as we found in our much larger total cohort. However, in our cohort 2 we found a slightly higher mutation detection of 13%. This might be due to the fact that the *RIT1* gene was not present in one of the panels used by Ali et al. (2017). The *RIT1* gene is an important gene in our cohort, with three of the 25 mutations

(12%) found in cohort 2 being in this gene. Additionally, microdeletions/duplications were not tested by Ali et al, which have been filtered out in our cohorts. More than 80% of our mutation-positive samples were *de novo*. In literature, the de novo rate has been estimated at 30%–75% (Tartaglia et al., 2002; Zenker et al., 2004; Jongmans et al., 2005). Our cases were prenatally detected and the higher percentage could be explained by the fact that milder cases might not show a prenatal phenotype and therefore are not tested in pregnancy. As well, hydropic fetuses have not always been tested for a rasopathy, which would then be missed out on *de novo* mutation detection.

Genes

In cohorts 1 and 2 combined only eight genes were involved in prenatally confirmed Noonan syndrome. Although no pathogenic variants were found in the *A2ML1, CBL, MAP2K1, NRAS, SPRED1* and *KRAS* genes in our total cohort, in literature a prenatal phenotype has been described for all these genes, except for the *SPRED1* gene. For the *A2ML1* gene, however, only one report has been published and it is debatable whether this gene is involved in the rasopathies (Croonen et al., 2013; Wong Ramsey et al., 2014; Bülow et al., 2015; Vissers et al., 2015; Hakami et al., 2016). Overall, the *PTPN11* gene is prenatally and postnatally the most prevalent gene in the panel; 67.5% of our samples with a rasopathy have a pathogenic *PTPN11* variant, which is in agreement with literature (Tartaglia et al., 2001; Maheshwari et al., 2002; Tartaglia et al., 2002).

In our study, *RAF1* and *RIT1* are the most frequently described genes involved in rasopathies after the *PTPN11* gene. *RIT1* has relatively recently been discovered and it also has an important role in prenatal rasopathies. This is possibly due to the presence of lymphatic malformations in this gene (Kouz et al., 2016), which is easily recognisable on fetal ultrasound. In our study, all three fetuses with a pathogenic *RIT1* mutation showed lymphatic problems. However, the indication of sending in samples for testing was increased NT or associated lymphatic problem; therefore, there is a bias in our cohort regarding this ultrasound feature. Only one of the three fetuses with a pathogenic *RIT1* variant in our cohort showed a cardiac defect. This is not significantly more than the fetuses with other pathogenic variants. Cardiac anomalies are frequently documented in patients with a pathogenic *RIT1* mutation (Aoki et al., 2013; Kouz et al., 2016). However, pulmonic valve stenosis and hypertrophic cardiomyopathy, the common cardiac defects described in Noonan syndrome, are difficult to visualise on fetal ultrasound. Therefore, they are easily overlooked in pregnancy and might not be confined to only fetuses with a *RIT1* mutation. *SOS1* has to our knowledge only been described a handful of times before in the prenatal setting (Hakami et al., 2016), and in our study only one fetus showed a pathogenic variant. This is possibly due to a milder phenotype, which might not be recognisable on prenatal ultrasound.

Clinical differences between the mutation-negative and mutation-positive group

The prenatal features persistent increased NT, JLS, hydrops fetalis, pleural effusion, cardiac anomalies and polyhydramnios are significantly more present in fetuses with confirmed rasopathy versus fetuses without a confirmed diagnosis. In both the mutation-positive and mutation-negative groups, an increased NT was the most frequent finding on ultrasound, and there was no significant difference between the groups (p-value 0.282). This is due to the fact that in most instances the indication for prenatal testing of a rasopathy was an increased NT. Therefore, it is difficult to confirm whether most prenatal rasopathy cases have indeed an enlarged NT. Ascites is not very specific for fetuses with a rasopathy with a p-value of 0.069. Renal anomalies are significantly more present in mutation-positive samples, but it is not a strong predictor (p-value 0.027). Both ascites and renal anomalies are always seen in combination with hydrops fetalis in our mutation-positive and mutation-negative cohort. Additionally, ascites is only detected in a small amount of mutation-positive and mutation-negative fetuses. The most common renal anomaly is pyelectasis. Although pleural effusion is significantly more present in fetuses with a confirmed rasopathy, this feature on its own does not predict a mutation. We did not find mutations in fetus with solely pleural effusion. Pleural effusion is almost always part of hydrops fetalis (Tables 3 and 4).

Clinical findings in the mutation-positive group

In the mutation-positive group, there were eight fetuses (20.0%) with just an isolated NT and only two of these fetuses turned into a term life birth. The other fetuses were terminated before 22 weeks of gestation. It is therefore difficult to determine whether these fetuses would have developed more anomalies in due course of the pregnancy. Twenty-three fetuses showed two or more ultrasound findings additional to the increased NT. Thus, the majority of samples with a confirmed rasopathy (23 of 40 fetuses, 57.5%) have multiple anomalies on ultrasound. We therefore recommend testing for a prenatal rasopathy when the NT is ≥3.5 mm and one of the following ultrasound anomalies is present: JLS, hydrops fetalis, pleural effusion, cardiac anomalies, polyhydramnios. It is debatable whether to test for a rasopathy when an enlarged NT is seen in combination with solely ascites or renal anomalies. The chance of finding a pathogenic variant is low, but not excluded.

A rasopathy cannot be excluded when only one ultrasound anomaly is seen, as our study shows pathogenic variants in eight fetuses with an isolated increased NT (with or without persistent NT enlargement). This has been described before (Hakami et al., 2016). The NTs of the fetuses were, however, large (5.5–13.0 mm). There is a statistical significant difference of the thickness of the NT between the mutation-positive versus

the mutation-negative samples with a cut-off at 5.0 mm. Therefore, we recommend testing for prenatal rasopathy in fetuses with an isolated increased NT of ≥5.0 mm. Additionally, in the presence of other ultrasound anomalies, the NT tends to be larger as well (Supplementary Table S2), which has been described before (Ali et al., 2017). Sixteen of the 40 fetuses (40%) with confirmed rasopathy had a cardiac defect. In literature, postnatally, approximately 80% of patients with established rasopathy show a cardiac anomaly (Calcagni et al., 2017). The difference is most likely due to the fact that the two most common heart defects in a rasopathy, pulmonic valve stenosis and hypertrophic cardiomyopathy, are difficult to detect on prenatal ultrasound. One fetus with a confirmed pathogenic *HRAS* variant had a NT of 3.4 mm. This is technically not an increased NT. However, this fetus showed multiple other ultrasound anomalies, fitting the prenatal phenotype of a rasopathy. Prenatal testing for a rasopathy when an increased NT is absent should be based on the remaining ultrasound anomalies. One can consider testing for rasopathies when multiple anomalies described in this study are present.

A genotype–phenotype correlation could not be established in our cohort. This might be due to the fact that many pregnancies, some at early gestation, were terminated when a mutation was found and therefore a complete prenatal and postnatal phenotype is lacking. The high percentage of pregnancy terminations (80%) in the mutation-positive group can have several reasons. An important reason might be that parents, who, due to cultural or religious beliefs, would not terminate a pregnancy in the first place, might not opt for genetic testing. Therefore, this could cause a bias in the percentage in termination of pregnancy. Another reason might be poor prognosis and parental anxiety either due to the enlarged NT or due to the combination of several ultrasound findings. For this reason, however, one would expect also a high percentage in termination of pregnancy irrespective of the presence of a mutation in a rasopathy gene. Unfortunately, we did not follow-up on the mutation-negative samples in our cohort. All parents were counselled by clinical geneticists, and in our opinion, a clinical geneticist is the expert physician for counselling a prenatal rasopathy. Parents will then be able to make a well-informed decision.

Several studies have documented a range of 21%–50% prenatal phenotype in patients with a postnatally confirmed rasopathy. These studies also showed a lack of an association between prenatal severity and postnatal outcome (Baldassarre et al., 2011; Gaudineau et al., 2013).

Challenges

The requisition forms for cohort 1 were not digitally available. As a result, we did not research the clinical phenotype of the mutation-negative samples in this cohort. In cohort 2, the requisition forms were digitally available and we based the prenatal phenotype on what the referring physician had written down. We trusted the information on the requisition form for the mutation-negative samples to be complete.

Our study did not include newborns with Noonan syndrome or an associated disorder. Additionally, a high percentage of terminations of pregnancy in our cohorts was established, which made postnatal follow-up impossible. Therefore, a complete genotype– phenotype correlation cannot be made. Additional research about the postnatal phenotype combined with the prenatal phenotype needs to be performed. Finally, the upcoming use of non-invasive prenatal testing (NIPT) presumably decreases the request for prenatal rasopathy testing as increased NT is an important ultrasound finding in the prenatal rasopathies, which is not measured anymore when performing NIPT.

Recommendations

This study is the most extensive study to date involving the largest cohort of prenatally tested rasopathy. We conclude that all but one confirmed prenatal cases of rasopathy show an enlarged NT. We recommend testing of fetuses with solely an increased NT after chromosomal abnormalities have been excluded when the NT is ≥5.0 mm. We also recommend testing when the NT is ≥3.5 mm and at least one of the following anomalies is present: distended JLS, hydrops fetalis, polyhydramnios, pleural effusion and cardiac defects. Ascites and to a lesser degree renal anomalies in combination with an increased NT are poor predictors to find a pathogenic variant and testing is then debatable. Research for better correlation between prenatal and postnatal phenotype should be performed.

In general, an NGS panel of known rasopathy genes should be used when a rasopathy is suspected. Although we did not find pathogenic variants in every gene in the panel, in all genes, a prenatal phenotype has been documented in literature. Therefore, a smaller panel is not advisable. However, in countries where an extensive panel is not available, testing for only *PTPN11* gene would catch at least 50% of the fetuses with a rasopathy.

We assume that adding recently discovered rasopathy genes (*PPP1CB, SOS2* and *LZTR1* genes) to the panel would increase the detection rate.

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SUPPLEMENTARY MATERIAL

Table S1 Class 3 variant positive samples in cohort 1 and 2 and their ultrasound findings

Case	Gene	Genomic change	Protein change	NGS panel	De novo	Pathogenicity	NT (mm)	Persistent NT	JLS	Hydrops fetalis	Pleural effusion	Ascites	Cardiac anomalies	Renal anomalies	Polyhydramnios	Other anomalies	Pregnancy outcome
41	RAF1	c.1928C>T	Pro643Leu	-	- pat	VUS	8.1	+	-	-	-	-	-	-	-	-	ToP at 14 weeks
42	RIT1	c.646C>T	Pro216Ser	+	- pat	VUS	not measured	-	-	-	-	-	VSD, dysplastic pulmonary valve, tricuspid valve stenosis, seen at 16 weeks	-	-	-	ToP>16 weeks
43	HRAS	c.488_507del	Ile163fs	+	?	VUS	7.2	n/a	-	-	-	-	-	-	-	-	ToP at 12 weeks
44	SOS1	c.3709C>G	Pro1237Ala	+	- mat	VUS	nuchal fold at 19 weeks	+	-	-	-	-	subaortal VSD	-	-	-	Live birth, healthy

Table S2 Correlation between increased NT and mutation presence

		NT_plain	Mutation_present
NT_plain	Pearson Correlation	1	-.486**
	Sig. (2-tailed)		.000
	N	110	110
Mutation_present	Pearson Correlation	-.486**	1
	Sig. (2-tailed)	.000	
	N	110	110

** Correlation is significant at the 0.01 level (2-tailed).

Chapter 7

Germline mutations in RYR1 are associated with foetal akinesia deformation sequence/ lethal multiple pterygium syndrome

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Acta Neuropathol Commun. 2014 Dec 5;2:148

ABSTRACT

Introduction Foetal akinesia deformation sequence syndrome (FADS) is a genetically heterogeneous disorder characterised by the combination of foetal akinesia and developmental defects which may include pterygia (joint webbing). Traditionally multiple pterygium syndrome (MPS) has been divided into two forms: prenatally lethal (LMPS) and non-lethal Escobar type (EVMPS) types. Interestingly, FADS, LMPS and EVMPS may be allelic e.g. each of these phenotypes may result from mutations in the foetal acetylcholine receptor gamma subunit gene (*CHRNG*). Many cases of FADS and MPS do not have a mutation in a known FADS/MPS gene and we undertook molecular genetic studies to identify novel causes of these phenotypes.

Results After mapping a novel locus for FADS/LMPS to chromosome 19, we identified a homozygous null mutation in the *RYR1* gene in a consanguineous kindred with recurrent LMPS pregnancies. Resequencing of *RYR1* in a cohort of 66 unrelated probands with FADS/LMPS/EVMPS (36 with FADS/LMPS and 30 with EVMPS) revealed two additional homozygous mutations (in frame deletions). The overall frequency of *RYR1* mutations in probands with FADS/LMPS was 8.3%.

Conclusions Our findings report, for the first time, a homozygous *RYR1* null mutation and expand the range of *RYR1*-related phenotypes to include early lethal FADS/LMPS. We suggest that *RYR1* mutation analysis should be performed in cases of severe FADS/LMPS even in the absence of specific histopathological indicators of *RYR1*-related disease.

INTRODUCTION

Foetal akinesia deformation sequence syndrome (FADS) is characterised by a variable combination of foetal akinesia, prenatal growth restriction, developmental defects (including cleft palate, cryptorchidism, cystic hygroma, heart abnormalities, intestinal malrotation and lung hypoplasia), arthrogryposis and, in some cases, limb pterygia, so that there is phenotypic overlap between FADS and severe cases of multiple pterygium syndrome (MPS) (Hall et al., 1982). Clinically MPS can be divided into the severe lethal form (LMPS) and the milder non-lethal Escobar type (EVMPS). MPS is most commonly inherited as an autosomal recessive trait though autosomal dominant and X-linked cases are described (Tolmie et al., 1987; McKeown and Harris, 1988; Prontera et al., 2006). Both MPS and FADS are genetically heterogeneous and although, in some cases, a diagnosis of a specific primary myopathy, metabolic or neurodevelopmental disorder can be made by clinical and pathological investigations, the underlying aetiology is unknown in the majority of cases (Cox et al., 2003). Previously, we and others have reported that germline mutations in genes encoding specific components of the acetylcholine receptor (AChR) complex at the neuromuscular junction may present with autosomal recessively inherited forms of FADS, LMPS and EVMPS (Hoffmann et al., 2006; Morgan et al., 2006). Thus mutations in *CHRNG* (which encodes the foetal gamma subunit of the acetylcholine receptor) have been associated with FADS, LMPS and EVMPS and mutations in genes that encode other subunits that make up the foetal acetylcholine receptor (*CHRNA1*, *CHRND*) or regulators of AChR function (e.g. *RAPSN* and *DOK7*) have been described in FADS/LMPS (Michalk et al., 2008; Vogt et al., 2008; Vogt et al., 2009). Interestingly, mutations in *CHRNA1, CHRND, RAPSN* and *DOK7* can also cause congenital myasthenia syndrome (CMS), a milder disorder that is characterised by muscle fatigability and, rarely, arthrogryposis (Brownlow et al., 2001; Burke et al., 2003; Beeson et al., 2005).

Identification of the underlying genetic cause of FADS/MPS facilitates clinical management by providing (a) precise genetic diagnosis, (b) enabling accurate predictions of recurrence risk and prognosis and (c) allowing the possibility of prenatal diagnosis. However, FADS and MPS are genetically heterogeneous and in many cases mutations in acetylcholine receptor-related genes cannot be identified. In order to characterise potential genetic causes of FADS/ MPS in such cases, we undertook molecular genetic investigations in cohorts of FADS, LMPS and EVMPS families that were enriched for autosomal recessively inherited forms of these disorders (i.e. enriched for parental consanguinity) and identified loss of function *RYR1* mutations as a cause of early lethal FADS/LMPS.

MATERIAL AND METHODS

Patients

66 families with features of FADS/LMPS/EVMPS and no known underlying genetic cause were investigated. In 36 families their clinical phenotype was FADS/LMPS and in 30 the phenotype was EVMPS. Consanguinity was recorded in 48% of the FADS/LMPS families and 20% of the EVMPS families. All families gave informed consent, the study was approved by the South Birmingham Research Ethics Committee and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki (Helsinki, 1964).

Molecular genetic analysis

Linkage analysis

A genome-wide linkage scan was carried out using the Affymetrix 250 K Human SNP Array 5.0 on DNA from stored foetal material of two affected siblings from a consanguineous family affected with FADS/LMPS. This scan excluded linkage to known FADS/LMPS genes and an ~10 Mb prime candidate region on chromosome 19 was identified and further evaluated by typing the parents and DNA from three affected foetuses with micro- satellite markers (details on request and see Figure 1A).

A

Marker	Location on Chr 19	Paternal genotype	Maternal genotype	Foetus 1 genotype	Foetus 2 genotype	Foetus 3 genotype
D19S222	33417730	239-239		239-242	239-242	239-242
D19S433	35108829	199-199	201-203	199-203	199-203	199-203
D19S430	36994291	127-127	127-127	127-127	127-127	127-127
D19S224	41219912	241-255	237-241	241-241	241-241	241-241
Chr19r2-15xAC41	41435658	242-242	242-244	242-242	242-242	242-242
D19S220	43123390	280-285	283-289	280-280	280-280	280-280
D19S228	43181340	203-205	203-205	205-205	205-205	205-205
D19S421	43562946	187-189	187-189	187-187	187-187	187-187
Chr19R2-15xTAT	44221282	164-164	164-175	164-164	164-164	164-164
Chr19r2-22xGT44	44659155	252-252	248-252	252-252	252-252	252-252

B

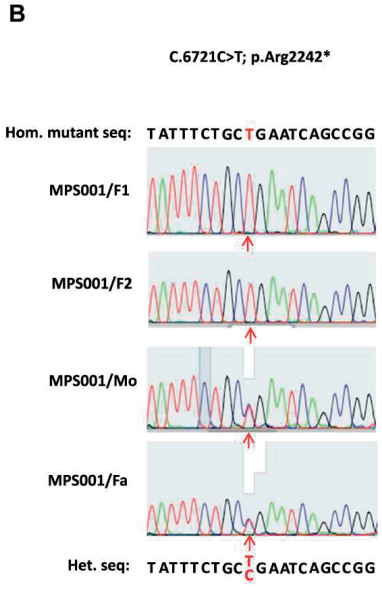

Figure 1 A: Mapping of a consanguineous family (MPS001) with lethal multiple pterygium syndrome to RYR1. The three affected foetuses shared a common homozygous region between 28,725,890 - 44,669,155 on chromosome 19. B: Chromatograms showing nonsense mutation (C.6721C>T; p.Arg2242*) at exon 42 in two affected foetuses (F1 and F2) and (in heterozygous state) in the parents (Mo and Fa).

RYR1 sequencing

RYR1 gene sequencing was performed after amplification of all 106 coding exons. Initially, sequencing was performed on whole genome amplified DNA (Qiagen REPLI-g kits) and candidate variants were then confirmed on stock DNA samples. Flanking primers were designed from genomic sequence 20–80 nucleotides upstream or down- stream from encoding exons. PCR products were sequenced in forward and reverse orientations using standard BigDyeR Terminator v3.1 cycle sequencing. Details of primer sequences are available on request. Sequence traces from each of the DNAs analysed was compared to the reference sequence from the ENSEMBL database (GRCh37: CM000681.1 - NM_000540; transcript ENST00000355481).

The segregation of sequence variants was checked in other family members (when available) by BigDyeR Terminator v3.1 sequencing. Frequency information for *RYR1* variants was sought from the NHLBI Exome variant server [http://evs.gs.washington.edu/EVS/] if avail- able and the prediction of possible effects of any amino acid substitution was achieved with the PolyPhen-2 tool [http://genetics.bwh.harvard.edu/pph2/].

Histopathological analysis

Histopathological analysis was performed on tissue obtained at autopsy from two fetuses of family MPS001 (12+6 and 14+0 weeks GA, respectively) and two age-matched controls (13+0 and 13+4 weeks GA, respectively) retrieved from the autopsy archive of the VU University Medical Center, Amsterdam, The Netherlands. Sixum thick formalin-fixed paraffin-embedded tissue sections were processed according to standard protocols (Bancroft, 2008). Histochemical staining included Hematoxylin & Eosin, Gomori trichrome and alizarin red S for calcium. After heat-induced antigen retrieval in 0.01 M citrate buffer (pH6), immunohistochemical staining was performed with antibodies against desmin (Abcam, 1:500), myosin heavy chain slow (Abcam, 1:100), active caspase 3 (Dako, 1:500), CD3 (Dako, 1:250), CD20 (Dako, 1:50) and CD45 (Dako, 1:100). Immunoreactivity was detected with 3,3′-diaminobenzidine as chromogen. Tissue sections were photographed using a Leica DM6000B microscope (Leica Microsystems). Omitting primary antibodies yielded no significant staining.

Ultrastructural analysis was performed on muscle tissue retrieved from formalin fixed material. The tissue was deparaffinised in xylene (60 minutes at 70°C), rehydrated, fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), post-fixed in 2% osmium tetroxide and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a FEI Technai 12 electron microscope.

The pictures were acquired as TIFF files and images were optimized for brightness and contrast using Photoshop, version 7.0 (Adobe systems, San Jose, CA).

RESULTS

Clinical and molecular genetic analysis in MPS001 family

Genome wide linkage analysis was performed on a consanguineous family from The Netherlands (see Family MPS001 in Table 1) that had had six pregnancies affected by FADS/MPS. The proband (F2) presented was the second pregnancy that was terminated at 12 + 6 weeks of gestation because of an increased nuchal translucency of 9 mm, fetal akinesia and joint contractures. There was no intrauterine growth restriction. At post mortem examination there was a cystic hygroma, lung hypoplasia, webbing of both elbows and knees and arthrogryposis. The calvaria were absent due to birth trauma however there were no skeletal abnormalities (histopathology results are described below). In the family history, the first pregnancy was terminated because of increased nuchal translucency of 12 mm, fetal akinesia and a unilateral club foot. Additionally, the third, fourth, sixth and seventh pregnancy were terminated, because of increased nuchal translucency and fetal akinesia. The fifth pregnancy ended in an early miscarriage.

After genotyping of DNA from two affected foetuses (first and second foetuses) by high resolution Affymetrix SNP arrays (Genome-Wide Human SNP Array 5.0) a large homozygous region on chromosome 19 was selected for further analysis. Genotyping with microsatellite markers (see Table 2), was then performed on parental DNA samples and DNA from three affected pregnancies. The additional genotyping defined the candidate autozygous region as Chr19; 35108829–44484993) (see Figure 1A). A microsatellite marker (D8S373) that mapped within the only other candidate autozygous region >2 Mb was demonstrated to be heterozygous in two affected foetuses and so excluded linkage to the chromosome 8 candidate interval (data not shown). At the time of analysis the candidate region on chromosome 19 contained 345 known or predicted genes including the ryanodine receptor 1 (skeletal) (*RYR1*) gene (Chr19;38924340–39078204).

Table 1 Clinical features of 66 probands in which *RYR1* mutation analysis was performed

Family number	Phenotype	Ethnicity	Consanguinity
MPS001	LMPS	White	Y
MPS002	LMPS	South Asian	Y
MPS003	LMPS	Middle Eastern	Y
MPS004	LMPS	South Asian	Y
MPS005	LMPS	South Asian	Y
MPS006	LMPS	South Asian	Y
MPS007	LMPS	Not available	N
MPS008	LMPS	White	N
MPS009	LMPS	White	N
MPS010	FADS	Middle Eastern	Y
MPS011	LMPS	Not recorded	Y
MPS012	LMPS	North African	Y
MPS013	LMPS	White	N
MPS014	LMPS	South Asian	Y
MPS015	LMPS	White	N
MPS016	LMPS	Not available	Y
MPS017	LMPS	North African	Y
MPS018	FADS	White	N
MPS019	LMPS	White	N
MPS020	LMPS	White	N
MPS021	LMPS	South Asian	Y
MPS022	FADS	Middle Eastern	Y
MPS023	LMPS	Middle Eastern	Y
MPS024	LMPS	White	N
MPS025	FADS	Mixed race	N
MPS026	FADS	White	N
MPS027	LMPS	Not available	Y
MPS028	LMPS	Not available	N
MPS029	FADS/LMPS	Not available	N
MPS030	FADS/LMPS	Not available	N
MPS031	LMPS	White	N
MPS032	LMPS	White	N
MPS033	LMPS	White	N
MPS034	EVMPS	Not available	Y
MPS035	EVMPS	South Asian	Y
MPS036	EVMPS	White	N
MPS037	EVMPS	Not available	N
MPS038	EVMPS	White	N
MPS039	EVMPS	White	N

Table 1 Clinical features of 66 probands in which *RYR1* mutation analysis was performed (*continued*)

Family number	Phenotype	Ethnicity	Consanguinity
MPS040	EVMPS	White	N
MPS041	EVMPS	White	N
MPS042	EVMPS	White	N
MPS043	EVMPS	South Asian	N
MPS044	EVMPS	South American	N
MPS045	EVMPS	South Asian	Y
MPS046	EVMPS	South Asian	Y
MPS047	EVMPS	White	N
MPS048	EVMPS	African	Y
MPS049	EVMPS	Not available	N
MPS050	EVMPS	White	N
MPS051	EVMPS	White	N
MPS052	EVMPS	White	N
MPS053	EVMPS	White	Y
MPS054	EVMPS	White	N
MPS055	EVMPS	White	N
MPS056	EVMPS	White	N
MPS057	EVMPS	South American	N
MPS058	EVMPS	White	N
MPS059	EVMPS	African	N
MPS060	EVMPS	White	N
MPS061	EVMPS	White	N
MPS062	EVMPS	White	N
MPS063	EVMPS	White	N
MPS064	LMPS	Middle Eastern	?
MPS065	LMPS	White	N
MPS066	LMPS	North African	Y

Clinical phenotype (FADS = Foetal Akinesia Deformation Sequence, LMPS = Lethal Multiple Pterygium Syndrome; EVMPS = Escobar Variant Multiple Pterygium Syndrome), ethnic origin and presence of parental consanguinity is recorded).

Table 2 Microsatellite markers employed in Mapping of chromosome 19 candidate region in consanguineous family (MPS001)

Marker	Genomic position	Source
D19S222	Chr.19-28,725,890-28,726,217 bp	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1341900
D19S433	Doesn't map to Chr.19 assembly	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1298168
D19S430	Chr.19-32,302,451-32,302,741 bp	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1657125
D19S224	Chr.19-35,493,932-35,494,196 bp	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1336138
Chr19r2-15xAC41	Chr.19-41,435,658-43,123,394	ftp://ftp.broad.mit.edu/pub/human_STS_releases/
D19S220	cHR.19-34,8798,595-34,879,871 BP	http://rgd.mcw.edu/rgdweb/search/markers.html?term=D19S220&speciesType=1
D19S228	Chr.19-34,937,645-34,937,798 bp	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1338234
D19S421	Chr.19-38,871,106-38,871,460 bp	http://rgd.mcw.edu/rgdweb/report/marker/main.html?id=1337182
Chr19R2-15xTAT	Chr.19-44,221,282-44,669,155	ftp://ftp.broad.mit.edu/pub/human_STS_releases/
Chr19r2-22xGT44	Chr.19-44,221,282-44,669,155	ftp://ftp.broad.mit.edu/pub/human_STS_releases/

RYR1 mutation analysis

Sequencing of *RYR1* in an affected foetus (F2) from Family MPS001 revealed a homozygous *RYR1* nonsense mutation (NM_000540.2(RYR1): c.6721C>T, p.(Arg2241*)). Further analysis demonstrated that a second affected foetus (F1) was also homozygous for c.6721C>T, whereas both parents were heterozygous for the nonsense mutation (see Figure 1B). Subsequently DNA from the F3, F4, F6 and F7 were also shown to be homozygous for the mutation (data not shown). The *RYR1* c.6721C>T mutation was previously detected (rs200563280), in the heterozygous state in 1 of 6503 individuals genotypes listed in the exome variant server (http://evs.gs.washington.edu/EVS) (no homozygous genotypes were detected at an average read depth of 49). In order to assess the potential role of recessive *RYR1* mutations in FADS, and/or MPS we proceeded to screen

a further 66 probands for germline *RYR1* variants. Two further potential candidate homozygous mutations were detected, a novel in-frame deletion of 27 nucleotides (NM_000540.2(RYR1): c.2097_2123del p.(Glu699_Gly707-del)) and an in-frame deletion of 3 nucleotides c.7043del- GAG p.(Glu2347del) (rs121918596)) (see Table 3). Each of the candidate mutations were detected in the homozygous state in individuals with FADS/MPS.

Table 3 Rare *RYR1* variants detected in families with FADS/LMPS phenotypes

Family ID	DNA change	Protein change	Genotype	Segregation in family?
MPS001	c.6721C > T	p.Arg2241^{STOP}	Homozygous	Yes
MPS002	c.2097_2123del	p.(Glu699_Gly707del)	Homozygous	Yes
MPS003	c.7043delGAG	p.(Glu2347del))	Homozygous	Yes

Clinical and molecular genetic analysis in MPS002 family

A novel in-frame deletion of 27 nucleotides (NM_000540.2 (RYR1):c.2097_2123del p.(Glu699_Gly707del)) was detected in Family MPS002 of Pakistani origin (Figure 2A).

The family presented at eighteen weeks of pregnancy with a female foetus with bilateral talipes and fixed flexion of the elbows. There was evidence of fetal hydrops on the ultrasound scan including a large cystic hygroma, subcutaneous oedema, ascites also pleural and pericardial effusions. A cardiac ventriculoseptal defect was also suspected. The pregnancy was terminated at 19 weeks of gestation. Post mortem examination revealed no intrauterine growth restriction, but the foetus was considered to be dysmorphic (protuberant eyes, hypertelorism, a flat nose, low set ears), with a complete cleft palate. There was fixed flexion of all the limb joints and a fracture of the proximal left humerus. Pterygia were present between the inferior margin of the mandible and the anterior chest wall and across the elbows. The heart was normal. There were no other congenital abnormalities noted. The brain was structurally and histologically normal. Muscle histopathological analysis is described below.

This candidate *RYR1* c.2097_2123del mutation was present in a homozygous state in two affected foetuses and was found to be heterozygous in both parents. The in-frame deletion was predicted to result in a missense substitution (p.(Glu699Asp)) followed by a deletion of 9 amino acids (GlyTrpGlyGlyAsnGlyValGlyAsp) from the SPRY2 domain of the *RYR1* gene product. All 9 of these deleted amino acids were conserved in vertebrate *RYR1* orthologues including zebrafish and 6 of 9 amino acids were conserved in C.elegans (see Figure 2A).

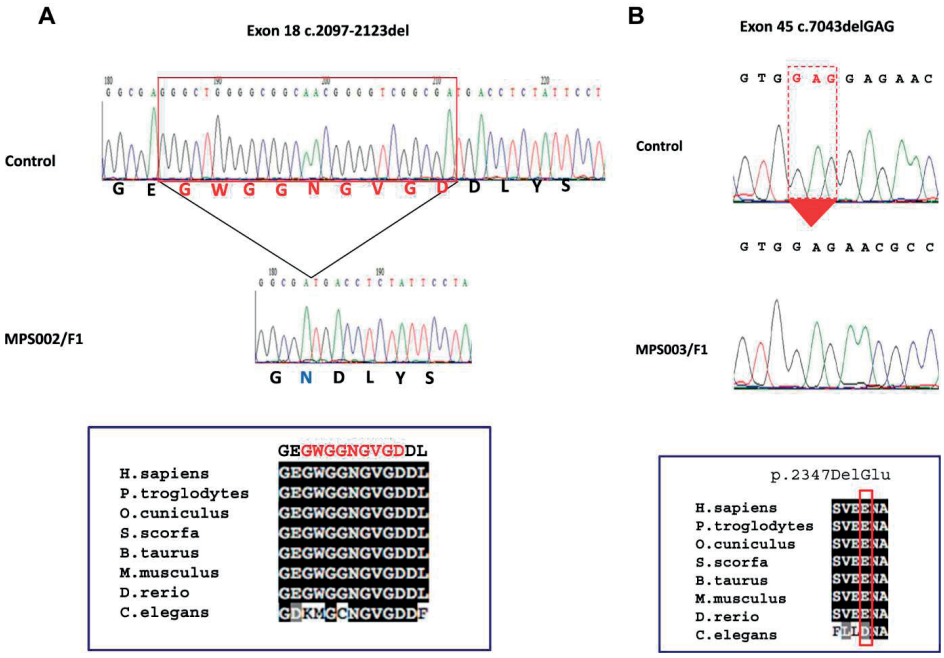

Figure 2 Identification of RYR1 in frame deletions. A. In the proband (F1) from Family MPS002 there is a homozygous deletion of 27 nucleotides (c.2097-2123del). The deleted/altered amino acids exhibited total conservation in zebrafish and partial conservation with 6/9 amino acids in C. elegans conserved. B: In the proband (F1) from Family MPS003 there is a homozygous deletion of 3 nucleotides (c.7043delGAG). The deleted amino acids (Glu/E) was conserved in the zebrafish with some degree of functional conservation in C.elegans (D/E).

Clinical and molecular genetic analysis in MPS003 family

In a consanguineous family (MPS003) with LMPS, a homozygous in-frame deletion (NM_000540.2(RYR1): c.7043delGAG, p.(Glu2347del) (rs121918596)) was identified in exon 45 in the proband (Figure 2B). This female foetus was the third pregnancy of a consanguineous Palestinian couple. In the pregnancy there was a suggestion of polyhydramnios. On ultrasound examination there were reduced foetal movements and joint contractures were present. The foetus had a cystic hygroma, a hydrothorax, a short neck, a kyphosis and a short trunk due to a scoliosis. The pregnancy was terminated at 23+3 weeks of gestation. On examination there was no evidence of intrauterine growth restriction. The foetus had craniofacial anomalies including downslanting palpebral fissures, hypertelorism, a broad nasal bridge, a small mouth and high arched palate, low set ears, a short broad neck and a scoliosis. There were flexion contractures of the shoulders, elbows, wrists, hips, knees and ankles. There were clenched hands but no finger contractures. There was webbing of the axillae, elbows, hips, knees and ankles, and rocker bottom feet. There was a severe scoliosis but no bony abnormalities identi-

fied on the radiograph. There was pulmonary hypoplasia. There were no abnormalities of the brain or spinal cord. The muscle in the extremities showed increased fibre size disproportion on microscopy (Figure 3). Cardiac muscle histology was normal.

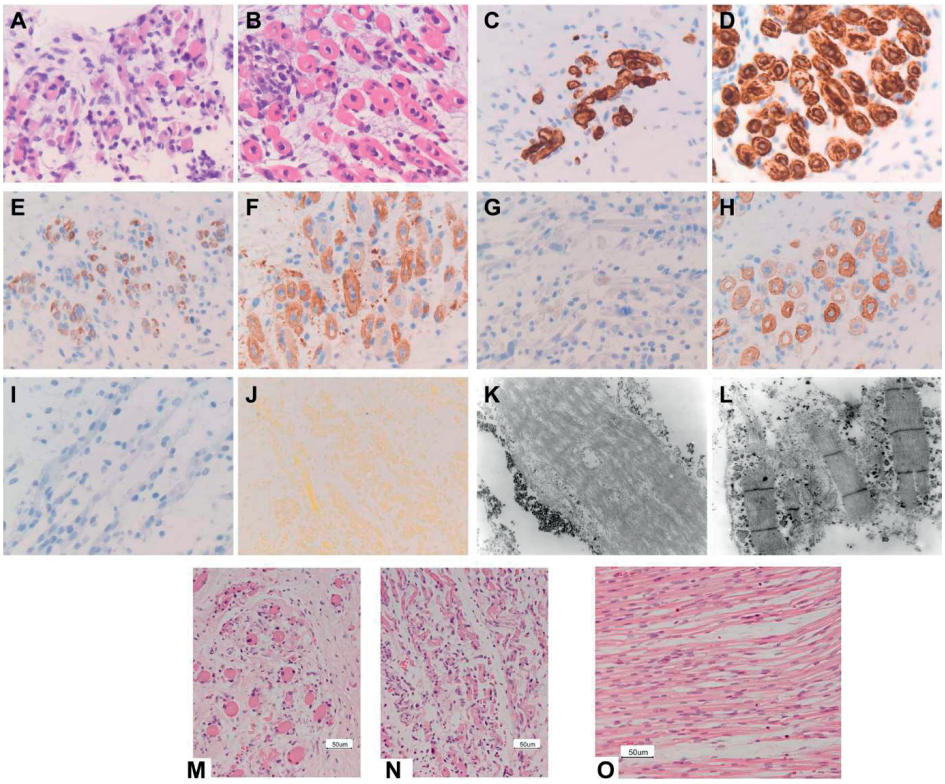

Figure 3 MPS001: Histological and ultrastructural findings in RYR1-mutant fetal skeletal muscle. (A,B) Hematoxilin & Eosin stain shows increased fiber size variability in the RYR1-mutant muscle (A, foetus F1) compared to an age-matched control (B). Some RYR1-mutant fibres have intensely eosinophilic cytoplasm (A, arrows). The nuclei are localized centrally in all fibres, compatible with the gestational age. The perinuclear clear halo present in many fibres is an artefact due to formalin fixation. (C,D) Labelling against desmin reveals a similar pattern of immunoreactivity and no evident core-like structures in both RYR1-mutant (C, foetus F1) and control tissue (D). (E-H) Labelling against the myosin heavy chain fast (E,F) and slow (G,H) shows that the numbers of myosin fast-positive type II fibres is comparable between the patient (E, foetus F2) and the control (F), whereas myosin slow-positive type I fibres are markedly reduced in RYR1-mutant (G, foetus F2) compared to control muscle (H). (I) Labelling for active caspase 3 is negative, excluding apoptosis, also in atrophic RYR1-mutant muscle fibres (foetus F2). (J) Alizarin red S staining shows no detectable accumulation of calcium inside the RYR1-mutant muscle fibres (foetus F1). (K,L) Ultrastructural analysis reveals profound myofibrillar disarray with disappearance of the Z-bands in the RYR1-mutant muscle fibres (K, foetus F2). By contrast, Z-bands are easily detected in control tissue (L). Magnifications: (A-J) 400x; K,L 30000x. (lower panels): histological findings in RYR1-mutant (Family MPS003) foetal skeletal muscle, GA 23 weeks. (M,N) Hematoxilin & Eosin stain of formalin fixed and paraffin embedded psoas muscle shows loss of fibres with increased fibre size variability and mild fibrosis in the RyR1-mutant muscle (M,N) compared to an age-matched control (O).

The karyotype was normal. *CHRNG, CHRNA1, CHRNB1, CHRND, RAPSN* and *DOK7* analysis was normal. Subsequently a fourth pregnancy of a male foetus was terminated at 21+3 weeks of pregnancy because of evidence of foetal akinesia. On examination of the foetus no growth restriction was noted. There was a cystic hygroma. The foetus had downslanting palpebral fissures, a broad nasal bridge, a small mouth with a high arched palate. There were contractures of the large and small joints with webbing of the axillae, elbows, hips and knees with pronounced kyphosis, 11 pairs of ribs, but no other bony abnormalities on radiography.

The foetus had lung hypoplasia and there was a small atrial septal defect. There were no abnormalities of the brain or spinal cord. Skeletal muscle microscopy showed increased fibre disproportion. Immunohistology of the structural proteins (alpha-actin, dystrophin 1,2 and 3, alpha, beta, delta, and gamma sarcoglycan, alpha and beta dystroglycan, dysferlin, caveolin-3, merosin laminin alpha-2 chain, merosin M-chain, myosin heavy chain and spectrin-1) did not identify the pathogenesis of the myopathy (data not shown). It was concluded that the foetus had a non-specific myopathy. The couple had one miscarriage and they had an older daughter with a severe undiagnosed metabolic disorder. There were no instances of malignant hyperthermia in the family and the mother had general anaesthesia on several occasions without any problems. The *RYR1* c.7043delGAG candidate mutation was present in both parents in the heterozygous state and there were no other relatives available for analysis. The c.7043delGAG deletion is predicted to result in loss of a glutamic acid residue at codon 2347 in the MHS/CCD hotspot region 2 of the *RYR1* gene product (Figure 4). This residue is conserved in zebrafish and although the amino acid sequence around this residue is divergent in C. elegans, the glutamic acid is conserved. Polymorphic variation at codon 2347 was not present in >13,000 *RYR1* alleles reported on the exome variant server (http://evs.gs. washington. edu/EVS), but the c.7043delGAG deletion was previously described, in the heterozygous

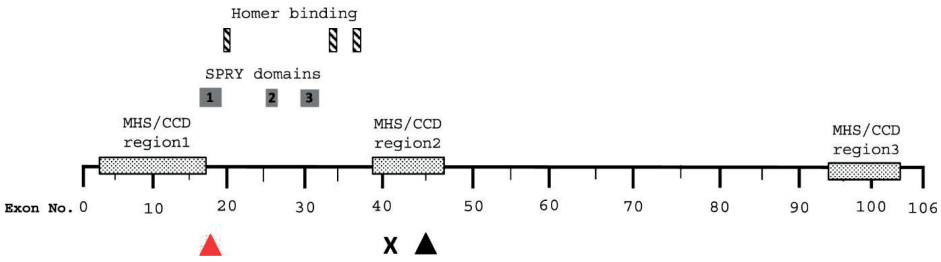

Figure 4 Location of nonsense mutation in MPS001 (X) and in frame deletions in Family MPS002 (red triangle) and Family MPS003 (black triangle) in relation to exon structure and RYR1 protein domains. Malignant Hyperthermia/central core disease mutation hot spots shown as stippled boxes, SPRY2 interacting domains 1,2 & 3 as grey boxfibres, Homer binding motifs as hatched boxes.

state, in affected members (total 5 cases) of two unrelated families that presented with malignant hyperthermia (Takeshima et al., 1994).

Histopathological studies

Family MPS001

Histopathological analysis was performed on intercostal skeletal muscle from the two foetuses of family MPS001 and two age-matched controls, as this was the only tissue available for both patients. Quantitative histological examination showed marked structural abnormalities in the *RYR1*-mutant skeletal muscle, including fibre loss, increased fibre size variability and increased endomysial spacing with fibrosis (Figure 3A,B). In agreement with the gestational age, muscle fibres of both patients and controls contained centrally positioned nuclei. Sparse muscle fibres with intense eosinophilic cytoplasm were detected in the patients, but not in the controls (Figure 3A,B) which was not due to calcium accumulation (Figure 3J). Staining against desmin, an intermediate filament protein typically expressed in muscle tissue, revealed no core-like structures (Figure 3C,D). Fibre type distribution, assessed by staining against the slow and fast myosin heavy chain, showed a marked reduction of myosin slow immunoreactivity, suggesting preferential hypotrophy of type I fibres (Figure 3E-H). Labelling for active caspase 3 excluded apoptotic loss of muscle fibres (Figure 3I). Ultrastructural analysis demonstrated hypotrophy with profound myofibrillar disarray and Z-disc loss (Figure 3K,L). Analysis of the spinal cord showed no loss of motor neurons or other pathological signs in the anterior horns and most proximal segments of the motor nerves, supporting the myogenic nature of the histological and ultrastructural findings.

Family MPS003

The muscle appearance was striking but non-specific. There was variation in myofibre size with larger hyalinised rounded fibres and smaller rounded atrophic fibres with an apparent increase in fibrous tissue. Fast and slow myosin heavy chains were co-expressed in a proportion of foetal myofibres. Occasional scattered chronic inflammatory cells were confirmed on CD3 and CD20 staining. Additional CD45 staining was interpreted as non-specific. Gomori trichrome staining was negative for nemaline rods and ragged red fibres. The couple had a previous intrauterine death at 23 weeks of gestation of a similarly affected male foetus (Figure 3M-O).

DISCUSSION

After undertaking genetic linkage studies in a consanguineous family with recurrent FADS/LMPS we mapped a large candidate autozygous region to chromosome 19 and then proceeded to identify a homozygous nonsense mutation in the ryanodine receptor 1 (*RYR1*) gene. *RYR1* encodes the largest known ion channel and the 2.3 MDa homotetrameric ryanodine receptor 1 structure is formed from the 565 kDa *RYR1* component proteins. Though the large size (15.3 kb coding sequence in 106 exons) of the *RYR1* gene makes mutation analysis challenging, *RYR1* mutations have previously been described in a variety of human disease phenotypes. Thus initially *RYR1* mutations were described in individuals susceptible to malignant hyperthermia (MHS) (Fujii et al., 1991; Gillard et al., 1991). *RYR1* mutations associated with MHS are typically heterozygous missense substitutions (Broman et al., 2011; Brandom et al., 2013; Kim et al., 2013). Subsequently *RYR1* mutations were described in the context of a variety of histological subtypes of congenital myopathies including central core disease, minicore/centronuclear myopathy with external ophthalmoplegia, centronuclear myopathy and congenital fibre-type disproportion (Treves et al., 2008; Clarke et al., 2010; Wilmshurst et al., 2010). Both dominant and recessive forms of *RYR1*-related congenital myopathies have been described and genotype-phenotype correlations have provided insights into likely clinical-functional relationships. Thus, in a large cohort of *RYR1*-associated myopathies, dominant mutations tended to be associated with milder phenotypes whereas recessively inherited cases had an earlier onset and a more severe course (Hwang et al., 2012; Klein et al., 2012; Maggi et al., 2013). In addition, though the age at presentation of recessive *RYR1* myopathies is variable, in a recent series all presented before age 10 years. Whereas *RYR1* mutations in dominantly inherited disease tend to cluster in specific protein domains e.g. C-terminal region (amino acids 4,550-4,940) in central core disease and N-terminal regions (amino acids 35–614 and 2,163-2,458) with MHS, recessively inherited mutations are widely distributed throughout the protein (Klein et al., 2012; Maggi et al., 2013). Typically the mutations found in patients with recessively inherited *RYR1*-myopathies are a combination of null mutation with a missense mutation, though two missense mutations can occur. Thus in a series of 118 patients with *RYR1*-related recessively inherited myopathies from four recent reports (Jungbluth et al., 2007; Klein et al., 2012; Amburgey et al., 2013; Bharucha-Goebel et al., 2013; Maggi et al., 2013), 61.5% of the cases had a truncating/in frame mutation combinations with only a single null mutation can cause a milder phenotype, (b) that mice homozygous for a Ryr1 mutation die in the perinatal period with gross abnormalities of skeletal muscle (Otsu et al., 1991) and (c) a history of foetal akinesia may be found with early onset autosomal recessive *RYR1*-related congenital myopathies (Klein et al., 2012). In addition, Romero et al. (Romero et al., 2003) reported seven foetuses/infants from six unrelated families

affected by central core disease in whom there was a history of foetal akinesia. Four cases from three families were found to harbour *RYR1* mutations: three cases (from two families) were compound heterozygotes for *RYR1* missense mutations and in one case only a heterozygous missense mutation was detected. Three of the four cases presented at birth and though in one case the foetus died at 32 weeks gestation (following termination of pregnancy after a previously affected sibling). Thus the phenotype in these cases was less severe than we observed and our findings demonstrate that the association between *RYR1* mutations and foetal akinesia extends to severe early onset lethal FADS and that histopathological evidence of central core disease is not a prerequisite for molecular investigation of *RYR1* in foetal akinesia.

The overall frequency of *RYR1*-related disease in our FADS/LMPS/EVMPS cohort was 4.5% (3/66; 95% CI: 0 to 9.5%) and 8.3% (3/36; 95% CI 0 to 19.5%) in our FADS/LMPS cohort. The case for pathogenicity of the two in frame deletions is supported by their absence from large repositories of genetic variation in control individuals, segregation with disease within the relevant families and evolutionary conservation of the mutated/deleted amino acid residues. *RYR1* is a key component of the excitation-coupling process in skeletal muscle such that opening of *RYR1* channels result in release of Ca^{2+} from the sarcoplasmic reticulum and initiation of muscle contraction. The novel in-frame deletion of 27 nucleotides (c.2097_2123del p.(Glu699_Gly707del)) detected in Family MPS002 is predicted to result in a missense substitution (p.E699N) followed by a deletion of 9 amino acids (GWGGNGVGD) within the SPRY2 predicted protein- protein interaction motif (Perálvarez-Marín et al., 2011) (highlighted in Figure 4). Previously missense substitutions within or adjacent to this deletion (c.2113G > C; p.Gly705Arg and p.Asp708Asn) have been reported in recessively inherited myopathies (Klein et al., 2012). The exon 45 in-frame deletion (c.7043delGAG) identified in Family MPS003 was predicted to result in loss of a glutamic acid residue at codon 2347. A missense mutation at a nearby residue (p.Arg2355Trp) has been reported in both dominantly and recessively inherited myopathies (Kim et al., 2013) and it is interesting that this deletion was previously described in the heterozygous state, in two unrelated families with malignant hyperthermia (Sambuughin et al., 2001). p.Glu2347 is contained within the MHS/CCD mutation hotspot in N-terminal region 2 (stippled box Figure 4),deletion/splice site mutation in combination with a missense mutation and 38.1% harboured two missense mutations. It is therefore striking that we identified a homozygous null mutation in affected individuals from Family MPS001. This observation is consistent with (a) our previous observation for RAPSN that homozygosity for a null mutation can cause FADS/LMPS other (Takeshima et al., 1994). Though no history of malignant hyperthermia syndrome was reported in Family MPS003, incomplete penetrance is well recognised in malignant hyperthermia and mutation carriers may not have been exposed to trigger events.

Though our findings establish recessive *RYR1* mutations as a cause FADS/LMPS, further work is required to fully establish the frequency of *RYR1* mutations in FADS/LMPS cohorts and to address how novel missense or in-frame deletions/insertions might be reliably interpreted in a clinical diagnostic setting. In a recent review of congenital myopathies treated at a single referral centre, a genetic diagnosis was established in two-thirds of cases and almost 60% of those with a genetic diagnosis had a *RYR1*-related myopathy (Maggi et al., 2013). Our findings suggest that *RYR1*-related neuromuscular disease may be a significant cause of FADS/LMPS. Though recessively inherited *RYR1*-related myopathies have been associated with certain histopathological subtypes such as minicore, centronuclear and congenital fibre-type disproportion myopathies, *RYR1* mutations may be associated with other histological subtypes or only nonspecific myopathic features (Maggi et al., 2013). Extrapolating from these observations, we suggest that, in cases of FADS/LMPS, *RYR1* mutation should be performed as part of a multigene diagnostic strategy (e.g. by second generation sequencing analysis) rather than being specifically targeted to cases with histopathological features that are considered characteristic of a *RYR1*-associated myopathy. The identification of *RYR1* mutations as a cause of familial LMPS/ foetal akinesia enables accurate reproductive risk prediction and reproductive options including prenatal diagnosis and pre-implantation diagnosis but also might lead to the identification of relatives at risk of malignant hyperthermia.

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Chapter 8

Discussion

In the more than ten years that have passed since this thesis was started, the field of (clinical) genetics has changed enormously: from performing conventional karyotyping and targeted diagnostic DNA-testing on a limited number of genes to performing whole exome sequencing and probably in the near future whole genome sequencing. This radical change did not only take place in postnatal genetics, but also in prenatal genetics. In this thesis the change in prenatal genetic care over those years was evaluated, in particular concerning an increased nuchal translucency (NT) in the first trimester of gestation. In this last chapter, the main findings of this thesis, together with their strengths and limitations, will be presented and discussed in the context of existing literature. After outlining recommendations for clinical implications and further research, this chapter closes with overall conclusions.

MAIN FINDINGS

To this day the etiology of an increased NT remains poorly understood. The most used theory on the development of an increased NT is an aberrant lymphatic development (Haak et al., 2002). In this thesis we showed that neural crest cells are involved in lymphatic development (Chapter 2) (Burger et al., 2014). Both directly and indirectly, neural crest cells, when differentiated into nerve cells, can act as a source for retinoid acid, which, in its turn causes nuchal edema when blocked from signaling.

The results in this thesis add to the studies on the (postpartum) clinical phenotype that have been published in the past (Bilardo et al., 1998; Souka et al., 1998; Souka et al., 2001; Senat et al., 2002; Souka et al., 2005; Bilardo et al., 2007; Axt-Fliedner et al., 2009; Bilardo et al., 2010; Ayras et al., 2013). Prenatal microarray in increased NT shows a diagnostic yield of approximately 5% additionally to QF-PCR (Quantitative Fluorescence Polymerase Chain Reaction (Chapter 3). Interestingly, no specific copy number variations (CNVs), either microdeletion or -duplication, were detected in fetuses with an increased NT (Chapter 4). The CNVs found in fetuses with an increased NT were compared to CNVs found in healthy adults and there was no significant difference in variation in CNVs between these groups. Besides chromosomal aberrations, fetuses with an increased NT also have an increased risk of structural and genetic anomalies (Nicolaides et al., 1992a; Pandya et al., 1995; Bilardo et al., 1998; Souka et al., 1998; Souka et al., 2001; Senat et al., 2002; Souka et al., 2005; Bilardo et al., 2007; Axt-Fliedner et al., 2009; Bilardo et al., 2010; Ayras et al., 2013). Although the spectrum of genetic syndromes is very wide and testing for single genes has shown not to be useful when no clear prenatal phenotype is present (VKGN, 2017), the association between RASopathies and an increased NT has often been described (Benacerraf et al., 1989; Nisbet et al., 1999; Achiron et al., 2000;

Baldassarre et al., 2011; Pergament et al., 2011; Hakami et al., 2016; Ali et al., 2017), though mostly in case reports. In this thesis, the association between an increased NT and RASopathies was confirmed by performing a retrospective study of all known cases in the Netherlands between 2011-2016 (Chapter 5 and 6). This leads to the formulation of recommendations when to perform DNA-diagnostics for RASopathy (Chapter 6).

DISCUSSION OF THE MAIN FINDINGS

Etiology of an increased NT

Lymphatic development in the fetus starts with forming jugular lymphatic sacs (JLS) from the internal jugular veins (Sabin, 1902) and it reaches finalization at the end of the first trimester of gestation. An altered lymphatic development can be explained as an important cause for nuchal edema, due to transient and local nature of an increased NT (Haak et al., 2002; Bekker et al., 2005a), which is measured at the end of the first trimester, and the presence of (distended) jugular lymph sacs in fetuses with an increased NT (Bekker et al., 2005a). Bekker et al. showed (Bekker et al., 2005b) that impaired neural crest cell migration could partly contribute to lymphatic maldevelopment, because aberrant lymphatic development coincided with significantly smaller cranial nerves surrounding the JLS. Cranial nerves are partly formed by migration and differentiation of cranial progenitor cells, called the neural crest cells (Muller and O'Rahilly, 2011). In this thesis, the involvement of neural crest cell migration in lymphatic maldevelopment was further explored showing that neural crest cells (NCCs) were observed in the JLS and in lymphatic endothelial cells (LEC) (Burger et al., 2014) (Chapter 2). This suggests involvement of NCCs in the formation of JLS and LEC and accordingly, the formation of nuchal edema. The involvement of NCCs in lymphatic development and subsequently nuchal edema may also explain the variety of structural malformations in fetuses with increased NT (Souka et al., 2001; Bilardo et al., 2007; Miltoft et al., 2012; Tahmasebpour et al., 2012; Ayras et al., 2013), because cardiovascular defects, craniofacial malformations and skeletal anomalies can all be related to disturbances in NCC migration or differentiation (Kirby and Waldo, 1995; Jiang et al., 2000; Huang et al., 2010; Van Ho et al., 2011). However, the exact potential of NCCs, whether differentiating directly or indirectly into lymphatic endothelial cells still needs to be further explored. Additionally, this study showed, that blocking retinoic acid signaling, which is an active metabolite of vitamin A and important in nervous and lymphatic vascular development (Lai et al., 2003; Niederreither et al., 2003; Niederreither and Dolle, 2008; Bowles et al., 2014), caused aberrant lymphatic development and nuchal edema in mouse embryos. However, the source of retinoid acid is still unknown. The most likely source are the nerve fibers adjacent to the

location where the LEC bud off from the cardinal vein to differentiate into mature LECs (van de Pavert et al., 2009; van de Pavert et al., 2014).

Clinical consequences of an increased NT

In 1992, Nicolaides proposed to introduce the measurement of the NT into the prenatal clinic to estimate the risk of a child with Down syndrome (trisomy 21) (Nicolaides et al., 1992b). Measuring the NT by ultrasound scan in combination with maternal age and testing of serum analytes PAPP-A and free beta-HCG, the so-called first trimester combined screening, has an estimated 82-87% (Malone et al., 2005) up to 94% (Engels et al., 2011) detection rate for trisomy 21 with a 5% false positive rate and approximately 90% detection rate for trisomy 18 and 13 (Kagan et al., 2015). It has therefore been a useful first-tier screening test for fetal chromosomal abnormalities in the years before the more accurate non-invasive prenatal testing (NIPT) was introduced. In addition to detecting aneuploidy, an increased NT is also associated with submicroscopic chromosomal aberrations (Grande et al., 2015), fetal structural anomalies (Pandya et al., 1995; Hyett et al., 1997) and genetic syndromes (Souka et al., 2001; Bilardo et al., 2007). This implies that further testing on a fetus with an increased NT and normal karyotype is warranted. Prenatal microarray, which detects small submicroscopic chromosomal deletions and duplications, is performed after Rapid Aneuploidy Detection (RAD) for trisomy 21, 18 and 13 is normal. Prenatal microarray shows an approximately 4-7% additional diagnostic yield to conventional karyotyping, as shown by Grande et al. (2015), depending on the associated anomalies. An isolated (without other fetal (structural) anomalies) increased NT has a lower yield on submicroscopic chromosomal abnormalities than non-isolated increased NT. Our study on prenatal microarray in fetuses with an isolated increased NT showed a 5% diagnostic yield (Chapter 3) and confirms the results from Grande et al.'s review (2015). We have shown that no specific or common deletions or duplications associated with an increased NT (Chapter 4), which adds to the variable structural defects and genetic syndromes that have been described to be associated with an increased NT (Bilardo et al., 1998; Souka et al., 1998) and possibly underlines the complex and multifactorial etiology of an increased NT.

When prenatal microarray is normal, the subsequent test to perform is DNA-testing for RASopathy (Croonen et al., 2013). This test is well established and is, in the Netherlands, offered as a gene panel of approximately 17 genes associated with Noonan syndrome or Noonan syndrome related disorders. In our recent study (Stuurman et al., 2019), we showed that the percentage of RASopathies in fetuses with an increased NT (isolated and non-isolated) is approximately 9% (Chapter 6). The detection chance is however, highly dependent on the thickness of the NT (Stuurman et al., 2019); mutation positive fetuses showed a (normally distributed) mean NT of 8.46 mm and mutation negative

samples showed a (non-normal distributed) median NT of 4.7 mm. It is therefore recommended to offer RASopathy testing in fetuses with an isolated increased NT of at least 5.0 mm or more. When the NT is between 3.5-5.0 mm the mutation detection for fetuses with an isolated increased NT is low (in our study 0%) and it is therefore recommended only to test for RASopathies in case of an increased NT below 5.0 mm when at least one of the following ultrasound anomalies is present as well: (distended) jugular lymph sacs, hydrops, pleural effusion, ascites, cardiac defects, renal anomalies or polyhydramnios.

Other genetic disorders associated with an increased NT are missed when only performing RASopathy diagnostic testing, but previously described genetic disorders found in fetuses with an increased NT are extremely diverse and numerous (Souka et al., 2005). Therefore, the association between an increased NT and genetic disorders other than the RASopathies seems to be rather weak (Souka et al., 2005; Pergament et al., 2011). Prenatal whole exome sequencing (WES) would capture these syndromes all at once, including the RASopathies. In WES all exons of all genes in the human genome are analyzed at once and this emerging technique has been introduced in the prenatal setting since 2014 (Carss et al., 2014; Drury et al., 2015; Vora et al., 2017; Lord et al., 2019; Petrovski et al., 2019). Initial studies on prenatal WES focused mainly on fetus with multiple structural anomalies (Carss et al., 2014; Drury et al., 2015) and not specifically on isolated increased nuchal translucency. However, in the last two years multiple studies reported on prenatal WES in fetuses with isolated increased NT (Choy et al., 2019; Daum et al., 2019; Lord et al., 2019; Petrovski et al., 2019; Xue et al., 2020). The diagnostic yield varies from 3% (Lord et al., 2019) to 13% (Xue et al., 2020) with an average of approximately 5%. It is an interesting discussion whether or not to perform prenatal WES for an isolated increased NT, because performing WES comes with additional challenges concerning reporting variants of unknown clinical significance (VUS), incidental findings not related to the clinical question (e.g. cancer heritability), costs and increased complexities for counseling (Harris et al., 2018). Additionally, a prenatal phenotype is different than a postnatal phenotype, which could make it harder to interpret whether the genotype fits the phenotype. A 3% diagnosis rate of WES in case of isolated increased NT, mentioned by Lord et al. (2019) and Petrovski et al. (2019) is low, but invasive prenatal testing is performed for lower percentages, such as testing in a pregnancy in which parents have a previous child with a random *de novo* pathogenic variant. In addition, the risk of fetal loss due to invasive testing is very low, ranging from 0.1%-0.3% depending on the test (chorionic villi sampling or amniocentesis) and the physician who is performing the test (Akolekar et al., 2015; Wulff et al., 2016; Salomon et al., 2019)(personal communication). Based on its low yield and large numbers of cases, prenatal WES in fetuses with an isolated increased NT is not (yet) commonly indicated (Lord et al., 2019). However,

different factors such as parental anxiety (Talati et al., 2021) and (obstetrical) medical history could contribute to consider prenatal WES in these cases.

Changing prenatal screening landscape

The use of genetic tests, including prenatal WES in fetuses with an increased NT will likely be more discussed, due to the implementation of an early structural anomaly scan ('13-week ultrasound scan') for all pregnant women in the Netherlands from September 1st 2021 onwards. In 2018, the Dutch Ministry of Health gave a green light for exploring the additional use of an early structural anomaly scan. This ultrasound scan should detect large structural anomalies at an early stage, which would otherwise be detected at 20 weeks of gestation in absence of this early scan (Edwards and Hui, 2018; Kenkhuis et al., 2018). Approximately 30-50% of fetal abnormalities could be picked up in the first trimester and these anomalies include, but are not limited to, cranial anomalies, (severe) heart defects and lower urinary tract obstructions (Rossi and Prefumo, 2013; Karim et al., 2017; Kenkhuis et al., 2018; Bardi et al., 2019; Syngelaki et al., 2019; Liao et al., 2021). The Dutch 13-week ultrasound scan will look at structural anomalies of the brain, skull and facial features, thorax, heart, abdominal defects, spine, extremities and also includes the NT measurement. The reasoning behind inclusion of the NT measurement as reported by the National Institute for Public Health and the Environment (Rijksinstituut voor Volksgezondheid en Milieu) is that: 1) the largest proportion of fetuses with an increased NT does not show structural anomalies in the first trimester, 2) improper measuring of the NT turns into subjective eyeballing and 3) an increased NT has always been a legitimate reason for referral to a maternal-fetal medicine department for invasive prenatal testing (RIVM, 2019b; Bardi et al., 2020).

Initially, the goal of measuring NT was to identify Down syndrome in the developing fetus (Nicolaides et al., 1992b). It has been extensively proven that NIPT is more reliable in detecting Down syndrome (sensitivity >95%) with a much lower false positive rate (0.1%) than first trimester combined screening (sensitivity 82-87%, false positive rate 5%) or measuring an increased NT alone (sensitivity 70%, false positive rate 5%) (Kagan et al., 2015; Bianchi and Chiu, 2018; ACOG, 2020). Therefore, measuring NT in the first trimester in order to identify (common) chromosomal anomalies is not needed when pregnant women choose NIPT for screening. NIPT is offered as first-tier screening test to all pregnant women in few countries including Belgium (Van Den Bogaert et al., 2021) and the Netherlands (TRIDENT-2 study) (van der Meij et al., 2019)].

An increased NT is also associated with submicroscopic deletions and duplications (Grande et al., 2015), which may be difficult or even impossible to detect with NIPT, depending on the specific test that is performed; in some countries commercial companies

offer specific testing for microdeletion syndromes through NIPT (Ravitsky et al., 2021). Berger et al. (Berger et al., 2020), however, concluded that a total of more than 4000 fetuses need to be screened for increased NT (with a cut-off of >3.0 mm) in order to detect a rare chromosomal abnormality. Some of these rare aneuploidies can, however, also be detected as an incidental finding when genome wide NIPT is used, as is currently done in the Netherlands with the TRIDENT studies (Van Opstal et al., 2018; van der Meij et al., 2019). Berger et al. (Berger et al., 2020) calculated a 5% positive predictive value for NT greater than 3.5 mm. An increased NT in the lower ranges with normal NIPT results most likely has a multifactorial origin, based on the high percentage of fetuses with good clinical postnatal outcome after chromosome abnormalities have been excluded (Bilardo et al., 2007). Therefore, at date, many invasive prenatal tests are performed without finding a clear cause for the increased NT, but increasing the likelihood of complications from the invasive test itself. If the cut-off value is lowered to 3.0 mm, as is suggested by some authors (Maya et al., 2017; Petersen et al., 2020; Sagi-Dain et al., 2021), the positive predictive value will even decrease to 2.6% (Berger et al., 2020), which in turn increases the (unnecessary) use of invasive testing with a risk of miscarriage and associated anxiety in parents.

An increased NT is associated with structural anomalies (Bilardo et al., 1998; Souka et al., 2001; Souka et al., 2005; Bilardo et al., 2007; Ayras et al., 2013) and of these anomalies, heart defects are the most common congenital anomaly associated with an increased NT (Hyett et al., 1997). However, at the 13-week fetal anomaly scan only approximately 50% of all heart defects will be picked up (Pike et al., 2014; Sarkola et al., 2015; Hernandez-Andrade et al., 2017; Syngelaki et al., 2019). If nuchal translucency measurement alone would be used to check for heart defects, only 30% would be detected (Makrydimas et al., 2003). As a result, measuring the NT on the 13-week scan seems of little additional value for detection of structural anomalies, including heart anomalies, when these anomalies are already part of the 13-week scan. The importance of the second trimester scan in addition to the first trimester scan should be mentioned to account for false-negative or false-positive results from the first trimester scan.

In clinical practice, health professionals are hesitant to exclude NT measurement out of fear of missing an anomaly or genetic diagnosis in the developing fetus, such as a RASopathy. However, in our paper on prenatal RASopathy testing (Stuurman et al., 2019), it is recommended to test for RASopathies when at least one of the following ultrasound anomalies in addition to an increased NT is seen (distended) jugular lymph sacs (JLS), hydrops fetalis, pleural effusion, ascites, cardiac and/or renal defects as this shows the highest diagnostic yield. In none of the fetuses in our study with an isolated increased NT below 5.0 mm the diagnosis of Noonan syndrome was confirmed (Stuur-

man et al., 2019). Even in the presence of abovementioned ultrasound anomalies, the NT tends to be larger in fetuses with a RASopathy (Ali et al., 2017; Stuurman et al., 2019). A severely increased NT (> 5.0 mm) is, even isolated, an indication for RASopathy testing in pregnancy (Chapter 6). It can be argued that fluid will be seen either on a dating scan or, when the dating scan is performed too early, visible on (first or second trimester) ultrasound scan without specifically measuring it due to the large amount of fluid visible ('eyeballing'). In our study most of the pregnancies with an isolated increased NT were terminated before 20 weeks of gestation, therefore it is difficult to determine whether more anomalies would have developed in due course.

It is thought that most pregnant women in the Netherlands will take the opportunity of a first trimester anomaly scan, comparable to the high uptake of approximately 90% of the second trimester ultrasound scan (Gitsels-van der Wal et al., 2014; RIVM, 2019a), because the scan is free of charge, is offered without an opportunity for another scan in second trimester and offers pregnant women a glance at their unborn baby (Gitsels-van der Wal et al., 2014). At the same time, the uptake of first trimester aneuploidy screening with NIPT (175 euros) is close to 50% (van der Meij et al., 2021). However, women who decide not to opt for NIPT, for example because they do not want to know whether their child has Down syndrome, might unexpectedly be confronted with an early detection of Down syndrome when opting for the 13-week ultrasound scan. This may cause parental distress for the remainder of the pregnancy.

The implementation of the first trimester ultrasound scan is part of the Dutch national prenatal screening program and NT measurement is embedded in this program. All screening has advantages and disadvantages and at all times one has to consider what is best at that moment. Although the measurement of an NT is a useful tool in detecting chromosomal anomalies, when NIPT and a detailed first trimester fetal ultrasound scan are performed, its use to detect additional anomalies seems only limited (Huang et al., 2018; Berger et al., 2020). With the introduction of the 13-week ultrasound scan in the Netherlands, starting in September of this year, it is interesting to see what will happen with the NT in terms of additive and/or predictive value.

STRENGTHS AND LIMITATIONS

Our study on the etiology of an increased NT is the first to identify neural crest cells in the jugular lymph sacs and managed to impose nuchal edema in mouse embryos by inhibiting retinoic acid signaling. This has not been described before and presents an interesting new insight in the complex etiology of an increased NT. However, with

an increased NT giving such a diverse (postnatal) phenotype, from severe syndromal intellectual disability to good clinical outcome, it is difficult to explain the lymphatic maldevelopment and neural crest cell migration as the sole cause of the diagnosis. Most likely many different factors contribute to the development of lymphatic problems, which challenge unravelling the etiology.

Our clinical studies on RASopathies contain the largest patient cohorts published on this subject with very complete prenatal data. Therefore, clear and significant recommendations could be given when testing for RASopathies is indicated. However, in the studies NT measurement was used as an important tool, which, with the implementation of NIPT and decreased uptake of first trimester combined screening, might challenge the prenatal detection of a RASopathy. As well, a high percentage of terminations of pregnancy in our cohorts was established, which made postnatal follow-up impossible. Therefore, a complete genotype–phenotype correlation cannot be made. Newborns with Noonan syndrome or a related condition were not included either.

Our clinical studies on the diagnostic yield of prenatal microarray and common CNVs did not involve large patient cohorts, such as the ones from Bilardo et al. (2010) and Souka et al. (2005), but our study on common CNVs in fetuses with increased NT is the first study to show the similarities in CNVs between fetuses with an increased NT and healthy unrelated adults. This confirms a multifactorial contribution to the formation of nuchal edema and shows no specific microdeletion or –duplication contributes to an increased NT.

CLINICAL IMPLICATIONS OF AN INCREASED NT

When an increased NT in pregnancy is confirmed, additional genetic testing through invasive prenatal testing (chorionic villi sampling or amniocentesis) is indicated. We have formulated the following recommendations based on the studies mentioned in this thesis:

- Rule out aneuploidy of chromosome 21, 18, 13 and the sex chromosomes with rapid aneuploidy detection (RAD)
- Perform prenatal array when RAD is normal to rule out submicroscopic deletions and duplications
    - o It is important to stress out the relatively low yield of this test, which is approximately 5%. (Chapter 3)
- Offer RASopathy testing in fetuses with an isolated increased NT of at least 5.0 mm or more. (Chapter 6)

- Offer RASopathy testing in fetuses with NT between 3.5-5.0 mm when at least one of the following ultrasound anomalies is present:
  - (Distended) jugular lymph sacs, hydrops, pleural effusion, ascites, cardiac defects, renal anomalies or polyhydramnios. (Chapter 5 and 6)
- Consider offering prenatal whole exome sequencing (WES), especially when multiple anomalies or other than the abovementioned structural anomalies are present. If offered, genetic counseling on incidental findings in prenatal WES is strongly advised.
- When all prenatal testing for increased NT is normal, the chance on a good outcome postnatal is high. Then the cause of an increased NT is unexplained and might be attributed to a complex multifactorial developmental process. (Chapter 2)

FUTURE RESEARCH

Although it seems likely that the development of nuchal edema is a complex and multifactorial process, linked to possible multiple embryonic pathways, the neural crest cells and lymphatic endothelial cells are prominent factors in this process. Further studies are required to gain more insight into the potential of the neural crest cells and their differentiation into lymphatic endothelial cells. Additional studies are also needed to establish that nerves are indeed the source of retinoic acid. Unravelling the etiology of an increased NT could ultimately help health care providers in differentiating between low or high risk on a good outcome of pregnancy.

In the Netherlands, the cut-off value of increased versus not increased NT is set at 3.5 mm (NVOG, 2012). However, as mentioned previously, some authors prefer 3.0 mm as the cut-off for invasive prenatal testing (Maya et al., 2017; Petersen et al., 2020; Sagi-Dain et al., 2021). The motive for this cut-off is that more large chromosomal anomalies will be detected prenatally. However, it lowers the positive predictive value (Berger et al., 2020) and as a result increases the percentage of women eligible for invasive prenatal testing which, in its turn, could increase complication risk and possibly anxiety in the parents-to-be. More research is needed whether lowering the cut-off value of the NT will benefit pregnant women.

With the implementation of the 13-week ultrasound and the (renewed) measurement of the NT, an increase of referrals to tertiary health centers is expected. A significant proportion of these referrals will turn out to be unnecessary due to the transient state of the found anomaly or being a false-positive anomaly, such as an isolated increased NT or certain cardiac defects respectively. These referrals could therefore create more distress in the future parents or cause unwanted complications if further invasive prena-

tal testing is performed. Additionally, when an increased NT is, by chance, detected on a dating scan, but resolved on the 13-week ultrasound scan, one might debate whether further invasive testing is warranted. Muller et al. (2004) showed that a disappearing enlargement is a favorable prognostic sign in chromosomal abnormalities and clinical outcome, although the results were not significantly different. Studies, such as the IMITAS study (www.13wekenecho.org), are initiated to investigate whether the benefits outweigh the disadvantages of the implementation of the 13-week ultrasound, including the NT measurement, by assessment of the detected structural anomalies, increased parental distress, unwanted complications and its involved costs.

CONCLUSIONS

The expanding importance of an increased NT from detection of Down syndrome to a wide variety of structural anomalies and genetic syndromes, its reduction in use and renewed interest in this, due to NIPT implementation and 13-week ultrasound scan respectively, show the complexity of (dealing with) this ultrasound anomaly. The diversity in which congenital structural anomalies and genetic syndromes occur, suggests that a single explanation for the development of nuchal edema is unlikely. New recommendations are proposed how to deal with a fetus with an increased NT. However, there are still answers to be found, especially on the etiology of an isolated and temporarily increased NT. In the coming years, the measurement of the NT in the 13-week ultrasound scan and the more widely use of prenatal WES for syndromal diagnosis in prenatal anomalies might give us new information on how to interpret an increased NT.

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Summary

Nuchal translucency (NT) refers to the translucent area in the neck region of the developing fetus and can be visualized by ultrasound scan between 11 and 13+6 weeks of gestation, after which it normally resolves. An increased NT represents nuchal edema and is defined at equal or greater than 3.5 mm (99th percentile) according to the Dutch Society of Obstetrics and Gynecology guidelines and is prevalent in 1% of all pregnancies. The etiology of an increased NT has not been elucidated thus far. An increased NT is associated with structural chromosomal abnormalities in the fetus and indicates further invasive diagnostic testing such as chorionic villi sampling or amniocentesis. However, when karyotype is normal after follow-up testing, the fetus is still at risk for structural anomalies and/or genetic syndromes. Although an increased NT, as part of first trimester combined screening, has been replaced by non-invasive prenatal testing (NIPT) in the Netherlands, a 13-week ultrasound scan will be implemented in the Dutch national prenatal screening program from September 2021 onwards. This 13-week ultrasound scan includes a NT measurement. This emphasizes the importance of research on (increased) NT.

Chapter 1 gives an overview of the origin of NT, its suggested etiology and sets out the aim of this thesis, which was to: 1) gain more insights into the etiology of the increased NT and 2) collect more clinical data on the association of an increased NT and postnatal outcome. Studies were performed to assess the contributing factors to lymphatic (mal) development and specifically to analyze the role of neural crest cell migration in the development of lymphatic endothelial cell differentiation and nuchal edema. Additionally, the clinical outcome of fetuses with an increased NT was assessed. In more detail, the diagnostic yield of prenatal microarray was explored as well as identifying specific copy number variations that could contribute to this yield. New genes that are involved in NT are described and last, the association between an increased NT and RASopathies was researched.

The most suggested theory on the development of nuchal edema has been abnormal lymphatic development. Lymphatic development starts with forming jugular lymph sacs (JLS) from the internal jugular veins. A disturbed differentiation of lymphatic endothelial cells (LEC) leads to distension of JLS and nuchal edema. In **Chapter 2** the contributing factors to lymphatic (mal)development and the role of neural crest cell migration in the development of lymphatic endothelial cell differentiation and nuchal edema were explored by examining mouse embryos. The study showed that neural crest cells (NCCs) were observed in the JLS and LEC, therefore suggesting involvement of neural crest cells in the formation of JLS and LEC and accordingly in nuchal edema development. The involvement of NCCs in lymphatic development and subsequently nuchal edema may also explain the variety of structural malformations in fetuses with increased NT,

because cardiovascular defects, craniofacial malformations and skeletal anomalies can all be related to disturbances in NCC migration or differentiation. Additionally, it was shown that blocking retinoic acid signaling, which is an active metabolite of vitamin A and important in nervous and lymphatic vascular development, caused aberrant lymphatic development (abnormal JLS) and nuchal edema in mouse embryos. Based on these findings, we conclude that neural crest cell disturbances can lead to lymphatic maldevelopment resulting in nuchal edema.

Invasive prenatal diagnostic testing through chorionic villi sampling or amniocentesis is indicated in a fetus with increased NT. When karyotype is normal after invasive diagnostic testing, prenatal microarray is performed to rule out submicroscopic deletions and/or duplications. **Chapter 3** describes the diagnostic yield (added value) of prenatal microarray to detect submicroscopic deletions and duplications in a cohort of 70 fetuses with isolated (without other structural anomalies on ultrasound scan) increased NT (≥3.5 mm). A 5% additional diagnostic yield was shown, which is in agreement with other studies (overall 4-7% diagnostic yield).

Prior studies on prenatal microarray in fetuses with increased NT did not mention any recurrent deletions or duplications. However, this has not been properly described in literature. Therefore in **Chapter 4** this was explored in more detail. Healthy unrelated adults (n=86) were compared for recurrent deletions and duplications with fetuses with increased NT (n=40), who had a normal postnatal outcome. No specific recurrent deletions or duplications were discovered in fetuses with an isolated increased NT and no differences exist between healthy unrelated adults and fetuses with isolated increased NT with regard to copy number variations that were found.

When karyotype and prenatal microarray are normal, a fetus with (isolated) increased NT is still at risk for structural anomalies and genetic syndromes. The list of associated syndromes is endless, but thus far only Noonan syndrome and its associated RASopathies have been evaluated systematically and proven to be of clinical significance in relation to increased NT. The RASopathies are a clinically defined group of genetic disorders caused by germline pathogenic variants in genes that encode components or regulators of the Ras/MAPK pathway. In **Chapter 5 and 6** the association between an increased NT and RASopathies is further explored by describing two of the largest studies on prenatal RASopathies. We showed that the percentage of RASopathies in fetuses with an increased NT (isolated and non-isolated) is approximately 9% (**Chapter 6**). The detection chance is however, highly dependent on the thickness of the NT; pathogenic variant positive fetuses showed a (normally distributed) mean NT of 8.46 mm and pathogenic variant negative samples showed a (non-normal distributed) median NT of 4.7 mm.

Based on these findings described in **Chapter 6** we formulated new recommendations when to test for RASopathies. We recommend offering RASopathy testing in fetuses with an isolated increased NT of at least 5.0 mm or more. When the NT is between 3.5-5.0 mm the pathogenic variant detection for fetuses with an isolated increased NT is low (in our study 0%) and it is therefore recommended only to test for RASopathies in case of an increased NT below 5.0 mm when at least one of the following ultrasound anomalies is present as well: (distended) jugular lymph sacs, hydrops, pleural effusion, ascites, cardiac defects, renal anomalies or polyhydramnios. These ultrasound anomalies are quite specific for Noonan syndrome and its related disorders in combination with an increased NT.

In **Chapter 7** a consanguineous family with recurrent pregnancies with severely increased NT, fetal akinesia and joint contractures is described. Chromosomal microarray showed a large homozygous region on chromosome 19 on which the *RYR1* gene is located. Sequencing this gene revealed a homozygous nonsense variant in all seven fetuses and parents were heterozygous carriers. Histopathological studies also showed structural abnormalities in the *RYR1*-mutant skeletal muscle. This study showed the involvement of *RYR1* variants in increased NT/fetal hydrops adding to the long list of seemingly associated syndromes in fetuses with an increased NT.

Chapter 8 summarizes and discusses all findings of the conducted studies in context of the existing literature and future perspectives. In conclusion, the expanding importance of an increased NT in associated structural anomalies over the years and its reduction in use and renewed interest in this, due to NIPT implementation and 13-week ultrasound scan respectively, show the complexity of (dealing with) this ultrasound anomaly. The diversity in which congenital structural anomalies and genetic syndromes occur, suggests that a single explanation for the development of nuchal edema is unlikely. New recommendations are proposed how to deal with a fetus with an increased NT. However, there are still answers to be found, especially on the etiology of an isolated and temporarily increased NT. In the coming years, the more widely use of newly discovered techniques such as prenatal whole exome sequencing (WES) for syndromal diagnosis in prenatal anomalies might give us new information on how to interpret an increased NT.

Nederlandse samenvatting

De nuchal translucency (NT), ook wel de nekplooi genoemd, verwijst naar het doorschijnende ('translucente') gebied in de nekregio van de foetus in het eerste trimester van de zwangerschap. De NT kan worden gevisualiseerd door echografie tussen de 11de en 14de week van de zwangerschap en deze verdwijnt normaliter daarna. Een verdikte NT bestaat uit vocht (nekoedeem) en wordt gedefinieerd als gelijk aan of groter dan 3,5 mm (99e percentiel) volgens de richtlijnen van de Nederlandse Vereniging voor Obstetrie en Gynaecologie. Een verdikte NT komt voor bij 1% van alle zwangerschappen. De etiologie van een verdikte NT is tot nu toe niet volledig opgehelderd. Als de NT verdikt is, bestaat er echter een verhoogde kans op structurele chromosomale afwijkingen bij de foetus en is er een indicatie voor invasieve prenatale diagnostiek door middel van een vlokkentest of vruchtwaterpunctie. Wanneer het chromosomenpatroon (karyotype) normaal is, bestaat er nog steeds een verhoogde kans op structurele afwijkingen en/of genetische syndromen bij de foetus. Hoewel een verdikte NT, als onderdeel van de combinatietest in het eerste trimester van de zwangerschap voor de screening op down-, edwards- en patausyndroom, in Nederland inmiddels is vervangen door de niet-invasieve prenatale test (NIPT), is vanaf september 2021 een 13 wekenecho ingevoerd in het landelijke prenatale screeningsprogramma. Binnen deze 13 wekenecho is een meting van de NT opgenomen. Dit benadrukt het belang van onderzoek naar de (verdikte) NT.

Hoofdstuk 1 geeft zowel een overzicht van de oorsprong als de mogelijke oorzaak van de NT en beschrijft tevens het doel van dit proefschrift. Het doel van dit proefschrift was om: 1) meer inzicht te krijgen in de etiologie van de verdikte NT, 2) meer klinische gegevens te verzamelen over de associatie van een verdikte NT en postnatale uitkomst. Er werden voor dit proefschrift verschillende studies, welke beschreven zijn in de verschillende hoofdstukken, uitgevoerd om te achterhalen welke factoren bijdragen aan een abnormale ontwikkeling van het lymfesysteem. In het bijzonder werd de rol van neurale lijstcellen bij de ontwikkeling van lymfatische endotheelceldifferentiatie en het ontstaan van nekoedeem bestudeerd. Daarnaast werd het klinisch beeld van de foetus met een verdikte NT onderzocht, waarbij zowel gekeken werd naar de diagnostische opbrengst van een prenatale microarray als naar het belang van verschillende veel in de bevolking voorkomende submicroscopische chromosoomveranderingen (copy number variations, CNVs). Tevens zijn er studies verricht waarbij nieuwe genen die betrokken zijn bij een verdikte NT, worden beschreven en werd de associatie tussen een verdikte NT en RASopathieën (Noonan syndroom en daaraan gerelateerde aandoeningen) onderzocht.

De theorie over de ontwikkeling van nekoedeem, die het meest aangehangen wordt, is abnormale lymfatische ontwikkeling. Lymfatische ontwikkeling begint met het vormen van jugulaire lymfezakken (JLS) uit de interne halsaderen (vena jugularis). Een verstoorde differentiatie van lymfatische endotheelcellen (LEC) leidt tot uitzetting van

JLS en het ontstaan van nekoedeem. In **Hoofdstuk 2** werden de factoren, die bijdragen aan een abnormale lymfatische ontwikkeling en de rol van de neurale lijstcel migratie bij het ontstaan van lymfatische endotheelcel differentiatie en het ontstaan van nekoedeem onderzocht in muisembryo's. De studie toonde aan dat neurale lijstcellen (NCC's) werden waargenomen in de JLS en LEC, wat suggereert dat neurale lijstcellen betrokken zijn bij de vorming van JLS en LEC en vervolgens bij de ontwikkeling van nekoedeem. De betrokkenheid van NCC's bij de lymfatische ontwikkeling en vervolgens het ontstaan van nekoedeem kan ook de verscheidenheid aan structurele afwijkingen bij foetussen met verdikte NT verklaren, omdat cardiovasculaire defecten, craniofaciale afwijkingen en skeletafwijkingen allemaal (gedeeltelijk) gerelateerd zijn aan verstoringen in NCC-migratie of -differentiatie. Bovendien werd aangetoond dat het blokkeren van de retinolzuur synthese (een actieve metaboliet van vitamine A en belangrijk bij de ontwikkeling van het zenuwstelsel en lymfatische bloedvaten) een abnormale lymfatische ontwikkeling (abnormale JLS) en nekoedeem tot gevolg had bij muizenembryo's. Op basis van deze bevindingen concluderen we dat verstoringen van de neurale lijstcellen kunnen leiden tot een abnormale lymfatische ontwikkeling, resulterend in nekoedeem.

Invasieve prenatale diagnostiek door middel van een vlokkentest of vruchtwaterpunctie is geïndiceerd bij een foetus met verdikte NT (≥3,5mm). Wanneer het karyotype vervolgens normaal is, wordt een prenatale microarray uitgevoerd om submicroscopische deleties en/of duplicaties uit te sluiten. **Hoofdstuk 3** beschrijft de diagnostische opbrengst (toegevoegde waarde) van prenatale microarray om submicroscopische deleties en duplicaties te detecteren in een cohort van 70 foetussen met geïsoleerde (dus zonder andere structurele afwijkingen op de echo) verdikte NT. De toegevoegde diagnostische ‚waarde bovenop karyotypering in deze studie is 5%, wat in overeenstemming is met andere onderzoeken (totaal 4-7% diagnostisch opbrengst).

Tot op heden is het onbekend of er specifieke steeds terugkerende, submicroscopische deleties of duplicaties voorkomen bij foetussen met een verdikte NT. Daarom is dit in **hoofdstuk 4** nader onderzocht. Bij foetussen met verdikte NT (n=40), van wie het bekend was dat ze gezond en zonder problemen geboren zijn, werden alle met prenatale microarray gevonden deleties en duplicaties vergeleken met die van gezonde niet-verwante volwassenen (n=86). Deze specifieke deleties en duplicaties zijn niet-ziekteveroorzakende chromosoomveranderingen, die veel in de algemene bevolking voorkomen (veel voorkomende of ook wel common copy number variations, CNVs) en over het algemeen geen fenotype geven. De onderzoeksvraag was of er specifieke CNVs waren die vaker voorkomen bij foetussen met een verdikte NT en waarin mogelijk genen liggen die betrokken zijn bij de ontwikkeling van het lymfatische systeem. Uit de studie kwam naar voren dat er geen specifieke terugkerende deleties of duplicaties zijn bij foe-

tussen met een geïsoleerde verdikte NT. Tevens zijn er geen verschillen tussen foetussen met geïsoleerde verdikte NT en gezonde niet-verwante volwassenen met betrekking tot deze CNVs.

Wanneer het karyotype en de prenatale microarray normaal zijn, bestaat er voor een foetus met (geïsoleerde) verdikte NT nog steeds een verhoogde kans op structurele afwijkingen en/of genetische syndromen. De lijst van geassocieerde syndromen is lang, maar de meest publicaties betreffen case reports. Tot nu toe zijn alleen het Noonansyndroom en de bijbehorende RASopathieën systematisch geëvalueerd. De RASopathieën zijn een klinisch gedefinieerde groep genetische aandoeningen die worden veroorzaakt door pathogene varianten in genen die coderen voor componenten of regulatoire elementen van de Ras/MAPK-pathway. In **Hoofdstuk 5 en 6** wordt de associatie tussen een verdikte NT en RASopathieën verder onderzocht door twee van de grootste studies over prenatale RASopathieën te beschrijven. We toonden aan dat het percentage RASopathieën bij foetussen met een verdikte NT (geïsoleerd en niet-geïsoleerd) ongeveer 9% is (**Hoofdstuk 6**). De detectiekans is echter sterk afhankelijk van de dikte van de NT; foetussen met een pathogene variant vertoonden een (normaal verdeelde) gemiddelde NT van 8,46 mm en foetussen met een verdikte NT zonder pathogene variant in een van de RASopathie-genen vertoonden een (niet-normaal verdeelde) mediane NT van 4,7 mm. Op basis van deze bevindingen hebben we nieuwe aanbevelingen geformuleerd voor het testen op RASopathieën bij foetussen met een verdikte NT. We raden aan om te testen op RASopathien bij foetussen met een geïsoleerde verdikte NT van ten minste 5,0 mm of meer. Wanneer de NT tussen 3,5-5,0 mm ligt, is de detectie van pathogene varianten voor foetussen met een geïsoleerde verdikte NT laag (in onze studie zelfs 0%). Daarom wordt aanbevolen om bij een foetus met een verdikte NT<5,0 mm alleen op RASopathieën te testen als minimaal één van de volgende echo-afwijkingen ook aanwezig is: (opgezette) jugulaire lymfezakken (JLS), hydrops, pleurale effusie, ascites, hartafwijkingen, nierafwijkingen of polyhydramnion. Deze echoafwijkingen zijn vrij specifiek voor de RASopathieen wanneer deze gezien worden in combinatie met een verdikte NT.

In **Hoofdstuk 7** wordt een bloedverwant (consanguin) echtpaar beschreven met meerdere zwangerschappen met een ernstig verdikte NT, foetale akinesie en gewrichtscontracturen. Chromosomale prenatale microarray toonde een groot homozygoot gebied op chromosoom 19 waarop het *RYR1*-gen zich bevindt. Sequentiebepaling van dit gen toonde een homozygote nonsense variant bij alle zeven foetussen. De ouders waren heterozygote dragers. Histopathologische studies toonden ook structurele afwijkingen in door *RYR1*-gemuteerde skeletspier aan. Deze studie toonde de betrokkenheid van *RYR1*-genvarianten bij verdikte NT/foetale hydrops, welke toegevoegd kan worden aan de lange lijst van mogelijk geassocieerde syndromen bij foetussen met een verdikte NT.

Hoofdstuk 8 vat alle bevindingen van de uitgevoerde onderzoeken samen en bespreekt deze in de context van de bestaande literatuur en toekomstperspectieven. De relevantie van een verdikte NT door de jaren heen, vervolgens de daling in gebruik en recent de hernieuwde belangstelling hiervoor als gevolg van respectievelijk de implementatie van NIPT en de 13weken echo, toont de complexiteit van (het omgaan met) deze echoafwijking. De diversiteit waarin aangeboren structurele afwijkingen en genetische aandoeningen voorkomen, suggereert dat één enkele verklaring voor het ontstaan van nekoedeem onwaarschijnlijk is. Er worden nieuwe aanbevelingen gedaan hoe om te gaan met een foetus met een verdikte NT. Er zijn echter nog steeds vragen te beantwoorden, vooral over de etiologie van een geïsoleerde verdikte NT. In de komende jaren kan het breder gebruik van nieuwe technieken zoals prenatale whole exome sequencing (WES) ons nieuwe informatie opleveren over hoe om te gaan met een verdikte NT.

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Curriculum vitae

Kyra Eva Stuurman werd geboren op 6 november 1978 te Tilburg. Na het gymnasium gevolgd te hebben aan het Mill Hill College te Goirle, begon zij, na een tussenstudie Medische Biologie, in 1999 met de studie Geneeskunde aan de Universiteit van Amsterdam te Amsterdam. Verschillende stages tijdens haar studie werden in het buitenland gelopen, zoals een verpleegstage in Malta. Haar wetenschappelijke stage verrichte zij in Toronto, Canada in 2003, alwaar zij onderzoek deed naar de lange termijn gevolgen van Langerhans Cell Histiocytose in kinderen. Nadat zij terugkwam uit Toronto en voordat zij begon met haar coschappen vertrok zij samen met een groep artsen vanuit de organisatie Medical Checks for Children onder leiding van Dr. Ines von Rosenstiel naar Nepal om bij kinderen (in weeshuizen) gezondheidschecks uit te voeren. Tegen het einde van haar coschappen in 2006 volgde zij zowel een keuze-coschap Klinische genetica in het AMC te Amsterdam als een keuze-coschap Tropische geneeskunde in Malawi. Tijdens het keuze-coschap Klinische genetica werd haar interesse voor dit vak gewekt en na een uitstap naar de farmaceutische industrie, begon zij als arts-assistent niet in opleiding (ANIOS) Klinische genetica in het AMC te Amsterdam. Zij volgde vervolgens haar opleiding tot klinisch geneticus in het VU medisch centrum te Amsterdam (opleider prof.dr. Hanne Meijers-Heijboer) en in BC Women's and Children's Hospital te Vancouver, Canada (opleider prof. Sylvie Langlois) en rondde de opleiding in 2016 af. Sindsdien werkt zij als klinisch geneticus in het Erasmus MC te Rotterdam. Gedurende haar opleiding tot klinisch geneticus werd, onder leiding van prof.dr. Hanne Meijers-Heijboer, reeds gestart met de verschillende studies, die beschreven staan in dit proefschrift. De afronding van het proefschrift vond plaats onder leiding van prof.dr. Lidewij Henneman en dr. Mariet Elting. Kyra woont met haar man Patrick en hun kinderen Noah, Lillooet en Max in Rotterdam.

Acknowlegdements

‘Een proefschrift schrijf je nooit alleen’, is een gevleugelde uitspraak. In de 12 jaar die de totstandkoming van dit proefschrift geduurd heeft, heeft het vaak wel zo gevoeld. Maar je schrijft een proefschrift inderdaad niet alleen, want dan komt er niet veel van terecht en duurt het dus heel lang :-). Goede begeleiding en support (en vooruit, een grote dosis doorzettingsvermogen) is key in het succesvol afronden van een proefschrift. Gelukkig is het hiermee goed gekomen en ik wil dan ook een aantal mensen bedanken, die hebben bijgedragen aan dit uiteindelijke resultaat.

Prof.dr. Hanne Meijers-Heijboer, beste Hanne, dank voor je vertrouwen in mij en het vrijlaten om het proefschrift in te vullen zoals ik dat voor me zag. Ik heb veel van je geleerd. Het is een mooi boekje geworden en ik hoop oprecht dat je het te zien krijgt.

Prof.dr. Lidewij Henneman, beste Lidewij, jouw bezielende begeleiding, optimisme en enthousiasme zijn werkelijk fenomenaal. Ik ken niemand die zo snel zo to the point aanvullingen geeft op mijn stukken. Het is erg prettig samenwerken met jou en ik ben ontzettend blij dat jij toegevoegd bent als een van mijn promotoren. Door jou is het proefschrift afgerond.

Dr. Mariet Elting, beste Mariet, tijdens mijn opleiding tot klinisch geneticus was je een voorbeeld voor me. Jouw kennis en prettige persoonlijkheid maken je een fantastisch klinisch geneticus. Dank voor je bijdrage in de laatste fase van het proefschrift.

Leden van de promotiecommissie, prof.dr. Cornel, prof.dr. Bilardo, prof.dr. Oostra, dr. Haak, dr. Galjaard en dr. Srebniak, dank voor het lezen en beoordelen van het proefschrift. Dat is erg gewaardeerd. Fijn dat jullie zitting willen nemen in de verdediging hiervan.

Natuurlijk wil ik tevens *alle patiënten*, die toestemming gaven voor de verschillende studies bedanken. In het bijzonder dank ik Mascha en haar prachtige gezin, die bijzonder geïnteresseerd waren vanuit hun eigen ervaring met een zwangerschap waarin een verdikte NT werd vastgesteld. Dank voor jullie vertrouwen in mij als (toen beginnende) dokter.

Daarnaast wil ik iedereen die meegeschreven heeft of anderszins heeft bijgedragen aan de verschillende studies danken voor de samenwerking. Zonder jullie geen publicaties. In het bijzonder veel dank aan Shama en Tuula voor de prettige samenwerking.

Drs. Shama Bhola, beste Shama, dank voor de fijne samenwerking door de jaren heen en bijdragen aan verschillende artikelen in dit proefschrift. Ik vind het ontzettend leuk dat we nog steeds contact hebben. Laten we dat nog lang voortzetten.

Dr. Tuula Rinne, beste Tuula, het was leuk om met jou samen te werken. We hebben toch maar een mooie paper samen neergezet.

Mijn paranimfen, Margot en Marieke, mijn kamergenoten. Het was niet meer dan logisch om jullie te vragen als mijn paranimfen en ik ben vereerd dat jullie naast me gaan staan op 15 december. Ik kan met een gerust gevoel de vragen aan jullie overdragen als ik het zelf niet weet :-). Margot, vanaf de eerste maand dat we samenwerkten tot het einde van mijn contract in het VUmc waren we kamergenoten. Lief en leed hebben we gedeeld, onze kinderen zijn even oud en de potten thee (en koekjes) niet aan te slepen. Marieke, je hebt Margots taak overgenomen, luisteren naar al mijn gezwets. Al bijna 5 jaar ben je mijn kamergenoot en je hebt me echt door het eerste moeilijke jaar heen getrokken. Jouw praktische houding heeft me al vaak geholpen. Hopelijk zitten we nog lang op dezelfde kamer.

Collega's van de afdeling Klinische genetica in het Erasmus MC, met name Yolande en Sarina, dank voor de mogelijkheid om het proefschrift tot een goed einde te brengen en de fijne tussendoormomenten om even stoom af te blazen.

Vrienden, family-in-law en familie, fijn dat jullie in mijn leven zijn. Phillis, dank voor de volgende zet. Zonder onze wandeling in Noordwijk had het proefschrift nog steeds als zwaard van Damocles boven mijn hoofd gehangen. Barrie and Sue, thank you for always showing support and interest in my work. I know it is not easy having one of your children on the other side of the world, but you have always made me feel welcome into your home and family. Did I loose one of my strikes now?

Mijn broers, Jonas en Hugo, ik geloof niet dat jullie ook maar enig idee hebben wat ik gedaan heb tijdens mijn onderzoek of zelfs maar wat ik in het dagelijks leven doe, maar ik vergeef het jullie :-). Het is gezellig als we samen zijn (veel te weinig) en ik weet dat ik altijd op jullie kan rekenen.

Mijn ouders, pap en mam, zonder jullie steun en liefde was ik niet gekomen waar ik nu ben. Dank voor alle mogelijkheden die jullie me gegeven hebben in het leven. Jullie hebben me vrij gelaten om het leven zelf te ervaren, bijgestuurd waar nodig en me laten opgroeien tot een wereldburger. Pap, wat zou je trots geweest zijn en wat ontzettend jammer dat je dit moment niet kan meemaken.

Patrick, my love, the one who knows me best, who stimulates me and who makes me feel comfortable. Your love and support means everything to me and I know I can always count on you. You are the only one who laughs about my jokes, even the kids don't do

that. After all these years together I have learnt one thing: it doesn't matter where we are in the world, as long as we are together all is fine. *Noah*, mijn gevoelige, slimme, oudste zoon, *Lillooet*, mijn prachtige, geestige, enige dochter en *Max*, mijn zonnestraaltje, mijn jongste zoon die veel te snel groot wordt, dank voor jullie liefde en aanwezigheid. Jullie maken alles goed in de wereld.