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ARTICLE

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

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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.
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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.[1–3] 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.[4,5] 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*.[6–13] Several of these genes are also involved in Cardio-Facio-Cutaneous (CFC) syndrome and Costello syndrome.[14–16] 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.[17–19]

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.[20–24] 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.[25] Lee *et al*[26] 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*.[27]

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.

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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.[24,27] 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 ($n=50$; 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.

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*[24] and cases 9, 12 and 13 have been described by Houweling *et al*.[27] 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)),[28] c.182A>G (p.(Asp61Gly)),[4] c.184T>G (p.(Tyr62Asp)),[5] c.205G>C (p.(Glu69Gln)),[28] c,417G>C (p.(Glu139Asp)),[5] and c.854T>C (p.(Phe285Ser)),[5] *RAF1* c.770C>T (p.(Ser257Leu)),[10] and *KRAS* c.173C>T (p.(Thr58Ile)).[6] One mutation has been previously described in a patient with the clinical diagnosis of LEOPARD syndrome: *PTPN11* (c.1381G>A, p.(Ala461Thr)).[29] The remaining three mutations had not been described in literature before the detection in this study: *RAF1* (c.775T>C, p.(Ser259Pro)), *PTPN11* (c.181G>C,

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]

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

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.[30] 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.[9]

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.

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.

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* ([5]), 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 ($\sim$100 for *RAF1*, $\sim$150 for *BRAF* and *MAP2K1*, and $\sim$1700 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.[31]

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*,[27] 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*[26] 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%.[27] The lower-positive test rate reported by Lee *et al*[26] 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, hydrothorax, 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*[24] 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

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, malalignment 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
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

Table 2 (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])*
Renal anomalies		No				No
Other features	Redundant nuchal skin, generalized skin edema	Severe hydrops, brachydactyly, virilized genital		Severe hydrops clubfeet		Hygroma colli
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	No
Distended JLS	No	Yes	Yes (7.1 × 8.7 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
Cardiac 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	Yes 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
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
Cardiac anomalies	No	Hypertrophy			Hypertrophy interventricular septum	Complete AVSD
Renal anomalies	No	Bilateral pyelectasis				
Other features	Severe skin edema	Nuchal edema			Loose nuchal skin	

Abbreviations: AVSD, atrio ventricular septal defect; GA, gestational age; HLH, hypoplastic left heart; JLS, jugular lymphatic 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.

et al[32] found most frequently polyhydramnios (38.3%) and increased NT (41%). The two most commonly reported indications for prenatal testing reported by Lee *et al*[26] were increased NT (44%) and cystic hygroma (48%). 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 worthwile.

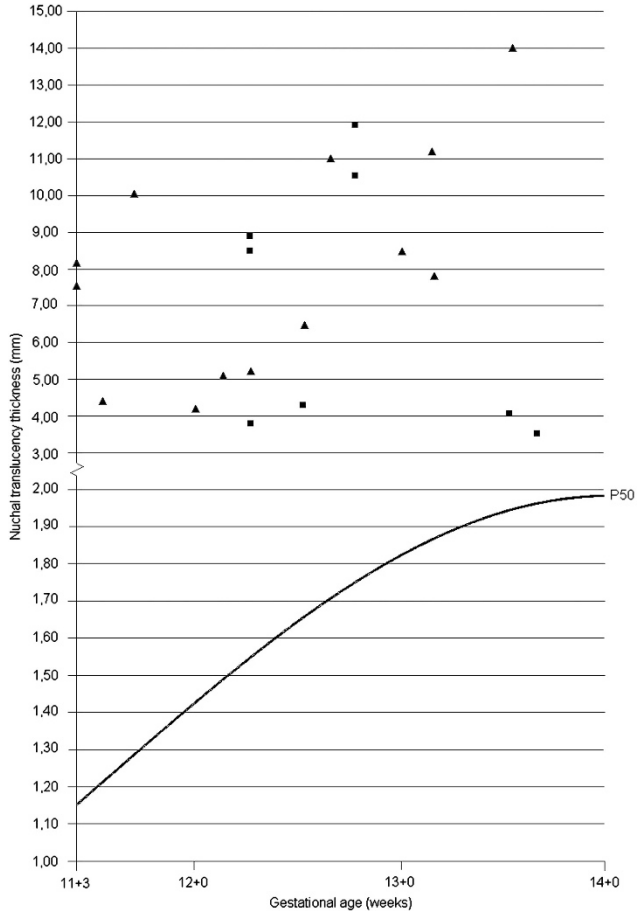

Figure 1 NT values of mutation-positive cases ($n=13$) and mutation-negative cases ($n=8$) from the diagnostic study group, according to GA. Only those cases for which NT values between 11–14 weeks of gestation were available, are shown. Mutation-positive cases are depicted by gray dots; mutation-negative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

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

	Total group		*Mutation-positive group*		*Mutation-negative group*		
Prenatal findings	*(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
Cardiac 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

Abbreviations: DNA, deoxyribonucleic acid; JLS, jugular lymphatic sacs; NT, nuchal translucency.
[a]Fisher's Exact test.

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,[33] 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%).[1,34–36] Also Menashe *et al*[22] 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.[37]

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 $\sim 2\,\mu g$ 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.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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