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“Everything you can imagine is real.”

Pablo Picasso

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals (I–VI), and previous unpublished data:

I. Muru K, Sitska M, Asser K, Ehrenberg A, Karro H, Õunap K, Reimand T. Prospective experience with contingent screening strategy for Down syndrome in Estonia. J Community Genet 2010; 1(3):133–138.

II. Muru K, Vals M-A, Sitska M, Asser K, Tammur P, Zilina O, Reimand T, Õunap K. Outcome of Children with Marked Changes in Maternal Screening Tests and Normal Karyotype. Hereditary Genetics 2014; 3:123.

III. Sitska M, Reimand T, Muru K. Prenatal diagnosis of fetal chromosomal anomalies: a summary of trimester II serum screening in Estonia (in Estonian). Eesti Arst 2008; 87(1):31−36.

IV. Muru K, Õunap K, Virro S, Kalev I. Genetic basis of congenital heart defects (in Estonian) Eesti Arst 2008; 87(5):357−366.

V. Kalev I, Muru K, Teek R, Zordania R, Reimand T, Köbas K, Õunap K. LEOPARD syndrome with recurrent *PTPN11* mutation Y279C and different cutaneous manifestations: two case reports and a review of the literature. Eur J Pediatr 2010; 169(4):469–473.

VI. Muru K, Kalev I, Teek R, Sõnajalg M, Kuuse K, Reimand T, Õunap K. A Boy with Holt-Oram Syndrome Caused by Novel Mutation c.1304delT in the *TBX5* Gene. Mol Syndromol 2011; 1(6):307–310

My contributions to the original articles are:

Publication I: study design, data analysis, writing the paper

Publication II: study design, data analysis, clinical evaluation of patients, data and sample collection, writing/co-writing the paper

Publication III: data collection, reviewing the manuscript

Publication IV: reviewing literature, writing the paper

Publication V: clinical investigation of patient 2, data and sample collection, writing/co-writing the paper

Publication VI: clinical investigation of patient, data and sample collection, writing the paper

ABBREVIATIONS

AC	amniocentesis
AD	autosomal dominant
AFP	α-fetoprotein
ANF	natriuretic peptide precursor A
APEX	arrayed primer extension
AS	aortic stenosis
ASD	atrial septal defect
AVSD	atrioventricular septal defect
BAV	bicuspid aortic valve
bp	base pair
CAH	congenital adrenal hyperplasia
cfDNA	cell free deoxyribonucleic acid
CHD	congenital heart defect
CLS	*café-au-lait* spots
CMA	chromosomal microarray analysis
CoA	coarctation of aorta
CRL	crown-rump length
CVS	chorionic villus sampling
DNA	deoxyribonucleic acid
DORV	double-outlet right ventricle
DR	detection rate
DS	Down syndrome (trisomy 21)
d-TGA	transposition of the great arteries
DV	ductus venosus
ECG	electrocardiography
ECHO	echocardiogram
ERK	extracellular signal-regulated kinases
fb-HCG	free β-human chorionic gonadotropin
FISH	fluorescence in situ hybridization
FMF	Fetal Medicine Foundation
FPR	false positive rate
HCG	human chorionic gonadotropin
HCM	hypertrophic cardiomyopathy
HGNC	HUGO Gene Nomenclature Committee
HLHS	hypoplastic left heart syndrome
HOS	Holt-Oram syndrome
HR-CGH	high-resolution comparative genomic hybridization
HRH	hypoplastic right heart
ISCN	International System for Human Cytogenetic Nomenclature
ISPD	International Society for Prenatal Diagnosis
IUGR	intrauterine growth restriction
LBW	low birth weight

LGA	large for gestational age
LS	LEOPARD syndrome
MAPK	mitogen-activated protein kinases
MI	mitral insufficiency
ML	multiple lentigines
MLPA	multiplex ligation-dependent probe amplification
MoM	multiples of median
NBL	nasal bone length
NF	nuchal skinfold
NF1	neurofibromatosis 1
NS	Noonan syndrome
NT	nuchal translucency
PA	pulmonary atresia
PAPP-A	pregnancy-associated plasma protein -A
PCR	polymerase chain reaction
PnD	prenatal diagnosis
PDA	persistent truncus arteriosus
PIH	pregnancy induced hypertension
PnS	prenatal screening
PS	pulmonary stenosis
SGA	small for gestational age
SNP	single nucleotide polymorphism
SRS	Silver-Russell syndrome
SV	single ventricle
TAPVC	total anomalous pulmonary venous connection
TCR	tricuspid regurgitation
TF	transcription factor
TOF	tetralogy of Fallot
uE3	unconjugated oestriol
US	ultrasound
VSD	ventricular septal defect

Nomenclature of genes in text

ABCC9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9 (HGNC_ID: 60)
BMPR1A	bone morphogenetic protein receptor, type IA (HGNC_ID: 1076)
BRAF	v-raf murine sarcoma viral oncogene homolog B (HGNC_ID: 1097)
CFC1	cripto, FRL-1, cryptic family 1 (HGNC_ID: 18292)
CHD7	chromodomain helicase DNA binding protein 7 (HGNC_ID: 20626)
CRELD1	cysteine-rich with EGF-like domains 1 (HGNC_ID: 14630)
CYP21A2	cytochrome P450, family 21, subfamily A, polypeptide 2 (HGNC_ID: 2600)
DHCR7	7-dehydrocholesterol reductase (HGNC_ID: 2860)
EVC	Ellis van Creveld syndrome (HGNC_ID: 3497)
FBN1	fibrillin 1 (HGNC_ID: 3603)
GATA4	GATA binding protein 4 (HGNC_ID: 4173)
HRAS	Harvey rat sarcoma viral oncogene homolog (HGNC_ID: 5173)
JAG1	jagged 1 (HGNC_ID: 6188)
KDM6A	lysine (K)-specific demethylase 6A (HGNC_ID: 12637)
KMT2D	lysine (K)-specific methyltransferase 2D (HGNC_ID: 7133)
KRAS	Kirsten rat sarcoma viral oncogene homolog (HGNC_ID: 6407)
MAP2K1	mitogen-activated protein kinase kinase 1 (HGNC_ID: 6840)
MAP2K2	mitogen-activated protein kinase kinase 2 (HGNC_ID: 6842)
MYH11	myosin, heavy chain 11, smooth muscle (HGNC_ID: 7569)
MYH6	myosin, heavy chain 6, cardiac muscle, alpha (HGNC_ID: 7576)
NF1	neurofibromin 1 (HGNC_ID: 7765)
NKX2-5	NK2 homeobox 5 (HGNC_ID: 2488)
NOTCH1	notch 1 (HGNC_ID: 7881)
NOTCH2	notch 2 (HGNC_ID: 7882)
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog (HGNC_ID: 7989)
PTEN	phosphatase and tensin homolog (HGNC_ID: 9588)
PTPN11	protein tyrosine phosphatase, non-receptor type 11 (HGNC_ID: 9644)
RAF1	v-raf-1 murine leukemia viral oncogene homolog 1 (HGNC_ID: 9829)
SHOC2	soc-2 suppressor of clear homolog (C. elegans) (HGNC_ID: 15454)
SMAD4	SMAD family member 4 (HGNC_ID: 6770)
SMN1	survival of motor neuron 1, telomeric (HGNC_ID: 11117)
SOS1	son of sevenless homolog 1 (Drosophila) (HGNC_ID: 11187)
ZIC3	Zic family member 3 (HGNC_ID: 12874)
TBX5	T-box 5 (HGNC_ID: 11604)

Glossary of used terms

Screening method	Description of the method
First trimester ultrasound screening	Ultrasound screening at first trimester usually comprise an ultrasound measurement of the width of an area of translucency at the back of the fetal neck – nuchal translucency; additionally there may be measurement of nasal bone length, fronto-nasal angel, tricuspid regurgitation etc.
First trimester serum screening	Risk calculation is usually based on the measurements of PAPP-A and fb-HCG together with maternal age
Second trimester serum screening	Risk calculation is usually based on the measurements of AFP, HCG, uE3 (triple test) and inhibin-A (quadruple test) together with maternal age
Combined test	Risk calculation is based on combining first trimester markers: NT and measurements of PAPP-A and fb-HCG together with maternal age
Integrated test	Risk calculation is based on the integration of measurements performed at the different times of pregnancy into a single test result. Different screenings integrate different markers. In Estonia it is usually integrated first trimester US marker – NT and second trimester serum markers (AFP, HCG, uE3)
Sequential screening	Screening in which a first-trimester test is performed (for example, the Combined test) and the result interpreted immediately. If it is positive, a diagnostic test is offered, but if it is negative, in second trimester serum markers are measured and the first-trimester markers (all or some) reused to form an Integrated test
Contingent screening	Screening in which a first-trimester test is used to triage the population of women screened into three groups: one group (high risk screen-positive) that is immediately offered a diagnostic test, a second group (screen-negative) that receives no future screening, and a third intermediate (or lower risk screen positive) group that has second-trimester markers measured and (all or some) first trimester measurements are reused to form an Integrated test.

1. INTRODUCTION

The prevalence of chromosomal aneuploidies and structural defects varies at different stages of pregnancy, from approximately 20% of pre-implantation embryos to 0.2–0.93% of fetuses at term. Most of these are incidental mistakes [Baird *et al.,* 1988; ESHRE 2008; Simpson 1990]. Trisomy 21 (Down syndrome, DS) is shown to be the most frequent chromosomal anomaly, with an incidence of 1 in 729 live born [Benn 2010]. Therefore, the development of prenatal screening and diagnostics is very important in clinical practice for detecting chromosomal anomalies as early as possible. The association between maternal age and the risk of having a DS pregnancy was first published in 1933 [Wald *et al.,* 1997]. Prenatal diagnosis (PnD) become feasible for women with a high risk of fetal chromosomal disorders from the early 1970s, after Steele and Breg had succeeded, in 1966, in culturing and karyotyping amniotic fluid cells [Elias 2010; Steele and Breg 1966].

Serum screening in the second trimester was introduced into routine clinical practice in the early 1990s [Haddow *et al.,* 1992]. Within a few years, first trimester markers, both ultrasound (US) and serum screening markers, came into use. First trimester combined screening is now the first choice in the routine care of pregnant women in many places. Nowadays already completely new screening tests are coming into clinical practice (e.g. cell free fetal DNA test) [Benn *et al.,* 2013].

In Estonia, prenatal screening (PnS) and PnD for fetal chromosomal disorders has become an essential part of the management of the pregnant woman. In 1990 Dr. Mari Sitska introduced PnD as a test for women with increased risk of fetal aneuploidy, and since 1995 PnD was implemented as PnS for women with high risk pregnancies (i.e. women aged above 35 years). Second trimester maternal serum screening (double or triple test) for women under 35 years of age was introduced at the end of 1998, and the first results were published in 2003 [Sitska *et al.,* 2003]. In 2005 the first clinical guidelines for prenatal diagnosis in Estonia were established (revised in 2008 and 2011) [Sitska 2008b]. First trimester US screening – nuchal translucency (NT) measurement has been available in some centres from 2001.

Birth prevalence of DS in Estonia has decreased with PnS and PnD from 1.17/1000 to 0.99/1000 from 1990 to 2005 [Reimand *et al.,* 2006a], and even more during recent years, but still every year a few babies with DS are born. In her thesis, Associate Prof. Tiia Reimand studied DS, its birth prevalence, its phenotype and associated medical problems and its influence to families' everyday life [Reimand *et al.,* 2006a; Reimand *et al.,* 2003; Reimand *et al.,* 2006b]. The results of her study are used in counselling families during the prenatal and postnatal counselling process.

Using second trimester screening protocol, the detection rate (DR) for DS is estimated to be about 60–70% (with a false positive rate (FPR) of 5%). With new screening protocols, the accepted minimum DR has risen to 75%

[Summers *et al.,* 2007], and therefore we needed to investigate new possibilities – toward first trimester screening and to find the most suitable protocol to use in Estonia.

Although the primary aim of the screening is to identify pregnancies at risk of aneuploidy, significant changes in markers may give insight into other adverse pregnancy outcomes, such as late pregnancy loss, preterm birth, intrauterine growth restriction, or may be markers of genetic disease [Dugoff and Society for Maternal-Fetal 2010; Gagnon *et al.,* 2008; Goetzl 2010; Miltoft *et al.,* 2012; Souka *et al.,* 2001; Summers *et al.,* 2003]. There are several studies about the long-term outcome of children with increased NT, showing that at the 2 years of age children didn´t have higher risk for developmental disorders [Miltoft *et al.,* 2012; Mula *et al.,* 2012; Senat *et al.,* 2007; Sotiriadis *et al.,* 2012]. At the same time there is nearly any information about of long-term outcomes of children with marked changes in maternal serum screening test, but mothers has been selected into high-risk group and they are worried about consequences about outcome. We had the aim to investigate long-term outcome of these children.

Increased NT, after exclusion of fetal chromosomal anomaly, is most often associated with fetal congenital heart defect (CHD) [Souka *et al.,* 1998]. CHD is the most common birth defect, affecting 3–13/1000 life births [van der Linde *et al.,* 2011]. CHD may by result in chromosomal abnormalities, or single-gene defect (e.g. Holt-Oram syndrome), or result in more complex mechanisms, possible involving interactions among different genes and other genetic and environmental modifications. Nevertheless, its clinical importance, the underlying genetic etiology of most CHDs remains unknown and is so-called "multifactorial". The understanding of fetal heart formation gives insight into the genetic etiology of CHD. Despite significant advances in the study of cardiac development, only a handful of human genes with mutations associated with CHD have been identified [Garg 2006].

Noonan syndrome (NS) is the most common non-chromosomal syndromic cause of CHD. NS is the eponymous name for the disorder described by pediatric cardiologist Jacqueline Noonan about 50 years ago. At the same time, a number of authors have suggested that the first reported patient with what is now called NS was a 20-year-old male with a webbed neck and several other typical features, reported by University of Tartu student Koblynski in 1883 [Mendez and Opitz 1985; Opitz and Pallister 1979].

For clinicians caring for children with CHD, it is very important to determine whether there is an underlying genetic pattern, because there may be other important organ system involvement, there may be prognostic information for clinical outcomes, there may be important genetic reproductive risks the family members should know about, and there may be other family members for whom genetic testing is appropriate [Pierpont *et al.,* 2007]. To the best of our knowledge, no previous studies have been performed to determine the genetic etiology of CHD in children in Estonia.

The aims of the study were to establish the first trimester screening for Down syndrome in Estonia, to evaluate the potential of the used contingent screening, and to investigate the long-term outcome of children born to mothers with marked changes in maternal first or second trimester serum markers and the first trimester ultrasound marker – NT. In addition, we specified the genetic causes of syndromic CHD and characterized some rare monogenic syndromes with CHD.

2. LITERATURE REVIEW

2.1. Prenatal screening and diagnosis

2.1.1. Epidemiology of aneuploidy

Aneuploidy is a common event in pregnancy, with a wide spectrum of medical consequences ranging from the lethal to the benign. The prevalence of chromosomal numerical abnormalities and structural defects varies at different stages of pregnancy. Many series of karyotyped miscarriages have confirmed that ~50% of all clinically recognized spontaneous miscarriages have a chromosome abnormality [ESHRE 2008; van den Berg *et al.,* 2012]. Of chromosomal aberrations, about 30% have trisomy, 10% are either triploid or tetraploid, 9% have a 45,X constitution, and 2% have a structural rearrangement [ESHRE 2008]. There are about 0.2–0.93% of fetuses with chromosomal aberrations at term [Baird *et al.,* 1988; ESHRE 2008; Simpson 1990].

The most frequent of aneuploidies which survives to term is DS, with a birth prevalence of 0.58–1.7 per 1000 live births [Hoshi *et al.,* 1999; Leonard *et al.,* 2000; Reimand *et al.,* 2006a; Rosch *et al.,* 2000]. DS is almost always the whole-chromosome trisomy, *i.e.* regular trisomy 21. It is usually the result of a nondisjunction in the first or the second stage of meiosis [Oliver *et al.,* 2008]. The most important risk factor for regular trisomy 21 is maternal age; birth prevalence increases rapidly with age, particularly after age 30 years [Hassold and Sherman 2000; Wald *et al.,* 1997]. DS is the most easily clinically recognized single chromosome abnormality, and it is also the most prevalent genetic cause of mental retardation in childhood. Consequently, DS is considered first and more extensive than both Edwards and Patau syndromes, which are respectively rare, and sex-chromosome aneuploidies, which are common but relatively benign.

2.1.2. Principles of prenatal screening

Screening tests are generally performed on healthy patients and are offered to the entire relevant population. They should therefore be cheap and easy to use and interpret; their sole function is to help define who is at higher risk for this condition in comparison to others. Screening is not just a test, but it should be part of the diagnostic system. The identification of risk cannot change outcomes unless an intervention follows and, conversely, specific interventions for higher risk individuals cannot happen without the widespread application of some initial test or inquiry to capture those at higher risk [Raffle and Gray 2009].

PnS is used to identify pregnancies at increased risk for certain birth defects or chromosomal disorders (predominantly risk for DS). PnS makes it possible to diagnose serious congenital anomalies and chromosomal disorders before a child's birth, which enables parents to make choices about the future.

PnS for aneuploidy has rapidly evolved, and PnS has now become an essential part of the modern management of pregnancy. Using second trimester screening protocol detection rate (DR) for DS is estimated to be about 60–70% (with false positive rate (FPR) 5%). With new screening protocols also the accepted minimum DR has been raised up to 75% [Summers *et al.,* 2007]. At the same time, PnS has become much more complicated due to the endeavour to improve detection rates at lower false positive rates [Mennuti and Driscoll 2003; Spencer 2007; Benn *et al.*, 2011].

PnS for DS combines the values of several screening markers (for example maternal age, α-fetoprotein (AFP), human chorionic gonadotropin (HCG) etc.). The risk calculations are given in odds (1:n); and are designated screen positive if the risk value exceeds a specified cut off (e.g. 1:270). PnS needs to be provided as an integrated service, counselling and diagnostic tests (an invasive procedure) should be offered to all women with positive screening results [Wald *et al.,* 1997].

Risk calculation depends on using a statistical model, and any model has limitations. Screening will miss some affected pregnancies (yielding false negative results) and will identify some unaffected pregnancies as screen positive (producing false positive results (FPR)). Statistically, the risk model will be specified by the means and standard deviations of the individual markers in affected and unaffected pregnancies and the correlation coefficients between all combinations. Determining the normal median values of the serum markers is a first step in risk estimation; it should be estimated from values obtained from all screened women. The concentration of each marker assayed for each woman is then divided by the normal median for women of the same gestational age in order to convert the concentration into a multiple of the normal median (MoM) [Wald *et al.,* 1997].

The performance of the screening test is measured by both the DR (the proportion of affected pregnancies with positive results) and the proportion of unaffected pregnancies with positive results (FPR).

2.1.3. Prenatal screening in the second trimester (Publication III)

In 1972 the association with raised AFP concentration in amniotic fluid and fetal neural tube defect was shown [Brock and Sutcliffe 1972]. In 1984 the first results on lower maternal AFP levels during ongoing DS pregnancy [Merkatz *et al.,* 1984] were published. Combining maternal age and maternal serum AFP level, we were able to identify about 35% of pregnancies with DS (with FPR 5%) [Cuckle and Wald 1987]. The higher level of HCG in second trimester serum markers during DS pregnancy compared with unaffected pregnancies was first published in 1987 [Bogart *et al.,* 1987]. Shortly thereafter, the third second trimester serum marker – unconjugated oestriol (uE3), proved to be about 25% lower in maternal serum during DS pregnancy [Canick *et al.,* 1988; Wald *et al.,* 1988a]. These three markers together with maternal age could

identify DS pregnancies with DR in about 60% (with FPR 5%) [Wald *et al.,* 1988b]. Second trimester serum screening (triple test) was introduced into routine practice in the early 1990s [Haddow *et al.,* 1992]. After adding a fourth second-trimester serum marker – dimeric inhibin A ("quadruple test") – the DR increased to 76% (with FPR 5%) [Van Lith *et al.,* 1992].

Many other different serum markers have been found to be associated with DS between 15 and 22 weeks of pregnancy: free beta HCG (fb-HCG), free alpha HCG (αHCG), seminal plasma protein 2 (SP2), carbohydrate antigen 125 (CA125), troponin, pregnancy-associated plasma protein – A (PAPP-A), placental growth factor (PGF) and the proform of eosinophil major basic protein (ProMBP) [Alldred *et al.,* 2012]. Some of these have also been introduced into clinical practice.

Serum screening (double/triple test) is widely accepted in Estonia; in 2006 about 91% pregnant women under the age of 37 were monitored [Sitska *et al.,* 2008a]. In the period 1999–2006 the DR of second trimester serum screening was 57.8%, with an FPR of 4.7% [Sitska *et al.,* 2008a].

US screening during the second trimester, the "genetic sonogram" has been used as a tool for aneuploidy screening [Breathnach *et al.,* 2007; Smith-Bindman *et al.,* 2001]. Up to 8 different markers have been included in risk estimation. A thickened nuchal fold is the most accurate marker, but a genetic sonogram in the second trimester should not be added to biochemical screening to detect fetuses with chromosomal abnormalities [Smith-Bindman *et al.,* 2007].

2.1.4. Prenatal screening in the first trimester

The use of US screening and placental markers for DS screening in the first trimester was first proposed by Nicolaides and colleagues in the early 1990s [Nicolaides *et al.,* 1992; Snijders *et al.,* 1998]. Most of the serum screening markers used in the second trimester have also been tested for use in DS screening during the first trimester [Wald *et al.,* 1997]. Two serum markers stand out as being effective in screening at 10–14 weeks – low PAPP-A and elevated fb-HCG [Brambati *et al.,* 1993; Brambati *et al.,* 1994]. The use of combination PAPP-A and fb-HCG with maternal age showed a screening DR of 78.9% [Brambati *et al.,* 1994]. The higher DR was shown when the first trimester US marker – NT was combined with first trimester serum markers (PAPP-A and fb-HCG), and a few years later, first trimester combined screening was introduced into routine screening [Wapner *et al.,* 2003]. In different combinations and strategies, the DR of PnS in the first trimester has been reached until 79–91% (with FPR 3%) [Benn *et al.,* 2011].

First trimester screening now involves both US screening and maternal serum screening. The first described US marker – NT (Figure 1), the sonolucent space evident at the back of a fetus's neck during first trimester, was included in prenatal screening about 20 years ago [Nicolaides *et al.,* 1992].

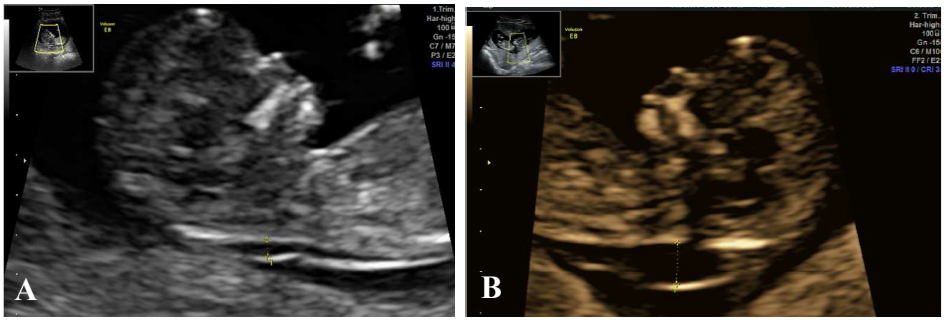


Figure 1. a) US picture of a fetus at 13+1 week of gestational age, with normal NT measurement (CRL 71 mm, NT 2.1 mm); b) US picture of a fetus at 12+2 week of gestational age, with an increased NT (CRL 57 mm, NT 5.1 mm, karyotype 46,XX, healthy after birth).

During 11 to 14 weeks of gestation (crown-rump length (CRL) 45–84 mm), measurement of NT is widely used, and it is a sensitive screening method for chromosomal abnormalities when it is measured in a defined way by sonographers and obstetricians who have taken part in a program of training and ongoing audit [Pandya *et al.,* 1994; Spencer 2007]. NT screening in clinical practice showed NT to be the most effective marker in single use, with NT ≥ 2.5 mm DR for DS reached near to 70% (FPR 5.9%) [Ghaffari *et al.,* 2012]. If NT is measured incorrectly by untrained and un-audited centers it actually has a negative impact on detection rates [Spencer 2007].

Increased NT is not only the marker of chromosomal abnormalities, but increased NT has also been shown to be a feature in a whole variety of genetic syndromes [Clur *et al.,* 2009; Muller *et al.,* 2007; Nicolaides 2003; Souka *et al.,* 2001; Souka *et al.,* 2005; Timmerman *et al.,* 2010; Timmerman *et al.,* 2013; van Huizen *et al.,* 2005]. The heterogeneity of conditions suggests that there may not be a single underlying mechanism for the collections of fluid in the skin of the fetal neck [Haak and van Vugt 2003]. Possible mechanism include: cardiac failure with association with abnormalities of the heart and great arteries [Clur *et al.,* 2009; Souka *et al.,* 2001]; venous congestion in the head and neck caused by constriction of the fetal body in amnion rupture sequence or superior mediastinal compression found in diaphragmatic hernia or the narrow chest in skeletal dysplasia [Chen *et al.,* 2012; Daskalakis *et al.,* 1997; Sebire *et al.,* 1997b; Souka *et al.,* 1998; Spaggiari *et al.,* 2012; Vimercati *et al.,* 2013]; altered composition of the extracellular matrix that may be attributed to gene dosage effects [Arigita *et al.,* 2011; Dempsey *et al.,* 2013; Pergament *et al.,* 2011; Souka *et al.,* 2002c; von Kaisenberg *et al.,* 1998]; abnormal or delayed development of the lymphatic system [Souka *et al.,* 2002b; von Kaisenberg *et al.,* 1999]; failure of lymphatic drainage because of impaired fetal movements in various neuromuscular disorders [Hyett *et al.,* 1997; Stiller *et al.,* 1999]; fetal anemia or hypoproteinemia [Souka *et al.,* 2002a; Souka *et al.,* 2002d; Tercanli *et al.,* 2001]; and congenital infections that act through anemia or cardiac

dysfunction [Petrikovsky *et al.,* 1996; Sebire *et al.,* 1997a]. As the possible underlying mechanism may be CHD or a genetic disorder, in follow-up it is suggested that fetal echocardiogram (ECHO) during the first and/or second trimester [Souka *et al.,* 2005] and genetic testing for specific genetic disorders be performed [Pergament *et al.,* 2011].

For genetic testing, the arrayed primer extension (APEX) system comprises a microchip containing DNA sequences in an ordered array, which enables the simultaneous analyses of hundreds of genetic markers. This has been available in Estonia (Asper Biotech) since 2010 (the test was implemented into prenatal testing in 2011). This microchip allows testing with one test for different genetic diseases: five genes for NS: *PTPN11* (32 point mutations), *SOS1* (24 point mutations), *KRAS* (10 point mutations), *RAF* (14 point mutations), and *MAP2K1* (1 point mutation); for Smith-Lemli-Opitz syndrome, the gene *DHCR7* for 130 mutations including splice site errors, deletions, and insertions; *CYP21A2* gene for congenital adrenal hyperplasia; and for spinal muscular hypertrophy the deletion mutation in *SMN1* was tested. Pergament *et al*., described the results of testing 120 pregnancies with increased NT (≥3 mm) and normal karyotypes using this microchip. Out of all of the tests, NS was diagnosed in 8 cases, and no other disease was diagnosed [Pergament *et al.,* 2011]. The approach used enables one to test only a few genetic diseases associated with increased NT [Souka *et al.,* 2005], and with the exception of NS, the frequency of other syndromes associated with increased NT remains unresolved in terms of their true clinical significance [Pergament *et al.,* 2011].

After normal US scan at 20 to 22 weeks of gestation, parents should be reassured of the risk of adverse perinatal outcome and postnatal developmental delay is not increased [Ayras *et al.,* 2013; Bijok *et al.,* 2013; Mula *et al.,* 2012].

In recent years, new additional first trimester US markers have been introduced and standardized: nasal bone, fronto-maxillary facial angle, ductus venosus and tricuspid valve Doppler evaluation [Sonek and Nicolaides 2010]. The addition of the newer markers serves the purpose of increasing DR while FPR decreases [Sonek and Nicolaides 2010].

2.1.5. Different strategies in prenatal screening

Different screening protocols and strategies are used in different countries (Table 1 and 2). The most commonly performed screening tests for DS screening use either second trimester maternal serum biochemical markers or a combination of first trimester maternal serum markers with ultrasound measurement of NT [Wright *et al.,* 2006].

Table 1. Various screening protocols in use. Adapted from ISPD statement [Benn *et al.,* 2011].

Protocol and subtype (completed weeks∗)		DR (%) at FPR 3%
1a	PAPPA+fb-HCG (10), NT (12)	82
1b	PAPPA+HCG (10), NT (12)	80
1c	PAPPA+fb-HCG (12), NT (12)	80
1d	PAPPA+HCG (12), NT (12)	79
2a	AFP+fb-HCG+uE3+InhA (15–19)	64
2b	AFP+HCG+uE3+InhA (15–19)	60
3a	PAPPA+fb-HCG (10), NT (12), contingent AFP+fb-HCG+uE3+InhA (15–19)	90
3b	PAPPA+HCG (10), NT (12), contingent AFP+HCG+uE3+InhA (15–21)	88
3c	PAPPA+fb-HCG (10), NT (12), stepwise AFP+fb-HCG+uE3+InhA (15–21)	92
3d	PAPPA+HCG (10), NT (12), stepwise AFP+HCG+uE3+InhA (15–21)	91
4a	PAPPA (10), NT (12), AFP+fb-HCG+uE3+InhA (15–19)	91
4b	PAPPA (10), NT (12), AFP+HCG+uE3+InhA (15–19)	89
4c	PAPPA+fb-HCG (10), NT (12), AFP+fb-HCG+uE3+InhA (15–19)	93
4d	PAPPA+HCG (10), NT (12), AFP+HCG+uE3+InhA (15–19)	91
5a	PAPPA+fb-HCG (10), NT+NB (12)	91
5b	PAPPA+fb-HCG (10), NT (12), contingent NB	91
5c	PAPPA+fb-HCG (10), NT (12), contingent TCR	88
5d	PAPPA+fb-HCG (10), NT (12), contingent DV	88
6a	PAPPA+fb-HCG (10), NT (12), anomaly (18+)	88
6b	PAPPA+HCG (10), NT (12), anomaly (18+)	86
7a	anomaly (18+)	56
7b	AFP+fb-HCG+uE3+InhA (15–19), anomaly (18+)	80
7c	AFP+fb-HCG+uE3+InhA (15–19), contingent anomaly (18+)	77
8a	PAPPA+fb-HCG (10), NT (12), AFP+fb-HCG+uE3+InhA (15–19), anomaly (18+)	96
8b	PAPPA+HCG (10), NT (12), AFP+HCG+uE3+InhA (15–19), anomaly (18+)	95

∗ Completed weeks, e.g. 10 = 10 weeks 0 days to 10 weeks 6 days. NT – nuchal translucency; NB – nasal bone absence; TCR – tricuspid regurgitation; DV – ductus venosus; NF – nuchal skinfold; NBL – nasal bone length; The rates specified are for the purposes of comparison of protocols and do not necessarily indicate optimal cut-offs. contingent = 1 in 50–1500 borderline risks, at term (equivalent to 1 in 38–1200 at mid-trimester), stepwise = borderline or lower risks, anomaly = major malformation, large NF, short femur, echogenic intracardiac focus, pyelectasis, echogenic bowel and ventriculomegaly. Predicted performance is based on published statistical parameters for NT and biochemical markers [Cuckle 2010], NBL [Cicero *et al.,* 2004], TCR and DV [Sonek and Nicolaides 2010], anomaly [Aagaard-Tillery *et al.,* 2009] and a standardized maternal age distribution [Cuckle *et al.,* 2004].

Table 2. National policies or recommendations for PnS for DS in place in 2004 in 18 European countries [Boyd *et al.,* 2008].

Countries	National screening policies or recommendations for DS screening test to be offered to all women	First-trimester screening actually offered		Second trimester biochemical screening offered	Maternal age at which CVS/AC is offered
		NT	NT + biochemistry		
Austria	No	+	+	–	≥ 35
Belgium	Yes	+		+	≥ 36 (charged if < 36)
Croatia	No	±	±	±	≥ 35
Denmark	Yes	–	+	–	CVS/AC not offered primarily on basis of maternal age
England and Wales	Yes*	±	±	±	CVS/AC not offered primarily on basis of maternal age
Finland	Yes	±	±	±	≥ 39
France	Yes	+	±		≥ 38
Germany	Yes	+ **	+ **	+**	≥ 35
Ireland	No	–	–	–	–
Italy	Yes	±	±	+	≥ 35
Malta	No	–	–	–	–
Netherlands	No	–	–	+**	≥ 36
Norway	No	±**	±**	±**	≥ 38
Poland	Yes	+	+	+	≥ 35
Portugal	Yes	+	±	±	≥ 35
Spain	No	±	±**	±**	≥ 35
Sweden	No	±	–	–	≥ 35
Switzerland	Yes	–	+	±***	CVS/AC not offered primarily on basis of maternal age

CVS – chorionic villus sampling; AC – amniocentesis
* – Screening policy was based on a detection rate, that is a screening test should be offered that had a DR for DS of >60% for an FPR of <5%
** – May be private
*** – Primary first-trimester screening, second-trimester screening for late bookers
+ – in place in all areas of the country
± – in place in some areas within the country

The first used screening protocols included a combination of different serum markers at second trimester combining with maternal age [Haddow *et al.,* 1992; Wald *et al.,* 1988b]. The DR with this protocol was 60% with FPR 5% [Haddow *et al.,* 1992; Wald *et al.,* 1988a].

The integrated test first described in 1999 by Wald *et al.* incorporates first- and second-trimester screening markers (US and/or serum markers) into a single test [Wald *et al.,* 1999]. The test result is given in the second trimester, and it is an example of a type of "nondisclosure" sequential testing. The integrated test has described DR as high as 85% with FPR 1% [Wald *et al.,* 1999], or with slightly different forms, DR varies from 89 to 93%, with FPR from 3% [Benn *et al.,* 2011]. It was concluded that integrated screening has been the simplest, most effective screening strategy, and this method has been shown to be feasible and acceptable in demonstration programs conducted in North America and Europe (United Kingdom) [Knight *et al.,* 2005; Palomaki *et al.,* 2005; Wald *et al.,* 2006]. At the same time, this strategy has a disadvantage from both a psychological and a medical point of view, as there are no possibilities for earlier PnD [Christiansen and Olesen Larsen 2002; Malone *et al.,* 2005].

Sequential screening policy, which makes it possible to offer an early diagnostic test to screen-positive women and second trimester screening to screen-negative women, can divided into two subtypes: step-wise (risk is estimated from up to seven markers) and independent (second trimester risk calculation uses only second trimester markers) [Cuckle *et al.,* 2005]. Using step-wise sequential screening, DR is estimated at 93% (with FPR 3%) and using the independent sequential screening model, estimated DR is 83% (with FPR 3%) [Cuckle *et al.,* 2005].

Contingent sequential screening can achieve similar performances to non-disclosure screening (integrated test), while only a fraction of women need second-trimester tests. Contingent screening, the most complex of the screening protocols, uses a first-trimester test to triage the population of women screened into three groups: high-risk screen-positive, i.e. women immediately offered a diagnostic test; screen-negative, women who receive no future screening; and lower-risk screen-positive, women whose first trimester results would be reused in the second trimester as part of a subsequent integrated test [Maymon *et al.,* 2005; Wald *et al.,* 2006; Wright *et al.,* 2006]. The estimated DR of this screening protocol is 92% (with FPR 3%) [Cuckle *et al.,* 2005].

Sequential and contingent screenings are not an application of single tests, but the application of a sequence of tests; there are many cut-off combinations in which different tests can be used. With these screenings, one of these screening parameters (DR, FPR, risk cut-off level) has to be specified for each of the component tests or specified for one test, and a screening parameter specified for the overall sequence. In addition, a lower risk cut-off to define screen-negatives on the first test must be specified for contingent screening [Wald *et al.,* 2006]. Both sequential and contingent screening have the advantage of achieving an earlier diagnosis in a varying proportion of women

[Wald *et al.,* 2006]. Independent sequential screening, which is widely used, is ineffective in providing invalid (independent) second trimester risk estimation [Cuckle *et al.,* 2005]. Among different types of screening policies, contingent screening is the most efficient, since a high DR could be achieved with the vast majority of women completing screening in the first trimester [Cuckle *et al.,* 2005].

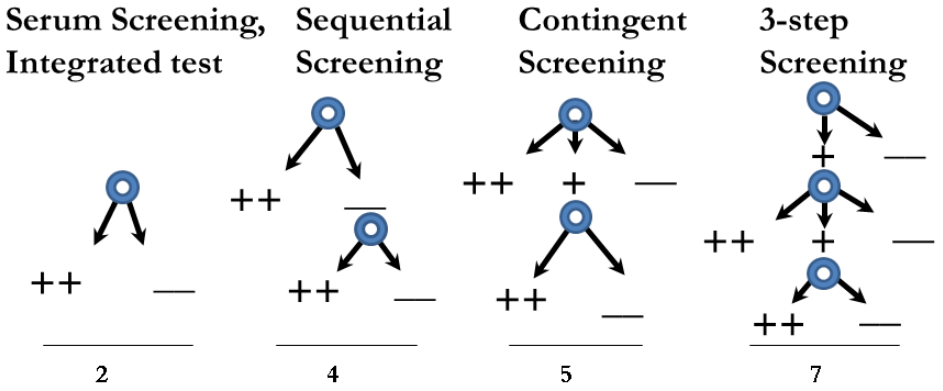


Figure 2. Different screening policies, showing the number of risk estimations (◎) and the number of result categories for the different screening methods [Wald *et al.,* 2006; Wright *et al.,* 2006].

The measurement of combination of markers in both the first and the second trimester provides the best screening performance [Malone *et al.,* 2005]. The difference in screening protocols is minor, as in different protocols up to 7 markers are used, and is less apparent with FPR 5% than with FPR 1%, because the DR is relatively high with all protocols [Malone *et al.,* 2005; Wyatt 2007] (Figure 2). The disadvantage of sequential protocols is that a first trimester risk needs to be calculated, but not necessarily reported in respect of nearly all patients. In sequential screening the major disadvantage may be that it precludes the performance of chorionic villus sampling (CVS) for early PnD in several cases and high FPR. At the same time, FPR is not high in contingent screening protocol, and there is the possibility of early PnD; in the group of women continuing to have the integrated test, however, anxiety is likely to be raised, because they have been specially identified as being in an intermediate group, and this could inadvertently channel more women towards invasive testing [Malone *et al.,* 2005]. This problem is avoided with sequential screening, but with this policy the screening performance is not materially better than contingent screening, and almost all women eventually have an integrated test, making it the least cost-effective policy [Cocciolone *et al.,* 2008; Wald *et al.,* 2006]. In different publications, studies have compared different screening protocols; each of them has specific advantages and disadvantages. Taking all of them together, the first trimester US is crucial in screening, and the

contingent/sequential approach should be favored, but local needs and possibilities should drive methodology for primary population screening strategy [Benn *et al.,* 2005; Breathnach and Malone 2007; Cuckle *et al.,* 2005; Wright *et al.,* 2004; Wyatt 2007] (Table 3).

Table 3. Assessment of different screening policies [Wald *et al.,* 2006].

	Screening policy		
Criteria	**Integrated test**	**Sequential screening**	**Contingent screening**
Administrative simplicity	+	–	–
Safety	+	–	–
Cost effectiveness	+	–	+
Early diagnosis	–	+	+
Avoids confusion from two risk estimates in the same pregnancy	+	–	–
Avoids unnecessary terminations (about one in five terminated pregnancies would have miscarried between the first and second trimester)	+	–	–
Avoids the risk of being missed, because some screening measurements are not performed in some women	+	–	–
Retention of AFP screening	+	–	–

At the same time, with all of the different screening options (some of which are only theoretical and others clearly implementable in practice), one should not lose sight of the fact that such complex strategies will need careful evaluation from a health care delivery aspect [Spencer 2007]. Many of the new models incorporating early US will also bring spin-offs in the detection of other chromosomal and structural anomalies and the identification of women at high-risk for many other potential problems of fetal-maternal health. High-quality US will become the foundation of early fetal-maternal assessment [Spencer 2007; Wyatt 2007].

Screening strategies change over time. Whereas there was initially only the option to perform PnD for the high-risk group (women with advanced maternal age), and first serum screening protocols were introduced about 25 years ago [Wald *et al.,* 1989]. In 2000 mothers were offered the choice between second trimester serum screening or first trimester combined screening [Mennuti and Driscoll 2003]. As of 2013 we have widely used different US and serum screening protocols and new choices, such as fetal cell free deoxyribonucleic acid (cfDNA) analysis from maternal serum, are already coming into clinical practice [Benn *et al.,* 2013].

In 2013 the Board of the International Society for Prenatal Diagnosis (ISPD) published a Statement Position for aneuploidy screening from the Aneuploidy Screening Committee [Benn *et al.,* 2013]:

1. Definitive diagnosis of DS and other fetal aneuploidies can only be achieved through amniocentesis or chorionic villus sampling;
2. The use of maternal age alone to assess fetal DS risk in pregnant women is insufficient;
3. A combination of US NT measurement and maternal serum markers in the first trimester should be made available to women who want an early risk assessment;
4. A four-marker serum test should be available to women who first attend prenatal care after 13 weeks 6 days of pregnancy;
5. Protocols that combine first trimester and second trimester markers are valid;
6. Second trimester ultrasound can be a useful supplement to other aneuploidy screening protocols;
7. Maternal cfDNA screening is an emerging technology that can provide highly effective prenatal screening for DS, trisomy 18, and possibly trisomy 13 in high-risk women. It is not a replacement for the analysis of amniotic fluid cells or CVS.

2.1.6. Principles of procedures and diagnostic tests for prenatal diagnosis

Diagnostic tests are designed to give a definitive answer to the question of whether or not the patient has a particular problem. These tests are generally complex and require sophisticated analysis and interpretation. They tend to be expensive, and are usually performed on patients considered to be "at risk". Identifying individuals with disease usually involves tests and procedures that are related with increased risks [Raffle and Gray 2009].

Definitive PnD of DS and certain other fetal aneuploidies through chromosome analysis of amniocytes or chorionic villus sample is an accepted part of prenatal care [Benn *et al.,* 2011]. Both are invasive procedures, necessitating a technique that requires entry into a body cavity or interruption of normal body functions.

2.1.6.1. Chorionic villus sampling

CVS is an invasive procedure carried out after 10 weeks of gestation. The procedure involves aspiration of trophoblastic tissue under continuous US monitoring. Trophoblastic tissue is needed for future analyzes of fetal chromosomes, DNA-tests or for enzymatic analysis. Data from randomized controlled trials as well as from systematic reviews and a large national registry study have estimated a procedure-related miscarriage rate of 0.5–1.0% [Eisenberg and Wapner 2002; Tabor and Alfirevic 2010]. Even with the estimated procedure

risk for CVS, the risk of miscarriage from invasive testing is inherently difficult to assess, as the procedure-related risk must be separated from the background rate. This background rate is likely to be higher than that of the general population due to the indications for testing, such as advanced maternal age and high-risk screening result. In conclusion, invasive procedures CVS and amniocentesis (AC) are considered equally safe, but the impact of operators' experience is substantial [Wapner 2005].

2.1.6.2. Amniocentesis

AC is an invasive procedure that requires taking a small sample of amniotic fluid transabdominally under ultrasound guidance, usually after 15 weeks of gestation, when it is safer and technically less demanding. The most common indication for AC is in the evaluation of fetal karyotype by cytogenetic analysis of amniotic fluid cells. AC as an invasive procedure has estimated a procedure-related risk for spontaneous miscarriage of 0.6% (P=0.0042; 95% CI, 0.19, 1.03) in one study [Seeds 2004], and no difference in another study [Towner *et al.,* 2007].

2.1.6.3 Diagnostic tests after invasive procedure

It is possible to perform different tests on direct biopsy or cultured cells. Most used tests are karyotyping, fluorescence in situ (FISH) analysis for specific chromosomal region, enzyme analysis and molecular testing. The diagnostic algorithm is decided by consulting doctor based of the screening results, US scanning and family history.

2.2. Associations of marked changes in maternal screening tests and outcome

Although the primary aim of PnS is to identify pregnancies at risk of aneuploidy, significant changes in markers may give insight into other adverse pregnancy outcomes.

2.2.1. The significance of increased NT during first trimester ultrasound screening

In normal fetuses, NT thickness increases with fetal CRL. About 5% of all fetuses in an unselected population show NT measurement above the 99th percentile for gestational age [Nicolaides 2004]. About 50% cases with NT $\geq$ 3.0 mm are associated with aneuploidy, and in the absence of associated anomalies, prognosis should be favorable [Bilardo *et al.,* 2007; Cha'ban *et al.,* 1996]. However, in euploid pregnancies with normal second trimester US

screening, a favorable outcome occurs in 97% of cases [Ayras *et al.,* 2013; Bilardo *et al.,* 2007]. At the same time, the underlying cause of an enlarged NT is unknown, and the associations of increased NT with chromosomal, non-chromosomal abnormalities and adverse pregnancy outcome has been studied for the past two decades [Adekunle *et al.,* 1999; Ayras *et al.,* 2013; Cha'ban *et al.,* 1996; Dugoff *et al.,* 2004; Goetzl 2010; Goetzl *et al.,* 2004; Krantz *et al.,* 2004; Miltoft *et al.,* 2012; Senat *et al.,* 2007; Spencer *et al.,* 2006; Spencer *et al.,* 2008b]. The prevalence of chromosomal defects and adverse pregnancy outcome including miscarriage, fetal loss, and fetal abnormalities increases exponentially with NT thickness [Bilardo *et al.,* 2007; Cha'ban *et al.,* 1996; van Huizen *et al.,* 2005]. Using, in addition, supplementary chromosomal analysis – high-resolution comparative genomic hybridization (HR-CGH) and multiplex ligation-dependent probe amplification (MLPA) for subtelomeric regions for the detection of small chromosomal aberrations does not increase the detection rate of genetic disease [Schou *et al.,* 2009].

In the case of enlarged NT and normal fetal karyotype, the fetus can be affected by a variety of structural and genetic disorders, of which CHD is the most common [Clur *et al.,* 2009; Hyett *et al.,* 1999; Souka *et al.,* 1998; Souka *et al.,* 2005]. Antenatal screening for major forms of CHD is possible, although there are different issues relating to its success, such as operator's skills, appropriate ultrasound equipment, and the time required for examination and ongoing audit [Sharland 2010]. The most common lesions in prenatally diagnosed series are hypoplastic left heart syndrome (HLHS) and atrioventricular septal defect (AVSD) [Sharland 2012]. In a group of enlarged NT and normal karyotype, the prevalence of CHD is estimated to be about 0.8–9.5% [Clur *et al.,* 2009; Muller *et al.,* 2007]. As with adverse outcome, the prevalence of CHD increases exponentially with increasing NT [Clur *et al.,* 2009]. In his study, Vogel *et al.* found that nearly half of prenatally diagnosed fetuses with CHD had increased NT [Vogel *et al.,* 2009]. Therefore, referral of all fetuses with increased NT for fetal ECHO is recommended [Clur *et al.,* 2009; Sharland 2012; Souka *et al.,* 2005; Vogel *et al.,* 2009]. At the same time, once prenatally CHD is diagnosed, the further evaluation of the fetus is recommended, because CHD may be also only one feature of genetic syndrome [Pajkrt *et al.,* 2004].

Several studies have been performed regarding the long-term outcome of children with increased NT. Some of these show a higher risk for developmental disorders [Adekunle *et al.,* 1999; Baumann *et al.,* 2005; Schou *et al.,* 2009; Senat *et al.,* 2002; Van Vugt *et al.,* 1998], whereas others do not show an increased risk for developmental delay at 2 years of age [Bilardo *et al.,* 2007; Cha'ban *et al.,* 1996; Hiippala *et al.,* 2001; Maymon *et al.,* 2000; Miltoft *et al.,* 2012; Mula *et al.,* 2012; Saldanha *et al.,* 2009; Senat *et al.,* 2007; Sotiriadis *et al.,* 2012] (Table 4).

Table 4. Long-term outcome of fetuses with enlarged NT, normal karyotype and normal examination at birth in the literature.

Reference	Number of subjects	NT cut off	Method	Control group	Age (months)	Lost (%)	Developmental delay
Cha´ban et al. (1996)	19	3.0 mm	questionnaire/phone	no	7–75	0	0/19 (0%)
Adekunle et al. (1998)	31	4.0 mm	questionnaire	no	13–38	26	2/23 (8.7%)
Van Vugt et al. (1998)	50	3.0 mm	questionnaire	no	7–75	32	1/34 (2.9%)
Maymon et al. (2000)	36	>95th percentile	questionnaire/phone	no	12–36	0	0/36 (0%)
Hiippala et al. (2001)	59	3.0 mm	clinical examination	no	24–84	15	1/50 (2%)
Senat et al. (2002)	58	4.0 mm	clinical examination/questionnaire	no	12–72	7	4/54 (7.4%)
Baumann et al. (2005)	179	3.0 mm	clinical examination	no	0–24	15.6	16/151 (10%)
Bilardo et al. (2007)	425	>95th percentile	questionnaire/phone	no	6–60	3.3	7/425 (1.6%)
Senat et al. (2007)	162	>99th percentile	clinical examination/questionnaire	yes	0–24	1.2	2/160 (1.2%)
Saldanha et al. (2009)	128	>95th percentile	phone/clinical examination	no	0–72	37.5	0/80 (0%)
Schou et al. (2009)	85	>99th percentile	clinical examination/questionnaire	no	8–33	6	3/80 (4%)
Mula et al. (2012)	124	>99th percentile	clinical examination/questionnaire	no	24	12.9	4/108 (3.7%)
Miltoft et al. (2012)	96	>99th percentile/ 3.5 mm	questionnaire	yes	24	16.7	1/80 (1.25%)

A recent systematic review by Sotiriadis *et al*. concluded that the rate of neuro-developmental delay in children with increased fetal NT, a normal karyotype, normal anatomy and no identifiable genetic syndromes does not appear to be higher than reported for the general population, but still more large-scale, prospective case-control studies would be needed [Sotiriadis *et al.,* 2012].

2.2.2. Significance of marked changes in markers in maternal serum screening

The combination of multiple abnormal markers and their association with adverse perinatal outcomes has been studied in a variety of ways since the introduction of multiple marker screening.

Both first trimester serum markers PAPP-A and fb-HCG are produced by the trophoblast, and therefore abnormal values of either analyte could suggest abnormal placentation. As long as 30 years ago, the association between low values of PAPP-A and fb-HCG and pregnancy loss have been documented in connection with threatened miscarriage [Westergaard *et al.,* 1983]. More recently, many different studies have shown associations between low first trimester serum markers PAPP-A (cut off < 0.4 MoM) or fb-HCG (cut off < 0.4 MoM) and adverse perinatal outcome, such as low birth weight [Dugoff *et al.,* 2004; Krantz *et al.,* 2004; Montanari *et al.,* 2009; Ong *et al.,* 2004; Pihl *et al.,* 2008a; Spencer *et al.,* 2008a], preterm delivery [Dugoff *et al.,* 2004; Smith *et al.,* 2002; Spencer *et al.,* 2008b] stillbirth [Dugoff *et al.,* 2004; Spencer *et al.,* 2006], miscarriage [De Leon *et al.,* 2004; Dugoff *et al.,* 2004; Ong *et al.,* 2000] and pregnancy-induced hypertensive disorders [Karahasanovic *et al.,* 2013; Poon *et al.,* 2009]. However, some studies did not find those associations or could only confirm some of them, and proposed that the efficacy of first trimester maternal serum markers in predicting adverse pregnancy outcome is low [Kavak *et al.,* 2006; Morssink *et al.,* 1998].

Associations with changes in second trimester serum markers AFP (cut off > 2.5 MoM), HCG (cut off > 2 to 4.0 MoM), and uE3 (cut off < 0.2 MoM) and adverse perinatal outcome (such as fetal death, preeclampsia, intrauterine growth restriction) have also been shown in different studies [Alleman et al., 2013; Baschat et al., 2002; Chandra et al., 2003; Cragun et al., 2004; Duric et al., 2003; Gagnon et al., 2008; Huang et al., 2005; Kang et al., 2008; Kashork et al., 2002; McPherson et al., 2011; Olsen et al., 2012; Sayin et al., 2008; Spaggiari et al., 2013; Spencer 2000; Summers et al., 2003]. Some authors have described the obstetrical data by looking at the screen-positive status [Huang *et al.,* 2005; Pihl *et al.,* 2008b; Summers *et al.,* 2003]; others have looked at combinations based on the unexplained elevations or reductions of the different markers [Benn *et al.,* 2000; Gagnon *et al.,* 2008; Morssink *et al.,* 1996a; Morssink *et al.,* 1996b; Olsen *et al.,* 2012; Zanini *et al.,* 1998]. Some studies have looked for an association with marked changes in serum markers and congenital malformations and/or genetic disorders. Associations have been

suggested between the high level of AFP and congenital nephrotic syndrome [Spaggiari *et al.,* 2013], or between low uE3 and steroid sulfactase deficiency, or Antley-Bixler syndrome and Smith-Lemli-Opitz syndrome [Bradley *et al.,* 1999; Cragun *et al.,* 2004; Kashork *et al.,* 2002]; or between low PAPP-A and Cornelia de Lange syndrome [Aitken *et al.,* 1999]. At the same time, one study showed an association between high levels of HCG and PAPP-A and hydrocele, but the other failed to find associations between high levels of HCG and congenital malformations [Celentano *et al.,* 2005; Hoffman *et al.,* 2008].

Evaluation and subsequent patient management must be based on the potential complications associated with the serum marker pattern [Alkazaleh *et al.,* 2006; Bromley *et al.,* 1994; Gagnon *et al.,* 2008; Ghosh *et al.,* 2006; Spaggiari *et al.,* 2013]. However, even after the optimization of cut-off values, these markers do not appear to be clinically acceptable as an effective tool for screening for adverse pregnancy outcomes [Kavak *et al.,* 2006].

To the best of our knowledge, there are nearly no published studies of postnatal follow-up of children born to mothers with marked changes in maternal serum screening markers.

2.3. Genetic causes of congenital heart anomalies (Publication IV)

2.3.1. Overview

CHD is the most common birth defect, affecting 3–13/1000 live born infants [Hoffman and Kaplan 2002; Pierpont *et al.,* 2007; van der Linde *et al.,* 2011]. The incidence of severe CHD requiring expert cardiologic care is about 2.5–3/1000 live births, and the moderately severe forms of CHD probably account for another 3 per 1000 live births [Gruber and Epstein 2004; Hoffman and Kaplan 2002]. The most common context for CHD is an infant with no other problems ("isolated" or "non-syndromal" CHD); CHD may, however, be just one component of a number of genetic, teratogenic or idiopathic childhood malformation syndromes ("syndromal" CHD).

It has been estimated that the population of adults with CHD is growing by about 5% per year, which predicts that the total adult CHD population likely reached 1 million by 2005 [Pierpont *et al.,* 2007]. Despite clinical importance of CHD, the underlying genetic etiology of most CHD remains unknown, and they are so-called "multifactorial" diseases. A hypothesis of multifactorial etiology was proposed 40 years ago [Nora 1968], and different environmental and genetic causes have been identified over the years [Harper 2010; Huang *et al.,* 2010; Jenkins *et al.,* 2007; Mitchell *et al.,* 2007; Nemer 2008; Pierpont *et al.,* 2007] (Figure 3).

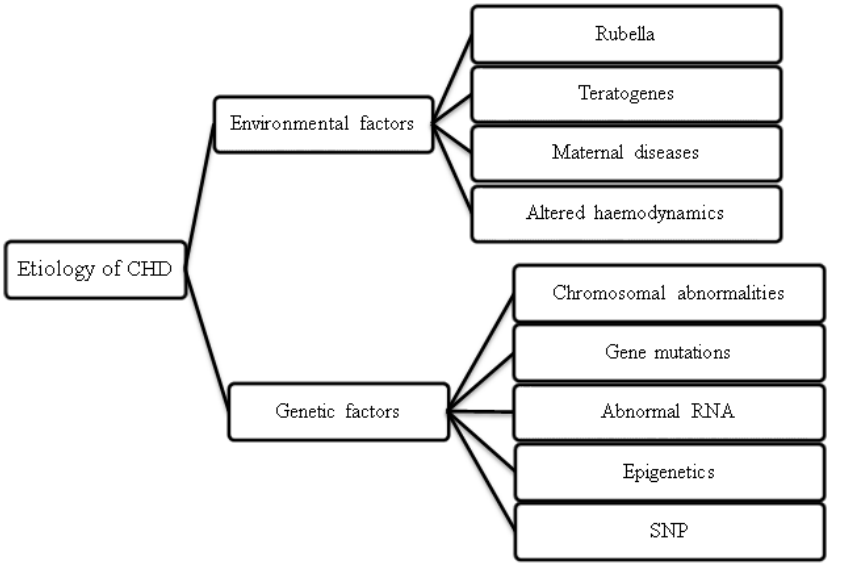


Figure 3. The diverse origins of CHD [Huang *et al.,* 2010].

RNA – ribonucleic acid; SNP – single nucleotide polymorphism.

The strong evidence supporting a genetic etiology for most CHDs in human beings. This is exemplified by the recurrence of CHD in the same family, associations with specific chromosomal abnormalities and the high occurrence of heart defects occurring in dysmorphic syndromes, which are often due to a genetic etiology [Anders *et al.,* 1965; Gill *et al.,* 2003; Goldmuntz 2004; Grech and Gatt 1999; Harper 2010; Johnson *et al.,* 1997; Loffredo *et al.,* 2004; Pierpont *et al.,* 2007] (Table 5).

The formation of the heart proceeds by sequential gene regulatory steps that dictate cell fates and organize specialized cell types into complex 3-dimensional units of structure and function. In order to explore the etiology of CHD, an approach focusing on the individual modular steps in cardiovascular morphogenesis is important, because most CHD results from abnormal morphogenesis in specific structural components of the developing heart and vessels [Kodo and Yamagishi 2011] (Figure 4).

Table 5. A selected group of genetic syndromes with CHD as one feature.

Disorder	**Usual heart defect**	**Inheritance**	**Known gene association**
Noonan syndrome	PS	AD	*PTPN11, SOS1, KRAS, RAF1, NRAS, BRAF, SHOC2* and *MAP2K1*
Holt-Oram syndrome	ASD, conduction defects	AD	*TBX5*
LEOPARD syndrome	PS, conduction defects	AD	*PTPN11*, *RAF1* and *BRAF*
Cardio-Facio-Cutaneous syndrome		AD	*BRAF*, *MAP2K1, MAP2K2* and *KRAS*
Costello syndrome		AD	*HRAS*
Alagille syndrome		AD	*JAG1*and *NOTCH2*
Ellis-van Creveld syndrome	VSD, single atrium	AR	*EVC*
Di-George syndrome/CATCH	conotruncal defects	AD	microdeletion in chromosome 22q11.2
Williams syndrome	supravalvular AS or PS	mostly sporadic	microdeletion in chromosome 7q11.23
Ivemark synrome	dextrocardia, DORV, ASD, VSD	mostly sporadic	*connexin43(?)*
VATER association	variable	sporadic	
CHARGE association	commonly conotruncal	AD	*CHD7*
Kabuki syndrome	CoA	AD	*KMT2D, KDM6A*
Goldenhar syndrome	variable	mostly sporadic	
Marfan syndrome	mitral valve prolaps, dilatated aortic root	AD	*FBN1*

AD – autosomal dominant; AR – autosomal recessive;
PS – pulmonary stenosis; ASD – atrial septal defect; VSD – ventricular septal defect; AS – aortic stenosis; DORV – double-outlet right ventricle; CoA – coarctation of aorta.

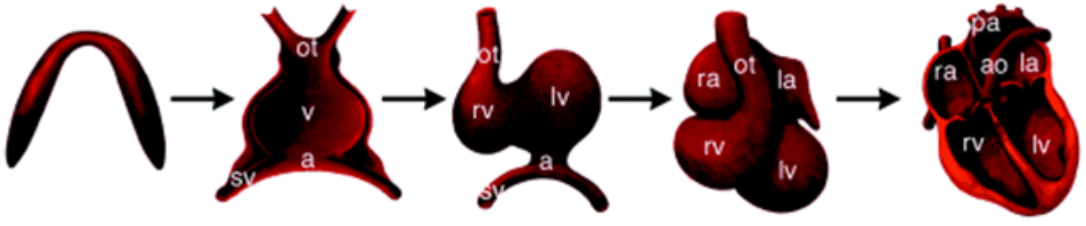
Stage	Cardiac crescent	Linear heart tube	Looping heart	Chamber formation	Maturation/septation
Mouse (embryo day):	7.5	8.5	9	10	12 - birth
Human (embryo day):	15	20	28	32	50 - birth
					
Important events	Cardiac differentiation	Migration to midline Heart tube forms A-P patterning Heart beats	Early cambers form Looping to the right	Chamber formation Trabeculation Cushions form Outflow tract system	Valves form Septation occurs Conduction system form Connection to great vessels
CHDs occurring at this stage	?	Cardia bifida Laterality defects	Laterality defects HLHS/HRH	Conotruncal defects Defective valves DORV AV canal defects TOF	ASDs/VSDs DORV AV canal defects TOF Conduction defects

Figure 4. An overview of heart development. The major steps in heart development outlined and potential CHD arising due to defects. This is an illustrative and not a comprehensive list of CHD and its relationship to major events in heart formation [Bruneau 2003; Nemer 2008].

Studies of familiar recurrence and transmission risk suggest that the etiology of CHD cannot be explained by simple single-gene inheritance, but must be explained by more complex mechanisms, possibly involving interactions among many genes of little effect and many potential environmental modifiers [McElhinney *et al.,* 2003; Nora and Nora 1976]. Despite significant advances in the understanding of cardiac development, the etiology of CHD remains largely unknown, and only a handful of human genes with mutations associated with CHD have been identified [Garg 2006; Grossfeld 2003; Mitchell *et al.,* 2007] (Table 6).

Table 6. CHD: types, frequency and associated genes [Gruber and Epstein 2004; Hoffman and Kaplan 2002; Mitchell *et al.,* 2007].

Defect	**Frequency**	**Gene Association**
Ventricular septal defect (VSD)	1:280	*NKX2-5, GATA4, TBX5*
Atrial septal defect (ASD)	1:1062	*NKX2-5, GATA4, TBX5, MYH6*
Atrioventricular septal defect (AVSD)	1:1372	*CRELD1*
Pulmonary stenosis (PS)	1:1372	
Persistent truncus arteriosus (PDA)	1:1252	*MYH11*
Tertalogy of Fallot (TOF)	1:2375	*NKX2-5, JAG1*
Aortic stenosis (AS)	1:2494	
Coarctation of aorta (CoA)	1:2445	
Hypoplastic left heart syndrome (HLHS)	1:3759	*NKX2-5*
Hypoplastic right heart (HRH)	1:4505	
Transposition of the great arteries (d-TGA)	1:3175	*CFC1, ZIC3*
Double-outlet right ventricle (DORV)	1:6369	*CFC1, NKX2-5*
Pulmonary atresia (PA)	1:7576	
Truncus arteriosus	1:9364	
Ebstein's anomaly	1:8772	
Bicuspid aortic valve (BAV)	1:73	*NOTCH1*
Tricuspid atresia	1:12658	
Single ventricle (SV)	1:9434	
Total anomalous pulmonary venous connection (TAPVC)	1:10638	

There are a number of genetic tests that can assist in diagnosing genetic alterations in children with CHD. Today these include cytogenetic and FISH analysis, chromosomal microarray analysis (CMA) and DNA analysis. For the clinician managing a child with CHD, it is very important to determine whether there is an underlying genetic pattern (e.g. deletions, duplications or mutations),

for the following reasons: there may be involvement of some other important organ system; there may exist prognostic information for clinical outcome; there may be important genetic reproductive risks that the family should know about; and there may be other family members for whom genetic testing is appropriate [Pierpont *et al.,* 2007]. With the rapid increase in knowledge about the genetics of CHDs, families and patients even with apparently isolated, non-familiar CHDs would benefit from a genetic consultation in the matter of the possible etiology and recurrence risk of CHD.

2.3.2. Syndromal congenital heart defect

In most cases CHD is isolated congenital defect, but in 1–4.6% of cases CHD is one feature of a genetic syndrome [Grech and Gatt 1999]. Here we describe some of the most common genetic syndromes in which CHD is one of the clinical symptoms.

2.3.2.1. Noonan syndrome

Noonan syndrome (NS; OMIM 163950) is an autosomal dominant (AD) disorder affecting the cardiovascular, craniofacial, skeletal, hematopoietic, lymphatic, and central nervous systems. The incidence of NS is estimated to be 1:1000–2500 live births [Nora *et al.,* 1974; Romano *et al.,* 2010; Tidyman and Rauen 2009]. NS is most common non-chromosomal syndromic cause of CHD [Marino *et al.,* 1999].

NS is genetically heterogeneous. In 1994 the first gene locus was described in region chromosome 12, and in 2001 the gene described in association with NS was *PTPN11* [Noonan 1994; Tartaglia *et al.,* 2001]. In about 40–50% of cases NS caused missense mutations in the *PTPN11* gene on chromosome 12, resulting in a gain-of-function of the protein SHP-2 (Src homology 2) [Croonen *et al.,* 2013a; Tartaglia *et al.,* 2002]. This enzyme is involved in the intracellular signal cascades, and is required in several developmental processes. Mutations in the N-SH2 and PTP domain affect the interaction, destabilizing the catalytically inactive protein conformation and results in a gain of SHP-2 function. During subsequent years molecular lesions of other genes (*SOS1, KRAS, RAF1, BRAF, MAP2K, SHOC2 and NRAS)* of the mitogen-activated protein kinases and extracellular signal-regulated kinases (MAPK/ERK) cascade has been described in association with NS [Cirstea *et al.,* 2010; Cordeddu *et al.,* 2009; Martinelli *et al.,* 2010; Pandit *et al.,* 2007; Razzaque *et al.,* 2007; Sarkozy *et al.,* 2009; Schubbert *et al.,* 2006; Serrano-Martin *et al.,* 2008; Tartaglia and Gelb 2005]. Recently, possible associations with mutations in the *CBL* gene and NS-like phenotype with predisposition to juvenile myelomonocytic leukaemia have been suggested [Martinelli *et al.,* 2010]. Nevertheless, in all genotyped cases it is possible to identify mutation in about 61% of clinical NS diagnosis; most often in the *PTPN11* gene (40.9%), and in fewer cases in

other associated genes – *SOS1* (11.1%), *RAF1* (4.7%), *SHOC2* (1.7%), *KRAS* (1.4%), *BRAF* (0.8%) and *NRAS* (0.2%) [Romano *et al.,* 2010].

NS has distinctive craniofacial features, including a broad forehead, hypertelorism, downslanted palpebral fissures, ptosis, posteriorly-rotated low-set ears and a high-arched palate [Tidyman and Rauen 2009]. The classical facial features do change with age: whereas after birth there is a typically tall forehead, hypertelorism, downslanted palpebral fissures, epicanthus, a short and broad nose with an upturned tip, a deeply grooved philtrum, wide peaks to the vermillion of the upper lip, a high palate, micrognathia, low-set and posteriorly-rotated dysmorphic ears and excessive nuchal skin with a low posterior hairline, over the years the face becomes more triangular with a broad forehead and a narrow pointed chin. The facial appearance often lacks expression; and in adulthood the neck is longer, with accentual webbing; the nasolabial folds are prominent, with seemingly transparent skin [Allanson 1987; Allanson *et al.,* 2010; Allanson *et al.,* 1985; Romano *et al.,* 2010].

CHD is diagnosed in about 80% of NS patients [Romano *et al.,* 2010]. NS has various cardiovascular phenotypes. PS (50–60%), hypertrophic cardiomyopathy (HCM) (20%), secondary ASD (6–10%) and partial AVSD are diagnosed most often, but several others, such as VSD, peripheral PS, AVSD, AS, CoA, TOF, mitral valve abnormalities and coronary artery anomalies, are have also been noted [Ishizawa *et al.,* 1996; Marino *et al.,* 1999; Roberts *et al.,* 2013; Romano *et al.,* 2010; Shaw *et al.,* 2007]. As left-sided obstructive lesions may develop in adulthood and pulmonary valve insufficiency and right ventricular dysfunction after earlier cardiac intervention, it is important to provide lifetime cardiac follow-up for NS patients [Danetz *et al.,* 1999].

Although short stature is one of the distinctive features of NS (affecting 50–70% of individuals), birth weight and length are typically normal, and some individuals will have normal growth and stature [Nora *et al.,* 1974; Ranke *et al.,* 1988]. The mean adult heights of European individuals with NS have been reported for women as ~ 153 cm and for men as ~ 162 and 167 cm [Ranke *et al.,* 1988; Shaw *et al.,* 2007]. The higher prevalence of short stature is reported in *PTPN11* mutation-positive individuals, and lower prevalence in *SOS1*-associated NS [Lepri *et al.,* 2011; Zenker *et al.,* 2004].

Feeding difficulties, such as poor sucking and recurrent vomiting sometimes require tube-feeding, mostly affects babies (75%) with NS [Sharland *et al.,* 1992]. Bleeding disorders are often mild, but affect about 30–65% of individuals with NS, and may become significant during surgical procedures [Sharland *et al.,* 1992; Witt *et al.,* 1988]. Abnormal lymphatic development is associated with NS, it can appear in different life periods, and has been estimated to be ~ 20% in NS individuals [Romano *et al.,* 2010]. Most often the manifestation is peripheral lymphedema during infancy, which typically resolves within a few years [van der Burgt 2007]. Cryptorchidism occurs in up to 80% of boys, but male gonadal dysfunction is suggested to be due to dysfunction of the Sertoli cells [Marcus *et al.,* 2008; Romano *et al.,* 2010].

Puberty is typically delayed and is characterized by a diminished pubertal growth spurt [Ranke *et al.,* 1988].

In individuals with NS the neurologic, cognitive and behavioral aspects are variable. In the first years there may be a general delay in milestones, which may be due to feeding difficulties and muscular hypotonia, but most individuals with NS at school age have normal intelligence, and only up to 40% require special education [Lee *et al.,* 2005; Sharland *et al.,* 1992; van der Burgt *et al.,* 1999].

Different studies have been published in genotype-phenotype correlations in patients with NS. Mutations in *PTPN11* are significantly associated with PS, short stature, bleeding diathesis and thorax deformities, and less with HCM and CoA [Roberts *et al.,* 2013; Zenker *et al.,* 2004; Tartaglia *et al.,* 2002]. Patients with the p.Asn308Asp or p.Asn308Ser mutations in the *PTPN11* gene have little or no intellectual disability [Roberts *et al.,* 2013]. Individuals with mutation in *SOS1* present pulmonary valve disease and ectodermal abnormalities, but they are also usually of normal height [Zenker *et al.,* 2004; Tartaglia *et al.,* 2007]. Mutations in *RAF1* are more closely associated with HCM and hyperpigmented cutaneous lesions [Pandit *et al.,* 2007; Razzaque *et al.,* 2007]. Mutations in other associated genes are quite rare. A gain-of-function mutation in *SHOC2*, p.Ser2Gly, has been identified as causative for a type of Noonan-like syndrome characterized by the presence of loose anagen hair [Komatsuzaki *et al.,* 2010]. The three main clinical features of NS – a typical, face, short stature and PS – are less frequently present in the group without a mutation [Croonen *et al.,* 2013a].

The diagnosis of NS can be made prenatally, when the pattern of anomalies is recognized, but US findings can be subtle and unspecific [Bakker *et al.,* 2011]. NS is the most frequently reported genetic syndrome connected with increased NT [Hiippala *et al.,* 2001; Pergament *et al.,* 2011; Souka *et al.,* 1998]. *De novo* mutation in either *PTPN11*, *KRAS* or *RAF1* was detected in 13 fetuses (17.3%) in pregnancies with an increased NT and at least one of the following additional features: polyhydramnios, hydrops fetalis, renal anomalies, distended jugular lymphatic sacs, hydrothorax, cardiac anomalies, cystic hygroma and ascites [Croonen *et al.,* 2013b]. When previously listed US findings or specific facial anomalies are present, additional targeted DNA analysis for NS is indicated [Bakker *et al.,* 2011]. The APEX array can also be used for prenatal analysis for NS [Pergament *et al.,* 2011]

Individuals with NS require follow-up for growth, development, cardiovascular function and for bleeding diathesis; there are guidelines for clinicians for better management with NS individuals [Roberts *et al.,* 2013; Romano *et al.,* 2010; van der Burgt 2007].

2.3.2.2. LEOPARD syndrome

LEOPARD syndrome (LS; OMIM 151100) is a rare AD multisystem disorder with full penetrance and variable expressivity [Gorlin *et al.,* 1969]. LEOPARD syndrome's acronymic name refers to its major features: **L**entigines, **E**CG conduction abnormalities, **O**cular hypertelorism, **P**ulmonic stenosis, **A**bnormal genitalia, **R**etardation of growth, and sensorineural **D**eafness [Gorlin *et al.,* 1971]. *PTPN11* was the first gene associated with LS [Digilio *et al.,* 2002; Legius *et al.,* 2002]. LS is mainly caused by missense mutations in the *PTPN11* gene. In more than 85% of LS cases, a heterozygous missense mutation is detected in *PTPN11* exons 7, 12 or 13. To date, only 11 heterozygous *PTPN11* mutations have been reported in LS patients (p.Tyr279Cys, p.Tyr279Ser, p.Ala461Thr, p.Gly464Ala, p.Thr468Met, p.Thr468Pro, p.Arg498Trp, p.Arg498Leu, p.Gln506Pro, p.Gln510Glu, p.Gln510Pro) [Martinez-Quintana and Rodriguez-Gonzalez 2012; Sarkozy *et al.,* 2008]. Most often, two recurrent *de novo* mutations, p.Tyr279Cys and p.Thr468Met are described [Conti *et al.,* 2003]; the mutation p.Thr468Met is described both with LS and NS phenotype [Aoki *et al.,* 2008; Lee *et al.,* 2011]. Whereas NS mutations in the *PTPN11* gene result in gain-of-function effects, LS mutations cause loss-of-function effects [Keyte and Hutson 2012]. The rate of detection of *PTPN11* mutations in patients with LS is about 88%, which is consistently higher than in patients with NS [Sarkozy *et al.,* 2004]. However, mutations in the *RAF1* [Pandit *et al.,* 2007] and *BRAF* [Koudova *et al.,* 2009; Sarkozy *et al.,* 2009] genes have recently been described as being associated with an LS phenotype.

LS and NS are allelic syndromes with considerable phenotypic overlap [Tartaglia *et al.,* 2011]. The clinical spectrum of LS associated with *PTPN11* mutations is markedly variable, with no specific pathognomonic features [Sarkozy *et al.,* 2004]. Diagnostic criteria for LS include multiple lentigines (ML) plus two additional recognized features or a first degree relative with ML plus three other features in the proband without ML, first proposed by Voron *et.al*. [Voron *et al.,* 1976]. Today, LS diagnostic clues for clinical diagnosis are similar: cutaneous manifestations, including ML and *café-au-lait* spots (CLS) as well as cardiovascular anomalies and deafness, especially during first year of life [Digilio *et al.,* 2006; Sarkozy *et al.,* 2004]. Distinct facial dysmorphism, including hypertelorism, ptosis, and large low-set auricles, occur in 90% of patients with *PTPN11* mutations [Sarkozy *et al.,* 2004; Sarkozy *et al.,* 2008], but they may only be expressed during infancy [Digilio *et al.,* 2006]. ML, described in most patients (up to 90%), presented as flat, black-brown macules anywhere on the trunk, but mostly on face, neck, and upper part of the trunk [Martinez-Quintana and Rodriguez-Gonzalez 2012; Sarkozy *et al.,* 2004]. CLS are observed (in about 61% of patients), alone or in association with ML, and CLS are similar to those found in Neurofibromatosis 1 (NF1) [Martinez-Quintana and Rodriguez-Gonzalez 2012]. CLS usually precede the appearance of ML, being present from the first months of life [Sarkozy *et al.,* 2008]. At the same time, ML are rare at birth and, classically, ML develop during childhood,

increasing in number until puberty and thereafter pigmentation of ML is increasing with years [Coppin and Temple 1997; Voron *et al.,* 1976]. Structural cardiac anomalies occur in 71% of probands with *PTPN11* mutations, most often HCM (in 80%), and less often PS or valve leaflet dysplasia [Sarkozy *et al.,* 2004]. As HCM may develop any time during infancy or adolescence, any patients with ML or LS should be observed carefully for HCM during childhood [Massoure *et al.,* 2012; Sarkozy *et al.,* 2004]. Hearing loss may be a feature in about 25% of patients, *PTPN11* mutations p.Tyr279Cys, p.Tyr279Ser are described with bilateral sensorineural deafness, while mutation p.Gln506Pro is associated with unilateral deafness [Sarkozy *et al.,* 2004]. Short stature or final height below the 25th centile is observed in 85% of patients [Sarkozy *et al.,* 2008]. Mental retardation is not a frequent feature of LS, and if it presents, it is usually mild [Martinez-Quintana and Rodriguez-Gonzalez 2012; Sarkozy *et al.,* 2008]. About 200 patients have so far been reported worldwide, although LS seems to be underdiagnosed or misdiagnosed due to its mild features and/or the absence of lentiginosis [Sarkozy *et al.,* 2008].

2.3.2.3. Holt-Oram syndrome

The Holt-Oram syndrome (HOS, OMIM 142900) is characterized by upper limb anomalies and CHD. HOS is inherited an AD trait and occurs in approximately 1:100,000 live births [Elek *et al.,* 1991]. HOS was first clearly described in 1960 by Holt and Oram, who observed ASD in members of 4 generations of a family, associated with "a congenital anomaly of the thumbs which lay in the same plane as the fingers, their terminal phalanges being curved inwards" [Holt and Oram 1960]. HOS is the most common of the heart-hand syndromes [McDermott *et al.,* 2005].

In 1994 a gene causing HOS was mapped into the distal long arm of chromosome 12 (12q21-qter) by linkage analysis [Bonnet *et al.,* 1994]. Some years later gene *TBX5* (a member of T-box transcription factors family) was identified [Basson *et al.,* 1997; Li *et al.,* 1997]. Different studies have shown low sensitivity (22–35%), which may be due to technical limitations or genetic heterogeneity [Basson *et al.,* 1997; Brassington *et al.,* 2003; Cross *et al.,* 2000; Li *et al.,* 1997]. At the same time, patients with familial or sporadic HOS, who were selected on the basis of strict diagnostic criteria, had mutations in the *TBX5* gene in up to 74% of cases [McDermott *et al.,* 2005]. The detected mutations are spread throughout the coding exons of the *TBX5* [Heinritz *et al.,* 2005], and most *TBX5* mutations are so far known to be truncation mutations [Basson *et al.,* 1997]. Nevertheless, in a significant proportion of typical HOS cases no mutation can be found within the *TBX5* coding region and flanking intronic sequences. Submicroscopic deletions within the *TBX5* gene can explain additional 2 % of clinical cases of HOS [Borozdin *et al.,* 2006].

The HOS phenotype has complete penetrance, but high intra- and inter-familial clinical expression variability [Newbury-Ecob *et al.,* 1996]. Upper limb

deformity may be bilateral, but it may also be asymmetric or even unilateral. All affected individuals exhibit upper limb abnormalities that range from phocomelia to clinodactyly or hypoplasia of the thenar eminence [Basson *et al.,* 1999; Newbury-Ecob *et al.,* 1996]. Narrow sloping shoulders may be a useful diagnostic pointer for HOS [Newbury-Ecob *et al.,* 1996]. Cardiac defects were seen in 95% of familiar cases, and include mostly ASD or VSD, although many other cardiac malformations have been reported, ranging from mitral valve prolapse to HLHS and/or cardiac conduction disease [Basson *et al.,* 1997; Bruneau *et al.,* 1999; Newbury-Ecob *et al.,* 1996]. Pulmonary vein defects are also common [Bohm *et al.,* 2008]. The incidence and severity of HOS limb and heart malformations caused by *TBX5* missense mutations depend on the specific amino acid residue altered [Basson *et al.,* 1999]. The lower limbs are not affected in HOS [Gruenauer-Kloevekorn and Froster 2003], and neither are postaxial upper limb, cranio-facial, pulmonary, genitourinary, gastrointestinal malformations and intellectual and sensory deficits features of HOS [Gruenauer-Kloevekorn and Froster 2003].

In the study of the facial phenotype of HOS, what Allanson and Newbury-Ecob subjectively describe as "gestalt" is a square face with a broad lower jaw and parietal bossing; a prominent and tall forehead, narrowing at the temples, hypotelorism; a relatively long nose with a wide base, and short columnella; there is, however, no objective evidence and no syndrome-specific pattern profile to facilitate the discrimination of HOS from other heart-hand syndromes [Allanson and Newbury-Ecob 2003].

Even if several of these symptoms are also described as exclusion criteria for HOS, atypical phenotypes have been described: isolated skeletal anomalies or cardiac defects [Lehner *et al.,* 2003], renal, craniofacial, axillary, tracheal and vertebral anomalies, deafness, and abdominal *situs inversus* [Brassington *et al.,* 2003; McDermott *et al.,* 2005], and also lower-limb malformation [Garavelli *et al.,* 2008].

Prenatal molecular diagnosis is feasible for families at risk, but the majority of cases result from *de novo* mutations [Basson *et al.,* 1999]. In US-screening, a finding of right atrial enlargement in the fetus may be a marker of HOS, and a thorough search for subtle upper limb abnormalities should also be performed if appropriate prenatal molecular testing is feasible [Paladini *et al.,* 2014].

2.3.3. Non-syndromal congenital heart defect

Cardiac development is a complex and highly regulated interplay of genes and cell-cell interactions, controlled by a highly conserved network of transcription factors (TFs) that connect signaling pathways with genes related to muscle growth, patterning, and contractility. The core TF network consists of NKX2, myocyte enhancing factor 2, GATA, TBX and transcription factor protein [Huang *et al.,* 2010]. The finding that complex regulatory circuits control heart development and the identification of the various regulators of cardiac

morphogenesis has provided a molecular explanation for the linkages of the same malformation to more than one gene [Bruneau 2003; Nemer 2008]. The mechanisms underlying the early stages of cardiogenesis are not fully understood; nevertheless, TF *GATA4* is emerging as the critical regulator of the earliest stages of cardiogenesis, TF *NKX2-5* is required for proper chamber specification and TF *TBX5* is essential for atrial formation [Nemer 2008; Srivastava 2006].

A mutation in any of these three genes can result in human cardiac septal defect and suggest that these three genes may work to direct common molecular pathways critical for cardiac septum formation [Bruneau 2008; Pashmforoush *et al.,* 2004; Stennard *et al.,* 2003]. *TBX5*, *GATA4* and *NKX2-5* function together to activate genes; also, mutations in *MYH6*, a downstream transcriptional target of *GATA4*, *TBX5* and *NKX2-5,* is associated with a cause of ASD [Bruneau 2008; Ching *et al.,* 2005; Garg 2006; Huang *et al.,* 2010].

2.3.3.1. *GATA4* gene

GATA4 (GATA binging protein 4) was identified as the genetic cause of non-syndromic ASD and VSD without conduction disturbances by studying large pedigrees with familiar CHD [Garg *et al.,* 2003]. The GATA-binding proteins are a group of structurally related transcription factors that control gene expression and differentiation in a variety of cell types. The *GATA4* gene is located in region 8p23.1 and belongs to a family of TFs that binds a consensus GATA DNA motif and contains two class IV zinc-finger domains [Arceci *et al.,* 1993; Garg *et al.,* 2003; Pehlivan *et al.,* 1999]. *GATA4* is expressed in the adult vertebrate heart, gut epithelium and gonads. During fetal development, *GATA4* is expressed in yolk sac endoderm and cells involved in heart formation [Arceci *et al.,* 1993]. The number of documented mutations in the *GATA4* gene associated with different CHDs does increase quickly, and associated CHD are mostly cardiac septal defects – ASD and VSD [Cheng *et al.,* 2011; Hatcher *et al.,* 2003; Hirayama-Yamada *et al.,* 2005; Kodo *et al.,* 2012; Rajagopal *et al.,* 2007; Salazar *et al.,* 2011; Sarkozy *et al.,* 2005b; Zhang *et al.,* 2008; Tomita-Mitchell *et al.,* 2007; Wang *et al.,* 2013; Xiong *et al.,* 2013; Yang *et al.,* 2012].

2.3.3.2. *NKX2-5* gene

The *NKX2-5* (*NK2 Homeobox 5*) gene belongs to the *homeobox* family and is mapped in region 5q35.1. Homeobox-containing genes play critical roles in regulating tissue-specific gene expression essential for tissue differentiation, as well as determining the temporal and spatial patterns of development [Shiojima *et al.,* 1995]. Targeted disruption of the murine homolog of "tinnman", *NKX2-5*, causes early embryonic lethality, with cardiac development arrest at an early stage [Lyons *et al.,* 1995]. Cardiac expression of *NKX2-5* continues throughout development and into adult life [Komuro and Izumo 1993; Olson 2004; Schott

et al., 1998]. The cardiac homeobox protein NKX2-5 is essential in cardiac development, and mutations in *NKX2-5* perturb later stages of cardiac development, such as cardiac septation. Mutation in *NKX2-5* may cause various CHDs, such as ASD, VSD, TOF, and/or cardiac conduction abnormalities [Goldmuntz 2004; Sarkozy *et al.,* 2005a; Schott *et al.,* 1998]. Some authors have suggested that patients with mutations in *NKX2-5* and ASD carry lifelong risk for the development of conduction defect and sudden death [Grossfeld 2003; Schott *et al.,* 1998]. Mutations in *NKX2-5* are described in only a small percentage (0.9–4%) of patients with various CHDs [Balci and Akdemir 2011; Gioli-Pereira *et al.,* 2010; Goldmuntz 2004; Kodo *et al.,* 2012; Liu *et al.,* 2011; McElhinney *et al.,* 2003; Posch *et al.,* 2008; Qin *et al.,* 2012; Rauch *et al.,* 2010; Reamon-Buettner and Borlak 2010; Wang *et al.,* 2011], which suggests that single gene mutation needs to be in combination with other genes and/or factors, as an inciting stimulus or an environmental or coexisting disease in order to establish CHD [Balci and Akdemir 2011; Posch *et al.,* 2008].

2.3.3.3. *TBX5* gene

The *TBX5* (*T-BOX 5*) gene belongs to the T-box (TBX) protein family, which are important developmental regulators that share a conserved 180 AA region (the T-domain or T-box) responsible for DNA binding. More than 20 members have been identified so far in mammals [Horb and Thomsen 1999; Nemer 2008]. Six members of the TBX TF family have been identified as crucial factors in distinct subprograms during cardiac regionalization [Greulich *et al.,* 2011]. The first evidence for a role of TBX proteins in the heart came from the finding that *TBX5* (mapped in region 12q24.1) is the gene mutated in HOS [Basson *et al.,* 1997; Li *et al.,* 1997]. Mutation in *TBX5* has also been associated with some cases of isolated CHD in sporadic or familiar cases [Bruneau 2008].

2.3.4. Microdeletions and congenital heart defect

Standard chromosome analysis may find chromosomal aberration in 8–13% of all neonates with CHD [Ferencz *et al.,* 1989]. With improved techniques and new approaches in cytogenetic analysis, the prevalence of chromosomal aberration in selected CHD groups is estimated much higher, especially in the identified 22q11.2 deletion [Johnson *et al.,* 1997].

Cytogenetic or CMA should be considered in connection with the phenotype of a recognizable chromosomal syndrome, multiple congenital anomalies, complicated family history additional health problems or developmental delay [Pierpont *et al.,* 2007].

2.3.4.1. 22q11.2 deletion syndrome

The 22q11.2 deletion syndrome is the most common microdeletion syndrome, with prevalence from 1:4000 live births to 1:6500 life births [Kobrynski and Sullivan 2007]. Various syndromes have been associated with the deletion of human chromosome 22q11.2 including DiGeorge syndrome, velocardiofacial syndrome and conotruncal anomaly face. The majority of patients have the same large (>3Mb) deletion encompassing about 30 functional genes. In several cases (8–28%), deletion syndrome is inherited from a parent [Digilio *et al.,* 1997; McDonald-McGinn *et al.,* 1999; Ryan *et al.,* 1997]. Clinical diagnosis is challenging because the phenotype varies from a clearly normal phenotype to a severe manifestation of different disease characteristics, such as CHD, palatal abnormalities, facial phenotype, learning difficulties and immune deficiency. CHD is present in about 74–80 % of patients with 22q11.2 deletion syndrome [Botto *et al.,* 2003; Momma 2007]. The most frequent anomalies are conotruncal defects of the outflow tract, such as TOF (20%), interrupted aortic arch (13%), VSD (14%), truncus arteriosus (6%), vascular ring (5.5%), ASD (3.5%), VSD/ASD (4%) [McDonald-McGinn and Zackai 2008]. During genetic counselling, FISH analysis for the 22q11.2 deletion should be a first choice for the child with conotruncal or septal CHD, because it may give lucidity about the etiology of CHD and insight regarding prognosis [McDonald-McGinn and Zackai 2008].

2.3.5. Prenatal diagnosis of congenital heart defect

Antenatal US screening for fetal cardiac abnormalities was introduced about 30 years ago, yet prenatal diagnosis of CHD is challenging. Most major forms of CHD, as well as some of the minor forms, have been described in the fetus and can be diagnosed in experience centers, but there are still certain lesions that cannot be predicted during fetal life [Sharland 2012]. Four-chamber view examination can detect different CHDs (e.g. HLHS, AS, TA, AVSD, etc.), yet there are several CHDs that require additional examination (e.g. TGA, DORV, TOF, pulmonary atresia with VSD etc.) [Sharland 2012]. Several studies have shown a great variability in detecting prenatally CHD [Acharya *et al.,* 2004; Acherman *et al.,* 2007; Dolk *et al.,* 2011; Friedberg *et al.,* 2009; Khoo *et al.,* 2008]. Even if in some types of CHDs DR of prenatal diagnosis is up to 75%, the median prenatal DR is about 30% [Sklansky *et al.,* 2009]. There is also the possibility of false positive results in prenatal diagnosis [Khoo *et al.,* 2008]. PnD for CHDs may improve newborn outcomes, but PnD leads parents into a difficult position regarding choices and decisions [Sharland 2012].

Up to 5% of CHDs are syndromic, and for the prenatal detection of the most common syndromic CHDs (NS, del22q11.2), it is possible to use the APEX-assay (described in detail in page 21) for prenatal testing in fetuses with NT>3 mm and normal karyotype, and FISH analysis for region 22q11.2.

3. AIMS OF THE PRESENT STUDY

The aims of the present study were:

1. To establish the first trimester screening for Down syndrome in Estonia;
2. To evaluate the potential of the used contingent screening;
3. To investigate whether chromosomally normal fetuses with marked changes in maternal first or second trimester serum markers and first trimester ultrasound marker NT have an increased risk of congenital or genetic anomaly or delayed development at 2 years of age;
4. To evaluate the genetic causes of syndromic congenital heart anomalies in Estonian children;
5. To characterize rare monogenic genetic syndromes with congenital heart defect.

4. MATERIAL AND METHODS

4.1. Study subjects

4.1.1. Study group of prenatal screening

All women seeking antenatal care at Tartu University Hospital during the first trimester were enrolled in the study group. The screening was completely voluntary; all women gave written consent before being enrolled in screening. The study period lasted from 1 February 2005 – 31 December 2008, during this period 3194 women agreed to participate.

At the beginning of the screening programme (1 February – 31 December 2005), 500 women also underwent routine second trimester serum screening during the 15^{th} to 18^{th} weeks of pregnancy. All women had routine second trimester ultrasound screening in the 19^{th} and 20^{th} weeks of pregnancy.

Two-step contingent screening was introduced from the beginning of year 2006. After first trimester combined screening, we triaged the population of screened women into three groups: a first group (high-risk screening-positive) to which a diagnostic test was immediately offered; a second group (screening-negative) that received no future screening; a third group (intermediate risk calculation) in which the first trimester ultrasound measurement result (NT) and the second trimester serum markers were integrated into the risk calculation.

The high-risk screening-positive group consisted of women with increased risk after first trimester combined screening. Their individual risk for trisomy 21 or trisomy 18 was higher than 1:50 or NT ≥3 mm and CVS or AC for fetal karyotyping were offered after the first trimester combined screening result. Provisional results from FISH analysis were available within 48 hours, and a final diagnosis using conventional karyotyping within two weeks.

The screening-negative group consisted of women whose individual risk calculation after the first trimester combined test was low (<1:5000) and who received no future screening.

The intermediate risk group included women with a medium risk calculation: a first trimester combined risk for DS of from 1:50 to 1:5000; or to whom we could not offer the combined screening method because of a lack of US scan data. The second trimester serum screening test (AFP; HCG and uE3) was offered and considered positive if the risk of trisomy 21 was ≥ 1:270 and for trisomy 18 ≥ 1:100. AC for fetal chromosome investigation was offered to women with a positive test result. In individual integrated risk calculation > 1:1000 for trisomy 21, the second trimester genetic sonogram for sonographic markers (increased nuchal skinfold, short humerus, short femur, echogenic bowel, pyelectasis, echogenic intracardiac focus, absence or hypoplasia of the nasal bone and choroid plexus cysts) were performed.

To identify DS infants born to screening-negative women who participated in our screening, we contacted all cytogenetic laboratories in Estonia.

4.1.2. Study group of children born to mother after marked changes of markers in prenatal screening tests

4.1.2.1. Prenatal study group

This study group was based on the screening test analysis of 5257 pregnant Caucasian women during a one-year period (from 16 February 2009 to 15 February 2010) at Tartu University Hospital, which covers approximately 1/3 of all prenatal screening tests performed in Estonia. According to the Estonian prenatal screening program, a contingent screening strategy for prenatal screening is used when possible [Muru *et al.,* 2010]. All obtained first trimester serum (1525 women) and ultrasound screening test (1589 women) and second trimester screening test (4410 women) results were analysed (Figure 5).

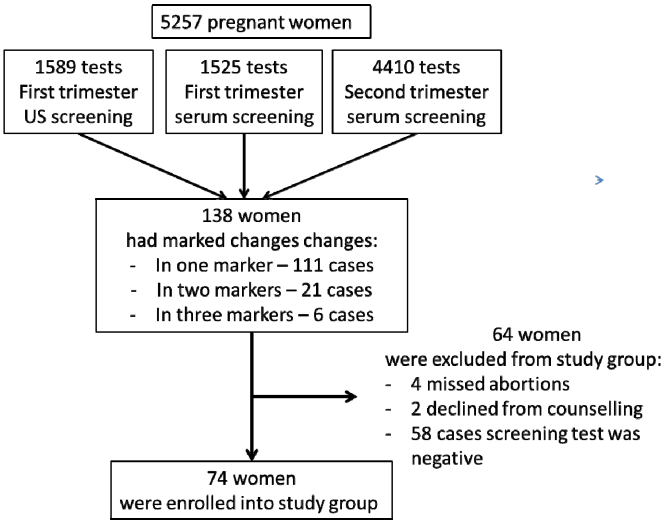


Figure 5. Prenatal study group.

Marked changes in biochemical and/or ultrasound markers were documented in 138 pregnant women, whereas a positive risk calculation using the Prisca software for chromosomal anomalies was evident in 80 of them. Women with positive Prisca risk calculation and marked changes in biochemical and/or ultrasound markers were included in the study group.

Seventy-four women were counselled by a geneticist and were included in our study (Figure 5). Four pregnancies had already spontaneously aborted by that moment, and two women refused to come to genetic counselling.

4.1.2.2. Postnatal study group

A total of 45 mothers (Figure 6) who were consulted by a geneticist and to whom additional prenatal investigations were suggested (ultrasound and/or fetal

chromosomal analysis), met the criteria previously described, had live births and were included in the postnatal study group. Ten of these women refused to bring their child to the follow-up. Thus the postnatal study group included 35 children born from mothers with marked changes in screening tests.

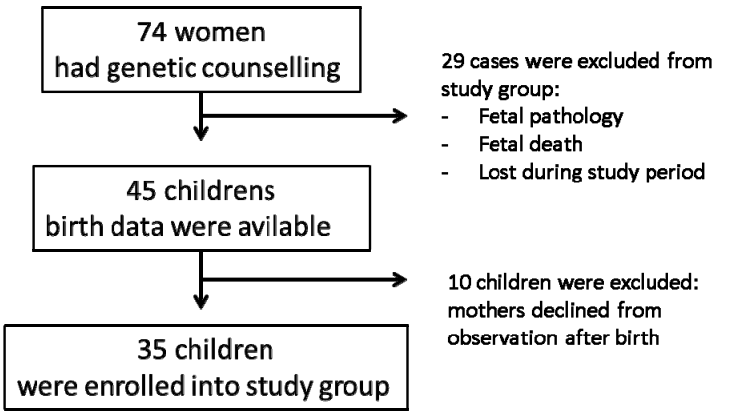


Figure 6. Postnatal study group.

4.1.3. Study group of children with congenital heart defect

The study group consisted of children with a postnatal diagnosis of CHD with positive family history (at least two affected individuals) or a clinically suspected genetic syndrome, one of the main feature of which being CHD (NS, LS or HOS). These children and their family members were referred to genetic counselling in the Department of Genetics at Tartu University Hospital during the years 2006–2010. Informed consent was obtained from the parents or legal guardians of the children for participation in the study.

Probands were examined by a medical geneticist; the collected date included family history, obstetrical information about pregnancy and birth, gestational age and growth parameters at birth, health problems after birth, as well as diagnosed congenital anomalies. On examination, a child's weight, height and head circumference were measured, developmental milestones were assessed and dysmorphic features were systematically sought. If there was a clinical suspicion of NS, Noonan's scoring criteria were used [van der Burgt 2007]. Conventional chromosomal analysis and FISH analysis for 22q11.2 micro-deletion was carried out on all patients. Blood samples for DNA were obtained from the proband, and if possible also from parents and siblings.

A total of 55 families were referred to genetic counselling (Figure 7). After genetic consultation, the study group was divided into subgroups: syndromic CHD (29): NS (23), LS (4) and HOS (2), and non-syndromic CHD (septal defect and/or right-left isomerism) with complicated family history (26).

Subjects in the NS group were enrolled with the help of the NS scoring system (Table 7).

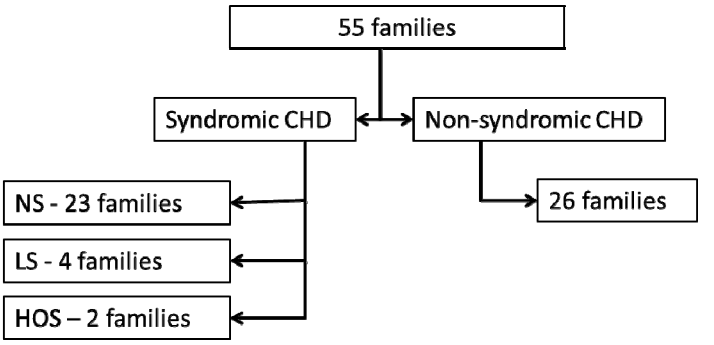


Figure 7. CHD study group.
CHD – congenital heart defect; NS – Noonan syndrome; LS – LEOPARD syndrome, HOS – Holt-Oram syndrome

Table 7. NS scoring system [van der Burgt 2007].

	Finding	**A** Major feature	**B** Minor feature
1	**Facial**	Typical face	Suggestive face
2	**Cardiac**	PS and/or typical ECG	CHD
3	**Height**	<3 percentile	<10 percentile
4	**Chest wall**	Pectus carinatum/ pectus excavatum	Broad thorax
5	**Other** **Mental retardation** **Cryptorchidism** **Lymphatic dysplasia**	All (in males)	One of features
6	**Family history**	1st-degree relative with definite NS	1st-degree relative with suggestive NS

Typical facial phenotype was considered: high forehead, hypertelorsim, downslanted palpebral fissures, dysmorphic ears, epicanthus, low-set and/or posteriorly rotated ears; wide, short or webbed neck, a low posterior hairline. Suggestive facial phenotype was considered if some but not all of the features were described.

Definite NS:
1A plus one of 2A–6A or two of 2B–6B;
1B plus two of 2A–6A or three of 2B–6B.

4.2. Methods

4.2.1. Prenatal screening tests

4.2.1.1. Maternal serum screening

First trimester serum screening was performed in gestational age of 10th to 13th weeks. All serum samples were assayed for PAPP-A and fb-HCG using Immulite DPC 2000 (distributed by Siemens Medical Solutions Diagnostics). For individual risk calculation (combined and/or serum-based risk) we used Prisca 4.0 software (distributed by Siemens Medical Solutions Diagnostics).

Second trimester serum screening was performed during the 15th to 18th weeks of pregnancy. Serum was analysed for AFP, HCG and uE3 using Immulite DPC 2000 (distributed by Siemens Medical Solutions Diagnostics). Serum-based risk and/or integrated risk were calculated using the Prisca 4.0 software.

Gestational age was calculated from the first day of the last menstrual period and confirmed using ultrasonography. All measured values were adjusted for maternal ethnicity, maternal weight, smoking, diabetes, multiple pregnancies, *in vitro* fertilization (IVF) and gestational age using MoM.

Prisca 4.0 software allows to give the risk estimation for trisomy 21 and trisomy 18, and in second trimester it is possible to give risk estimation for neural tube defect. Prisca 4.0 allows to give risk based on serum markers (independently based 1st trimester or 2nd trimester serum markers, but not serum integrated risk); combined risk (NT with PAPP-A and fb-HCG) and integrated risk (NT with AFP, HCG and uE3). Screening is considered to be positive if risk for trisomy 21 is above 1:270, and risk for trisomy 18 above 1:100.

Marked changes in markers were defined as above 3.0 MoM for serum markers AFP and HCG, below 0.25 MoM for PAPP-A, fb-HCG, HCG and uE3.

4.2.1.2. Ultrasound screening

First trimester screening – NT thickness was measured, if possible, at the gestational age of between 11–13 weeks of pregnancy (CRL 45–84 mm) using standard procedures by a Fetal Medicine Foundation (FMF) certified sonographer or by a well-trained sonographer working under the supervision of a certified one. A mini anomaly scan was also performed. The used NT cut-off was 3.0 mm.

All women had routine second trimester ultrasound screening in the 19th and 20th weeks of pregnancy.

4.2.1.3. Prenatal cytogenetic investigation

Cytogenetic investigation was carried out in the Department of Genetics at Tartu University Hospital with amniotic or chorionic villi cultured cell analysis

using the routine protocol (GTG-banding). The chromosomes were classified according to the International System for Human Cytogenetic Nomenclature (ISCN, 1995). Results are available in 10–12 days.

In interphase Fluorescent *in situ* hybridization (FISH) analysis, prenatal commercial test probes for chromosome 21, 13, 18 and X and Y are used. Results are available in 24–48 hours.

4.2.2. Postnatal clinical examination

Children were examined by a pediatrician or medical geneticist at the age of two years. The collected data included obstetrical information about pregnancy and birth, gestational age and growth parameters at birth, children's health problems including diagnosed congenital malformations in the neonatal period or later, maternal health problems prior to pregnancy and family history. On examination, a child's weight, height and head circumference were measured, developmental milestones were assessed and dysmorphic features were systematically sought.

Fenton Intrauterine Growth Curves and Estonian age- and gender-specific growth curves were used to evaluate growth parameters at birth and later. Low birth weight (LBW) was defined as birth weight of less than 2500 grams regardless of gestational age. Small for gestational age (SGA) was defined as birth weight below the 10^{th} percentile, and large for gestational age (LGA) was defined as birth weight above the 90^{th} percentile for gestational age. On examination, growth parameters between the 3^{rd} to 97^{th} percentiles were considered normal. Preterm and post-term births were defined as birth at less than 37 gestational weeks, or more than 42 gestational weeks respectively. The age, growth parameters and developmental milestones were corrected until two years of age in all children born less than 32 gestational weeks.

If growth failure, delayed developmental milestones, more than three dysmorphic features and/or congenital malformations were diagnosed, further diagnostic tests were indicated. These included CMA, metabolic investigations and in one case genetic analysis for NS.

4.2.2.1. Chromosomal microarray analysis

In order to detect the copy number variations in selected patients of the postnatal study group of children born to a mother after marked changes in markers of prenatal screening tests, whole-genome genotyping was performed using HumanCNV370-Quad or HumanCytoSNP-12 BeadChips (Illumina Inc., San Diego, CA, USA), allowing an effective resolution of 49 and 62 kb respectively (ten consecutive SNP markers). The genotyping procedures were performed according to the manufacturer's protocol. Genotypes were called by BeadStudio v.3.1 or GenomeStudio v2009.1 software (Illumina Inc.), and further CNV analysis and breakpoint mapping was conducted using the

QuantiSNP v1.1 or v2.1 software [Colella *et al.,* 2007]. The discovered regions were compared with the reference sample-set population-based samples from Estonia [Nelis *et al.,* 2009] to exclude population-specific variations.

4.2.2.2. APEX–array analysis for Noonan syndrome

DNA samples in selected patients of the postnatal study group of children born to a mother after marked changes in markers of prenatal screening tests were tested with an APEX assay [Kurg *et al.,* 2000]. The test was ordered commercially from ASPER Biotech, Tartu, Estonia.

Five genes were assayed for NS: *PTPN11* (32 point mutations); *SOS1* (24 point mutations); *KRAS* (10 point mutations); *RAF* (14 point mutations); and *MAP2K1* (1 point mutation).

4.2.2.3. APEX-array and MLPA analyses for congenital adrenal hyperplasia syndrome (17-hydroxylase deficiency)

DNA samples in selected patients of the postnatal study group of children born to a mother after marked changes in markers of prenatal screening tests was tested with an APEX assay [Kurg *et al.,* 2000]. The test was ordered commercially from ASPER Biotech, Tartu, Estonia.

In *CYP21A2* gene 21 specific known mutations and deletion/duplication in the region were analysed.

4.2.2.4. Metabolic investigations

Metabolic investigations in selected patients of the postnatal study group of children born to a mother after marked changes in markers of prenatal screening tests included amino acid and organic acid analysis by gas chromatography/ mass spectrometry and acylcarnitine analysis using tandem mass-spectrometry.

4.2.3. Sequencing of selected genes (TBX5 and PTPN11)

DNA was extracted from whole blood (with a K_3EDTA tube) using the standard salt-precipitation method. Candidate genes were selected on the basis of literature review. All laboratory work was done by I. Kalev in the Department of Human Biology and Genetics in the Institute of Biomedicine and Translation Medicine at University of Tartu. All methods are described in detail in Publication V and VI, and in Kalev *et al*. [Kalev 2008].

4.2.4. Statistical analysis

For statistical analysis the descriptive statistics (mean, median, and range etc.) were used.

The performance of the proposed prenatal screening protocol is measured by the DR, the proportion of affected pregnancies with positive results, and also by the FPR, the proportion of unaffected pregnancies with positive results.

$$\text{DR (\%)} = \frac{\text{All cases of DS (pre- and postnataly diagnosed DS)}}{\text{All cases of DS after positive screening test}} \text{ x } 100\%$$

$$\text{FPR (\%)} = \frac{\text{All positive PnS tests} - \text{true positive cases of DS}}{\text{All PnS tests}} \text{ x } 100\ \%$$

4.2.5. Ethical considerations

These studies were approved by the Ethics Committee on Human Research of the University of Tartu. Informed consent was obtained from the parents or legal guardians of the children for participation in the study.

5. RESULTS AND DISCUSSION

5.1. Contingent screening strategy for Down syndrome in Estonia

5.1.1. The results of first trimester screening for chromosomal abnormalities (Publication I)

During the study period (2005–2008), 3194 women agreed to participate in routine first-trimester screening. At the beginning of the screening program, 500 women also underwent routine second-trimester serum screening during the 15th to 18th weeks of pregnancy. From the beginning of 2006, two-step contingent screening was introduced (Figure 8).

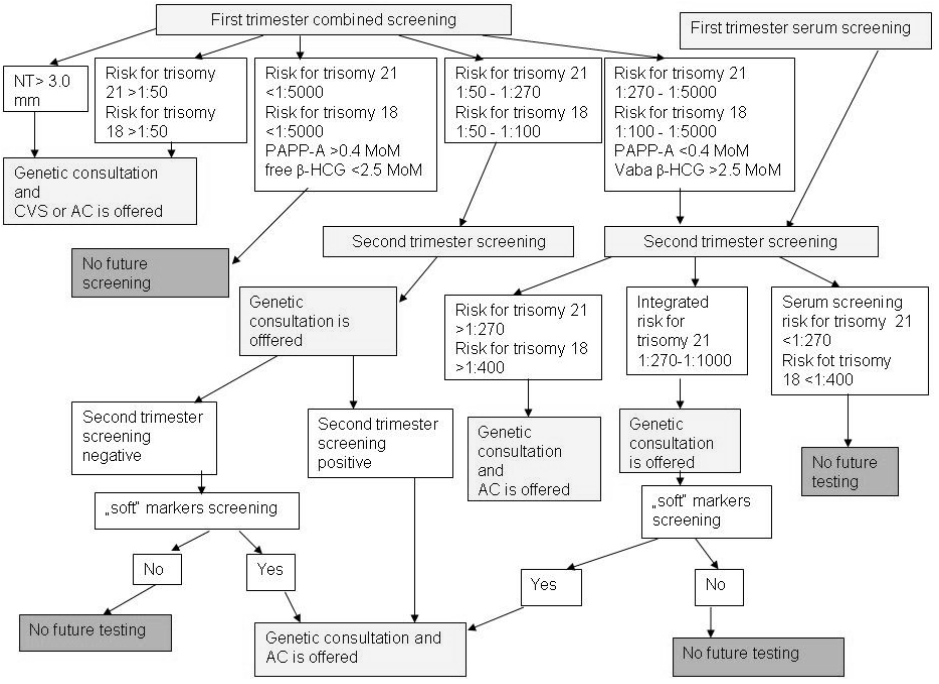


Figure 8. The used screening protocol 2006–2008.

The average age at giving birth was 28.5 years, (mean age at delivery in Estonia was 28.2 years [http://www.tai.ee]), and 355 women (11.1%) were older than 35 years of age. We were able to give to 2718 women (85.1% of all those who tested) a combined risk calculation (NT with first trimester serum screening). In 14.9% of tests, NT was either not measured properly or CRL was less than 45 mm at the time of NT measurement. The median CRL was 61.5 mm (ranging from 45–83 mm), and the median NT was 1.63 mm (ranging from 0.2–8.8 mm).

In 32 cases (1.2%), NT was greater than 3 mm. The median gestational age at the time of blood sampling was 12+2 weeks (with a range of 8–13 weeks).

Table 8. Distribution of women in different risk groups and ascertained chromosomal aberrations.

First trimester results	**High risk ≥1:50**	**Intermediate risk 1:51 – 1:5000**			**Low risk ≤1:5000**
Number of women	30 (0.93%)	1777 (55.64%)			1387 (43.44%)
Second trimester screening	0	1712 (96.3%)*			0
Second trimester results		High risk ≥1:270	Intermediate risk 1:270 – 1:1000	Low risk ≤1:1000	
Number of women		79	167	1466	
Karyotype normal	12	75	166	1465	1387
Karyotype abnormal	18	4	1 **	1 **	0
	DS 13 cases (76.5% of DS) ES 2 cases TS 2 cases Triploidy 1 case	DS 2 cases (11.8% of DS) TS 1 case *de novo* translocation 1 case ***	DS 1 case (5.9% of DS)	DS 1 case (5.9% of DS)	

* – 3.7% of women missed second trimester screening by their own choice
** – false negative cases, as there were no indications to offer prenatal diagnosis
*** – 46,XX,t(9;17)(q22;q23) *de novo*
DS – Down syndrome; ES – Edwards syndrome, TS – Turner syndrome

High-risk group

Of the 3194 women screened in the first trimester, 30 (0.93%) had a combined risk higher than 1:50. Twelve women accepted an invasive procedure on the basis of a first trimester risk calculation after counselling by a medical geneticist. CVS was the procedure chosen by seven and AC by five women. Eighteen of the high-risk women decided to repeat serum screening in the second trimester. In ten of these women the risk remained high (>1:50), and nine of the women accepted an invasive procedure. Low risk in the second

trimester was reported in eight women, and a second-trimester genetic sonogram was the first choice of these women. Among the high risk group, 18 women (60 %) were carrying a baby with a chromosomal abnormality (Table 8): DS was diagnosed in 13 cases, Edwards syndrome in two cases, triploidy (69,XXX) in one case and Turner syndrome or its variations in two cases.

NT was greater than 3 mm in 32 cases (1.2%). Chromosomal abnormality or congenital defect was diagnosed in ten of them (32%): DS in six cases, and Edwards syndrome, Turner syndrome, diaphragmal hernia or CHD in one case each.

Low-risk group

Of the women screened in the first trimester, 1387 (43.4 %) had a combined risk lower than 1:5000. No false negative cases were registered.

Intermediate risk group

In 1777 women (55.7%), we found intermediate risk in the first trimester. Of these, 65 women (3.7%) did not pass the second trimester by their own choice. In 1712 women (96.3%), second trimester serum screening was performed. An invasive procedure was offered to 79 women (2.5%) after second trimester screening due to a high-risk test result. In the intermediate risk group, six aneuploidies (0.3%) were ascertained (Table 8): DS in two cases, a Turner syndrome variation in one case and a *de novo* translocation in one final case. There were two cases in the intermediate risk group that resulted in the birth of a child with DS and were counted as false negative test results (Table 8). In the first case the first trimester screening result was intermediate (1:3611), and she had the negative result at the second trimester (1:2492). In the second case the first trimester result was 1:3534 and she also had intermediate risk at the second trimester (1:607). A genetic sonogram was offered to her, and as it was normal, according to our screening policy the invasive procedure was not offered to the family.

The DR in the study period with the developed screening program was 88.3%, the FPR 3.4% (0.9% after first-trimester screening and 2.5% after second-trimester screening). The DR and FPR are comparable to some reported results from other published screening programs [Crossley *et al.,* 2002; Okun *et al.,* 2008; Rozenberg *et al.,* 2007; Wortelboer *et al.,* 2009], but performance was nevertheless lower than the published results of large prospective studies [Nicolaides *et al.,* 2005; Spencer and Nicolaides 2003]. The lower performance rate may be due to the small study group, in which every case played a major role in DR calculation, and also the median maternal age in our study group was 3 years lower than in large studies. In time period 2005–2012 the DR was 92% with FPR 2.9% (unpublished data), confirming that new strategies need time to prove itself and there is association between performance rate and size of the study population.

Also the cut-off for the first trimester (risk ≤1:5000) we used was much lower than that presented in the literature [Benn *et al.,* 2005; Christiansen and Olesen Larsen 2002; Cocciolone *et al.,* 2008; Vadiveloo *et al.,* 2009; Wald *et al.,* 2006], and this made it possible to offer closure screening to about 40% of women. We choose to implement a very low screening-negative cut-off because we needed to calculate our own multiple of medians (MoMs), but our laboratory through-put was low. In 2011 we changed screening-negative cut-off level to 1:1500. New strategy allowed offering closure of screening after first trimester up to 75% of women, with same DR and FPR (unpublished data).

5.1.2. The effectiveness of the used contingent screening protocol (Publication I)

We performed a prospective cohort study including non-selected pregnancies during the program of first-trimester screening for DS in a 4-year period at a single centre, with the main purpose of evaluating the potential of contingent screening in the population of pregnant women.

Table 9 shows advantages and disadvantages of the introduced contingent screening policy compared with a second trimester screening protocol. It is showing that contingent screening has the potential to considerably reduce second-trimester sampling, with little impact on overall screening performance when compared to a policy of nondisclosure screening, where all markers are measured on all women [Guanciali-Franchi *et al.,* 2012; Wright *et al.,* 2006]. When one-step screening (e.g. integrated or serum screening) is easy to perform and all participants receive a risk calculation under the same conditions, the result of screening and PnD is available four weeks later, and one should not overlook the human cost [Wald *et al.,* 2006]. Contingent screening is not a single test, but the application a sequence of tests; there are many cut-off combinations in which the different tests can be used, and local needs and possibilities should be adapted [Wald *et al.,* 2006]. Considering our local possibilities and need, we initially used a low screening-negative cut-off, and only raised our screening-negative cut-off to 1:1500 after 5 years' experience. With subsequent years' experience, we can say that this was reasonable (DR and FPR have remained stable).

Table 9. Assessment of two different screening policies used in Estonia, adapted from [Wald *et al.,* 2006].

Criteria	Screening policy	
	Second trimester serum screening	**Contingent screening**
Administrative simplicity	+	–
Safety	–	+
Cost effectiveness	–	+
Early diagnosis	–	+
Avoids confusion from two risk estimates in the same pregnancy	+	–
Avoids unnecessary terminations (about one in five pregnancies terminated would have miscarry between the first and second trimesters)	+	±
Avoids the risk of being missed, because some screening measurements are not performed in some women	+	±
Retention of AFP screening	+	–

Using a very low cut-off, however, means that more women had to wait for second-trimester screening results, which may create more anxiety. Therefore we realized the importance of the fact that in counselling patients with intermediate risks, it is important to indicate that these are not "high-risk" results, but simply a sub-group for whom a second round of testing could possibly be beneficial [Benn *et al.,* 2005]. Infrequently, two independent test results may give a discrepant individual risk calculation that is difficult to interpret. A multi-step screening protocol generates additional subsets of patients who have high-risk results; potentially, all such women could request invasive testing [Cocciolone *et al.,* 2008; Huttly *et al.,* 2006]. Benn *et al.* found little evidence that 2-step screening resulted in additional invasive testing [Benn *et al.,* 2007]. In our study the women who had high risk in the first trimester but low risk in the second trimester chose the US scan and not invasive testing for additional investigation. This suggests that women did find the revised risk to be reassuring [Benn *et al.,* 2007]. In that group we did not find any DS, but our numbers are small. With appropriate patient counselling, contingent screening can potentially provide most patients with early reassurance or diagnosis, while engaging the power of additional testing for those patients who would reap the most benefit [Benn *et al.,* 2005]. At the same time in screening with several steps there is the possibility of being missed from one step [Wald *et al.,* 2006]. In our study, among women in the intermediate risk group, 65 (3.7%) women did not pass the second trimester due to their own choice, and to the best of our knowledge there were no DS in this group, but the numbers are small.

The contingent screening protocol offers first trimester low-risk group women earlier reassurance that they are not at risk of fetal chromosomal abnormalities. At the same time, contingent screening has the advantage of achieving an earlier diagnosis in a varying proportion of women. In the first trimester, high-risk results (individual risk ≥1:50) represented 0.9% of all tests, and with only first trimester screening we were able to detect 76.5% (13 of 17) of DS prenatally during the study period. The contingent screening strategy we used added two more cases to prenatally diagnosed DS and raised DR to 88.3%, with FPR of 3.4%. This is a better result than the second trimester screening with a detection rate of 67% and a false positive rate of 4.7% which was used earlier in Estonia [Sitska *et al.,* 2008a].

Earlier diagnosis allows women greater privacy and safer termination of affected pregnancies [Mennuti and Driscoll 2003]. Even when we know that about 30% pregnancies with DS will miscarriage after the 12th week of pregnancy [Bray and Wright 1998], it may be better to know the reason for miscarriage or have your own decision of termination. Knowing the reason is also important for the estimation of recurrence risk, as the recurrence risk for chromosomal abnormalities is low, but higher than in the general population [De Souza *et al.,* 2009; Morris *et al.,* 2005]; and knowing this, we can give future parents the assurance of low recurrence risk and the possibility of prenatal testing during their next pregnancy.

Nearly half of women did not have AFP screening for neural tube defect. Even if in Estonia the accessibility of the US scan before the 21st week of pregnancy is high – 93.7% (in 2005–2008) [http://www.tai.ee], the US scan at the 19th or 20th week depends on the operator's competence. The operator should also be informed of the fact that AFP screening has not been performed. Similarly, as screening for chromosomal disorder is moving into the first trimester, screening for fetal neural tube defects is moving into the first trimester [Bernard *et al.,* 2013]. Unfortunately, neither AFP screening or US screening are able to detect prenatally all affected cases [Hildebrand *et al.,* 2010].

In Estonia the essential criteria for using a wider contingent screening policy is the feasibility of measuring NT, which at the moment is only possible in a few centers. There is a need to have more ultrasound operators who have a license to perform the 1st trimester US scan (NT measurement).

In conclusion, our study has demonstrated that contingent screening is effective and feasible in clinical practice in local conditions. It is a better choice for DS screening in Estonia instead of the previously used second trimester screening, and offers the advantage of earlier diagnosis.

5.2. Risk of chromosomally normal fetuses with marked changes in maternal first or second trimester serum markers and first trimester ultrasound marker NT having a congenital or genetic anomaly or delayed development at 2 years of age (Publication II)

5.2.1. Prenatal study group

A total of 5257 women underwent prenatal screening tests during the study period, and 240 of them had a positive screening result (4.6%). In total, 1525 first trimester (PAPP-A and/or fb-HCG) and 4410 second trimester (AFP and/or HCG and/or uE3) serum screening tests, and an additional 1589 first trimester ultrasonography marker NT measurements, were performed at Tartu University Hospital during the one-year investigation period.

The postulated marked changes were present in 138 pregnant women. A total of 165 significant changes were documented. Deviations in only one marker were observed in 111 cases, in two markers in 21 cases and in three markers in six cases (Figure 9). AFP elevation and/or low uE3 were the most frequent changes in solitary markers. In 80/138 cases (1.4% of all tested women), the Prisca risk calculation was positive for chromosomal disorders or neural tube defects. Of those 80 women, 74 were referred to genetic counselling, as four pregnancies had already spontaneously aborted by that moment, and two women refused to come to genetic counselling. The remaining 58 women had Prisca risk calculations that were negative for chromosomal disorders and, therefore, they were not evaluated further. However, we have one genetic service center in Estonia and at least three years later the children born from these 58 pregnancies have no clinical indication of a need to perform chromosomal analysis, so we may be quite sure that these tests were truly negative.

Of the 74 counseled women, deviation in three markers was documented in 6 cases, in two markers in 21 cases and in one marker in 47 cases. During prenatal diagnostics (ultrasound and/or fetal karyotyping), genetic pathology was diagnosed in 12 cases (16%): trisomy 21 (3 cases), trisomy 13 (1 case), triploidy (1 case), anencephaly (2 cases), *spina bifida* (1 case), gastrochisis (1 case), renal agenesis (1 case), HLHS (1 case), and fetal hydrops (1 case) (Table 10). Fetal death was diagnosed in 12 cases (16%). In the subgroup with three marker changes, fetal pathologies were diagnosed in all cases (6/6): fetal death (4), triploidy (1) and genetic pathology diagnosed after birth (1). In the subgroup with two markers, fetal pathologies were diagnosed in 8/21 (38%) of cases: fetal death (4), anencephaly (1), DS (1), trisomy 13 (1), fetal hydrops (1) (Table 11).

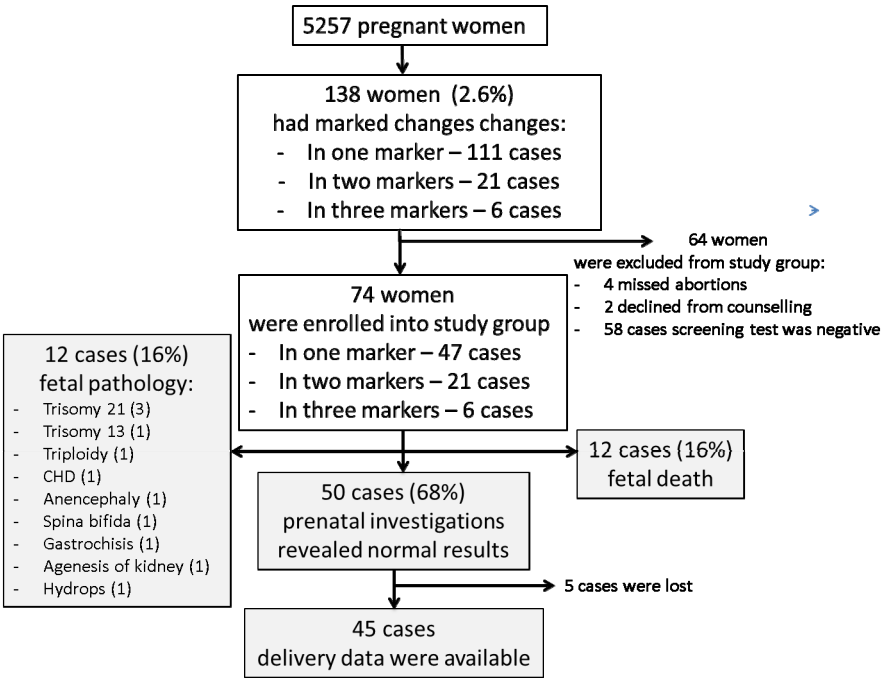


Figure 9. Flow diagram of results of prenatal study group.

Table 10. Prenatally diagnosed pathology and screening results.

NT (mm)	PAPP-A (MoM)	fb-HCG (MoM)	AFP (MoM)	HCG (MoM)	uE3 (MoM)	Diagnosed pathology	Pregnancy outcome
1.7	**0.23**	0.51	1.72	1.33	**0.13**	trisomy 13	terminated
6	1	2.4	–	–	–	trisomy 21	terminated
4.4	**0.22**	**4.04**	–	–	–	trisomy 21	terminated
–	–	–	0.53	2.44	**0.18**	trisomy 21	terminated
2.2	**0.07**	**0.09**	0.34	**0.02**	0.27	triploidy	terminated
3.7	0.9	0.36	–	–	–	HLHS**	terminated
–	–	–	**4.97**	0.62	0.3	anencephaly	terminated
–	–	–	**12.01**	1.87	**0.23**	anencephaly	terminated
–	–	–	**7.67**	0.52	1.31	gastroschisis	terminated
1.4	**0.24**	**0.14**	–	–	–	hydrops*	terminated
–	–	–	**4.19**	1.1	1.44	bilateral renal agenesis	terminated
–	–	–	**5.12**	0.78	1.36	neural tube defect	terminated

* karyotype 46,XY
** HLHS – hypoplastic left heart syndrome, karyotype – 46,XY

Table 11. Association in outcome of pregnancy and number of changed markers.

	Subjects	Fetal death	Fetal pathology	Subjects*	Threatened miscarriage	PIH	Platcental pathology	Preterm birth	Postterm birth	SGA	LGA	Postnatally diagnosed genetic disease
extreme value in one marker	47	4	7	36(81%)	2	1	5	2	4	4	1	2***
extreme value in two marker	21	4	4	13(62%)	1	1	1	1	0	1	0	1****
extreme value in ≥ 3 markers	6	4	1	1(17%)	0	0	1	1	0	1	0	1*****
Total	**74**	**12**	**12**	**50****	**3**	**2**	**7**	**4**	**4**	**6**	**1**	**4**

SGA – small for gestational age; LGA – large for gestational age; PIH – pregnancy induced hypertension;
* – number of women carried on with pregnancy; ** – 5 of them declined from future participating in this study, study group consisted of 45 women; *** – one congenital heart disease, one congenital adrenal hyperplasia; **** – one congenital heart disease, ***** – one Silver-Russell syndrome

The diagnosed complications through pregnancy and delivery were evaluated in the remaining 45 pregnant women; no information was available about five pregnancies (Table 11). Pregnancy complications (threatened miscarriage or pregnancy induced hypertension (PIH)) were documented in 5/45 cases. Complications during delivery were documented in 9/45 women: premature birth (3 cases), induction of premature labour due to fetal distress and oligohydramnion (1 case), emergency Cesarean section (1 case) and post-term delivery (4 cases). Placental pathologies were documented in 7/45 cases (small placenta for gestational age was the most common pathology observed).

Abnormal levels of serum markers and elevated NT in prenatal screening for the most common aneuploidies have been associated with adverse pregnancy outcomes [Gagnon *et al.,* 2008; Goetzl 2010; Huang *et al.,* 2005; Ilagan *et al.,* 2004; Miltoft *et al.,* 2012; Spencer 2000; Spencer *et al.,* 2008a; Spencer *et al.,* 2006; Summers *et al.,* 2003].

Increased NT during first trimester ultrasound screening was documented in nine cases (in all cases the NT measured between 3 and 6 mm), which is less than expected. The reason may be that women with enlarged NT did not choose serum screening, and instead immediately opted for PnD. During subsequent prenatal investigations, chromosomal anomalies were diagnosed in two cases, and a critical heart defect (HLHS) was diagnosed in one case; in all of these cases, the pregnancy was terminated. Pregnancy outcome was documented in 6 cases: normal pregnancy and delivery in two cases, whereas threatened miscarriage was documented in 3 pregnancies, and in one case LGA was diagnosed at birth. One mother declined to come to the follow-up consultation with her child after birth.

Low PAPP-A was documented in 14/1525 cases (0.92%), and of those, eight were enrolled in the study group. Prenatal investigations diagnosed fetal pathology in 4/8 cases: triploidy, DS, trisomy 13 and fetal hydrops. In one case SGA was documented at delivery, but the others had normal birth weight. In our small study group, an association between low PAPP-A and SGA was documented in 1/4 of cases. An association between low PAPP-A and SGA or preterm delivery has been found in several published studies [Dugoff *et al.,* 2004; Spencer *et al.,* 2008a; Spencer *et al.,* 2008b]; the association between PAPP-A and SGA seems to be stronger that between fb-HCG and SGA [Spencer *et al.,* 2008a].

Low fb-HCG during 1st trimester screening was documented in 18/1525 cases (1.2%), and six of these were included in the study group. With PnD 2/6 fetal pathology was diagnosed (triploidy, fetal hydrops); one child was born from induced preterm labour in the 30th week of pregnancy due to oligohydramnion, in one case there was a post-term delivery, and two children were healthy at birth. Similarly to low PAPP-A, low fb-HCG is associated with adverse outcome (late pregnancy loss, preterm birth) of pregnancy; women with marked changes in PAPP-A and/or fb-HCG should undergo subsequent evaluation [Dugoff *et al.,* 2004; Goetzl 2010; Krantz *et al.,* 2004]. At the same

time, elevated fb-HCG with elevated HCG during the 2nd trimester was documented in only 3/1525 cases, prenatal investigation revealed normal results and children were born with normal birth parameters after normal pregnancies.

Elevated AFP (>3.0 MoM) was documented in 26/4410 cases (0.6%); in 19 cases the AFP was the only changed marker in the screening test. 24 women came to genetic counselling, and during ultrasound examination, fetal death was diagnosed in 8/24 cases (33%) and structural anomalies in 5/24 cases (20.8%). Pregnancy outcome was observed in 11 cases with elevated AFP, pregnancy without any complications and normal birth was documented in 6/11(55%) cases, whereas the remaining cases were complicated by preterm birth (in 2/11 cases), SGA (in 2/11 cases), placental pathology (in 5/11 cases) and PIH (1). The AFP level is associated with a high rate of pregnancy complications [Alleman *et al.,* 2013; Duric *et al.,* 2003; McPherson *et al.,* 2011; Milunsky *et al.,* 1989; Spaggiari *et al.,* 2013; Spencer 2000]. According to the National Institute for Health Development in Estonia, the rate of spontaneous abortions was 2–6 % per year during 2009–2010 [http://www.tai.ee]. In our study group, fetal death was found to be 16%, and in the subgroup with elevated AFP value the percentage was as high as 33%, which is much higher than in the general population. Pregnancy and delivery without complications after elevated AFP during second trimester and with normal ultrasound results was documented in only 55% of cases. AFP elevation in maternal serum during the second trimester is a risk factor for adverse pregnancy outcome, and additional ultrasound during the third trimester is recommended [McPherson *et al.,* 2011]. Spaggiari *et al.* have also suggested monitoring the maternal serum AFP level twice to select a subgroup that is at higher risk for fetal death, preeclampsia, and/or intrauterine growth restriction [Spaggiari *et al.,* 2013].

Low uE3 (<0.25 MoM) was detected in 43/4410 cases (0.98%), and in 36 cases low uE3 was the only changed marker in the screening test. The screening test was considered positive in 35 cases, of which 33 came to genetic consultation. During ultrasound examination, fetal death was detected in 10/33 cases (30%); fetal structural anomaly was diagnosed in one case, and fetal chromosomal anomaly in two cases. Pregnancy outcome was observed in 20 cases. Pregnancy without any problems and birth at term were documented in 15/20 cases (75%), but SGA was diagnosed in two cases. Of remaining one pregnancy complicated with induced prenatal labour due to intrauterine growth restriction and oligohydramnion, four children were born post-term (4/20 cases). Low uE3 in maternal serum during the second trimester has been associated with adverse perinatal outcome (including intrauterine death) and with several genetic conditions, such as congenital adrenal hypoplasia, steroid sulfactase deficiency and the Smith-Lemli-Opitz syndrome [Bradley *et al.,* 1999; Dugoff and Society for Maternal-Fetal 2010; Duric *et al.,* 2003; Gagnon *et al.,* 2008; McPherson *et al.,* 2011]. In our study group, fetal death was found in 16%, in the subgroup with low uE3 value, the percentage was considerably higher (30%), and in two of these cases uE3 was the only significantly changed

marker. Pregnancy loss during the second trimester was much higher in the study group than in the pregnant population as a whole [http://www.tai.ee]. In our small study group, congenital adrenal hyperplasia was postnatally diagnosed in one case, whereas no cases with the Smith-Lemli-Opiz syndrome or steroid sulfactase deficiency were found. However, in implemented prenatal screening program, cases with low uE3 are managed as recommended in the literature [Dugoff and Society for Maternal-Fetal 2010; McPherson *et al.,* 2011]. In addition, in our study group, we diagnosed postnatally Silver-Russell syndrome (SRS); during 1st trimester there was low fb-HCG (0.1 MoM) and during 2nd trimester screening there was very low uE3 (0.09 MoM) and HCG (0.18 MoM) in maternal serum. Low values for PAPP-A, fb-HCG, HCG and uE3 in maternal serum may be markers of genetic syndrome, with IUGR as one feature.

Prenatal screening tests are valuable not only for aneuploidy screening, but may also be predictors for complications during pregnancy, delivery and in the postnatal period. However it should be kept in mind, even after the optimization of cut-off values, these markers do not appear to be clinically as an effective tool for screening for adverse pregnancy outcomes [Kavak *et al.,* 2006]. Nevertheless, women with a positive screening test and marked changes in the screening markers and normal prenatal investigations could benefit from closer monitoring regarding possible complications during pregnancy and delivery; but evaluation and subsequent patient management must be based on the potential complications associated with the serum marker pattern [Alkazaleh *et al.,* 2006; Bromley *et al.,* 1994; Gagnon *et al.,* 2008; Ghosh *et al.,* 2006; Spaggiari *et al.,* 2013].

5.2.2. Postnatal study group

The postnatal study group consisted of 35 children. Four children had postnatally been diagnosed with congenital anomalies and/or syndromes: two had CHD – ASD and VSD with PDA, one had SRS and one had congenital adrenal hyperplasia (CAH). On follow-up examination, further diagnostics was additionally indicated in eight children, while one mother refused to permit investigations. In one case previously clinically diagnosed CAH was confirmed by DNA test. All other results of the performed analyses (CMA, APEX-array for NS, and metabolic investigations) were normal.

The rate of CHD in our postnatal study group was 5.7%. Two heart defects were diagnosed postnatally (ASD and VSD with PDA). Both children were born as SGA, and had more than three dysmorphic features and delayed milestones on examination. Nevertheless, both had normal karyotypes on prenatal testing and also normal CMA on examination. These children have had surgical repair of CHD and are still undergoing follow-up with a cardiologist. As the incidence of CHD may vary among studies depending on the timing of echocardiograms and the population included, their defects can be classified as

moderate lesions, which together with severe CHD have an average incidence of 6 per 1000 live births [Hoffman and Kaplan 2002]. Both children have the most common forms of CHD. In addition, to our knowledge at least one heart anomaly (HLHS) with normal karyotype and no 22q11.2 microdeletion was diagnosed prenatally, and the pregnancy was ended due to congenital malformation. In this case fetal NT was 3.6 mm. The relationship between increased NT and CHD has been described, and a recently published meta-analysis found that approximately 44% of major CHD in fetuses with normal karyotype have NT of more than 2.5 mm [Sotiriadis *et al.,* 2013]. In our study group, 9 fetuses had NT ≥3 mm, and only one case with major CHD was found. If we exclude the two cases with fetal chromosomal anomalies, only one of the 7 fetuses (14%) with NT ≥3 mm had CHD. As for the other two postnatally diagnosed CHD cases, NT was 1.8mm in one child, and one child did not have an NT scan during its fetal life. Nevertheless, fetuses with increased NT should be screened for major CHD during pregnancy. Unfortunately, not all women in Estonia have access to an NT scan, as not all sonographers have the FMF certificate.

Five children belong to the postnatal subgroup with increased NT. Three of them had more than three dysmorphic features, but none had delayed developmental milestones or structural defects. Similar between increased NT and normal long-term neurodevelopmental outcomes have been described in several other studies [Bilardo *et al.,* 2007; Hiippala *et al.,* 2001; Maymon *et al.,* 2000; Miltoft *et al.,* 2012; Mula *et al.,* 2012; Saldanha *et al.,* 2009; Schou *et al.,* 2009; Senat *et al.,* 2007; Van Vugt *et al.,* 1998]. At the same time, five children (14%) in postnatal subgroup with marked changes in maternal serum markers had delayed milestones at the age of 2 years. Nevertheless, one cannot draw any conclusions about the links between serum markers and children's developmental outcome, as our postnatal study group is small, the affected serum markers were different and the evaluation of development is based on questioning the mothers and on the examiners' subjective opinions.

In conclusion, of the offspring of 74 pregnant women (1.4% of all screened women) with postulated marked changes and positive Prisca risk calculation, prenatal or postnatal genetic abnormality was diagnosed in 16 cases (21.6%), fetal death occurred in 12 cases (16.2%) and the child was healthy at the age of 2 years in 31 cases (41.9%) (Table 12). Genetic abnormalities were diagnosed prenatally in 3/4 of cases and postnatally in 1/4 of cases. It was not possible to get the final information about pregnancy outcome in 15 cases (20.3%).

Table 12. Outcome of pregnancies with postulated marked changes in prenatal screening.

During a one-year evaluation period among 5257 pregnant women
74 pregnant women (1.4%) with postulated marked changes and a positive Prisca risk calculation
Prenatally confirmed genetic or structural abnormality – 12 cases (16.2%) Fetal death – 12 cases (16.2%) Postnatally confirmed genetic abnormality – 4 cases (5.4%) Healthy child at the age of 2 years – 31 cases (41.9%) Lost during prenatal or postnatal evaluation – 15 cases (20.3%)

One limitation of our study was the small and mixed study population. A strict follow-up group with extensive follow-up is possible in bigger centers. Nevertheless, a correlation between significant changes in prenatal screening markers and adverse pregnancy outcome can still be seen.

Our study confirmed the fact that prenatal screening tests are not only valuable for aneuploidy screening, and children born to these mothers should be followed by a pediatrician for additional consultation after birth.

In our study group we postnatally diagnosed genetic disorders in two cases. We describe in detail the changes in maternal serum markers and pregnancy-related and postnatal outcomes of CAH.

5.2.2.2. Case report – Congenital adrenal hyperplasia

The patient's mother's 1st trimester screening marker levels were in the normal range (PAPP-A 0.59 MoM and fb-HCG 1.58 MoM, NT was 1.8 mm), but she had a high risk for trisomy 21 (1:82). She also wanted to perform the 2nd trimester screening test before making a final decision about invasive procedures. In the 2nd trimester screening test, the risk for trisomy 21 was also high (1:15) and uE3 was very low (0.15 MoM). Fetal karyotype was normal – 46,XY, and ultrasound investigation in the second trimester showed normal fetal development. Molecular testing for CAH was not available in Estonia in 2009, and due to low uE3, postnatal screening for CAH was suggested. The child was born from a normal birth at term with normal birth weight and length, and neonatal period was without problems. After birth, the measurement of 17-OH-progesterone revealed a marked elevation (95.7 nmol/L), and CAH was diagnosed. The diagnosis was confirmed by DNA analysis, which showed compound heterozygosity: there was a chimeric gene involving the *CYP21A2* gene and a pseudogene in one allele, and the deletion of exon 3 of *CYP21A2*

was discovered in the second allele. At the age of two years he was a normally developed boy.

From 2012, in Estonia, prenatal testing for CAH is suggested in the case of low uE3 (<0.2 MoM) in maternal serum and normal karyotype.

5.3. Genetic causes of congenital heart anomalies in Estonian children

The main aim of our study was to identify the molecular etiology of genetic dysmorphic syndromes, one of whose main feature is CHD. We selected individuals from the study group of familial CHD (55 families). After careful clinical investigation, the genetic syndrome causing CHD was suspected in 35 patients from 29 families: NS in 29 cases, LS in 4 cases and HOS in 2 cases.

5.3.1. Noonan syndrome

Our study group consisted of 29 patients with clinical suspicion of NS: 23 probands and their six family members. Clinical diagnosis was confirmed based on the diagnostic criteria proposed by van de Burgt [van der Burgt 2007] (Table 7). At the time of examination, patients were in the age range from 3 months to 36 years, and the sex ratio was equal (F/M ratio 14/15). In five families was more than one affected family member.

5.3.1.1. Phenotype evaluation of probands with clinical diagnosis of NS

CHD and short stature were the main clinical complaints for probands upon referral to genetic consultation. Height of less than 3 percentiles was documented in approximately half of cases (12/23), and CHD was a feature in two-thirds of them (15/23). Six of them had complex CHD (6/15); PS (7/15) was diagnosed most often. The classical facial phenotype of NS was described in half of cases (11/23). Hypertelorism (18/23), low-set dysmorphic ears (15/23) and a wide or short neck (17/23) were observed most often. Among additional features, hypotonia (9/23) and pectus excavatum/broad thorax (15/23) were described occasionally. In two male cases (13%), cryptorchidism was diagnosed. Hearing loss was diagnosed in 2/23 probands. In this group, developmental delay or mental retardation was documented in 13/23 (50%) cases, and in 3 (12.5%) cases cognitive delay was observed. In five cases the familiar NS was suspected and in four families other family members were added to the clinical study group.

Patients' clinical features, NS scoring results and molecular findings are described in table (Appendix I).

5.3.1.2. Molecular testing for *PTPN11* gene

In all 23 probands, exons 2, 3, 4, 7, 8, 12, and 13 of the *PTPN11* gene (NM_002834.3) were tested. The mutation was detected in 4/23 cases (17%). Mutations were found in exon 3 (c.172A>G; p.Asn58Asp), in exon 8 (c.923A>G; p.Asn308Ser), in exon 12 (c.1403C>T; p.Thr468Met) and in exon 13. (c.1510A>G; pMet504Val) (Figure 10). Mutation p.Thr468Met in exon 12 is described in cases of clinical diagnosis of both NS and LS [Aoki *et al.,* 2008; Lee *et al.,* 2011], but in our case the clinical features suggested NS. In the case of detected mutation in the *PTPN11* gene, genetic testing was offered to family members (with or without clinical diagnosis of NS), and 9 family members from 4 families were tested. In two families the proband inherited the mutation from a parent (NS was not previously molecularly confirmed, but clinically suspected at genetic counselling) and in two cases, thereafter, diagnosis of NS was also molecularly confirmed in siblings (Figure 11).

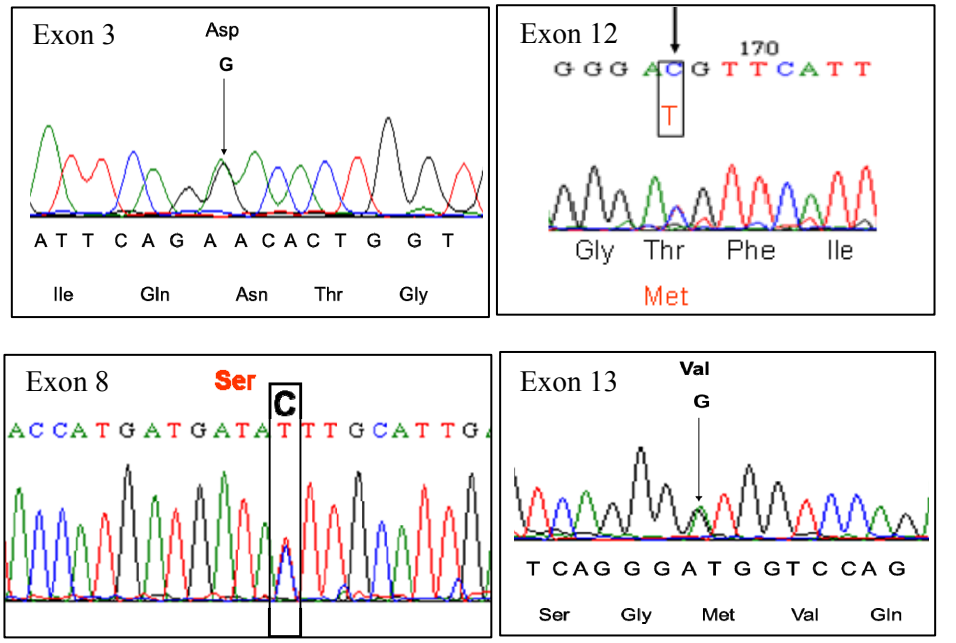


Figure 10. Sequencing chromatograms of the *PTPN11* mutation.

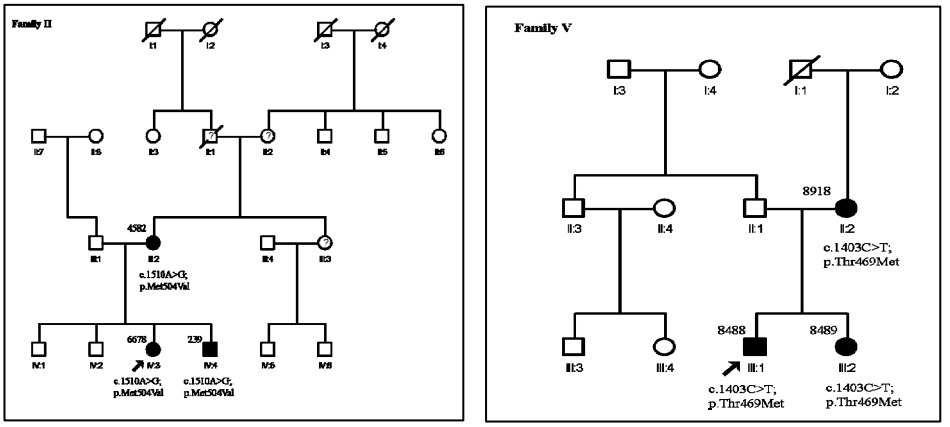


Figure 11. Pedigrees of families II and V.

5.3.1.3. Genotype-phenotype correlation of the patients with NS

In our study group, mutations in *PTPN11* were detected in 8/29 cases (in 28% of patients with clinical suspicion of NS). Detection of mutation in *PTPN11* in other published studies is higher, approximately 33–45% [Jongmans *et al.,* 2005; Kosaki *et al.,* 2002; Lee *et al.,* 2011; Romano *et al.,* 2010; Yoshida *et al.,* 2004a]. This may be due to the fact that selection bias plays a role here. Firstly, we may have less experience in clinical diagnosis of NS, and therefore we may have also included in the study group patients with lower NS scoring points. At the same time, other authors may have stronger clinical criteria. However, the mutation in *PTPN11* was detected in the case of low NS scoring points (family V), which confirms the extremely variable phenotype of NS.

When the usual reason for referring to genetic consultation was CHD with dysmorphic feature and/or short stature, then the decisive trait for selection into study group was typical or suggestive facial dysmorphism. At the same time, typical facial dysmorphism was described in only 55% of all cases, but in the subgroup with mutation in *PTPN11*, all patients had typical facial dysmorphism as hypertelorism, downslanted palpebral fissures, low-set dysmorphic ears, a wide/webbed neck, and prominent forehead in infants and a more triangular face in adults.

Our study group contained five cases of familial NS. The phenotype in these families was variable, and although short stature and dysmorphic features are distinctive features of NS, CHD was often the reason for children being referred to genetic consultation. The *PTPN11* mutation was detected in two familial cases (p.Met504Val, p.Thr468Met).

Table 13 summarises the distribution of several characteristics of NS, grouped by mutation-positive and negative.

Table 13. Frequency of characteristic NS findings grouped by *PTPN11* mutation-positive and negative test results.

Clinical symptoms	**Patients with clinical diagnosis of NS (n=29)**		**Patients with mutation in *PTPN11* (n=8)**		**Patients without mutation in *PTPN11* (n=21)**	
Sex: male	15	52%	4	50%	11	52%
Short stature (<3 percentile)	15	52%	3	37.5%	12	57%
Facial phenotype						
Typical	16	55%	8	100%	8	38%
Mild	13	45%			13	62%
CHD	17	58%	5	62.5%	12	57%
Complex CHD	6	21%	2	25%	4	19%
PS	9	31%	3	37.5%	6	28.6%
HCM	1	3%			1	4.8%
ASD	3	10%	2	25%	1	4.8%
Valvular insufficiency	4	14%	2	25%	2	9.5%
AVSD	1	3%			1	4.8%
FOA	2	7%			2	9.5%
TOF	1	3%			1	4.8%
Dilatative cardiomyopathy	1	3%			1	4.8%
PDA	1	3%			1	4.8%
Thorax anomalies	17	59%	6	75%	11	52%
Cryptorchidism	2	13%			2	13%
Hearing loss	3	10%			3	14.3%
Ocular anomalies	11	38%	5	62.5%	6	14.3%
Strabismus	4	14%	1	12.5%	3	14.3%
Ptosis	7	24%	4	50%	3	14.3%
Myopia	1	3%			1	4.8%
Hypoplasia *n. optici*	1	3%			1	4.8%
Renal pathology	3	10%	1	12.5%	2	9.5%
Hypotonia (during infancy)	11	38%	4	50%	7	33%
Cognitive delay	3	10%	1	12.5%	2	9.5%
Developmental delay	13	45%			13	62%

Short stature (stature <3 percentile) was documented in 52% of cases, but it occurred less in the mutation-positive group. The reason for the less pronounced short stature in the mutation-positive group may be that 2 patients were less than 1 year old at the time of observation, and short stature is not so obvious at that age [Digilio *et al.,* 2011]. In our study group the percentage with short stature was similar to other studies, in which approximately 50–70% of individuals with NS have short stature [Croonen *et al.,* 2013a; Shaw *et al.,* 2007]. In our group we had five adult women, and in four of them height was less than 3 percentiles (155–156 cm), which is comparable with mean adult height (153 cm) in European individuals [Ranke *et al.,* 1988; Shaw *et al.,* 2007].

The prevalence of CHD was less in our study group (58%) than in the literature (80–85%) [Kosaki *et al.,* 2002; Sznajer *et al.,* 2007]. The prevalence of CHD was the same in mutation-positive and mutation-negative group (5/8 versus 12/21). PS was the most prevalent CHD, diagnosed in 31% of patients, and ASD and valvular insufficiency occurred in 10% and 14% patients respectively. As in other studies, PS was the most common CHD in NS patients, following by ASD [Croonen *et al.,* 2013a; Jongmans *et al.,* 2005; Shaw *et al.,* 2007; Sznajer *et al.,* 2007; Tartaglia *et al.,* 2010].

Thorax anomalies (broad thorax and/or pectus excavatum) were described in 59% of cases, and in 75% of mutation-positive cases. Mutation in *PTPN11* is associated with a higher presence of chest deformity [Jongmans *et al.,* 2005; Zenker *et al.,* 2004; Yoshida *et al.,* 2004a]. Renal anomaly, with little medical significance, was diagnosed in 3 patients (10%), which is comparable with other studies [Romano *et al.,* 2010].

Feeding difficulties and bleeding diathesis are quite common features in NS, but in our study there was no documentation of such symptoms. Lymphedema in the perinatal period was documented in two cases (in the group without mutation). Cryptorchidism was also documented in only two cases, which is markedly less than in other studies [Jongmans *et al.,* 2005; Lee *et al.,* 2011; Tartaglia *et al.,* 2002].

In the mutation-positive group, hypotonia during infancy was more prevalent. At the same time, developmental delay or mental retardation was less common in patients with *PTPN11* mutation, whereas children with *PTPN11* mutation were younger (age ranged from 3 months to 9 years). Despite no developmental problems at the assessment, they need follow–up during development [Roberts *et al.,* 2013; Romano *et al.,* 2010].

NS is the most frequently reported genetic syndrome associated with prenatally enlarged NT during the 1st trimester [Pergament *et al.,* 2011; Souka *et al.,* 1998], but unfortunately 1st trimester US scan was not widely feasible in Estonia, and only one child without CHD had documented increased NT during fetal life. Still, as we have access to perform prenatal NS testing with the APEX-array in Estonia, we recommend it after normal karyotype results to all women with increased NT. Recent studies have shown that there is a higher detection rate for NS if the fetus has some additional US features (as persistent

nuchal fold, cystic hygroma, hydrops fetalis, pleural effusion) to enlarged NT [Bakker *et al.,* 2011], and therefore we should process our own work results, and if appropriate make changes to our protocols.

During our study period (2006–2010), two cases from the mutation-negative subgroup with clinical diagnosis of NS, another diagnosis of genetic syndrome was confirmed: Cantu syndrome in one case, and microdeletion 10q23.1–23.3 in another case. This demonstrates how difficult it can be to confirm the diagnosis of a genetic syndrome on clinical ground alone.

In the first case (6048), Cantu syndrome was also suspected due to hyperthrichosis, which is a quite unusual feature for NS [Roberts *et al.,* 2013; Tartaglia *et al.,* 2011]. However, mutational analysis could only be performed after the mutations in the *ABCC9* gene were associated with Cantu syndrome in 2012 [Harakalova *et al.,* 2012]. *De novo* mutation c.3347G>A; p.Arg1116His in the *ABCC9* gene was detected in this patient by sequencing analysis, and a diagnosis of Cantu syndrome was confirmed (personal communication Prof. K. Õunap).

In the second case (6258), the hypothesis of microdeletion syndrome was raised after the diagnosis of juvenile polyposis at the age of 4 years. Before that, this patient had a clinical diagnosis of NS (NS scoring points – 2A+B). Approximately 20% of individuals with juvenile polyposis have mutations in the *BMPR1A* gene, and approximately 20% of them have mutations in the *SMAD4* gene. Both genes are located in chromosomal region 10q23.1–23.3. Therefore CMA was performed in this index patient, and a 5.1Mb size deletion in the region 10q23.1–23.3 was detected, which confirmed that this deletion is responsible for the patient's polyposis [Reimand 2011]. The deleted region also includes also part of the *PTEN* gene. A germline mutation of the *PTEN* gene is associated with the PTEN hamartoma tumor syndrome encompasses four major clinically distinct syndromes – Cowden, Bannayan-Riley-Ruvalcaba, Proteus, and Proteus-like syndrome [Hobert and Eng 2009]. Because our patient also presents several features of these syndromes, such as macrocephaly, developmental delay and polyposis, they cannot be ruled out.

In the present study, *PTPN11* mutations were not identified in a significant portion of patients (21 out of 29) who had fulfilled the diagnostic criteria for NS. Phenotypic features were mostly comparable in the mutation-negative and mutation-positive group (Table 13). This points to genetic heterogeneity in NS, and future analysis in the subgroup of patients with classical features of NS should be continued. It may also point to the complexity of clinical diagnosing NS, as NS overlaps with several genetic syndromes. Genotyping can also aid with diagnosis in the case of mild or atypical features, when genotyping could establish the diagnosis, and enable better management and improve genetic counselling.

5.3.2. LEOPARD syndrome (Publication V)

Our study group contained 4 cases (age at clinical examination was from 7 days to 16 years) with clinically suspected LS (Appendix II). The *PTPN11* gene was tested in all cases, and mutation in the *PTPN11* gene was found in only one case (LS-I) in an exon 7 mutation c.836A>C (p.Tyr279Cys) with complete clinical features, but distinct changes in skin pigmentation.

The patient (LS-I) was a 4-year-old girl who was the second child in the family. She was born from induced labour due to polyhydramnion, with a birth weight of 3230 g and a birth length of 50 cm. Due to repeated vomiting and respiratory distress, she was re-hospitalized in the second week of life; cardiac investigation showed mild mitral prolaps and regurgitation. At the age of one month, HCM was diagnosed. She was closely followed for HCM etiology, mainly due to a suspicion of metabolic diseases (fatty acid oxidation, mitochondrial defects and Pompe disease). At the age of 4 years she presented slight growth retardation: her height was in the 10^{th} percentile, her weight was in the 25^{th} percentile and her head circumference was below the 10^{th} percentile. Psychomotor development was normal. She showed facial dysmorphism with a prominent forehead and hypertelorism; she had a wide neck with a pterygium, wide trunk and small umbilical hernia. She has been treated with beta-blockers and her HCM status has not progressed; she has been followed by a cardiologist every 4 months. Although her first lentigines appeared at birth, their rapid growth began at the age of 3 years. They were located mostly in the joint areas of mostly the lower but also the upper extremities, but not on the face or upper trunk (Figure 12). The rapid growth of lentigines made it possible to shift the diagnosis toward LS.

The diagnosis was confirmed by molecular analyses. The bi-directional direct sequencing of *PTPN11* mutation hot-spot exons, including 7, 12 and 13, and their flanking intron boundaries, revealed one of the most frequent *PTPN11* mutations, p.Tyr279Cys, in exon 7. Molecular analysis of the parents revealed *de novo* mutation. Cytogenetic analysis yielded a normal result.

Molecular analysis of the patient revealed one of the most common mutations, Y279C, in the *PTPN11* gene. In genotype-phenotype correlation, the mutation p.Tyr279Cys is more frequently associated with short stature, deafness and HCM [Sarkozy *et al.,* 2004]. Although more than half of p.Tyr279Cys patients have short stature, we did not notice this in our patient [Digilio *et al.,* 2002; Digilio *et al.,* 2006; Keren *et al.,* 2004; Legius *et al.,* 2002; Limongelli *et al.,* 2007; Limongelli *et al.,* 2008; Sarkozy *et al.,* 2004; Yoshida *et al.,* 2004b]. This could be explained by the fact that our patient was only 4 years old, and this symptom may be more evident later in childhood. Deafness is a frequent characteristic of p.Tyr279Cys in LS, with about 20–25% of patients' exhibit hearing loss [Sarkozy *et al.,* 2004]. Although hearing was checked in our patient and acoustic investigation showed normal hearing at the time of examination, periodic hearing assessment is recommended. Indeed, one of the main health problems of LS patients carrying the *PTPN11* mutation p.Tyr279Cys is HCM,

and in all children in whom LS was diagnosed during the first year of life [Digilio *et al.,* 2006]. Our patient had severe HCM in the neonatal period, and metabolic diseases that cause HCM were strongly suspected. Although in our patient HCM has remained in a stable condition, follow-up is needed, because it may become more discernible over time [Yoshida *et al.,* 2004b].

Most patients with the p.Tyr279Cys mutation exhibit ML, which usually appears at the age of 2–3 years and is located predominantly on face and upper trunk [Digilio *et al.,* 2006; Sarkozy *et al.,* 2004; Tartaglia *et al.,* 2010; Yoshida *et al.,* 2004b]. Our proband presented her first lentigines at birth, but their rapid growth began at the age of 2–3 years. Distribution of the ML was unusual, as they were located mainly on the extremities and in the joint regions, while the upper part of the trunk and the face were spared (Figure 12). To the best of our knowledge, only one earlier report has described ML predominantly on the lower extremities [Adriaenssens *et al.,* 2007], although unfortunately there are no comments about the distribution of lentigines in the most published cases. Therefore it is speculative to say that this is a special feature. Furthermore, the number of ML tends to increase until puberty, and therefore the involvement of face and trunk may become more intense over time.

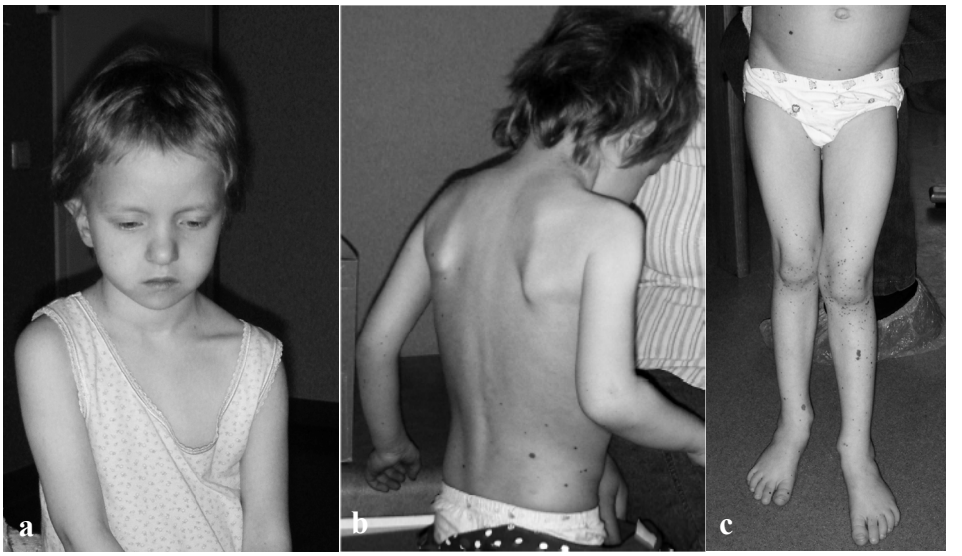


Figure 12. Phenotype and location of lentigines of the proband (LS-I) at the age of 4 years: the face and the upper trunk have been spared from lentigines (a, b). ML are located mostly in the joint areas and especially in the lower extremities (c).

Even if LS is a rare disorder, it has been less often described in Northern Europe [Adriaenssens *et al.,* 2007; Legius *et al.,* 2002; Yoshida *et al.,* 2004b]. This may be due to lower testing and research availability, especially in countries of the former Soviet Union.
Thus it seems that LS is a genetically quite homogenous disease, as 88% of clinically diagnosed LS cases have a mutation in the *PTPN11* gene [Sarkozy *et al.,* 2004], and it cannot always be confirmed after clinical diagnosis. In our study group, only one of four clinically suspected cases of LS was confirmed with molecular testing. This may be due to the fact that LS is phenotypically very variable and has marked overlap with related disorders: Noonan and Costello syndromes, neurofibromatosis type I (NF1) and cardio-facio-cutaneous syndrome (Table 14). Confirmation of the clinical diagnosis of LS using molecular tools is greatly needed. In two cases (LS-II, LS-III) from our study group, the main hypothesis after excluding LS is NF1, and in one case (LS-IV) the observation is ongoing.

The long-term prognosis of LS patients is generally favourable and most adults do not require special medical care. Patients need annual hearing and cardiologic assessment, as well as follow-up of growth parameters and development [Sarkozy *et al.,* 2008].

About 200 patients have so far been reported worldwide, although LS seems to be underdiagnosed or misdiagnosed due to its mild features and/or the absence of lentiginosis [Sarkozy *et al.,* 2008], and therefore the clinicians should give more consideration to rare genetic syndromes, especially in the case of symptoms from different clinical areas.

Table 14. Related disorders: neuro-cardio-facial-cutaneous syndromes and their molecular and clinical characteristics.

Disorder	Inheritance	Causative genes	Prevalence	Phenotype
Noonan syndrome	autosomal dominant	*PTPN11, SOS1, RAF1, BRAF, KRAS, MAK2K,SHOC2, NRAS*	about 1:1000–1:2500	Short stature, CHD, cardiopmyopathy, broad or webbed neck, pectus deformities, characteristic facies, bleeding diathesis, developmental delay of variable degree
LEOPARD syndrome	autosomal dominant	*PTPN11, RAF1, BRAF*	rare	Short stature, CHD, cardiomyopathy, characteristic facies, deafness, bleeding diathesis, developmental delay of variable degree. ML, CLS, ECG abnormalities are distinctive from related syndromes.
Costello syndrome	*de novo* autosomal dominant mutation	*HRAS, KRAS, BRAF, MEK1*	rare	Short stature, CHD, characteristic facies with more coarse features, mental retardation is more severe. Skin involvement is distinctive – curly, sparse hair, loose soft skin, papillomata
Cardio-facio-cutaneous syndrome	*de novo* autosomal dominant mutation	*KRAS, BRAF, MEK1, MEK2*	rare	Short stature, CHD, cardiomyopathy, characteristic facies, mental retardation is more severe. Hyperceratotic dry skin and sparse, curly hair are distinctive. Phenotype overlaps most with NS syndrome, but facial appearance tends to be coarse. Cardiac problems mostly overlap with LS syndrome.
Neurofibro ma-tosis type 1	autosomal dominant	*NF1*	about 1:3000	Short stature, CLS, cutaneous neurofibromas, intertriginal freckling, iris Lish nodules, developmental delay of variable degree
Watson syndrome	autosomal dominant	*NF1*	rare	Short stature, CHD, mental retardation, skin pigment changes and involvement are similar to NF1

CHD congenital heart defect*, ML* multiple lenitignes, *CLS café-au-lait* spots, *ECG* echocardiography

5.3.3. Holt-Oram syndrome (Publication VI)

The CHD study group contained two cases with clinical suspicion of HOS.

The first case was a newborn boy with prenatally suspected and postnatally confirmed CHD (AVSD) and a dysmorphic phenotype. He was born as a second child to his mother; his half-brother died at the age of 1.5 months (unknown reasons), and he also had a healthy half-sister. He was born at term, with a birth weight of 3150 g (25–50 percentiles), a length of 52 cm (50 percentiles), a head circumference of 34 cm (<3 percentiles), and an Apgar score of 8/8. Dysmorphic features included downslanted palpebral fissures, hypertelorism, epicanthal folds, high palate, dysmorphic ears and triphalangeal thumbs bilaterally. Cytogenetic and FISH analyses from peripheral blood lymphocytes revealed a normal male karyotype, 46,XY ish 22q11.2 (HIRAx2). DNA analysis of *TBX5* gene revealed no mutation in the eight analysed coding exons and their flanking regions. The child died at the age of 1 month due to heart failure and a conduction defect (atrioventricular blockade).

The second case was a boy with CHD and dysmorphic features. He was the second child of young, healthy, non-consanguineous parents. At the time of the child's birth his mother was 22 years old and his father 24 years old. He was born at 38 weeks of gestation from induced labour. His birth weight was 3680 g (25–50 percentiles), length 52 cm (75–90 percentiles) and head circumference 37 cm (25–50 percentiles), and his Apgar score was 4/7/8. At 25 weeks of pregnancy, bradycardia of the fetus was observed. Using a prenatal US scan, VSD was diagnosed, and 0.2 cm of liquid was ascertained in the pericardial cavity; postnatal studies also revealed ASD and PDA. From birth, sinus bardycardia and a conduction defect was presented. At the end of the first week, heart failure evolved, and treatment with diuretics was commenced. Dysmorphic features included dolichocephaly, dysmorphic ears, frenula of the tongue and upper lip, a structurally normal thumb with distal displacement, short distal phalanges of the fingers, a simian crease in the left hand and a sacral dimple. Radiological investigation showed no structural changes in the metacarpal bones. An operation for PDA closing was done at the age of 3 months; the postoperative period was complicated due to the conduction defect. At the age of 8 months, echocardiography revealed muscular VSD, sinus-venosus type ASD, dilatation of the right cavities, hypertrophy of the right ventricle (left ventricle 22–23 mm) and paradoxical moving of the septum. Conduction defect, sinus bradycardia, supraventricular rhythm migration and the first degree atrioventricular blockade were also present. At the age of 1 year, asthma was diagnosed.

At the age of 15 months his weight was 10.3 kg (10–25 percentiles), length 80.5 cm (50 percentiles), head circumference 47 cm (10–25 percentiles), and psychomotor development was in normal range. He showed minimal facial dysmorphism with a prominent forehead, narrow and peculiar shoulders (with an "angel wing" appearance when seen from the back), pectus excavatum, brachydactyly and a distally placed thumb (Figure 13). He did not have an index

finger nipper-function, and his elbow and shoulder joints had contrariwise movement (Figure 13). X-rays showed delayed bone age (the *capitulum humeri* corresponded to 6 months) and the existence of *cubitus valgus* (Figure 14). He also had bilateral hypotrophy of the muscles in the regions of the shoulder and ulna.

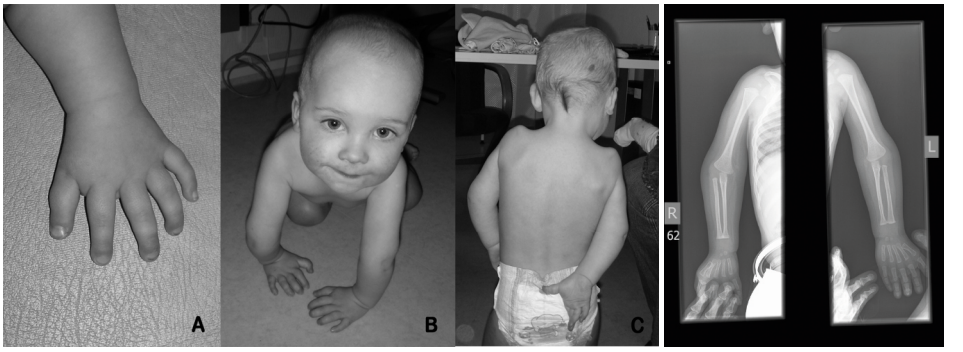


Figure 13. The proband at the age of 15 months.
Figure 14. X-rays of the upper limbs of the patient at the age of 15 months (R=right, L=left). Note the delayed bone age (the small *humerus capitulum* corresponded to 6 months), the absence of *capitulum humeri* and the existence of *cubitus valgus.*

At the age of 2 years he had a radical heart operation: debanding and closing of the ASD and VSD. The post-operative period was complicated by pneumonia and cardiovascular decompensation. Due to his upper limb anomaly he has been consulted by orthopedic doctors.

Cytogenetic and FISH analyses from peripheral blood lymphocytes revealed a normal male karyotype, 46,XY ish 22q11.2 (HIRAx2). Through DNA analysis of the *TBX5* gene (NM_000192.3) we identified a novel heterozygous frameshift mutation (c.1304delT, p.L435fsX146) in exon 9 of the *TBX5* gene in our patient (Figure 15). The mutation was verified by resequencing. The germline origin of the mutation was confirmed by the occurrence of the same mutation in the patient's buccal mucosal epithelial cells. Molecular analysis of his parents showed normal results (Figure 15). Due to the frameshift lesion, the coding sequence is predicted to encode for an elongated protein characterized by 84 miscoding (codons 435–519) and 62 supernumerary (codons 519– 581) amino acid residues at the C-terminus. This mutation was considered to be pathogenic, as it altered a region of the protein that is required for proper TBX5 function [Bohm *et al.,* 2008].

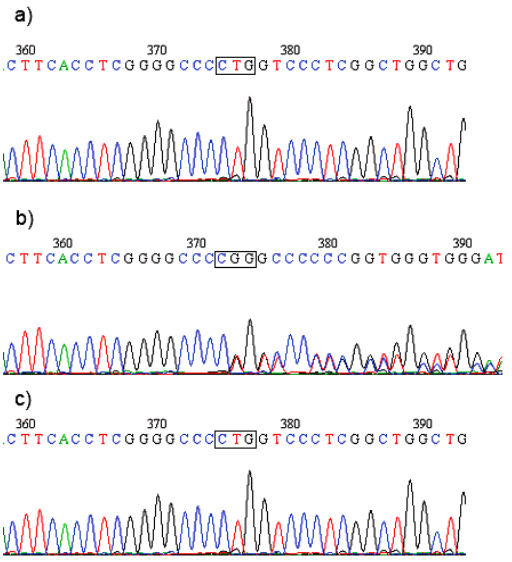


Figure 15. The identification of a novel frameshift mutation predicting an elongated *TBX5* protein in HOS. Chromatograms of *TBX5* exon 9 showing the c.1304delT deletion (p.L435fsX146). (**a**) unaffected mother, (**b**) the heterozygous index patient, (**c**) unaffected father.

The *TBX5* gene belongs to the Brachyury (T) family, which includes transcription factors with a common DNA-binding domain, the T-box [Wessels and Willems 2010]. T-box genes are essential in embryogenesis, including specification of the mesoderm, as well as limb and heart morphogenesis [Liberatore *et al.,* 2000]. The mutations in human *TBX5* that cause HOS include deletions, missense, nonsense, splice site and frameshift mutations and rearrangements [Heinritz *et al.,* 2005]. Most of the mutations in the *TBX5* known so far lead to truncated protein unable to bind and activate target DNA. Truncation mutations seem to produce a null allele, resulting in a haploinsufficiency of the TBX5 protein [Gruenauer-Kloevekorn and Froster 2003; McDermott *et al.,* 2005]. It is claimed that *TBX5* haploinsufficient mutations are the most significant cause of HOS [Basson *et al.,* 1997]. To the best of our knowledge, on only one earlier occasion has an elongation-caused mutation in *TBX5* (c.1333delC, p.H445fsX136) been reported, by Böhm et al. [Bohm *et al.,* 2008]. We found a deletion/frameshift mutation c.1304delT, p.L435fsX146 in *TBX5*, which is predicted to produce a compositionally similar elongated protein of 580 amino acids instead of a normal protein product of 518 amino acids.

Amino acids 1–54 and 238–518 in the TBX5 protein are the most important for effective DNA binding [Brassington *et al.,* 2003]. Our findings confirm this assumption – a mutation in protein position 435 (p.L435fsX146) causes a classic HOS phenotype.

The TBX5 protein is associated with other cardiac transcription factors including GATA4 and NKX2.5, and synergistically activates different cardiac effector target genes [Garg *et al.,* 2003; Hiroi *et al.,* 2001; Wessels and Willems 2010]. A mutation in *TBX5*, *GATA4* and *NKX2.5* genes may result in congenital heart defects. Moreover, *TBX5* has been shown to be required for the normal cardiac conduction system [He *et al.,* 2004]. Our patient demonstrated septal defects (secondary ASD, muscular VSD) and a conduction defect (first step atrioventricular block). A patient with a similar *TBX5* mutation c.1333delC described by Böhm *et al.* [Bohm *et al.,* 2008] had muscular VSD and total ASD and an atrioventricular block requiring a pacemaker. It is not known exactly if the severity of limb and heart defects varied only due to mutation positions, or whether there are any other age-related factors and/or mechanisms, for example anticipation [Fan *et al.,* 2003]. As our patient is so young, it is possible that a more severe conduction defect may develop. Therefore he must be carefully observed by a cardiologist.

Table 15. Comparison of clinical features of patients with deletion/frameschift mutations in the *TBX5* exon 9 resulted in very similar elongated TBX5 proteins.

Patient	Age (yrs)	Sex	Heart anomalies	Limb anomalies	Other features
c.1333delC [Bohm *et al.,* 2008]	4	M	Bradycardia due to severe atrioventricular block; muscular VSD; total ASD; right hypoplastic lung and pulmonary veins	Bilateral triphalangeal thumbs; bilateral hypoplastic clavicles and radii	Micrognathia; long philtrum
c.1304delT (a present case)	1.3	M	Bradycardia due to I step atrioventricular block, conduction defect; muscular VSD; ASD; PDA	Bilateral structurally normal thumbs with distal displacement; narrow shoulders; hypotrophia of muscles in region of shoulders	Prominent forehead, frenula of tongue and lip; simian crease in the left hand; cubitus vagus hands; pectus excavatum

It is tempting to speculate that the c.1304delT mutation generally causes the same effect at the phenotype level as the previously mentioned c.1333delC mutation, because of the high compositional similarity of the mutated proteins. Despite the fact that p.L435fsX146 has ten miscoding amino acids more than p.H445fsX136 (between positions 435 – 445), there are predicted to be 62 C-terminal supernumerary amino acids in both of the resulting mutated proteins: as ascertained by Böhm *et al.* [Bohm *et al.,* 2008], an elongation effect

abrogates activation of the TBX5 binding site containing *ANF* (or alternatively *NPPA* – natriuretic peptide precursor A) promoter [Hiroi *et al.,* 2001] (Table 15). Like ANF, natriuretic peptides are implicated in the control of extracellular fluid volume and electrolyte homeostasis. For example, mutations in this gene have been associated with atrial fibrillation of familial type 6 [Ren *et al.,* 2010]. It has been shown that transcriptional regulation activity requires specific motifs between protein C-terminal residues 238–518 [Brassington *et al.,* 2003; Conlon *et al.,* 2001], and perhaps supernumerary amino acids modulate the protein conformation by masking the TBX5 transcriptional activator domains [Bohm *et al.,* 2008]. Thus both truncation and elongation mutations in *TBX5* may result in nonfunctional proteins.

The main phenotypic impairments of upper body skeletal structures and the heart in a c.1304delT patient are commensurable enough with an earlier referred c.1333delC patient (Table 15). It is tempting to note that our patient had no structural skeletal changes, although limb anomalies such as triphalangeal thumbs are referred to in an earlier case [Bohm *et al.,* 2008]. It generally seems that the type and exonic position of the mutation would be manifested in HOS phenotypic specificities, whereas the exact exonic positioning would be associated with the severity of impairments.

There are other disorders with diagnostic features that overlap with HOS – Rothmund-Thomson and Okihiro syndromes, UMS (ulnar-mammary syndrome), thrombocytopenia absent radius syndrome, acro-renal-ocular syndrome and VACTERL (vertebral anomalies, anal atresia, cardiac malformations, tracheoesophageal fistula, renal anomalies, limb anomalies) [Kohlhase *et al.,* 2003]. Therefore *TBX5* molecular diagnostics can be used as an informative tool in HOS study for precise clinical diagnosis and genetic counseling.

This present sporadic observation confirms that mutations in the *TBX5* gene can be delineated as the underlying defect in HOS. Our results support the idea that any *TBX5* mutation not prominently in the T-box region has the potential to affect the development of the heart and limbs in HOS. However, the severity of impairments seems to depend on the precise location of the mutation. There may, of course, be additional genetic factors, which could synergistically modulate the individual HOS phenotype.

6. CONCLUSIONS

1. Contingent screening protocol was introduced in Estonia in 2006. This protocol reduced considerably second-trimester sampling with little impact on overall screening performance. The high-risk group of pregnant women achieved an earlier diagnosis and the low-risk group of pregnant women received earlier reassurance of normal results.

2. Contingent screening is a better choice for DS screening in Estonia than the previously used second trimester screening. The contingent screening we performed demonstrated DR of 88.3% and FPR of 3.4%, which are better than previously used second trimester screening's DR (57.8%) and FPR (4.7%).

3. Among the offspring of 74 pregnant women with postulated marked changes and positive Prisca risk calculation, prenatal or postnatal structural or genetic abnormality was diagnosed in 16 cases (21.6%), fetal death occurred in 12 cases (16.2%), and the child was healthy at the age of 2 years in 31 cases (41.9%). Structural or genetic abnormalities were diagnosed prenatally in 3/4 of cases, and postnatally in 1/4 of cases. In 15 cases (20.3%) we were unable to obtain the final information about pregnancy outcome.

4. Four children from the postnatal study group had been diagnosed with congenital anomalies and/or syndromes: two had CHD – atrial septal defect and ventricular septal defect with patent ductus arteriosus, one Silver-Russell syndrome and one congenital adrenal hyperplasia.

5. Our study confirmed that prenatal screening tests are valuable not only for aneuploidy screening, but may also be predictors for other structural or genetic abnormalities. Children born to these mothers should be followed by a pediatrician for additional consultation after birth, as they have a 5.4% risk of having a congenital or genetic abnormality.

6. The most prevalent genetic syndrome with CHD was NS, which was clinically diagnosed in approximately half of the investigated cases. The disease that caused a mutation in the *PTPN11* gene was found in 28% of clinical NS cases, which is less than in the literature (33–45%). We may have less experience in the clinical diagnosis of NS, or it could be the result of the extreme variable phenotype, as in one family with low NS scoring points, the mutation in *PTPN11* was detected.

7. All patients in *PTPN11* mutations-negative group fulfilled diagnostic criteria for NS. Phenotypic features were mostly comparable in the mutation-negative and mutation-positive group. This shows the heterogeneity of NS; further investigations should be undertaken in the subgroup of patients with classical features of NS.

8. Two cases from the mutation-negative subgroup with clinical diagnosis of NS another diagnosis was confirmed: one case of Cantu syndrome and one case of 10q23.1–23.3microdeletion. This shows the overlap of NS with several other genetic syndromes and the importance of the molecular confirmation of the diagnosis.

9. Four cases of LS were clinically diagnosed, and in one of them a recurrent mutation p.Tyr279Cys in the *PTPN11* was detected. Despite the fact that with the detected mutation multiple lentigines were present in most patients (93.9%), the clinical picture of our patient was distinct from previously reported cases. Multiple lentigenes were located mainly on the extremities and in the joint regions, whilst the upper part of the trunk and the face were spared. To the best of our knowledge, only one earlier report has described multiple lentigines predominantly in the lower extremities.

10. HOS was clinically confirmed in two cases, with both patients exhibiting CHD and upper limb anomalies. Molecular analysis identified a novel and unusual heterozygous frameshift mutation (c.1304delT, p.Leu435fsX146) in exon 9 of the *TBX5* gene in one of these. Upper limb anomalies in this patient were relatively mild and unusual for HOS – distally displaced thumbs, narrow shoulders and hypotrophy of the muscles in the shoulder region. This mutation is predicted to cause an elongated TBX5 protein with 84 miscoding amino acids and 62 supernumerary C-terminal amino acids. To the best of our knowledge, only one such type of elongation mutation has thus far been reported in the *TBX5* gene. Our results support the hypothesis that any *TBX5* mutation not prominently in the T-box region has the potential to affect the development of the heart and limbs in HOS.

7. APPENDIX

Appendix I. Clinical and molecular features of patients with clinical suspicion of NS.

Patient ID number	Family number *	Sex	Age	Height (percentile)	Clinical features		Other features	Developmental delay/mental retardation	NS scoring points**	Analysis of *PTPN11* gene
					Facial dysmorfism	Congenital heart defect				
5336	**I**	F	34 years	<3	Mild	PS	She has two sons with similar phenotype	No	2A + B	Normal result
6678	**II**	F	3 months	75–90	Typical	PS; ASD	Hypotonia, pectus excavatum	No	3A + B	c.1510A>G; p.Met504Val
4582	II	F	35 years	<3	Typical	ASD	Mild ptosis	No	2A + B	c.1510A>G; p.Met504Val
239	II	M	5 months	10–25	Typical	PS	Hypotonia, pectus excavatum, mild ptosis	No	3A	c.1510A>G; p.Met504Val
4316	**III**	M	1 year	<3	Mild	TOF	Hypotonia, cryptorchidism, broad thorax, mild ptosis	Developmental delay	A + 6B	Normal result
4320	III	F	29 years	<3	Mild	None	Renal pathology, mild ptosis	No	A + 3B	Normal result
5808		M	9 years	<3	Mild	None	Pectus excavatum	Mild	2A + 2B	Normal result
5975	**IV**	F	7 years	25–50	Typical	None	Hypotonia, broad thorax, strabismus, myopia	Mild	A + 3B	Normal result
5976	IV	F	36 years	<3	Typical	None	Hearing loss	No	2A + B	Normal result
6009		M	9 years	<3	Typical	PS, valvular insufficiency	Hypotonia, pectus excavatum, renal pathology	Mild cognitive delay	3A + B	c.172A>G; p.Asn58Asp
5978		F	5 years	10–25	Typical	PS	Hypotonia, pectus excavatum, renal pathology	Mild cognitive delay	3A + B	Normal result

Patient ID number	Family number *	Sex	Age	Height (percentile)	Clinical features		Other features	Developmental delay/mental retardation	NS scoring points**	Analysis of *PTPN11* gene
					Facial dysmorfism	Congenital heart defect				
6048		M	12 years	25–50	Mild	ASD, dilatative cardio-myopathy	Hypertrichosis, postaxial extra digit, prenatally polyhydramnion and LGA	Mild cognitive delay	3B	Normal result
6258		M	3 years	50–75	Typical	PS, AVSD	Hypotonia, macrocephaly	Developmental delay	2A + B	Normal result
6703		F	3 years	50–75	Typical	None	Hypotonia, pectus excavatum, lymphatic dysplasia in perinatal period	No	2A + 2B	Normal result
6627		F	6 years	<3	Mild	None	Pectus excavatum, hearing loss, bilateral hypoplasia of n. optici	Mild	2A + 2B	Normal result
7244		M	2 years	<3	Typical	PS	Hypotonia, pectus excavatum, several ML, peculiar hair structure	Developmental delay	4A + 2B	Normal result
6905		F	7 years	<3	Typical	PDA, valvular insufficiency	Broad thorax, lympatic dysplasia in perinatal period	No	2A + B	Normal result
7843		M	9 years	<3	Typical	Valvular insufficiency	Hypotonia, broad thorax, bilateral cryptorchidism (operated), strabismus	No	2A + 2B	c.923A>G; p.Asn308Ser
7700		M	1 year	25–50	Mild	None	Broad thorax, strabismus	Developmental delay	3B	Normal result

Patient ID number	Family number *	Sex	Age	Height (percentile)	Clinical features		Other features	Developmental delay/mental retardation	NS scoring points**	Analysis of *PTPN11* gene
					Facial dysmorfism	Congenital heart defect				
6851		M	3 years	50–75	Mild	PS, FOA	Hypotonia, strabismus, partial ptosis	Developmental delay	A + 2B	Normal result
8584		M	14 years	<3	Mild	FOA	Broad thorax, several ML	Mild	A +3B	Normal result
8488	**V**	M	7 years	10–25	Typical	None	Broad thorax, mild ptosis, CLS	No	A + B	c.1403C>T; p.Thr468Met
8489	V	F	6 years	10–25	Typical	None	Broad thorax, mild ptosis, CLS	No	A + B	c.1403C>T; p.Thr468Met
8918	V	F	33 years	10–25	Typical	None		No	A + B	c.1403C>T; p.Thr468Met
9355		M	15 years	<3	Mild	None	Diagnosed growth hormone deficiency, atopic dermatitis	Mild	A + 2B	Normal result
9576		F	10 years	<3	Mild	Valvular insufficiency	Bilateral hearing loss, palatochichis	Mild	A + 2B	Normal result
9656		M	17 years	3	Mild	HCM	His mother has similar phenotype	Moderate	A + 3B	Normal result
301		F	17 years	<3	Typical	PS, arrhythmia	Broad thorax, several ML, partial ptosis	No	3A + B	Normal result
367		M	4 years	50–75	Mild	None	Broad thorax, CLS	Developmental delay	3B	Normal result

* familiar cases marked with Roman numbers (I–V); probands in **bold**
** – NS scoring system proposed by van der Burgt

Appendix II. Clinical features of patients with clinically suspected LS.

Proband	Sex	Age	Height (percentile)	Congenital Heart Defect	Phenotype	Multiple Lentigines	*Café-au-lait* spots	Additional information
LS-I	F	4 years	10th	HCM	Prominent forehead, hypertelorism, wide neck with the pterygeum, a wide trunk	Several, mostly on extremities	Few	Hypotonia, normal hearing
LS-II	M	16 years	3–10th	No	Mild synophris, prominent nasal root, high palate, dysmorphic ears, a wide trunk	Several, mostly on trunk	>5	Hypermetropia, hemangioma on right leg
LS-III	F	4 years	50th	No	Prominent forehead, down-slanting palpebral fissures, hypertelorism, dysmorphic ears, wide neck with the pterygeum, widely spaced nipples	Several, mostly on trunk	>15	Normal hearing, hypotonia, developmental delay
LS-IV	M	7 days	>97th	ASD, VSD, HCM of left ventricle	Hypertelorism, dysmorphic ears	No	No	LGA -birth weight 4880 g (> 97th percentile), normal development at the age of 7 months

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10. SUMMARY IN ESTONIAN

Sünnieelsete sõeluuringute strateegiad, kaugtulemused lastel skriiningtestide muutuste korral ja sagedasemad sündroomsed südamerikked Eestis

Sissejuhatus

Sünnieelne diagnostika on saanud üheks kindlaks osaks rasedate jälgimises. Geneetiliste haiguste aspektist kasutatakse sünnieelset diagnostikat kõige enam kromosoomiaberratsioonide leidmiseks lootel. Kromosoomide arvulisi ja struktuurseid muutusi esineb inimesel sageli, kuid sagedus varieerub raseduse staadiumist sõltuvalt. Nii leitakse kromosoomimuutusi preimplantatsiooni faasis u. 20% embrüotest, aga sünnil on sagedus 0,2–0,93% [Baird *et al.,* 1988; ESHRE 2008; Simpson 1990]. Kromosoomihaigustest diagnoositakse sünnijärgselt kõige sagedamini trisoomia 21 e. Downi sündroomi (DS), mille sünnisageduseks hinnatakse 0,58–1,7 juhtu 1000 sünni kohta [Hoshi *et al.,* 1999; Leonard *et al.,* 2000; Reimand *et al.,* 2006a]. Seost ema sünnitusvanuse tõusu ja DS sünnisageduse vahel kirjeldati juba 1933. aastal [Wald *et al.,* 1997]. Sünnieelne diagnostika (SD) jõudis kliinilisse praktikasse 1970. aastate alguses, peale seda, kui 1966. aastal olid Steele ja Breg õnnestunult kultiveerinud ja karüotüpiseerinud amnionirakke [Elias 2010; Steele and Breg 1966].

Sünnieelsete sõeluuringute eesmärgiks on hinnata riski kromosoomihaigusega lapse sünniks käesoleva raseduse ajal. Seerumskriining raseduse teisel trimestril (kaksik- või kolmiktest: AFP – α-fetoproteiin, HCG – inimese kooriongonadotropiin, uE3 – vaba östriool) võeti kasutusele kliinilises praktikas 1990. aastate algul [Haddow *et al.,* 1992]. Juba mõne aasta pärast lisandusid sõeluuringusse ka esimese trimestri markerid: nii ultraheli (NT – *nuchal translucency* ehk kuklapiirkonna läbikumavus) kui ka vereseerumi markerid (nt. PAPP-A – rasedusega seotud plasmaproteiin-A, fb-HCG – HCG vaba β-alaühik). Samuti on välja töötatud väga erinevaid skriiningu protokolle ja strateegiad [Canick 2012]. Tänapäeval on laialt kasutusel I trimestri kombineeritud skriiningtestid. Lisaks on juba ka täiesti uudsel metoodikal põhinevad skriiningtestid (nt. loote rakuvaba DNA analüüs ema verest) tulemas kasutusse kliinilises praktikas [Benn *et al.,* 2013].

Eestis alustati loote kromosoomianalüüside teostamisega 1990. aastal dr. Mari Sitska eestvedamisel. Alates 1995. aastast hakati pakkuma sünnieelseid uuringuid sõeluuringuna kõrge riskiga rasedatele (nt. vanus > 35 aasta) ning 1998. aasta lõpul juurutati II trimestri seerumskriining (kaksik- või kolmiktest). Esimese trimestri ultraheli skriininguga (NT mõõtmisega) alustati suuremates keskustes 2001. aastal. Rasedusaegsed sõeluuringud on Eestis laialdaselt kasutusel: II trimestri seerumskriininguga oli 2006. aastal kuni 37 aastastest naistest hõlmatud 91% [Sitska *et al.,* 2008a]. Ajavahemikul 1999–2006 oli kasutatava skriininguprotokolli avastamise määr 57,8% (vale-positiivsuse määr 4,7%);

2006. aastaks oli avastamise määr tõusnud juba 67%-ni, mis on sarnane publitseeritud teadusuuringutes eeldatavaga [Haddow *et al.,* 1992; Sitska *et al.,* 2008a]. Samas on uute markerite ja skriiningstrateegiate kasutusele võtmisega tõusnud ka eeldatav skriiningu avastamismäär 75%-ni [Summers *et al.,* 2007] ning seetõttu oli ka meil eesmärgiks töötada välja uus skriiningstrateegia ning leida meie tingimustes efektiivne skriininguprotokoll.

Sõeluuringutes hinnatavad markerid ei ole spetsiifilised ainult kromosoomihaigustele, vaid leitud muutused markerites võivad viidata raseduse halvale prognoosile (raseduse katkemine, enneaegne sünnitus, üsasisene kasvupeetus) või olla markeriks loote pärilikule haigusele [Aitken *et al.,* 1999; Androutsopoulos *et al.,* 2013; Benn *et al.,* 2000; Dugoff *et al.,* 2005; Dugoff and Society for Maternal-Fetal 2010; Gagnon *et al.,* 2008; Goetzl 2010; Kashork *et al.,* 2002; Kowalczyk *et al.,* 1998; Sayin *et al.,* 2008; Souka *et al.,* 2001; Spaggiari *et al.,* 2013; Summers *et al.,* 2003]. Mõnedes teadusuuringutes on leitud, et lastel, kellel looteeas tuvastati NT suurenemine, esineb sagedamini kaasasündinud arengurikkeid, pärilikke haigusi ning arenguprobleeme 2 aasta vanuses [Adekunle *et al.,* 1999; Baumann *et al.,* 2005; Schou *et al.,* 2009; Senat *et al.,* 2002; Van Vugt *et al.,* 1998], kuid samas on ka avaldatud uuringutulemusi, kus kõrgenenud riski ei leitud [Bilardo *et al.,* 2007; Cha'ban *et al.,* 1996; Hiippala *et al.,* 2001; Maymon *et al.,* 2000; Miltoft *et al.,* 2012; Mula *et al.,* 2012; Saldanha *et al.,* 2009; Senat *et al.,* 2007; Sotiriadis *et al.,* 2012]. Samas ei ole publitseeritud uuringuid, mis hindaksid laste tervist ja arengut, kui raseduse ajal on emal esinenud märgatavad muutused vereseerumi markerites.

Suurenenud NT-d seostatakse peale kromosoomihaiguste väljalülitamist kõige sagedamini kaasasündinud südameriketega (KSSR) [Souka *et al.,* 1998]. KSSR on kõige sagedasem kaasasündinud arengurike, esinemissagedusega 3–13 juhtu 1000 sünni kohta [van der Linde *et al.,* 2011]. KSSR põhjuseks võib olla kromosoomihaigus, ühe-geeni defekt (nt. Holt-Orami sündroom) või kompleksne mehhanism, kus on seotud erinevate geenide ja keskkonnafaktorite koostoime. Siiski jääb enamikel juhtudel KSSR etioloogia ebaselgeks ning seega räägitakse nn. multifaktoriaalsest etioloogiast. Geneetilisi põhjuseid peetakse KSSR korral siiski määravateks, mida toetab KSSR kordumine ühes ja samas perekonnas ning samuti KSSR sage esinemine erinevate kromosoomipatoloogiate ning geneetiliste sündroomide ühe sümptomina [Anders *et al.,* 1965; Goldmuntz 2004; Pierpont *et al.,* 2007]. Hoolimata olulistest avastustest südame arengu mõistmisel, on siiani seostatud vähe konkreetseid geene KSSR-ga. [Garg 2006; Grossfeld 2003; Mitchell *et al.,* 2007].

Noonani sündroom (NS, OMIM163950) on kõige sagedasem mitte-kromosomaalne sündroomne KSSR põhjus. NS on autosoom-dominantse (AD) pärandumisega haigus, mis haarab väga erinevaid organsüsteeme: kardiovaskulaarset, kraniofatsiaalset, skeleti-, vereloome-, lümfi- ja kesknärvisüsteemi. NS kliiniline pilt on väga heterogeenne. Kõige sagedasemateks sümptomiteks on KSSR (prevaleerivalt pulmonaalstenoos) ja väike kasv [Tartaglia *et al.,* 2011]. Umbes 61% kliinilise diagnoosiga patsientidel tuvastatakse muutus ühes

järgnevatest geenidest: *PTPN11* (40,9%), *SOS1* (11,1%), *RAF1* (4,7%), *SHOC2* (1,7%), *KRAS* (1,4%), *BRAF* (0,8%) ja *NRAS* (0,2%) [Romano *et al.,* 2010]. Kliiniliselt väga sarnane ning geneetiliselt alleelne NS-iga on LEOPARD sündroom (LS; OMIM 151100), mida eristavad NS-st vähem väljendunud näo düsmorfism, naha muutused ning KSSR-st prevaleeruv hüpertroofiline kardiomüopaatia [Sarkozy *et al.,* 2004]. Eelmistest kliiniliselt eristuv Holt-Orami sündroom (HOS, OMIM 142900) on kõige sagedasem süda-käsi sündroomide grupis [McDermott *et al.,* 2005]. HOS-i iseloomustab KSSR ja käte arengurike. HOS on AD päranduvuse ning täieliku penetrantsusega, kuid väga varieeruva ekspressiivsusega haigus [Newbury-Ecob *et al.,* 1996]. HOS sümptomaatikaga on seostatud osadel juhtudel mutatsioone *TBX5* geenis [Basson *et al.,* 1997; Brassington *et al.,* 2003; Cross *et al.,* 2000; McDermott *et al.,* 2005].

KSSR võimaliku geneetilise etioloogia tuvastamine on oluline, sest tegemist võib olla mitmeid organsüsteeme haarav haigusega, mille etioloogia tuvastamine annab infot võimaliku prognoosi ning KSSR kordusriski osas perele, kusjuures täpsest diagnoosist võib tuleneda ka näidustus teiste pereliikmete uurimiseks [Pierpont *et al.,* 2007]. Eestis pole varem KSSR geneetilist etioloogiat süstemaatiliselt uuritud.

Töö eesmärgid

1. Juurutada Eestis kombineeritud skriining kromosoomhaiguste suhtes esimesel trimestril.
2. Hinnata kontingent-skriiningu strateegia efektiivsust meie populatsioonis.
3. Uurida, kas sünnieelsetel sõeluuringute markerites esinevad märgatavad muutused annavad kõrgema riski kaasasündinud või geneetilise patoloogia esinemiseks ning lapse arenguprobleemideks 2 aasta vanuses.
4. Hinnata sündroomsete KSSR geneetilisi põhjuseid Eesti lastel.
5. Kirjeldada KSSR-ga seotud harvaesinevaid monogeenseid geneetilisi sündroome.

Uuringugrupid ja uurimismeetodid

Sünnieelse skriiningu strateegia väljatöötamise uuringugrupp

Rasedatele, kes pöördusid ajavahemikul 01.02.2005 – 31.12.2008 Tartu Ülikooli Kliinikumi günekoloogi vastuvõtule raseduse esimesel trimestril (10^{+6}–13^{+6} rasedusnädalal), pakuti I trimestri sõeluuringuid: I trimestri UH-uuringut (NT mõõtmiseks) ja I trimestri vereseerumi sõeluuringut (PAPP-A ja fb-HCG määramiseks). Saadud tulemuste alusel hinnati riski trisoomia 21 ja trisoomia 18 suhtes Prisca 4.0 arvutitarkvara kasutades. 2005. aastal pakuti kõikidele naistele ka II trimestri seerumskriiningut (kolmiktest – AFP, HCG, uE3). Alates 2006. aastast rakendati kontingent-skriiningu strateegiat, kus I trimestri kombineeritud riskihinnangu alusel jaotati skriiningu läbinud naised kolme gruppi: kõrge risk (neile pakuti koheselt diagnostilisi uuringuid), madal risk (skriining-

uuringud lõpetati) ja vahepealne riskihinnang (korrati sõeluuringut II trimestri seerumskriiningu markereid kasutades). Rakendatavat skriiningu strateegiat hinnati järgmiste parameetrite alusel: avastamismäär (*detection rate*) ja valepositiivsuse määr (*false positive rate*).

Sünnieelsete sõeluuringumarkerite märgatavate muutuste kaugmõju hindamise uuringugrupp

Uuringugrupi moodustamiseks analüüsiti 5257 naise I ja II trimestri sõeluuringu markereid, mis olid hinnatud ajaperioodil 16.02.2009–15.02.2010 Tartu Ülikooli Kliinikumis. Määratud märgatavad muutused skriiningu markerites (AFP ja HCG > 3,0 MoM, PAPP-A, fb-HCG, HCG, uE3 < 0,25 MoM ja NT ≥ 3,0 mm) esinesid 138 naisel. Uuringugruppi kaasati 74 naist, kellel sõeluuring oli positiivne (riskihinnang oli piirväärtusest kõrgem trisoomia 21, trisoomia 18 või neuraaltoru defektide suhtes), ning kes käisid geneetiku konsultatsioonil ja sünnieelsetel uuringutel. Andmed sünnituse kohta saadi 45 naiselt, kelle lapsed kaasati uuringugruppi ning kutsuti 2 aasta vanuses konsultatsioonile, 10 ema ei soovinud vastuvõtule tulla ning seega laste uuringugrupi moodustasid 35 last. Konsultatsioonil koguti andmed pereanamneesi, raseduse, sünnituse, sünniparameetrite, esimese ja teise eluaasta arengu ning diagnoositud arengurikete kohta. Vastuvõtul mõõdeti kaalu, pikkust ja peaümbermõõtu, hinnati arengut ning samuti uuriti lapsi süstematiseeritult düsmorfsete tunnuste osas. Näidustustel (kasvupeetuse, arenguprobleemid, kolme või enama düsmorfse tunnuse esinemisel) tehti lisauuringuid (submikroskoopiline kromsoomianalüüs, ainevahetuse uuringud, ühel juhul molekulaarne testimine NS suhtes). Tulemuste statistiline analüüs polnud õigustatud uuringugrupi väiksuse tõttu.

KSSR laste uuringugrupp

Uuringugrupi moodustasid ajavahemikul 2006–2010 Tartu Ülikooli Kliinikumi geneetiku konsultatsioonile suunatud patsiendid, kellel oli sünnijärgselt diagnoositud KSSR ning kellel oli KSSR diagnoositud veel vähemalt ühel pereliikmel või kellel kliiniliselt kahtlustati geneetilist sündroomi, mille üheks tunnuseks on KSSR. NS kahtluse korral kasutati kliinilise diagnoosi panemisel NS punktisüsteemi [van der Burgt 2007]. Peale geneetilist konsultatsiooni jaotati uuringugrupp kaheks: mittesündroomne KSSR (26 probandi), sündroome KSSR (29 probandi). Sündroomne KSSR jaotus omakorda: NS (23 probandi, lisaks 6 kliinilise NS diagnoosiga pereliiget); LS (4 probandi), HOS (2 probandi). NS ja LS korral analüüsiti *PTPN11* geeni ning HOS korral *TBX5* geeni. Molekulaardiagnostilised analüüsid teostati Tartu Ülikooli bio- ja siirdemeditsiini instituudis vanemteadur I. Kalevi poolt.

Uuringu peamised tulemused

1. Eestis alustati kontingent-skriininguga 2006. aastal. Kontingent-skriiningu strateegia vähendas oluliselt vajadust pakkuda uuringugrupi naistele II trimestri seerumskriiningut. Samas ei ilmnenud negatiivset mõju skriiningu avastamismäärale ega ka valepositiivsuse määrale. Kõrge riskiga naistele pakuti varem sünnieelset diagnostikat ning madala riskigrupi naised said varem kindlust, et lisauuringud ei ole vajalikud.

2. Kontingent-skriining on parem valik võrreldes varem Eestis kasutatud II trimestri seerumskriininguga. Kasutatud kontingent-skriiningu avastamismäär uuringuperioodil oli 88,3% (valepositiivsuse määr – 3,4%), mis on märgatavalt kõrgem varem kasutatud II trimestri seerumskriiningu tulemustest (ajaperioodil 1999–2006 oli avastamismäär 57,8% ja valepositiivsusmäär 4,7%).

3. Sõeluuringutes esinevate märgatavate muutuste ja positiivse sõeltesti korral (74 naist) diagnoositi lootel/lapsel geneetiline patoloogia või arenguanomaalia sünnieelselt või sünnijärgselt 16 juhul (21,6%), raseduse katkemine või loote surm 12 juhul (16,2%) ja laps oli terve 2 aasta vanuses 31 juhul (41,9%). Info sünnituse ja lapse arengu kohta jäi saamata 15 juhul (20,3%). Geneetiline patoloogia või arenguanomaalia diagnoositi sünnieelselt 3/4 juhtudest ning sünnijärgselt 1/4 juhtudest.

4. Lastel, kelle emal oli sünnieelsetel sõeluuringute markerites esinenud märgatavaid muutusi, diagnoositi sünnijärgselt kaasasündinud arengurike ja/või geneetiline sündroom neljal juhul (5,4%): kahel juhul KSSR – kodade vaheseina defekt ja vatsakeste vaheseina defekt koos avatud arterioosjuhaga, ühel juhul Silver-Russelli sündroom ja ühel juhul kongenitaalne adrenaalne hüperplaasia.

5. Käesolev uuring kinnitas, et sünnieelsed sõeluuringud ei hinda riski mitte ainult kromosoomihaiguste suhtes, vaid võivad anda viidet ka arenguriketele või geneetilisele patoloogiale. Lapsed, kelle emal esines raseduse ajal tehtud sõeluuringutes märgatavaid muutusi, peaks olema sünnijärgselt süvendatud jälgimisel lastearsti juures, sest neil on 5,4% tõenäosust omada kaasasündinud arenguriket või geneetilist patoloogiat.

6. Kõige sagedamini diagnoositi kaasasündinud südamerikete korral NS, mida kliiniliselt diagnoositi umbes pooltel geneetiku konsultatsioonile suunatud juhtudest. NS haigustpõhjustav muutus leiti *PTPN11* geenis 28%-l kliiniliselt diagnoositud NS-ga patsientidel. Mutatsiooni esinemissagedus meie uuringugrupis oli madalam kui kirjanduses (33–45%). Põhjuseks võib olla NS väga varieeruv kliiniline pilt (ühes peres tuvastati haiguspõhjuslik muutus *PTPN11* geenis ka väheväljendunud fenotüübi korral) või meie väiksem kliiniline kogemus NS osas.

7. Kõik uuringugruppi kaasatud NS diagnoosiga patsiendid täitsid NS kliinilise diagnoosi kriteeriumid. Kirjeldatud fenotüüp oli võrreldav mutatsioon-positiivsete ja mutatsioon-negatiivsete patsientide grupis, mis näitab NS kliinilise pildi variaabelsust ja heterogeensust. NS-ga seotud teiste geenide osas testimist tuleks jätkata patsientidel, kellel esineb klassikaline NS fenotüüp.

8. Kliinilise NS diagnoosiga *PTPN11* mutatsioon-negatiivses grupis kinnitus hiljem kahel juhul teine diagnoos: ühel juhul Cantu sündroom ja ühel juhul mikrodeletsioon regioonis 10q23.1–23.3. See viitab NS-i kliinilise pildi variaablsusele ja kattuvusele mitmete teiste geneetiliste sündroomidega ning kliinilise diagnoosi molekulaarse kinnitamise olulisusele.

9. LS diagnoositi kliiniliselt neljal juhul ning ühel juhul kinnitus diagnoos molekulaarselt, kui *PTPN11* geenis tuvastati varem kirjeldatud mutatsioon p.Tyr279Cys. Hoolimata sellest, et tuvastatud muutus *PTPN11* geenis on kõige sagedasem muutus LS korral, esines meie patsiendil eristuv kliiniline pilt: pigmentlaigud ja sünnimärgid esinesid peamiselt jäsemetel ning enam liigeste piirkonnas, samas rindkere ja nägu olid puutumata. Varasemalt on kirjanduses vaid ühel korral kirjeldatud pigmentlaikude ja sünnimärkide eelistatud paiknemist alajäsemetel.

10. HOS diagnoositi kliiniliselt kahel juhul, kui patsientidel esines KSSR ja ülajäsemete arengurike. Molekulaarne analüüs tuvastas ühel juhul varem kirjeldamata heterosügootse muutuse: raaminihke mutatsiooni (c.1304delT, p.Leu435fsX146) *TBX5* geeni 9. eksonis. Ülajäsemete haaratus sellel patsiendil oli suhteliselt väheväljendunud ja HOS-i korral harvaesinev – distaalse paigutusega pöidlad, kitsad õlad ning õlavöötme lihaste hüpotroofia. Tuvastatud mutatsioon *TBX5* geenis põhjustab TBX5 valgu pikenemise. Varem on kirjanduses publitseeritud sarnast valku pikendavat raaminihke muutust ühel korral. Meie tulemused kinnitavad, et ka mutatsioonid väljaspool *T-box* piirkonda mõjutavad südame ja käte arengut HOS korral.

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12. PUBLICATIONS

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Education:

1977–1988	Tallinn Secondary School No 46
1988–1994	University of Tartu, Faculty of Medicine
1994–1995	Children Hospital in Tallinn, postgraduate training (internship) in Pediatrics
1998–2008	University of Tartu, Faculty of Medicine, postgraduate specialist medical training (residency training) in Medical Genetics
2008–2014	University of Tartu, Faculty of Medicine, PhD studies

Professional employement:

1987–1988	Women's Clinic of Pelgulinna Hospital in Tallinn, Assistant nurse
August 1991	Danderyd Sjukhus (Sweden, Stockholm), Department of the General Surgery, Assistant nurse
1991–1993	Women's Clinic of Pelgulinna Hospital in Tallinn, Children's Intensive Care Department, Medical nurse
1997–1998	Women's Clinic of Pelgulinna Hospital in Tallinn, Paediatrician
July–Aug. 2000	Women's Clinic of Pelgulinna Hospital in Tallinn, Paediatrician
2002–2006	Estonian Genome Project Foundation, Project Manager
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2004–…	Tartu University Hospital, United Laboratories, Department of Genetics, Clinical geneticist

Special courses:

2002	Course "Good Clinical Practice in clinical Trials"
2006	First European course in Clinical Dysmorphology "What I know best", Rome (2 days)
2007	"First course in Clinical Dysmorphology", European School in Genetic Medicine, Bologna (4 days)

2007	Three weeks training in syndrome diagnostic and genetic counselling in the Medical Genetics Clinic at the Family Federation, Helsinki
2008	Second European course in Clinical Dysmorphology "What I know best", Rome (2 days)
2009	European School of Genetic Medicine, 9th Course in "Genetic counselling in practice", Bologna (6 days)
2009	Third European course in Clinical Dysmorphology "What I know best", Rome (2 days)

Scientific work:

A total of 9 publications and 18 presentations at international scientific conferences.

The subject of my research activities have been genetic disorders and dysmorphology, prenatal diagnostics and screening; and genetic etiology of congenital heart defects.

Member of Junior Doctors´ Association, Estonian Society of Human genetics and Estonian Perinatal Society.

List of publications:

Begić F, Tahirović H, Kardašević M, Kalev I, Muru K. Leopard syndrome: a report of five cases from one family in two generations. Eur J Pediatr. 2014 Jan 9. [Epub ahead of print]

Muru K, Vals M-A, Sitska M, Asser K, Tammur P, Zilina O, Reimand T, Õunap K. Outcome of Children with Marked Changes in Maternal Screening Tests and Normal Karyotype. Hereditary Genetics 2014; 3:123; [Epub ahead of print].

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K. Muru, M. Sitska, K. Asser, A. Ehrenberg, H. Karro, K. Õunap, T. Reimand. 2010 Prospective experience with contengent screening strategy for Down syndrome in Estonia J Community Genet. 1(3):133–138I.

Kalev, K. Muru, R. Teek, R. Žordania, T. Reimand, K. Köbas, K. Õunap. 2010 LEOPARD syndrome with recurrent PTPN11 mutation Y279C and different cutaneous manifestations: two case reports and a review of the literature Eur J Pediatr. Apr;169(4):469–73.

K.Muru, K. Õunap, S. Virro, I. Kalev. 2008 Genetic basis of congenital heart defects (in Estonian). Eesti Arst 87(5):357–366.

M. Sitska, T. Reimand, K. Muru. 2008 Prenatal diagnosis of fetal chromosomal anomalies: a summary of trimester II serum screening in Estonia (in Estonian). Eesti Arst, 1:31–36

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1997–1998	Tallinna Pelgulinna Haigla Sünnitusmaja, pediaater
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2004–2007	AS LTKH, Pelgulinna Sünnitusmaja naistenõuandla, arst-konsultant
2002–2006	SA Eesti Geenivaramu, projektijuht
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Erialane täiendus:

2002	Kursus „Head kliinilised tavad kliinilistes katsetes”
2006	I düsmorfoloogia kursus “What I know best”, Rooma (2 päeva)
2007	Kursus „Kliiniline düsmorfoloogia”, European School in Genetic Medicine, Bolonga (4 päeva)
2007	Kolmenädalane meditsiinigeneetilise koolituse alane praktika Soome Perekeskuse Meditsiinigeneetika osakond, Helsingi
2008	II düsmorfoloogia kursus “What I know best”, Rooma (2 päeva)
2009	European School of Genetic Medicine “Genetic counselling in practice”, Bologna (6 päeva)
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Teadustegevus:

Kokku on ilmunud 9 publikatsiooni ja 18 ettekannet rahvusvahelistel konverentsidel.

Teadustöö on seni olnud reotud järgmiste valdkondadega: inimese geneetika ja düsmorfoloogia, pärilike haiguste sünnieelne diagnostika, perekondlikud kongenitaalsed südamerikked.

Eesti Nooremarstide Ühenduse, Eesti Inimesegeneetika Ühingu ja Eesti Perinatoloogia Seltsi liige.

Publikatsioonide loetelu:

Begić F, Tahirović H, Kardašević M, Kalev I, Muru K. Leopard syndrome: a report of five cases from one family in two generations. Eur J Pediatr. 2014 Jan 9. [Epub ahead of print]

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K.Muru, K. Õunap, S. Virro, I. Kalev. 2008 „Kaasasündinud südamerikete geneetilised põhjused". Eesti Arst 87(5):357–366.

M. Sitska, T. Reimand, K. Muru. 2008 „ Loote kromosoomihaiguste sünnieelne diagnostika: kokkuvõte II trimestri vereseerumi sõeltesti tulemustest Eestis" Eesti Arst, 1:31–36

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