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New diagnostic methods for early detection of inborn errors of metabolism in Estonia

UNIVERSITY OF TARTU
Press

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Dissertation is accepted for the commencement of the degree of Doctor of Philosophy (in medicine) on September 19th, 2018 by the Council of the Faculty of Medicine, University of Tartu, Estonia.

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Commencement: December 18th, 2018

Publication of this dissertation is granted by the University of Tartu.

ISSN 1024-395X
ISBN 978-9949-77-887-4 (print)
ISBN 978-9949-77-888-1 (pdf)

University of Tartu Press
www.tyk.ee

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LIST OF ORIGINAL PUBLICATIONS

I Reinson K, Künnapas K, Kriisa A, Vals M-A, Muru K, Õunap K. (2018). High incidence of low vitamin B12 levels in Estonian newborns. Molecular Genetics and Metabolism Reports, 15, 1–5.

II Puusepp S, Reinson K, Pajusalu S, Murumets Ü, Õiglane-Shlik E, Rein R, Talvik I, Rodenburg RJ, Õunap K. (2018). Effectiveness of whole exome sequencing in unsolved patients with a clinical suspicion of a mitochondrial disorder in Estonia. Molecular Genetics and Metabolism Reports, 15, 80–89.

III Reinson K, Õiglane-Shlik E, Talvik I, Vaher U, Õunapuu A, Ennok M, Teek R, Pajusalu S, Murumets Ü, Tomberg T, Puusepp S, Piirsoo A, Reimand T, Õunap K. Biallelic *CACNA1A* mutations cause early onset epileptic encephalopathy with progressive cerebral, cerebellar, and optic nerve atrophy. Am J Med Genet A. 2016 Aug;170(8):2173–6.

IV Thompson K, Majd H, Dallabona C, Reinson K, King MS, Alston CL, He L, Lodi T, Jones SA, Fattal-Valevski A, Fraenkel ND, Saada A, Haham A, Isohanni P, Vara R, Barbosa IA, Simpson MA, Deshpande C, Puusepp S, Bonnen PE, Rodenburg RJ, Suomalainen A, Õunap K, Elpeleg O, Ferrero I, McFarland R, Kunji ER, Taylor RW. Recurrent De Novo Dominant Mutations in *SLC25A4* Cause Severe Early-Onset Mitochondrial Disease and Loss of Mitochondrial DNA Copy Number. Am J Hum Genet. 2016 Oct 6;99(4):860–876.

V Reinson K, Kovacs-Nagy R, Õiglane-Shlik E, Pajusalu S, Nõukas M, Wintjes LT, van den Brandt FCA, Brink M, Acker T, Ahting U, Hahn A, Schänzer A, Haack TB, Rodenburg RJ, Õunap K. Diverse phenotype in patients with complex I deficiency due to mutations in *NDUFB11* (Submitted)

VI Reinson K, Ilo U, Künnapas K, Vals M-A, Muru K, Kriisa A, Õunap K. Expanded newborn screening by using tandem mass-spectrometry in Estonia: a review of 18-month'experience (In Estonian) Eesti Arst, 2016. 95 (8), 506–514.

Contribution of the author to the preparation of the original publications:

Publication I:	Participation in the study design; collecting, analyzing, and interpreting data; and writing the manuscript.
Publication II:	Participation in the study design; collecting and analyzing clinical data; and preparing the manuscript
Publication III:	Participation in the study design; collecting and analyzing clinical data; and writing the manuscript.
Publication IV:	Collecting and analyzing clinical data; and preparing the manuscript.
Publication V:	Participation in the study design; collecting and analyzing clinical data; and writing the manuscript
Publication VI:	Participation in the study design; collecting, analyzing, and interpreting data; and writing the manuscript.

ABBREVIATIONS

3MCCD	3-methylcrotonyl carboxylase deficiency
AD	Anno Domini
ADP	Adenosine diphosphate
adPEO	Autosomal dominant progressive external ophthalmoplegia
ARG	Argininemia
Arg	Arginine
ATP	Adenosine triphosphate
BWA	Burrows-Wheeler Aligner
C0	Free carnitine
CI	Complex I
C2	Acetylcarnitine
C3	Propionylcarnitine
C4	Butyrylcarnitine
C5	Isovalerylcarnitine
C5DC	Glutarylcarnitine
C5OH	3-OH-isovalerylcarnitine
C6	Hexanoylcarnitine
C8	Octanoylcarnitine
C10	Decanoylcarnitine
C14	Myritoylcarnitine
C14:1	Tetradecenoylcarnitine
C14:2	Tetradecadienoylcarnitine
C16	Palmitoycarnitine
C16OH	3-OH-plamitoylcarnitine
C18	Stearylcarnitine
C18:1	Oleylcarnitine
C18:1OH	3-OH-oleylcarnitine
C18:2	Linoleylcarnitine
C18OH	3-OH-stearoylcarnitine
CACT	Carnitine-acylcarnitine translocase deficiency
CAG	Cytosine-adenine-guanine
CAH	Congenital adrenal hyperplasia
CDC	Centers for Disease Control and Prevention
CIT I	Citrullinemia type I
Cit	Citrulline
CHT	Congenital hypothyroidism
CM	Cardiomyopathy
CPT I	Carnitine palmitoyltransferase I deficiency
CPT II	Carnitine palmitoyltransferase II deficiency
Crea/GA	Creatinine and guanidinoacetate ratio
CSF	Cerebrospinal fluid
CUD	Carnitine uptake deficiency

dATP	Deoxyadenosine triphosphate
DBS	Dried blood spots
DD	Developmental delay
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EA2	Episodic ataxia type 2
EchoCG	Echocardiography
EEG	Electroencephalogram
ERNDIM	European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited Disorders of Metabolism
ExAC	Exome Aggregation Consortium
FAOD	Fatty acid β-oxidation defects
FHM1	Familial hemiplegic migraine type 1
FPR	False positive rate
GA I	Glutaric aciduria type 1
GA II	Glutaric aciduria type 2
GATK	Genome Analysis Toolkit
Hcy	Homocysteine
HCY	Homocystinuria
HPA	Hyperphenylalaninemia
ID	Intellectual disability
IEM	Inborn errors of metabolism
IQ	Intelligence quotient
IVA	Isovaleric aciduria
KO	Knockout
LCHADD	Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency
Leu	Leucine
MCADD	Medium-chain acyl-CoA dehydrogenase deficiency
MD	Mitochondrial disorder
MELAS	Mitochondrial encephalopathy, lactic acidosis and stroke-like episodes
Met	Methionine
MLPA	Multiplex ligation-dependent probe amplification
MMA	Methylmalonic acid/ methylmalonic aciduria
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Mass spectrometry
mSAC	Serum acylcarnitines from mother
MSUD	Maple syrup urine disease
mtDNA	mitochondrial DNA
mU/UCS	Milliunits per unit of citrate synthase
NADH	Nicotine amide adenine dinucleotide reduced form
NBS	Newborn screening
nDNA	nuclear DNA

NGS	Next generation sequencing
OA	Organic acidurias
OMIM	Online Mendelian Inheritance in Man
OXPHOS	Oxidative phosphorylation system
Phe	Phenylalanine
PKU	Phenylketonuria
PPA	Propionic aciduria
PPV	Positive predictive value
R4S	Region 4Stork
RNA	Ribonucleic acid
SAA	Serum amino acids
SAC	Serum acylcarnitines
SCA6	Spinocerebellar ataxia type 6
SCADD	Short chain acyl-CoA dehydrogenase deficiency
SMA I	Spinal muscular atrophy type I
SOA	Serum organic acids
SucA	Succinylacetone
tHcy	Total homocysteine
Tyr	Tyrosine
TYR I	Tyrosinemia type I
UAA	Urine amino acids
UOA	Urine organic acids
US	United States
Val	Valine
VLCADD	Very long chain acyl-CoA dehydrogenase deficiency
WES	Whole exome sequencing
WISC-III	Wechsler Intelligence Scale for Children III

1. INTRODUCTION

The measurement of single metabolites as a source of information related to health and disease has a long history. Ancient Chinese cultures (1500–2000 BC) recognized urine as an important material for assessment well-being, for example, sweet-tasting urine was indicative of a disease, which now is known as diabetes. However, the word metabolism originates from the Greek word "μεταβολή", which means "change" and the concept of metabolism was first mentioned by Ibn al-Nafis 1260 AD, who stated that "the body and its parts are in a continuous state of dissolution and nourishment, so they are inevitably undergoing permanent change" [van der Greef et al. 2013].

The next revolution in the field of metabolism was the discovery of enzymes by German chemist Eduard Buchner at the beginning of the 20th century. This realization led to the focus on intracellular chemical reactions and inspired the development of biochemistry, like enzymatic reactions and intracellular biochemical pathways. The following fundamental change came right after, when the British physician Sir Archibald Garrod proposed the detection of changes in metabolic pathways caused by a single inherited gene defect. This concept became the basis for the field of inborn errors of metabolism [van der Greef et al. 2013]. Most of these inherited conditions are not clinically evident at birth, because accumulating metabolites cross the placenta and are cleared by the mother during gestation, therefore affected infants usually appear normal at birth but lead to significant irreversible harm or death.

Some conditions, like phenylketonuria (PKU), are effectively treatable if detected early enough. Therefore another breakthrough in diagnosing metabolic disorders took place at the beginning of the 1960s, when Dr Robert Guthrie described a method of testing newborns for PKU for the first time [Guthrie and Susi 1963]. This testing was performed during the asymptotic period and it is valued as the beginning of the newborn screening (NBS).

A crucial impact in the advancements of NBS came in the 1990s with the adaption of electro-spray ionization tandem mass spectrometry (MS), which examines multiple metabolites in one analytical run. Therefore, many biochemically related disorders could be detected in one test, allowing screening for extremely rare disorders that might not otherwise have been considered suitable for separate testing, and more likely allow to better characterize a particular metabolic disease or other iatrogenic influence such as total parenteral nutrition. Consequently, this approach left behind the principle of a one-method, one-metabolite, one-disease conception [Chace D.H. 2005].

In Estonia, the NBS program was initiated in 1993 for only one disorder – PKU, which belongs to the group of aminoacidurias. Phenylalanine (Phe) was measured from dried blood spots (DBS) by a modified fluorometric method based on enhancement of the fluorescence of a phenylalanine-ninhydrin reaction product by L-leucyl-L-alanine [Ounap et al. 1998]. The first thorough systematic review in the field of inborn error of metabolism (IEM) in Estonia was composed by Õunap et al., who concluded that the incidence of PKU in Estonia identified during retrospective studies was lower than the identified

prospectively during newborn screening – the incidence of PKU was estimated 1:6,010 live births [Ounap 1999]. Congenital hypothyroidism (CHT) was added to the screening program three years later [Mikelsaar et al. 1998] and the above described screening program lasted in Estonia for more than 20 years.

Nowadays, in Europe, most of the countries are using tandem MS for newborn screening and the number of disorders included in nation-wide screening programs were expanded up to 29, like in Austria [Burgard et al. 2012]. One of the most important group of disorders in the NBS program seems to be fatty acid β-oxidation defects (FAOD). The first diagnostic test in Estonia for FAOD was molecular genetic testing for the common mutations in genes responsible for medium-chain acyl-CoA dehydrogenase deficiency (MCADD) and long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD). The carrier frequency for the mutation c.985A>G (p.K329E) in the *ACADM* gene, responsible for the MCADD, is 1:220 in Estonia, and the frequency of possibly affected homozygotes is 1:193,000 [Lillevali 2000]. The carrier frequency for the mutation c.1528G>C (p.E510Q) in the *HADHA* gene, responsible for the LCHADD, is much higher than expected – 1:173 [Joost et al. 2012a]. Therefore, taking into account that the c.1528G>C mutation makes up 87.5% of disease alleles in Estonian LCHADD patients, the estimated prevalence of LCHADD in Estonia would be 1:91,700 [Joost et al. 2012a]. The prevalence of other disorders which are presented in most of the NBS programs are unknown in Estonia.

However, not all inherited metabolic disorders that begin in childhood can be screened and treated in the asymptomatic period. For example, in case of mitochondrial disorders, where genetic analysis, counselling and prenatal diagnosis in further pregnancies is essential; yet it is difficult to reach molecular genetic result considering that this IEM may present an extremely nonspecific clinical and biochemical picture at any age and the phenotype varies tremendously among family members.

Joost et al. in 2012 worked out a diagnostic algorithm for better identification of patients with possible mitochondrial disorder (MD) among Estonian pediatric neurology patients [Joost 2012]. This algorithm is based on clinical presentation specified with instrumental investigations and the biochemical phenotype, which resulted in single gene sequencing [Joost et al. 2012b]. During the study period (2003–2009), the live-birth prevalence for childhood onset MDs was 1:20,764 in Estonia [Joost 2012; Joost et al. 2012b]. However, the landscape of diagnosing MDs has been changing within the last couple of years due to the discovery of novel diagnostic methods like whole exome sequencing (WES), which were not being mentioned and used in the previous study by Joost et al.

The aims of our study were to evaluate new diagnostic methods and their effectiveness of expanded NBS by tandem MS in Estonia; and to determine the effectiveness of WES in clinical practice with patients suspected to have childhood onset MD. In addition, we have added three interesting and thoroughly investigated cases to illustrate the research processes and the outcome, as well as to confirm the effectiveness of WES.

2. REVIEW OF LITERATURE

2.1 Newborn screening for inborn errors of metabolism by tandem MS

2.1.1 Tandem MS introduction to expanded NBS programs

The introduction of tandem MS technology to newborn screening in the 1990s gave a tremendous impulse to expand and develop the range of disorders to be included in the standard NBS panel in many countries [Millington et al. 1991]. Therefore, the use of this methodology broke the traditional paradigm in NBS of one test for one disorder and permitted a range of conditions to be identified using a single analytical run to identify newborns with severe disorders that are relatively preventable and treatable (or controllable).

Tandem MS high sensitivity and specificity can be used for qualitative and quantitative analysis of many analytes, such as amino acids and acylcarnitines, organic acids, homocysteine, orotic acid, purines and pyrimidines, steroids, and vitamin D to name a few, with appropriate internal standards [Ombrone et al. 2016]. Therefore, the usage of tandem MS gives an opportunity to get the references for more than 50 different conditions [Therrell et al. 2015].

Still, tandem MS just provide the method for analysis, but NBS does not include only testing, it is a thoroughly elaborated system, which requires additional equipment, transport and tracking the test results, supplying special diets and care; personnel for clinical follow-up and counseling parents, experts like lab technicians, chemists, biologists and nutritionists. Besides, they all need constant training.

The expansion of NBS gives us an opportunity to identify more affected children, but it produces also some difficulties like the increase in the recall rate [Tarini et al. 2006]. One of the reasons is that some metabolites that are markers for diseases, have proven to have poor specificity [Ombrone et al. 2016] and for some metabolites have an overlap between controls and patients ranges [McHugh et al. 2011]. At the same time, one should not forget that in the period of introducing the NBS program, there is no experience (including no in-house cutoff values).

Whereas, the experiences in expanded NBS area is also accompanied by identification of more affected individuals than previously known from clinical ascertainment [Joost et al. 2012a; Ounap et al. 1998; Wilcken et al. 2003]; by unpredictable prognosis [Wilcken 2008]; and by insufficient knowledge of natural history [Fernhoff 2009; Levy 2010; McCabe and McCabe 2008]. It is also important to note that some presently screened disorders may involve little benefit or seem to be benign and their inclusion in NBS detection might be reconsidered as perhaps unnecessary and even harmful. Some publications describe the anxiety and the potential medicalization for these families [Landau et al. 2017; Wilcken 2008]. The false-positive screening results are also causing

needless parental stress and may influences the child–parent relationship [Bonham 2014].

On the other hand, all NBS laboratories have to face a number of false-negative cases [Estrella et al. 2014; Wilcken et al. 2003], which highlights the next cornerstone on the NBS – the range and calculation of cutoff values for screening. The result of this test should be a good compromise between sensitivity and specificity. A good sensitivity could result in a high false positive rate, while having high specificity could cause false negative results. Some screening programs first set their cutoffs for mean ± 2SD [la Marca 2014] or establish initial cutoffs from 1st to 99.0th (or 0.05th to 99.95th) percentile [Couce et al. 2011; Lindner et al. 2011; Lund et al. 2012; McHugh et al. 2011; Vilarinho et al. 2010]. Most of the screening laboratories report that all cutoff values were adjusted over time to optimize the performance, including false positive rate, and to compensate for changes in analytical method [la Marca 2014; Lund et al. 2012; Vilarinho et al. 2010].

2.1.2 Worldwide standards and practice for NBS by tandem MS

There is only some limited advice available from national advisory committees and national medical or public health professional organizations regarding NBS policies. Even the conditions and the criteria for an acceptable screening program are still controversial; giving arises to greatly varying worldwide NBS panels. Different economic environment of countries, but mostly the way how national decisions are made in relation to determining newborn screening policy, have led to divergent choices in different countries. Those administrations in which decisions were strongly influenced by metabolic physicians and scientists or geneticists including Australia and the United States (US) tended to use a wide selection expanded programs at an early stage [Hoffmann et al. 2014; Landau et al. 2017; Therrell et al. 2015]. Whereas countries, including most in Europe, where the policy decisions are determined more by public health officials, the NBS programs include a relatively limited number of disorders [Burgard et al. 2012; Loeber et al. 2012; Therrell et al. 2015].

Therefore, divergence has resulted in significant disparities in screening services available to newborns in all over the world and there is no worldwide "golden standard" for NBS [Bonham 2014; Burgard et al. 2012; Lehotay et al. 2011; Loeber et al. 2012; Pollitt 2006].

A positive example of a unity in the field of NBS has been created in the US, where currently 49 congenital metabolic disorders are in the core and secondary NBS panel using tandem MS [Ficicioglu 2017; Janeckova et al. 2012; Rinaldo et al. 2008]. The prerequisites for that kind of accordance was made by creating the Recommended Uniform Screening Panel (RUSP) that is accepted and recommended by the US Secretary of Health and Human Services Advisory Committee of Heritable Diseases in Newborns and Children (SACHDNC) for

all the NBS programs in the US. [Ficicioglu 2017; Landau et al. 2017]. This screening program is available to all newborns born in US.

The opposite situation is in Europe, where there is little unity. For example, the screening situation in Italy is very fragmented – several regions regulate their NBS programs in place and list of coverage conditions vary between 20 and 40 (Tuscany, Umbria, Liguria, Sardinia, Emilia Romagna, Sicily, Veneto), while some other regions have experimental programs ongoing (Lazio). Approximately one third of newborns currently undergo expanded screening in Italy [Burlina and Corsello 2015]. Belgium has been divided into two separate jurisdictions (Flemish respectively French Community), where different screening programs are used, and The Liechtenstein newborns are screened in Switzerland [Loeber et al. 2012].

The nearest countries in our regions like Finland, Latvia and Lithuania, continue to have little experience in the field of expanded NBS. Latvia are screening newborns only for PKU and CHT [Loeber et al. 2012], Lithuania added galactosemia and congenital adrenal hyperplasia (CAH) to the NBS panel at the beginning of the 2015, while screening for PKU and CHT continues as before [personal contact with J. Songailiene]. In Finland, until January 1, 2015 the only disorder screened for comprehensively in the whole country was CHT, which was performed from the umbilical cord blood; and just recently the screening program expanded by five congenital metabolic disorders: glutaric aciduria type 1 (GAI), MCADD, LCHADD, PKU and CAH [Therrell et al. 2015].

Therefore, the NBS is taking place to some extent in most European countries [Burgard et al. 2012; Loeber et al. 2012] and most of these programs claim to be based on the screening principles first developed by Wilson and Jungner on behalf of the World Health Organization in 1968 [Wilson and Jungner 1968]. At the same time, many expert groups have turned their attention to screening panels which include a large number of disorders that may not meet all of these criteria and therefore, these principles have been revisited in regard to medical advances [Dhondt 2007; Dhondt 2010; Pollitt 2006; Pollitt 2007]. The incidence of a disease is a less compelling criterion when the disorder can be detected at no additional cost with multiplex technology, but inclusion of diseases with no effective treatment remains questionable [Dhondt 2010]. In the light of this knowledge, it is also important to point out that the list of the screened disorders is unlikely to be final in the long run. Many programs have re-evaluated this list because of the added experience and knowledge [Lindner et al. 2011; Lund et al. 2012]. For example, 3-methylcrotonyl carboxylase deficiency (3MCCD) was thought to be a rare disorder with severe neurological pathology, but during the NBS, this disorder was detected in an appreciable number of newborns and in their clinically unaffected mothers [Stadler et al. 2006]. Although, there is also some contradiction, because some reports are describing patients with 3MCCD having acute metabolic decompensation [Ficicioglu and Payan 2006; Grunert et al. 2012]. A similar discussion takes place with short-chain acyl-CoA dehydro-

genase deficiency (SCADD), which is considered as benign condition, while there is reports that suggest being aware of hypoglycemia in patients with SCADD to prevent adverse consequences [Waisbren et al. 2008].

Therefore, many screening programs have made reassessments for one reason or another. Like Germany, where nationwide expanded newborn screening was started in 2000 with more than 29 conditions [Lindner et al. 2011; Maier et al. 2005; Schulze et al. 2003]. In 2005, German health authorities decided to limit the number of disorders to be detected by tandem MS up to ten and decided that all results of metabolites, which are not needed for this purpose, must be suppressed or deleted immediately after analysis [Bodamer et al. 2007; Lindner et al. 2011]. This highlights the next difference between the approaches in NBS – the full-profile approach or the limited access approach (like in Germany). The full-profile approach allows to use the maximum amount of information without the regard to the distinction between appropriate and inappropriate target conditions. This is accompanied by a disadvantage – the full-profile mode increases the risk of incidental detection of abnormal conditions, for which the clinical significance of a positive screening result is very much in doubt [Dhondt 2010].

Furthermore, the length of DBS storage varies considerably, which is between 3 months (Germany) to more or less indefinite (Denmark, Norway and Sweden) [Loeber et al. 2012]. While it is known that most of the metabolites are degraded in months [Adam et al. 2011], therefore the DBS can be used for DNA extraction only. According to this, maybe the storage of full-profile of metabolites is useful by giving availability for retrospective investigation.

Each NBS performing center or country in Europe can consider these aforementioned topics and their drawbacks as well as advantages to make the most appropriate decision for them. However, nobody doubts that NBS is a very important and extraordinarily positive initiative, especially for rare and serious inherited disorders. This is also confirmed by medical specialist/scientists and families where the affected child grows. For example, a short-term assessment, based on a parental survey, found that patients diagnosed clinically showed a higher incidence of intellectual disability (ID) and their parents experienced greater stress than when the disorder was detected by NBS [Waisbren et al. 2013; Waisbren et al. 2002]. In a longer-term outcome, evaluation reported fewer deaths and fewer clinically significant disabilities at 6 years of age when detection resulted from NBS [Wilcken et al. 2009]. Moreover, NBS is also evaluated as an economically advantageous engagement [Schoen et al. 2002].

Lessons learned from current ongoing worldwide NBS programs indicate that we are still far from perfectionism. We believe that in the future NBS area expands and overlaps with many other fields of science. The approach will change because of rapidly developing technology and the addition of genetic tests with extra biomarkers into NBS will make it possible to detect many more genetic conditions [Solomon et al. 2012]. Besides, the genomic sequencing could open up new opportunities for increasing the specificity of current tandem MS screening tests by bringing out only the true-positive results and enabling to

screening the rare disorders that currently lack methods for conventional biochemical NBS [Berg et al. 2017]. Furthermore, our knowledge is insufficient for completely understanding the complexity of diseases. Therefore, we believe, that the collaboration between the area of genes as genomics, messenger ribonucleic acids (RNAs) as transcriptomics, proteins as proteomics, and metabolites as metabolomics are inevitable.

2.2 Expanded newborn screening for detection of vitamin B12 deficiency

2.2.1 The distribution of vitamin B12 deficiency

Vitamin B12 is a water-soluble vitamin, which is a micronutrient essential for cellular growth, differentiation and development. Humans are unable to synthesize it by themselves and thus rely on dietary intakes and a complex intracellular route for vitamin B12 processing and delivery [Hannibal et al. 2009].

Vitamin B12 deficiency seems to be worldwide problem, especially in developing countries where deficiency is common in all age groups [Allen 2009]. In developed countries, it is most frequently found in the elderly age group; however, the prevalence in all age groups may be higher than previously thought [Allen 2009; Irevall et al. 2017]. This condition is frequently underdiagnosed in pregnancy and in infants from mothers having insufficient levels of this micronutrient [Sarafoglou et al. 2011; Wheeler 2008].

The worldwide spread of NBS programs using tandem MS has also greatly contributed to highlighting the high incidence of vitamin B12 deficiency even in industrialized countries [Irevall et al. 2017], and it has also been pointed out that non-inherited conditions are mainly secondary to maternal deficiency [Dror and Allen 2008; Hinton et al. 2010; Marble et al. 2008; Refsum et al. 2004; Sarafoglou et al. 2011; Scolamiero et al. 2014].

Maternal vitamin B12 deficiency during pregnancy is a strong predictor of neonatal vitamin B12 deficiency as the active transport of cobalamin in the fetus serum is carried out through the placenta [Duggan et al. 2014; Monsen et al. 2001]. Mothers' inadequate dietary intake and malabsorption are the most common causes of congenital acquired vitamin B12 deficiency. Dietary patterns with reduced intake of animal sourced foods, such as vegetarian, vegan, or low-cost diets, and religiously or culturally motivated avoidance of animal products, are associated with an increased risk for above described condition [Allen 2008; Koebnick et al. 2004; Murphy and Allen 2003]. Additionally, gastrointestinal disorders, gastric bypass and pernicious anemia can lead to malabsorption and deficiency of vitamin B12 [Allen 2008; Hinton et al. 2010]. Nowadays, the maternal B12 deficiency can be subclinical, which is defined as a total serum B12 concentration of 150–249 pmol/L [Carmel 2012; Carmel 2013]. These mothers may not be anemic and their vitamin B12 levels are normal or low-normal, therefore, the diagnosis and management of it, is a matter of great

interest, due to its much higher prevalence (up to 40% of the population in western countries) compared to clinical deficiency [Carmel 2012; Carmel 2013].

An Ontario study group reported that 10% of pregnant women (> 28 days) are vitamin B12 deficient [Ray et al. 2008]. Increased rates of up to 51% vitamin B12 deficiency during pregnancy have been found for India [Duggan et al. 2014], Australia [Hure et al. 2012] and Mexico [Allen et al. 1995], potentially leading to a high prevalence of congenital acquired vitamin B12 deficiency. Therefore, it seems that this problem is quite common in different regions and treatment is relatively simple – the only question is how to find patients in need of treatment as quickly as possible.

2.2.2 The vitamin B12 deficiency related changes in NBS profile

Along with folate, vitamin B12 is necessary for the synthesis of DNA, lipids and proteins, and an essential step in this process of conversion the homocysteine (Hcy) to methionine (Met) [Rasmussen et al. 2001]. These processes are crucial during the growth and brain development [Dror and Allen 2008]. In addition, this vitamin has a significant role in odd-chain fatty acid and branched chain amino acid metabolism for isomerization of methylmalonyl-CoA to succinyl-CoA. Intracellular B12 deficiency leads to an increase in methylmalonyl-CoA, which converts into methylmalonic acid (MMA) and is released into the blood stream. Therefore, plasma MMA concentration may serve as a functional biomarker of vitamin B12 status and reflects intracellular deficiency [Carmel 2011; Hannibal et al. 2016; Refsum et al. 2004; Savage et al. 1994]. While it must be remembered that the measurement of total circulating vitamin B12 alone may not be enough to give an assessment of the actual situation, because ~80% is bound to haptocorrin, and therefore it is not bioavailable for cellular uptake and does not reflect the cellular vitamin B12 status [Carmel 2000; Devalia et al. 2014; Lysne et al. 2016; Solomon 2005].

The acylcarnitine profile of DBS samples from NBS includes a propionylcarnitine (C3), a compound of odd-chain fatty acid metabolism [Thompson 1992], which is widely used analyte as a marker for propionic- and methylmalonic acidemia, but at the same time it may also be indicative for vitamin B12 deficiency. Therefore, some newborn screening programs have started to use C3 as the first marker for congenital acquired vitamin B12 deficiency. [Hinton et al. 2010; Sarafoglou et al. 2011; Scolamiero et al. 2014]. However, it is important to emphasize that the C3 is a primary maker used to detect organic acidurias and remethylation defects and its level alone is not sensitive or specific enough to detect all newborns with congenital acquired vitamin B12 deficiency. Moreover, C3 levels may not be sufficiently high during the first few days of life when the DBS is collected [Campbell et al. 2005]. Therefore, there is some evidence that the C3 to acetylcarnitine (C2) ratio and/or C3 to palmitoycarnitine (C16) ratio together with C3 are better than C3 level alone for detecting or identifying the babies with nutritional B12 deficiency [Armour et

al. 2013; Sarafoglou et al. 2011; Scolamiero et al. 2014]. The MMA, measured from DBS as a secondary test, might also add value to increase sensitivity and specificity in detection of vitamin B12 deficiency [Sarafoglou et al. 2011; Schroder et al. 2016]. Some authors like Gramer et al. have also suggested using total homocysteine (tHcy) from DBS as a first marker to detect remethylation disorders and additionally congenital acquired vitamin B12 deficiency. The second-tier markers like Met, Met/Phe, C3 and C3/C2 should be evaluated afterwards to confirm the initial hypothesis [Gramer et al. 2017]. Considering that this topic is recently increasingly debated, it is very likely that new approaches will be available in the near future.

2.2.3 The congenital acquired vitamin B12 deficiency diagnosed by NBS

The results of congenital acquired vitamin B12 deficiency diagnosed by NBS, published for so far (intentionally or coincidentally found), have shown a relatively high incidences (Table 1). Sarafoglou et al. described a population-based study of 363,649 infants born in Minnesota. During the six-year study period, 11 newborns with vitamin B12 deficiency were found, therefore the incidence was 3.02/100,000 live births (1:33,113) and the lower cutoff level of C3 was 5.25 μmol/L [Sarafoglou et al. 2011]. Higher incidence was detected in the Italian population, where a six-year expanded metabolic newborn screening pilot project showed an incidence of congenital acquired vitamin B12 deficiency of 20/100,000 (1:5,000) and the lower cutoff level of C3 was 3.16 μmol/L, [Scolamiero et al. 2014]. The most exceptional statement was proposed by Refsum et al., who concluded that 10% of screened newborns had low vitamin B12 levels [Refsum et al. 2004]. Therefore, numerous recent publications show that NBS with tandem MS has great potential to identify congenital vitamin B12 deficiency, an important and treatable condition in asymptomatic stage [Campbell et al. 2005; Hinton et al. 2010; Marble et al. 2008; Sarafoglou et al. 2011; Scolamiero et al. 2014].

Table 1. Summary of publications about the congenital vitamin B12 deficiency.
* – calculated results; n.a. – not available

Study group	No. of screened newborns	No. of newborns with congenital acquired vitamin B12 deficiency	Incidence of congenital acquired vitamin B12 deficiency
Hinton et al.[2010]	n.a.	n.a.	**0.88/100,000 (1:113,636*)**
Sarafoglou et al.[2011]	363,649	11	**3.02/100,000 (1:33,113*)**
Gramer et al.[2017]	26,202	5	**19.08/100,000* (1:5,241*)**
Scolamiero et al.[2014]	35,000	7	**20/100,000* (1:5,000)**
Reinson et al.[2017]	41,453	14	**33.8/100,000 (1:2,959)**
Refsum et al.[2004]	4,992	~10% (500*)	**10,000/100,000* (1:10*)**

Early detection and intervention of vitamin B12 deficiency is critical to prevent irreversible neurologic damage. Unrecognized neonatal vitamin B12 deficiency worsens if the infant is exclusively breastfed by a vitamin B12 deficient or sub-clinical deficient mother due to decreased fetal stores and insufficient intake through breastmilk [Rasmussen et al. 2001]. Infantile vitamin B12 deficiency often presents with nonspecific symptoms, such as muscle hypotonia, irritability, or failure to thrive [Dror and Allen 2008; Rasmussen et al. 2001]. It is difficult to make the diagnosis on the clinical ground and there tends to be substantial diagnostic delay [Graham et al. 1992], which can easily be avoided.

Prolonged vitamin B12 deficiency during infancy has been associated with negative long-term health outcomes in children [Molloy et al. 2008], including impaired cognitive function and developmental delay (DD) [Bhate et al. 2008; Garcia et al. 2009], however, the extent and degree of disability depend on the severity and duration of the deficiency [Rasmussen et al. 2001]. Therefore, it would be beneficial to improve NBS, so that most of those children could be detected in the symptom-free phase.

2.3 Diagnostic approach to mitochondrial disorders

2.3.1 Mitochondrial function and genes regulating it

Mitochondria are double-membrane-bound organelles, which are of bacterial origin, and during the historical symbiotic relationship, they became normal component of eukaryotic cells [Ochman and Moran 2001]. They are essential for a wide range of cellular processes, including iron-sulfur cluster formation, amino acid and fatty acid synthesis and degradation, the tricarboxylic acid cycle, heme synthesis, and production of adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS). The primary MDs are considered to describe diseases with a primary disorder in the entire route of the OXPHOS.

The origin of mitochondria remains exposed with their multicopy genetic material – mitochondrial DNA (mtDNA), which is exclusively maternally inherited. The mtDNA copy number varying greatly between individuals and across different tissues from the same individual [Giles et al. 1980]. The multicopy nature of mtDNA gives rise to heteroplasmy, a condition in which mutant and wild-type mtDNA molecules coexist. While, homoplasmy occurs when all of the mtDNA molecules have the same genotype. Heteroplasmic mutations often have a variable threshold – a level to which the cell can tolerate defective mtDNA molecules [Stewart and Chinnery 2015]. But it is not just about it – more than 289 nuclear genes are currently known to be associated with MDs [Wortmann et al. 2017] and this list is not finished yet.

Mutations in these genes may result in a defect in the corresponding subunit(s), causing an isolated or complex deficiency in the OXPHOS enzymes. Hundreds of different proteins are required for the biosynthesis of the OXPHOS, including assembly factors and mitochondrial translation factors

[Koopman et al. 2012; Mimaki et al. 2012; Nouws et al. 2012; Smits et al. 2010]. A defect in one of these proteins often leads to a deficiency of OXPHOS enzymes as well. Furthermore, a large number of defects outside the OXPHOS have been described that directly or indirectly affect the mitochondrial oxidative ATP production.

Taking the above mentioned into account, mitochondrial dysfunction is implicated in a broad spectrum of diseases, affecting various tissues. The clinical spectrum of MDs is extremely broad and the underlying biochemical and genetic defects are heterogeneous [Koopman et al. 2012; Morava et al. 2006b; Munnich et al. 1996]. Identical clinical signs and symptoms can be caused by mutations in different mitochondrial or nuclear genes and the same mutation can lead to different phenotypes. However, the majority of children with MD presents at least one or more neurological signs or symptoms (e.g., muscle weakness, peripheral neuropathy, ophthalmoplegia, movement disorder, epilepsy or migraine), which are all non-specific [Munnich et al. 1996].

Therefore, it is often a major challenge to diagnose MDs and discover their molecular cause, but the introduction of new diagnostic methods has greatly contributed to this.

2.3.2 Traditional diagnostic approach for MDs

For a long period of time the MDs diagnostic was initially based on measurement of the biochemical markers like lactic acid in serum and cerebrospinal fluid (CSF), high serum alanine levels, and increased excretion of the metabolites of the tricarboxylic acid cycle – these were represented in patients with mitochondrial dysfunction [Rubio-Gozalbo et al. 2000], but not always as they were not specific to MDs.

For example, lactic acidemia may be present only in 50% of patients [Koenig 2008] and up to 87% of patients with neonatal onset of MD [Honzik et al. 2012]. Valuable information can be obtained from brain magnetic resonance imaging (MRI) and muscle biopsy: light microscopy for structural changes, evaluated by histochemical, immune-histochemical and ultrastructural investigations with biochemical analysis of respiratory chain enzymes [Kisler et al. 2010]. This approach has already been thoroughly described in advance [Joost 2012] and a number of guidelines and algorithms have been developed to improve the diagnostic field [Joost et al. 2012a; Morava et al. 2006b; Nissenkorn et al. 1999; Wolf and Smeitink 2002], but none of them guaranteed the detection of most of the patients with MDs. For example, the molecular diagnosis by using Sanger sequencing were achieved only in 11% of the patients with suspected MD [Neveling et al. 2013]. Besides, mitochondrial dysfunction may occasionally be a secondary phenomenon, which occurs alongside to a primary genetic syndrome and/or neuromuscular disorder [Valenti et al. 2014; Wortmann et al. 2009]. Therefore, the final diagnosis could take years.

2.3.3 WES as a new diagnostic approach for MDs

Nowadays, many scientists have raised a question – would the WES make a difference and be as a first-line diagnostic test in patients suspected to have MD; especially in children to save the time and avoid the need for invasive procedures like skin and muscle biopsy.

WES is the analysis of all coding gene regions, which comprises approximately 1.6% of the human genome and this technology is increasingly used in clinical practice [Bamshad et al. 2011; Fokstuen et al. 2016; Kaname et al. 2014; Majewski et al. 2011]. This method analyzes about 30 million base pairs that are translated into functional proteins. Mutations in base pairs may impair protein expression or function, thereby causing disease [Ficicioglu 2017].

Compared with traditional diagnostic testing for MDs, which are described by Joost et al. [Joost 2012], the recent evaluations of WES for diagnosing MDs have shown that this approach is more likely to provide a final diagnosis. Moreover, it seems to be quicker and cheaper as the amount of genetic information that can be obtained in a single test is considerably larger compared to previous approach, which required several different tests. This is also indicated by studies that report the success rate of big exome sequencing up to 40% and in selected subgroups it can be up to 60% [Haack et al. 2012; Neveling et al. 2013; Wortmann et al. 2015]. Still, it is important to note that most of the above described work with WES is research based, particularly the studies describing novel genetic defects [Galmiche et al. 2011; Haack et al. 2014; Yarham et al. 2014]; or selected patient cohorts who have beforehand confirmed OXPHOS enzyme deficiency in fibroblasts or muscle [Calvo et al. 2012; Ohtake et al. 2014; Taylor et al. 2014].

Publications about WES as a tool for routine diagnostic work have just recently been issued. The first of them described the findings in 109 patients with a suspected MD [Wortmann et al. 2015]. This cohort consisted of patients with ID (most frequent finding), DD, myopathy/exercise intolerance, and mitochondrial dysfunction in muscle; the genetic diagnosis was confirmed in 42 patients (39%). This research also has been emphasizing the patient's thorough investigation before performing the WES. For these patients, the inclusion criteria were suspicion of a MD by the referring physician, absence of large scale mtDNA deletions, mtDNA point mutations and lacking of copy number variations [Wortmann et al. 2015].

Several studies have attempted to evaluate how successful WES is for the molecular diagnosis of MDs and come to the conclusion that this method has its own advantages and disadvantages, and often we need biochemical and functional studies too. For example, 42 infantile mitochondrial patients with biochemically proven OXPHOS deficiency, the molecularly confirmed diagnosis was made in 24% of patients and almost half of the patients remained without a diagnosis or a prioritized candidate [Calvo et al. 2012]. A significant number of undiagnosed cases might indicate a very wide spectrum of genetic disorders in patients suspected of MD. Therefore, even WES makes sometimes a clinical

and molecular diagnosis challenging. Still, there are authors who recommend the approach using exome sequencing in leukocyte extracted DNA in all individuals suspected of MD, irrespective of age, gender, and stage of the disease [Wortmann et al. 2017].

Concurrently, the functional and biochemical studies (including a muscle and skin biopsy) remain important due to unknown variants of uncertain pathogenicity in genes not reported earlier, although they may lead to MD. Even variants in known disease causing genes may require functional validation, for example, in the case of an unusual clinical presentation. This validation may consist of a relatively simple test, such as a Western blot or a specific enzyme assay. As well as genetic complementation studies, in which the wild type gene is introduced in patient's cultured cells to test if this restores the cellular or biochemical defect [Danhauser et al. 2011; Huigsloot et al. 2011; Jonckheere et al. 2013]. All of these approaches have also been used extensively by our study group.

2.4 *CACNA1A* -related disorders and genotype-phenotype associations

The *CACNA1A* gene (OMIM 601011) encodes the pore-forming alpha-1A subunit of the calcium channel $Ca_v2.1$ acting as an ion pore and a voltage sensor [Strupp et al. 2007; Vahedi et al. 2000]. These channels have been shown to have an essential role to mediate synaptic release from a variety of neuronal cell types, both excitatory and inhibitory, in the cortex, hippocampus, thalamus and cerebellum [Damaj et al. 2015]. Particularly in the *CACNA1A* gene, several mutations like nonsense mutations, missense mutations and expansion of cytosine-adenine-guanine (CAG) repeats have been described earlier and they lead to variable clinical phenotypes – including episodic ataxia type 2 (EA2, OMIM 108500), familial hemiplegic migraine type 1 (FHM1, OMIM 141500) and spinocerebellar ataxia type 6 (SCA6, OMIM 183086). It is important to bring out that there is no strong correlation between phenotype and genotype [Strupp et al. 2007], although expansion of the CAG repeats causes mainly SCA6 [Jen et al. 2001]. However, channel function and its relation to phenotype are not well studied in most of the cases, leaving a need for functional annotation of *CACNA1A* and its variants in a model organism [Luo et al. 2017].

Over times, the descriptions of *CACNA1A*-related phenotypes only expand and an increasing number of publications have been associating it with epilepsy. This may occur during severe hemiplegic migraine attacks or as an independent epileptic event [Haan et al. 2008; Holtmann et al. 2002; Jouvenceau et al. 2001]. There are also rare case reports from patients having been diagnosed with epileptic encephalopathy [Damaj et al. 2015; Hayashida et al. 2017; Hino-Fukuyo et al. 2015; Ohmori et al. 2013]. In addition, ID has also been previously described [Freilinger et al. 2008; Kors et al. 2003] causing mental

dysfunction varying from a profound [Curtain et al. 2006; Vahedi et al. 2000] to a mild ID [Damaj et al. 2015].

All the above-mentioned conditions are inherited in an autosomal dominant pattern (Table 2).

Table 2. *CACNA1A*-related disorders in the OMIM database

Location	Phenotype	Phenotype OMIM number	Inheritance
19p13.13	Epileptic encephalopathy, early infantile, 42	617106	AD
	Episodic ataxia, type 2	108500	AD
	Migraine, familial hemiplegic, 1	141500	AD
	Migraine, familial hemiplegic, 1, with progressive cerebellar ataxia	141500	AD
	Spinocerebellar ataxia 6	183086	AD

Our workgroup is describing for a first time the clinical picture and molecular finding of patients with the semidominantly inherited mutations in the *CACNA1A* gene, which are likely to cause early epileptic encephalopathy, progressive cerebral, cerebellar, and optic nerve atrophy with severe muscular hypotonia and reduced lifespan [Reinson et al. 2016b]. Therefore, this is a novel finding, which expands the *CACNA1A*-related disorders phenotypic spectrum.

2.5 A novel mutation is causing the third distinct phenotypic group associated with *SLC25A4* gene

Humans have four tissue-specific isoforms of ADP/ATP translocase, which are encoded by four closely related nuclear genes [Dolce et al. 2005]. Solute carrier family 25, member 4 (*SLC25A4*) gene (OMIM 103220) is located on the sub-telomeric region of chromosome 4q and encodes the AAC1 (also known as ANT1) protein. This gene is expressed at high level in heart, skeletal muscle, brain and in organs with low mitotic regeneration, but at low level in proliferating cells such as myoblasts during muscle development [Clemencon et al. 2013]. AAC1 protein is homodimer of 30-kD subunits embedded in the mitochondrial inner membrane. This dimer forms a gated pore, which plays a central role to import ADP across the inner membrane into the mitochondrial matrix and export ATP from the matrix into the intermembrane space, which is confluent with the cytosol. In this way, the ADP/ATP ratio is regulated in mitochondrial oxidative phosphorylation [Fiore et al. 1998; Klingenberg 1989].

Several mutations in *SLC25A4* have been linked to mitochondrial disorders and fall into two distinct clinical phenotypes. Firstly, null recessive mutations causing a mitochondrial DNA depletion syndrome 12B presenting myopathy and cardiomyopathy phenotype in childhood or early adulthood and it is

characterized by lactic acidosis, fatigue and exercise intolerance (OMIM 615418) [Echaniz-Laguna et al. 2012; Korver-Keularts et al. 2015; Strauss et al. 2013]. Secondly, several single heterozygous mutations are reported in cases of adult-onset autosomal dominant progressive external ophthalmoplegia (adPEO, OMIM 609283) [Deschauer et al. 2005; Kaukonen et al. 2000; Siciliano et al. 2003].

The advent of WES has greatly improved the prospect of achieving a genetic diagnosis for patients suspected of MD and therefore, the spectrum of mitochondrial disease causing mutations continues to expand [Taylor et al. 2014]. One of the latest accomplishments by using WES was previously undocumented, recurrent, *de novo*, dominant mutations in *SLC25A4* gene [Thompson et al. 2016], which is causing the third distinct phenotypic group and is discussed in detail in results section.

2.6 Diverse phenotype in patients with mutations in the *NDUFB11*

The mammalian complex I (NADH-ubiquinone oxidoreductase) is the largest multi-subunit enzyme of the mitochondrial electron-transport chain and catalyses the first step in the respiratory chain, transferring two electrons from NADH to ubiquinone, coupled to the translocation of four protons across the membrane [Carroll et al. 2002].

Complex I (CI) is the most frequently observed single enzyme deficiency causing OXPHOS disorders [Rodenburg 2011]. Usually, it does not cause only a deficiency of the NADH:ubiquinone oxidoreductase enzyme activity, but it also leads to several additional defects at the cellular level, including altered mitochondrial network morphology, altered membrane potential, changes in intracellular calcium homeostasis and elevated formation of mitochondrial reactive oxygen species (ROS) production [Luo et al. 1997; Verkaart et al. 2007]. This gives rise to the pathogenesis of numerous hereditary and degenerative disorders [Hoefs et al. 2012] with no clear genotype-phenotype correlation [Koene et al. 2012; Pagniez-Mammeri H. 2012; Tucker et al. 2011].

The most recently identified gene defects causing CI deficiency are in the *NDUFB11* gene, which is located in the short arm of the X-chromosome (Xp11.23) [Rodenburg 2016]. This gene encodes a relatively small integral membrane protein, which belongs to the supernumerary group of subunits, and proved to be essential for the assembly of an active CI [Fassone and Rahman 2012]. Over the last three years, there have been published few papers with patients whose clinical problems are presumably caused by mutation in *NDUFB11* gene – six females and seven males with four asymptomatic female carrier. Our study group reported the clinical, biochemical, and molecular characterization in additional two male patients without skin manifestation.

2.7 Summary of the literature

The NBS program has remained unchanged in Estonia for more than 20 years. At the same time, the tandem MS has been used and far more than two disorders are tested in most of European countries. Therefore, we believe that the next reasonable step in Estonia would be to expand the newborn screening program and start to use tandem MS method. Whereas, the method has been available in Estonia since 2008 for measuring the acylcarnitines form serum in symptomatic patients [Joost et al. 2012a]. Besides, our knowledge about the FAOD and intoxication type of metabolic disorders like aminoacidurias and organic acidurias (OA), are limited in Estonia. At the same time, the need for early identification is important, as the clinical outcome in some of those disorders is generally favorable, but they are related with significant morbidity and mortality if they remain undiagnosed [Sim et al. 2002].

In the field of IEM there are also a large number of disorders that can not be diagnosed by NBS, although they start in childhood and often need an urgent intervention. One of the largest such group consists of MDs, which are complicated to diagnose, because of lack of specific markers and a highly variable phenotype. For so far, many different algorithms are used for that, but they tend to be time-consuming, require invasive procedures, and more than half of the cases do not achieve a molecularly validated diagnosis. While, a novel diagnostic method like WES seems to be suitable for replacing the old approach. At the same time, only very few groups have applied it in clinical practice, although, we believe that in this particular field, the introduction of this method can bring the greatest benefits.

3. AIMS OF THE PRESENT STUDY

The aims of the present study were:

- To evaluate the methodology and the effectiveness of the expanded newborn screening program in Estonia (Paper I and VI);
- To assess the effectiveness of the whole exome sequencing analysis in clinical practice in Estonia in patients with an unsolved suspected mitochondrial disorder (Paper II);
- To investigate the clinical effect of compound heterozygous mutation in the *CACNA1A* gene (Paper III);
- To ascertain the functional and clinical effect of a novel *de novo* mutation in the *SLC25A4* gene (Paper IV);
- To characterize the *NDUFB11*-related phenotypes and functional studies (Paper V).

4. MATERIALS AND METHODS

4.1 Study subjects

4.1.1 Expanded newborn screening for selected inborn errors of metabolism in Estonia (Paper I and VI)

In Estonia, a population-based expanded newborn screening for congenital metabolic disorders by using tandem MS was introduced on January 1, 2014. For the first 12 months (01/01/2014-31/12/2014) it was a pilot project and since 2015 this project was financed by the Estonian Health Insurance Fund. During the four years (2014–2017), we have screened 54,899 newborns and >99% of them were Caucasian. Parents were provided with information leaflets and booklets during the pregnancy and before the bloodspots were obtained, without the need to sign an informed consent form. If the parents did not allow their child to participate in the newborn screening program, an empty and signed screening card were sent to the screening laboratory.

For the first 12 months, we conducted screening as a pilot project and investigated 13,643 newborns. Forty-nine parents (0.36% of all births) refused to participate in the project, and thus expanded NBS during the pilot study included 99.64% of the newborns in Estonia [Reinson et al. 2016a]. In the year 2015 the number of screened patients was 13,915 and 58 (0.42%) parents refused; in the year 2016 it was 13,881 newborns and 42 (0.3%) parents refused; and last year we screened 13,460 newborns and 61 (0.45%) parents refused.

Screening samples consisted of capillary blood drawn from heel prick, and were collected on Whatman 903® filter paper. The samples were dried and sent by mail or courier from local hospitals to the Department of Clinical Genetics, Tartu University Hospital for analysis. This is the only laboratory in Estonia, which performs the NBS. The recommended age for obtaining a screening sample was 3rd to 5th day of life; most of the bloodspots were collected between 2nd to 7th day of life.

After three years of expanded NBS in Estonia, we re-evaluated thoroughly all the cases of congenital acquired vitamin B12 deficiencies, which were diagnosed in our laboratory. Therefore, the prevalence of vitamin B12 deficiency is calculated based on 3-year (2014–2016) data and the prevalence of all other screened disorders is based on 4-year data.

4.1.2 Study group of patients with suspicion of mitochondrial disorders, including three patients who are characterized in detail (Paper II–V)

The retrospective study group (N=21) was selected from a database of 181 patients whose fibroblast cell cultures have been stored during January 2003 – December 2013 in the Department of Clinical Genetics, Tartu University

Hospital. All those patients were consulted by a clinical geneticist and had a definite diagnosis or a suspicion of a metabolic disease. The fibroblasts were cultivated from a skin biopsy either to carry out necessary enzyme analyses or for future diagnostic purposes. In our study group, we included patients in whom referring physician suspected a mitochondrial disease, but the exact etiology was not established. Four patients were excluded due to consent withdrawal, loss of clinical symptoms, no DNA or no contacts with the family. The WES analysis was not performed to any of these patients as it was not available in Estonia before 2013. The prospective study group (N= 11) was selected during January 2014 – March 2016. The main inclusion criteria were the childhood onset of the symptoms and a strong clinical suspicion of MD, which were made by consulting clinical geneticist or pediatrician working in the Department of Clinical Genetics, Tartu University Hospital. In total, we had 28 patients in our study group.

We re-examined all the medical history charts of each included patient to obtain their clinical signs and symptoms, results of metabolic and imaging studies and other medical investigations including a muscle biopsy. Those retrospective patients who were alive were invited for a follow-up genetic consultation. Afterwards, we scored each patient according to the mitochondrial disease criteria (MDC) developed by Wolf and Smeitink, 2002 [Wolf and Smeitink 2002] and Morava et al. 2006 [Morava et al. 2006a].

4.2 Methods

4.2.1 Method for expanded newborn screening for selected inborn errors of metabolism in Estonia (Paper I and VI)

Amino acids and acylcarnitine analysis of dried blood spots were performed on a Xevo TQD Triple Quadrupole Mass Spectrometer (Waters, Milford, MA, USA) using *MassChrom Kit* (Chromsystems, Grafeling, Germany), which is a commercial in vitro semi-quantitative diagnostic kit. This method enables to determinate 13 amino acids, 30 acylcarnitines (including free carnitine). The kit consists of 27 stable isotope labelled internal standards for calibration and results are derivatized. Since tyrosine (tyr) alone is not sufficient to diagnose tyrosinemia type I (TYR I), we were convinced that our method choice should also allow the determination of succinylacetone (SucA). During the preparation and analysis of samples, the manufacturer's protocol was followed; the exact parameters of the method and mass transfer are available on the manufacturer's website: https://www.chromsystems.com/products/newborn-screening.html – 11/12/2017.

The disorders included in our NBS panel are: phenylketonuria (PKU), maple syrup urine disease (MSUD), tyrosinemia type I (TYR I), homocystinuria (HCY), argininemia (ARG), citrullinemia type I (CIT I), isovaleric aciduria (IVA), propionic aciduria (PPA), methylmalonic aciduria (MMA), glutaric

aciduria type I (GA I), congenital vitamin B12 deficiency, medium chain acyl-CoA dehydrogenase deficiency (MCADD), long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD), very long chain acyl-CoA dehydrogenase deficiency (VLCADD), carnitine uptake deficiency (CUD), carnitine-acylcarnitine translocase deficiency (CACT), carnitine palmitoyltransferase I deficiency (CPT I), carnitine palmitoyltransferase II deficiency (CPT II) and glutaric aciduria type II (GA II).

All the cutoff values for amino acids and acylcarnitines (including C3) which we used in the first months, were originally taken from the literature [Lindner et al. 2011; Lund et al. 2012; McHugh et al. 2011; Vilarinho et al. 2010]. After screening for the first thousand newborns, we calculated the first in-house cutoff values: 1st and 99th percentile. Mostly we use only upper cutoff values, except for arginine (Arg), citrulline (Cit), methionine (Met), free carnitine (C0), acetylcarnitine (C2), palmitoycarnitine (C16), oleylcarnitine (C18:1) and stearylcarnitine (C18), in which, for diagnostic purposes, we also needed a lower cutoff values. For data processing, we used two programs: Excel (Microsoft Corp., Redmond, Oregon, USA) and SAS 9.2. (SAS Institute Inc., North Carolina, USA).

Above described calculation for cutoff values was performed repeatedly to improve the quality of newborn screening and last time it was done based on almost 30,000 newborn's screening results, which are currently in use.

In the second half of the pilot project, we joined with the worldwide collaboration project – Region 4Stork (R4S) [McHugh et al. 2011] and all flagged results were compared with this database and the likelihood of occurrence of the suspected disorder was calculated. All newborns, whose calculation results indicated the possible presence of the disease, were called out.

The ratios of some metabolites like C3/C2 and C3/C16 were still taken from the literature [Scolamiero et al. 2014]. Folate and vitamin B12 were measured on Cobas 601 Immunoassay Analyzer (Roche Diagnostics GmbH, Mannheim) and tHcy measurement was performed by enzymatic assay on Cobas Integra 400 Plus Analyzer (Roche Diagnostics GmbH, Mannheim) and their age-appropriate reference intervals were taken from the CALIPER database (http://www.sickkids.ca/caliperproject/index.html – 22/03/2017).

4.2.2 DNA and mtDNA investigations performed to the patient with suspicion of mitochondrial disorders (Paper II–V)

The DNA of all the patients was extracted from blood lymphocytes. The WES analysis was carried out using different methods due to availability and cost-effectiveness at different time points. The WES enrichment kits were SureSelect XT Human All Exon v5 for patient with *CACNA1A* mutations – Paper III, v4 for patient with *SLC25A4* mutation – Paper IV (Agilent Technologies, Santa Clara, CA, USA) or Nextera Rapid Capture Exome 37 Mb kit (Illumina Inc., San Diego, CA, USA) for patient with the *NDUFB11* mutation – Paper V.

All patients were sequenced as proband only, except the family with a patient having mutation in the *NDUFB11*, in whom offspring-parent trios were sequenced. After the sequencing, the reads were aligned to hg19 reference genome using Burrows-Wheeler Aligner (BWA) [Li and Durbin 2010]. Further data processing, variant calling, and annotation was performed following Genome Analysis Toolkit (GATK) best practice guidelines [Van der Auwera et al. 2013] using Picard, GATK [DePristo et al. 2011; McKenna et al. 2010], Annovar [Wang et al. 2010] and SnpSift [Cingolani et al. 2012]. All reported variants were confirmed, and familial segregation analysis was done by using Sanger sequencing.

The mtDNA analysis was performed to all patients. Each patient's reads generated during standard WES, but mapped to mtDNA, were specifically analyzed to detect mtDNA variants, and to assess the feasibility of investigating mtDNA from standard WES reads. In order to study mtDNA, SAMtools [Li et al. 2009] were used to extract reads mapped to chrM. These reads were aligned to the b37 reference genome (GRCh37 including rCRS mitochondrial sequence) using BWA MEM [Li and Durbin 2009], and subsequently sorted and marked for duplicates by Picard. The variants were called by combining SAMtools [Li et al. 2009] mpileup with VarScan [Koboldt et al. 2009], and then annotated using Annovar [Wang et al. 2010]. The analysis of guided variants along with haplogroup calling was guided by Mitomap and Mitomaster [Lott et al. 2013]. The mtDNA coverage was calculated using the GATK DepthOfCoverage tool. The only exception was made by a patient with *SLC25A4* mutation, whose complete mtDNA was screened for mismatches and rearrangements using Long Template PCR and Affymetrix Human Mitochondrial Resequencing array 2.0 (Santa Clara, CA, USA).

Mitochondrial respiratory chain complex activities were measured spectrophotometrically in cultured fibroblasts or muscle, using previously described methods [Rodenburg 2011].

4.3 Statistical analysis

The statistical analysis for descriptive statistics (mean, median, and range etc.) and data processing were made with two programs: Excel (Microsoft Corp., Redmond, Oregon, USA) and SAS 9.2. (SAS Institute Inc., North Carolina, USA).

The annual live-birth data was obtained from the database of Statistics Estonia, which is a government agency in the area of administration of the Ministry of Finance in Estonia (https://www.stat.ee – 01/03/2018) to calculate the incidence of screened disorders.

4.4 Ethics

At the beginning of 2014, the newborns screening in Estonia was firstly introduced as a pilot project (Papers I and VI), which was approved by the Research Ethics Committee of the University of Tartu (approval date 18/11/2013 and number 231/T-5) in agreement with the Declaration of Helsinki. Testing of healthy newborns involves many risks, but few doubt the necessity and usefulness of newborn screening programs. However, this field is becoming slightly more complicated as the range of disorders being tested increases, and new technologies becoming available. Therefore, many bioethics have increasingly highlighted three principles that are still important to follow when adding new diseases to the screening list or changing the approach. Firstly, the natural history of the condition, including development from latent to declared disease should be adequately understood. Secondary, there should be a suitable test or examination for diagnosing the disorder and an agreed policy concerning whom to treat as patients. Thirdly, there should be an accepted and available treatment for patients with recognized disease [Dhondt 2010; Johnston et al. 2018]. Therefore, adding any new disease or technology to the newborn screening program requires careful consideration. All disorders that were added to the newborn screening panel in Estonia meet the criteria described above.

The study of diagnostic approach to mitochondrial disorders (Papers II–V) was approved by the Research Ethics Committee of the University of Tartu [approval date 17/11/2014 and number 242/M-10; renewal date 20/11/2017 number 242/M-11(2)] in agreement with the Declaration of Helsinki. An informed consent was obtained from all the patients, their parents and/or legal guardian (including three cases, which were characterized in detail), except from those who had deceased. Participants in this study group already had symptoms, so the implementation of the new diagnostic method was indispensable, since previous studies had not identified a molecularly confirmed diagnosis. Still, it is important to point out that while reporting the results, the recommendations of the American College of Medical Genetics and Genomics were followed [Green et al. 2013].

5. RESULTS AND DISCUSSION

5.1 Newborn screening for inborn errors of metabolism by tandem MS in Estonia (Paper VI)

During four years, we screened 54,899 newborns and confirmed the diagnosis of 29 children, therefore the overall prevalence of a metabolic disorders (including congenital acquired vitamin B12 deficiency) identified on newborn screening in Estonia is 1:1,893 newborns.

In the first year (pilot project), the diagnosis was confirmed in eight children (Table 3) and surprisingly, the congenital acquired vitamin B12 deficiency was identified in seven children. This diagnosis continues to be most common in tested disorders and this topic will be discussed more widely in the next chapter.

In the four-year summary, the second most commonly diagnosed condition was hyperphenylalaninemia (HPA) (including classical phenylketonuria, tetrahydrobiopterin-deficient HPA and benign HPA). This result was expected because it was previously known that the incidence of this condition in Estonia is relatively common – 1:6,700 newborns [Lillevali et al. 2017]. In the course of four years, we also diagnosed glutaric aciduria in one case.

Table 3. Disorders diagnosed on NBS during the four-year screening period.

	2014	**2015**	**2016**	**2017**	**Total**
No. of tested newborns	13,643	13,915	13,881	13,460	54,899
Disorder					
Congenital acquired vitamin B12 deficiency	7	3	4	7	21
HPA	1	0	4	2	7
Classical PKU	0	0	3	2	5
Tetrahydrobiopterin-deficient HPA	1	0	0	0	1
Benign HPA	0	0	1	0	1
Glutaric aciduria type I	0	1	0	0	1

None of the diagnosed child had any symptoms at the first appointment, and all diagnosed patients remained symptomatic free for 48 observed months. The amount of incidence is slightly higher compared to other regions like in Germany, where it is 1:2,712 screened newborns [Harms and Olgemoller 2011] [Schulze et al. 2003]; in Portugal it is 1:2,396 [Vilarinho et al. 2010], and in Austria it is 1:2,855 [Kasper et al. 2010]. However, at the moment there are still too few children screened to draw a long-term conclusion about the incidence of screened disorders in Estonia.

As expected, the incidence of HPA in Estonia is relatively high. Over the four years, we have diagnosed seven cases, including one patient with tetrahydrobiopterin-deficient form [c.1222C>T (p.R408W)/ c.782G>A (p.Arg261Gln)], and one with benign HPA [c.1222C>T (p.R408W)/ c.1208C>T (p.Ala403Val)]. Four of five patients with PKU (severe form of HPA) were homozygotes for the p.R408W mutation in the *PAH* gene and one child were compound heterozygous for c.143T>C (p.L48S)/ c.838G>A (p.E280K) mutations in the *PAH* gene. Our results overlap with the study of retrospective overview of Estonian PKU patients which revealed that our region has exceptionally high genetic homogeneity, as 80% of all PKU alleles carry the p.R408W mutation which is typical of Eastern Europe [Lillevali et al. 2017].

In one case, the phenylalanine remained altered at first post-NBS evaluation, but later became spontaneously normal and were considered as transitory HPA. Her *PAH* gene was sequenced and she was carrier of the p.R408W mutation. The multiplex ligation-dependent probe amplification (MLPA) analysis was also performed and no duplication or deletion was found in the *PAH* gene. She will stay under our observation until the end of her third year. At the last evaluation, when she was two years old (without any treatment), her development was age appropriate and biochemical markers (including amino acids in the serum) were without aberrances.

In the NBS, we also detected a child with glutaric aciduria type I and she was compound heterozygous for c.1204C>T (p.Arg402Trp)/ c.1262C>T (p.Ala421Val) mutations in the *GCDH* gene. The older child in this family, who was born before the initiation of the expanded NBS, received an etiologic diagnosis after confirmation of a younger child's diagnosis. At that point, she was at the age of six and it was thought that her moderate physical disability was caused by an intrauterine infection. Her motor function improved noticeably after the onset of a reduced lysine-containing diet and administration of L-carnitine.

Based on four-year screening, we can affirm that the incidence of MCADD in Estonia remains rare as it was described before by Lillevali et al. [Lillevali 2000]. During that period, we did not diagnose any newborn with this diagnosis, which distinguish us from the rest of Europe where the MCADD seems to have quite high incidence. For example, in Portugal the incidence is 1:9,036 [Vilarinho et al. 2010], in Denmark and Faroe Islands it is 1:9,164 [Lund et al. 2012], in Germany it is 1:10,610 [Harms and Olgemoller 2011] and in Austria it is 1:24,900 [Kasper et al. 2010]. Unfortunately, we do not have any relevant data about this disorder in neighboring countries, because there is no nation-wide expanded newborn screening or it was initiated in 2015 and the data has not been published yet.

Regrettably, we have to report that in one case LCHADD was not diagnosed with NBS, because the child died prior to collection of DBS (before the age of 72 hours). This information came to us because the parents of the deceased child turned to the Department of Clinical Genetics, Tartu University Hospital for obtaining recurrence risk. Based on anamnesis, the consulting geneticist had a suspicion of LCHADD, therefore, the parents were examined for the presence of the common c.1528G>C mutation in the *HADHA* gene, and both were

carriers. Thereafter, their child's autopsy material was further investigated and it revealed the homozygosity for the c.1528G>C mutation in the *HADHA* gene, which confirmed the persistence of LCHADD of deceased child.

In the first year, due to the abnormalities, found in the primary results of the newborn screening test, 33 newborns with their guardian were called out to additional analyses. During the first 6 months of the pilot project, the number of these patients was 22, while the sum of screened children was 6344. The cases, in which biochemical markers were abnormal at NBS but normal upon diagnostic testing, were considered as false positives. The false positive rate (FPR), during that period, was 0.28% and the positive predictive value (PPV) was 18%. Within the next 6 months, the number of children called out was 11 and total of screened children during that period were 7299. The FPR decreased to 0.09% and the PPV increased up to 36%. Summarizing the results of both half-year the total of false positive results were 25 and the FPR were 0.18%, while the PPV were 24%. In four years' time, the FPR has remained to 0.1% and the PPV has risen to 36.7%. In the last (fourth) year, the FPR was 0.07% and the PPV 52.6%. An overall specificity is 99.9% and since we still have not diagnosed any false negative case, then the overall sensitivity of tandem MS screening remains 100%. At the same time, we will continue our annual analysis of data and make conclusions to introduce improvements in our program.

When comparing our results with other NBS programs, we can find similarities. The FPR in Germany over a 42-month period was 0.33% therefore the specificity was 99.67% and the PPV was found to be 11.31% [Schulze et al. 2003]. In Austria, during the first 93 months, the overall PPV was 12.62% and specificity was 99.76% [Kasper et al. 2010]. Significantly higher PPV were calculated in Galicia in Spain, where the summary of 10-year data showed that the PPV was 76.11%, sensitivity was 97.16% and the specificity was 99.98%, [Couce et al. 2011]. These results also indicate that the longer the screening is ongoing, the more experience will be added, and therefore the PPV will increase and the FPR will decrease.

Before the nation-wide expanded newborn screening could be started in Estonia, a thorough preliminary work was needed. In Estonia, we did not have any overview of most of the diseases, which are being screened in the USA and European countries, due to the small population size – 1.3 million inhabitants (https://www.stat.ee – 01/06/2018) and recent availability of accurate diagnostic methods. Therefore, the panel of the tested disorders in Estonia was put together according to the literature and experience of the closest countries, where extensive screening has been ongoing and detailed articles have been published, such as Denmark [Lund et al. 2012] and Germany [Lindner et al. 2011; Schulze et al. 2003]. Both regions started the NBS with a wide range of diseases to be test, and this approach is compatible with our team's perspective as well. It was important for us to learn from the experiences of others to avoid screening such disorders, which seemed to involve little or no benefit; at the same time, the list should cover most of the disorders, which can be screened by tandem MS method. The expected incidence of diseases were taken from literature [Garg

and Dasouki 2006] and it had a modest impact on our decision, since adding one disease to the list does not increase the total cost of the test.

Therefore, in addition to PKU, 18 new congenital metabolic disorders (Table 4) were added to the list of the diseases to be screened in Estonia. It was a consensus decision among most of the specialists working in the field of IEM in Estonia.

Taking into account the relatively low birth rate in Estonia – the decision was to use a commercial kit to determinate 13 amino acids, 30 acylcarnitines and SucA. The NBS strategy was right from the beginning to apply a full-profile approach, which means that we gathered maximum information form one sample in one run, without regard to the distinction between appropriate and inappropriate target conditions. This decision was thoroughly discussed and the decisive factor was the fact that in Estonia, there is only one laboratory that is responsible for NBS and it is part of the Department of Clinical Genetics, Tartu University Hospital, therefore, the trained specialists, including pediatricians specialized in congenital metabolic disorders, work in the same center. In Estonia, all children's genetic counseling, including those which involve IEM and the follow-up of those disorders, were made by same center, therefore we believe that wide profile of metabolites are rather an advantage that enables faster and more accurate information exchange. This does not mean that we do not follow strictly the rule to inform newborns' families only for disorders that we screen, while in an emergency situation the consulting specialist can have full-profile immediately. Another positive aspect for storage of full-profile of metabolites is the availability for retrospective investigation, because most of the metabolites are degraded in months [Adam et al. 2011], and after that, the DBS can only be used for DNA extraction. According to this, we have stated that we will keep the DBS for at least 25 years; therefore, we have stored all of them.

It was important to compose an optimal list of the analyses which should be performed at the first appointment after being called out (Table 5), to prevent excessive work and cost for the medical team; and take into account the socio-economic capacity of the families. Therefore, one of the principal decisions was to take the sample for DNA extraction already in the first appointment to carry out molecular-genetic analyses when necessary, since we do not have the opportunity to perform enzyme analyses in our own laboratory.

We are aware that the correct interpretation of metabolic profiles calls for well-balanced cutoffs and it is often necessary to accept a certain number of false positives in order to ensure sufficient sensitivity. Therefore, the next step was technical interpretation. A decision limit (cutoff value) for each metabolite was initially set according to the literature [Lindner et al. 2011; Lund et al. 2012; McHugh et al. 2011]. Each metabolite crossing the cutoff value was flagged automatically. These values could not be used for long period, because the population-based differences and differences in laboratory methods could be observed. We therefore used a multistep process to establish in-house cutoff values.

Table 4. Disorders screened in Estonia since 2014.
Arg – arginine, C0 – free carnitine, C2 – acetylcarnitine, C3 – propionylcarnitine, C4 – butyrylcarnitine, C5 – isovalerylcarnitine, C5DC – glutarylcarnitine, C5OH – 3-OH-isovalerylcarnitine, C6 – hexanoylcarnitine, C8 – octanoylcarnitine, C10 – decanoylcarnitine, C14 – myritoylcarnitine, C14:1 – tetradecenoylcarnitine, C14:2 – tetradecadienoylcarnitine, C16 – palmitoycarnitine, C16OH – 3-OH-plamitoylcarnitine, C18 – stearylcarnitine, C18:1 – oleylcarnitine, C18:1OH – 3-OH-oleylcarnitine, C18:2 – linoleylcarnitine, C18OH – 3-OH-stearoylcarnitine, Cit – citrulline, Leu – leucine, Met – methionine, Phe – phenylalanine, SucA – succinylacetone, Tyr – tyrosine, Val – valine, & – [Garg and Dasouki 2006] ; * – [Ounap et al. 1998]; ** – [Joost et al. 2012a].

Metabolic disorder	Abbre-viation	OMIM	Expected incidence[&]	Key metabolites
Aminoacidurias				
Phenylketonuria	PKU	261600	1:10,000 1:6,010*	↑ Phe
Maple syrup urine disease	MSUD	248600	1:200,000	↑ Val; ↑Leu
Tyrosinemia type I	TYR I	276700	1:100,000	↑ SucA (↑ Tyr)
Homocystinuria	HCY	236200	1:150,000	↑ Met
Argininemia	ARG	207800	1:350,000	↑ Arg
Citrullinemia type I	CIT I	215700	1:60,000	↑ Cit; ↓ Arg
Organic acidurias				
Isovaleric aciduria	IVA	243500	1:100,000	↑ C5
Methylmalonic aciduria	MMA		1:75,000	↑ C3
Propionic aciduria	PPA	606054	1:75,000	↑ C3
Glutaric aciduria type I	GA I	231670	1:50,000	↑ C5DC
Congenital vitamin B12 deficiency				↑ C3
Beta oxidation defects				
Medium chain acyl-CoA dehydrogenase deficiency	MCADD	201450	1:15,000	↑ C6; ↑ C8; ↑ C10
Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency	LCHADD	609016	1:75,000 1:94,864**	↑ C16OH; ↑ C18OH; ↑ C18:1OH
Very long chain acyl-CoA dehydrogenase deficiency	VLCADD	201475	1:75,000	↑ C14:1; ↑ C14; ↑ C14:2
Carnitine uptake deficiency	CUD	212140	1:75,000	↓ C0; ↓ C16; ↓C18
Carnitine-acylcarnitine translocase deficiency	CACT	212138	1:1,000,000	↑ C16; ↑ C18:1 ↑ C18; ↑ C18:2
Carnitine palmitoyl-transferase I deficiency	CPT I	255120	1:1,000,000	↑ C0; ↓ C16; ↓ C18
Carnitine palmitoyl-transferase II deficiency	CPT II	255110	1:1,000,000	↑ 16; ↑ C18; ↑ C18:1; ↑ C18:2
Glutaric aciduria type II	GA II	231680		↑ C5DC; ↑C4; ↑C5; ↑ C5OH

The initial cutoff values were set on the 1st and 99th percentile based on data collected and analyzed from 1,000 sequential and healthy newborn samples. These cutoffs were firstly modified after six months of screening; next were after 12 months, then after 15 months, as more clinical data became available. Currently, we are using the calculation, which is based on almost 30 000 newborn's screening results.

Table 5. An optimal list of confirmatory analyses for each suspected disorder.
DNAs – DNA for storage; mSAC – serum acylcarnitines from mother; SAA – serum amino acids; SAC – serum acylcarnitines; SOA – serum organic acids; UAA – urine amino acids UOA – urine organic acids; * added in 2018

Metabolic disorder	**An optimal list of confirmatory analyses**
Aminoacidurias	
PKU	SAA, DNAs, urine sample for storage
MSUD	SAA, UAA, UOA, DNAs
TYR I	SAA, UOA, DNAs
HCY	SAA, UAA, UOA, DNAs, vitamin B12, homocysteine
ARG	SAA, UAA, UOA, DNAs
CIT I	SAA, UAA, UOA, DNAs
Organic acidurias	
IVA	UOA, SAC, DNAs
GA I	SAA, SAC, UOA, DNAs
MMA	SOA*, SAC, UOA, DNAs, vitamin B12, folic acid, homocystein, holotranscobalamin*; vitamin B12, folic acid, homocystein from mother
PPA	SOA*, SAC, UOA, DNAs, vitamin B12, folic acid, homocystein, holotranscobalamin*; vitamin B12, folic acid, homocystein from mother
Congenital Vitamin B12 deficiency	SOA*, UOA, DNAs, vitamin B12, folic acid, homocystein, holotranscobalamin*; vitamin B12, folic acid, homocystein from mother
Beta oxidation defects	
MCADD	SAC, UOA, DNAs
LCHADD	SAC, UOA, DNAs
VLCADD	SAC, UOA, DNAs
CUD	SAC, UOA, DNAs, mSAC
CACT	SAC, UOA, DNAs
CPT I	SAC, UOA, DNAs
CPT II	SAC, UOA, DNAs
GA II	SAC, UOA, DNAs

In the first 6 months when the results of the primary analyses were clearly aberrant and suggestive of a severe disorder, the patient was immediately referred to our clinical unit. If the results lay outside the cutoff range but were not as aberrant as to constitute clear proof of a severe disease, a second sample was requested form the same test card but from another blood spot. If the second sample also tested positive, the patient was called out. Such tactics was necessary in order to gain experience and make sure the result was correct, although it was not the best solution, since the number of false positives remained high. Therefore, the first year passed mainly by collecting experiences, which is also reflected in the percentages of FPR and PPV.

The whole world is moving in the direction of cooperation, since the occurrence of diseases is relatively rare, and experience is therefore worth sharing. Our improvement of the recall rate and the FPR of the second half of the pilot project year was strongly influenced by the introduction of worldwide collaboration project R4S, which was launched in 2005 [McHugh et al. 2011]. The R4S project was initiated as a large collection of information to generate data for NBS programs to improve tandem MS screening by achieving uniformity of tandem MS testing panels, improving analytical performance, and reducing false-positive and false-negative screening results. This is a custom-designed and -coded application for the collection and reporting of possibly any type of newborn screening data based on numerical results. The system is a web-based application and tools are generating a score reflecting the likelihood of a diagnosis based on condition-specific disease ranges of all informative analytes. Site-specific customization of these tools is available to correct for differences in analyte panels and sample preparation (derivatized versus underivatized method). [McHugh et al. 2011]. The international participation in the R4S project includes 1,050 NBS programs from 64 countries, and the true-positive database has exceeded up to 17,000 cases, with more than 1.2 million NBS results. The results of Estonian newborns are also included in the database.

All newborn screening laboratories are strongly recommended to participate in quality control networks, because most programs actually never encounter 30–80% of the conditions that they are testing for [McHugh et al. 2011]. Therefore, since 2014, we have also participated annually in external quality control specimens for acylcarnitines and amino acids. The testing panels were received from the Newborn Screening Quality Assurance Program organized by the US Centers for Disease Control and Prevention (CDC), our laboratory number is 0606, and the European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited Disorders of Metabolism (ERNDIM), our laboratory number is 0322. Every year, we have passed the Newborn Screening Quality Assurance Program organized by CDC; and in most ERNDIM quality control schemes, we have achieved the maximum score or lost only one point.

Since the 2015, our laboratory is also internationally accredited to perform NBS and our screening program results meet the criteria of adequate analytical and postanalytical performance, which were developed by Rinaldo et al. This

includes detection rate at least 1:3,000 or higher, PPV >20%, and FPR <0.3% [Rinaldo et al. 2006].

Many NBS laboratories carry out fundamental changes after a certain amount of data has been analyzed. For example, in Denmark, after seven years of NBS, the time interval for obtaining samples was changed from 4 to 9 days to 2 to 3 days postpartum and biotinidase deficiency with TYR I were added to the screening panel. In addition, because of the high FPR and late screening results compared with the rapid progression of the disease, screening for CIT I was stopped at that point [Lund et al. 2012]. Since 2018, we have also added some extra analyses for newborns, who were called out because of increased C3 and according to the new protocol, a serum holotranscobalamine and organic acids should be measured (Table 5). Nevertheless, in the long run, the changes in our screening panel will definitely not be limited.

For a better understanding of the advantages and effectiveness of NBS for certain entities, there should be longer-term studies of disorders screened and cases diagnosed in our population, however the first four years of expanded NBS has given very important knowledge of the incidence of some diseases in our region and expanded the phenotype of these disorders. Therefore, the continuation of the developed scale of screening seems to be beneficial and in the future, it is worth looking at ways to expand the range of screened diseases.

5.2 Congenital acquired vitamin B12 deficiency among Estonian newborns (Paper I)

The first thorough analysis about the congenital acquired vitamin B12 deficiency was conducted after three years of screening in Estonia and the result was unexpected – the incidence of this condition is 33.8/100,000 (1:2959) newborns.

We identified all newborns with a C3 value higher than 4.31 μmol/L (up to a year and a half the cutoff value of C3 was 4.6 μmol/L) on DBS analysis, with or without altered C3/C2 (abnormal value > 0.18) and/or C3/C16 (abnormal value > 1.80) ratios. They underwent pediatric evaluation and further laboratory testing, including the measurement of vitamin B12, folate and tHcy in serum, and the MMA content in urine. At that period, we could not implement the MMA measurement as a second-tier test from the same DBS. Instead, this was done from fresh urine samples collected during the first pediatric consultation. MMA from serum was measured posteriorly in eight of them, in the rest, there was not enough serum left. In these eight newborn's serums contained elevated amount of methylmalonic acid: 2.41 – 17.02 μmol/L (abnormal value >0.3) and these results are reported in Table 6.

Table 6. Biochemical and clinical parameters of the 14 newborns and their mothers, who underwent paediatric evaluation and further laboratory testing.
* – Scolamiero et al.; / – not measured.

No	Sex	C3 (µmol/L) (abnormal value >4.31; 99th percentile)	C3/2 (abnormal value >0.18)*	C3/C16 (abnormal value >1.8)*	Methionine (µmol/L) (reference intervals: 4.23–44.30; 1st to 99th percentile)	Vitamin B12 (pmol/L) (reference intervals: 216–891)	Folate (nmol/L) (reference intervals: 7.0–46.5)	Hcy (µM) (abnormal value: >10)	MMA (Mmol/mol Cr) in urine (abnormal value: >10)	MMA (µmol/L) in serum (abnormal value: >0.3)	Exclusively breast-fed	Mother's vitamin B12 (pmol/L) (reference intervals: 141–489)	Mother's prenatal folic acid supplemen-taton ≥ 400µg/day
1.	M	**4.8**	**0.19**	1.23	8.52	**162**	27.7	**15.7**	8.75	/	yes	251	yes
2.	M	**5.27**	**0.3**	**2.17**	9.01	**176**	38.1	6	not detected	/	yes	316	yes
3.	M	**5.88**	**0.23**	1.8	11.89	**184**	32	**10.3**	1.5	/	yes	Normal (auto-immune disease)	yes
4.	F	**5.91**	**0.23**	**2.46**	14.93	**110**	36.9	**12.2**	7.36	/	yes	Normal	yes
5.	F	**5.96**	0.08	0.47	9.09	**143**	31.2	**22.9**	**62.08**	**6.71**	yes	174	yes
6.	M	**7.07**	0.18	**2.67**	6.44	**103**	38.5	**14.1**	**132**	**4.92**	yes	Normal	yes
7.	M	**7.27**	**0.3**	**2.43**	6.27	**60**	59,9	**23.6**	**24.07**	**4.0**	yes	Low (vegetarian)	n.a.
8.	M	**7.35**	0.17	**2.19**	13	**201**	/	/	4.97	/	yes	168	yes
9.	M	**7.94**	0.14	**1.42**	20.21	**184**	67.2	8	2.51	**17.02**	yes	404	yes
10.	M	**8.08**	**0.45**	**2.21**	21.27	**135**	43	**14**	not detected	/	yes	Normal	yes
11.	M	**8.45**	**0.21**	**2.52**	8.4	**187**	/	/	7.7	**3.19**	yes	Normal	yes
12.	M	**8.94**	0.18	**2.46**	10.6	**188**	79	**10.3**	4.6	**14.74**	yes	246	yes
13.	M	**9.91**	0.17	**1.52**	10.7	**208**	35.6	**11.3**	9.01	**2.41**	yes	347	yes
14.	M	**12.16**	0.16	**1.58**	18.99	**168**	30.4	6.3	1.31	**14.9**	yes	230	yes

None of the newborns had any clinical symptoms associated with vitamin B12 deficiency before the treatment. All biochemical markers normalized after treatment, which strongly suggests the presence of impaired vitamin B12 status. After a thorough prospective clinical follow-up and laboratory testing, we can confirm that all well-known genetic disorders that could cause neonatal onset of elevated MMA were also excluded in all of the treated children.

As the most common cause for vitamin B12 deficiency among newborns is maternal vitamin B12 deficiency, the blood tests for mothers were also performed. Most of the mothers showed values of vitamin B12 within the reference interval (141–489 pmol/L) with tendency towards lower normal limit and they described themselves as healthy and nourished, without serious chronic medical conditions, except for one mother who had been diagnosed with systemic lupus erythematosus. Her autoimmune disease was well-controlled during the pregnancy. In our study, only one mother had low vitamin B12 level without any clinical complaints and she was mainly vegetarian for religious reasons. All mothers, except one, used prenatal folic acid supplements, mostly 400 micrograms per day (Table 6), which is quite widespread practice in Estonia.

Although the study period was quite short, we believe that the three-year experience is long enough to reflect the current situation of this condition in Estonia since Italy's newborn screening program showed that the incidence of congenital acquired vitamin B12 deficiency remained similar over the years [Italian Society for the Study of Inborn Metabolic Diseases and Newborn Screening 2015].

Our incidence of congenital acquired vitamin B12 deficiency is 33.8/100,000 (1:2959), which is higher than reported in most of the literature [Hinton et al. 2010; Italian Society for the Study of Inborn Metabolic Diseases and Newborn Screening 2015; Sarafoglou et al. 2011; Scolamiero et al. 2014]. Sarafoglou et al. described a population-based study of 363,649 infants born in Minnesota. During the six-year study period, 11 newborns with vitamin B12 deficiency were identified, thus the incidence was 3.02/100,000 live births (1:33,113). All of them had secondary changes due to maternal vitamin B12 deficiency [Sarafoglou et al. 2011]. Higher incidence was detected in the Italian population, where a six-year expanded metabolic newborn screening pilot project showed an incidence of congenital acquired vitamin B12 deficiency of 20/100,000 (1:5,000) and evidenced that this condition is also mainly a consequence of maternal vitamin B12 deficiency [Scolamiero et al. 2014]. Unfortunately, we did not measure any other biochemical parameters for most of the mothers in our study group. Therefore, we could miss the borderline vitamin B12 deficient mothers.

The most exceptional hypothesis was proposed by Refsum et al., who concluded that 10% of screened newborns had vitamin B12 levels below 150 pmol/L, which is the lower reference limit for adults [Refsum et al. 2004]. Therefore, it would be beneficial to detect most of those children in the symptom-free phase. For this purpose, some newborn screening programs have lowered their cutoff level of C3 and started to use more actively the ratios of

some metabolites. For example, Minnesota Department of Health, Mayo Clinic, and University of Minnesota markedly lowered the cutoff level of C3 from 9.2 μmol/L to 5.25 μmol/L and the vitamin B12 detection rate increased more than three times [Sarafoglou et al. 2011]. A similar decision was also made by Scolamiero et al. [Scolamiero et al. 2014]. Although, Campbell et al. pointed out that the C3 is a maker used to detect only organic acidurias and remethylation defects and its level alone is not sensitive or specific enough to detect all newborns with congenital acquired B12 deficiency, moreover C3 levels may not be sufficiently high during the first few days of life when the DBS is collected [Campbell et al. 2005]. Therefore, there is evidence that the C3 to C2 ratio and/or C3 to C16 ratio together with C3 are better than C3 level alone for detecting or identifying the babies with nutritional B12 deficiency [Armour et al. 2013; Sarafoglou et al. 2011; Scolamiero et al. 2014]. Some authors have also suggested measuring tHcy from the first DBS and if it is elevated, then evaluate the second-tier markers like Met, Met/Phe, C3 and C3/C2, which is considered to be valuable method to detect remethylation disorders and vitamin B12 deficiency [Gramer et al. 2017]. The MMA, measured from DBS as a secondary test, might also add value to increase sensitivity and specificity in detection of congenital acquired vitamin B12 deficiency [Sarafoglou et al. 2011].

During the study period in Estonia, we could not implement the second-tier testing of MMA from DBS; however it will be introduced in our laboratory in the near future. We measured the MMA level in freshly collected urine (most of the urine samples were collected at least a week after the first DBS) and only three of the fourteen children with congenital acquired vitamin B12 deficiency had elevated MMA in urine. The MMA concentration in serum were measured posteriorly, therefore, there were not enough serum left from all newborns who underwent the first pediatric evaluation but still it is notable that all eight newborns, with whom we could measure serum MMA, had it elevated. (Table 6).

For a long time, the causes of maternal B12 deficiency were considered to be associated with a strict vegan diet, poverty and malnutrition, occult pernicious anemia, previous gastric bypass surgery, and short gut syndrome [Kuhne et al. 1991]. Nowadays, the maternal B12 deficiency can be subclinical, mothers may not be anemic and their vitamin B12 levels are normal or low-normal. Maternal vitamin B12 concentrations during pregnancy are thought to be closely associated with fetal [Muthayya et al. 2006; Obeid et al. 2006] and early infant [Deegan et al. 2012; Finkelstein et al. 2014] vitamin B12 status. Some authors even suggest that maternal dietary intake during pregnancy is a stronger determinant of infant vitamin B12 status than are maternal vitamin B12 stores [Duggan et al. 2014].

Interestingly, most of mothers included in our study group had normal or low-normal levels of serum vitamin B12, which is similar to reports providing evidence of subclinical vitamin B12 deficiency in presence of normal serum B12 levels [Klee 2000]. This phenomenon is explained by the fact that tissue levels become depleted before serum levels. Some people with borderline or

low-normal serum vitamin B12 levels may have symptoms that resolve with B12 treatment, suggesting that levels previously considered borderline or normal may represent deficiency in some patients [Lindenbaum et al. 1988]. Therefore, it is important to highlight that all 14 children were exclusively breast-fed.

In Estonia, a majority of women uses folic acid supplementation during pregnancy, as recommended by clinicians. The standard dose is 400 micrograms of folic acid per day and most of them are taking their supplements conscientiously. That is reflected in our study group as well – most of the mothers had taken pure folic acid during their pregnancy. Only in one case, we could not verify mother's supplementation status (Table 6, Pt. No 7). Selhub et al. suggested that both pathways of vitamin B12 metabolism are adversely affected by high serum folate, despite the fact that folate is directly involved only in methionine synthase activity [Selhub et al. 2007]. A similar finding was described by Monsen et al., concluding that high serum folate during infancy is attributable to low B12 status and resulting the methyl folate trap phenomenon [Monsen et al. 2003]. More extensive studies are needed for specifying the precise connection between folic acid supplementation during pregnancy and newborns' low vitamin B12 level to make any fundamental conclusions.

In addition, we should not forget that vitamin B12, folate, tHcy and MMA undergo marked changes during childhood. In the first weeks of life, there is a considerable decrease in serum vitamin B12 level, accompanied by a marked increase in plasma tHcy and MMA [Minet et al. 2000; Monsen et al. 2001]. The lowest vitamin B12 levels and the highest tHcy and MMA levels in childhood are seen in infants 6 weeks to 6 months of age [Monsen et al. 2003]. Therefore, the use of correct age-appropriate reference values is indispensable to prevent hyper-diagnostics. Although, we believe that significantly more harm can be done by diagnostic delay, since vitamin B12 deficiency is associated with biomarkers of genomic instability. Global DNA hypomethylation and decreased purine and pyrimidine synthesis impair genomic stability, causing chromosomal breaks and aberrations [Fenech 2012]. A study in Turkey noted that DNA damage was increased in vitamin B12 deficient children and their mothers [Minnet et al. 2011]. Equally important is the fact that vitamin B12 deficiency is associated with retardation of neural myelination in some studies [Black 2008; Lovblad et al. 1997; McCaddon 2013], and long-term consequences of neurological deterioration may persist even after vitamin B12 deficiency has been treated [Celiker and Chawla 2009; Graham et al. 1992; von Schenck et al. 1997]. In addition, it is difficult to make the diagnosis on the clinical ground and there tends to be substantial diagnostic delay [Graham et al. 1992] .

In the light of the results obtained, we will continue to screen the congenital acquired vitamin B12 deficiency among our NBS program. Therefore, each child with aberrant C3 and C3/C2 or C3/C16 will be thoroughly examined to exclude congenital acquired vitamin B12 deficiency, which can easily be corrected in most cases.

5.3 Effectiveness of WES as a diagnostic method in clinical practice for patients with suspicion of mitochondrial diseases (Paper II)

To evaluate the effectiveness of WES in Estonia, we analyzed 28 patients with a suspected MD who had been referred to a clinical geneticist in the Department of Clinical Genetics, Tartu University Hospital during the years 2003–2016. A disease-causing variant was found in 16 patients (57%) by WES in nuclear DNA. A mitochondrial disorder was detected in four patients (14%) with variants in *SLC25A4, POLG, SPATA5* and *NDUFB11* genes. Other variants found were associated with a neuromuscular disease (*SMN1, MYH2* and *LMNA* genes), a neurodegenerative disorder (*TSPOAP1, CACNA1A* and *ALS2* and *SCN2A* genes), a multisystemic disease (*EPG5, NKX1-2, ATRX* and *ABCC6* genes), and one in an isolated cardiomyopathy causing gene (*MYBPC3*). An mtDNA point mutation was found in one patient in the *MT-ATP6* gene in the mtDNA analysis. Therefore, the overall efficiency of WES was 61% (Table 7).

In addition, we analyzed the mtDNA of 27 patients using exome sequencing data. The coverage of mtDNA was highly dependent on the enrichment kit used, thus all samples analyzed using the SureSelect enrichment kits had markedly lower coverage. For the samples sequenced with the SureSelect kits, the 1x coverage ranged from 96.5% to 99.9% of the mtDNA nucleotides, with one outlier of 38.3%, but the 20x coverage ranged only from 0–9%. Regarding the Nextera kits, the sequence depth of 1x was achieved in 100% of the mtDNA positions, and the 20x coverage ranged from 63% to 100%, with 12/15 samples achieving the 20x coverage of > 95%. In two sibs sequenced using the TruSeq kits, the 1x coverage was 100%, and the 20x coverage was 51% and 76%. We detected one disease-causing variant in the *MT-ATP6* gene (Table 7, Pt No 3), although the sample was sequenced after SureSelect enrichment, so the coverage was lower (10 reads out of 12 supported the variant).

The patient selection bias in our study group is quite low since the Department of Clinical Genetics, Tartu University Hospital is the only department in Estonia, offering the counseling of clinical genetics, and our routine approach to patients with an unsolved suspected MD is to take a skin biopsy for further studies. Although, we have to admit that our cohort is rather small and in the retrospective study group, we preliminary excluded patients who have an MD formerly been confirmed by molecular genetic studies. Therefore, this could influence the final results.

Table 7. The list of the patients investigated in our study with detailed description of the phenotype-related gene and protein function (BOLD font marks the mitochondrial disorder).

Pt no	Sex	Current age	Onset	MDC	Gene	Function of the gene and protein, disease causality
1	**F**	**6y**	**At birth**	**11**	***SLC25A4*** **(NM_001151.3)**	**Encodes the ADP/ATP translocase 1 protein, which imports ADP into the mitochondrion and exports ATP into the intermembrane space. Its dysfunction causes insufficient adenine nucleotide availability for deoxyadenosine triphosphate (dATP) synthesis and imbalanced deoxynucleotide triphosphate (dNTP) pools, leading to mtDNA depletion. Its defects are associated with mitochondrial DNA depletion syndrome type 12 (OMIM 615418), adPEO (OMIM 609283) and a third distinct phenotypic group described by us [Thompson et al. 2016].**
2	**M**	**25y**	**6y**	**10**	***POLG*** **(NM_002693.2)**	**Encodes the DNA polymerase subunit gamma-1 protein, which is essential for mtDNA replication and repair. Its deficiency can cause mtDNA depletion and/or multiple deletions and is associated with Alpers syndrome (OMIM 203700), Mitochondrial neurogastrointestinal encephalopathy syndrome (OMIM 613662), mitochondrial recessive ataxia syndrome (OMIM 607459), adPEO (OMIM 157640) and autosomal recessive PEO (OMIM 258450) [Hikmat et al. 2017].**
3	**F**	**24y**	**2y**	**8**	***MT-ATP6*** **(NC_012920.1)**	**Encodes subunit alpha of the ATP synthase or complex V which produces most of the ATP in human cells. Its defects are associated with neuropathy, ataxia, retinitis pigmentosa (OMIM 551500), Leigh syndrome (OMIM 256000) and mitochondrial infantile bilateral striatal necrosis (OMIM 500003) [Kucharczyk et al. 2010].**
4	F	Died at 3m	Pre-natal	8	*SMN1* (NM_000344.3)	Encodes the survival motor neuron protein which directly and indirectly via spliceosomal snRNP biogenesis (is critical for snRNP assembly) is involved in general cellular RNA processing [Lorson and Androphy 1998]. Its deficiency is associated with spinal muscular atrophy (OMIM 253300).
5	**F**	**Died at 4y11m**	**Early infancy**	**7**	***SPATA5*** **(NM_145207.2)**	**Encodes the spermatogenesis-associated protein 5, which is a member of the ATPases associated with diverse activities protein subfamily. SPATA5 deficiency leads to imbalance of mitochondrial fusion-fission events, mitochondrial shortening, decreased ATP production at axonal endings and impaired axogenesis in primary cortical neurons *in vitro*, and results in an autosomal recessive syndrome with severe global developmental delay, severe speech impairment, hearing loss, abnormal EEG and microcephaly (OMIM 616577) [Puusepp et al. 2018].**
6	M	5y	After birth	7	Unsolved	
7	**M**	**2y8m**	**At birth**	**6**	***NDUFB11*** **(NM_019056.6)**	**Encodes the NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 11, a component of the respiratory chain complex I. Its defects are associated with isolated mitochondrial complex I deficiency (OMIM 252010) and linear skin defects with multiple congenital anomalies (OMIM 300952). (Paper V)**

Pt no	Sex	Current age	Onset	MDC	Gene	Function of the gene and protein, disease causality
8	M	9y	3 weeks	6	*MYH2* (NM_017534.5)	Encodes the myosin-2 protein, a heavy chain isoform that is expressed in fast type 2A muscle fibers and is essential for muscle contraction. Its defects are associated with autosomal recessive and autosomal dominant proximal myopathy and ophthalmoplegia syndrome (OMIM 605637) and an unusual phenotype of neonatal-onset congenital myopathy matching our patient's phenotype [D'Amico et al. 2013].
9	M	Died at 1y	After birth	6	*EPG5* (NM_020964.2)	Encodes the ectopic P granules protein 5 homolog protein, which has a key role in the autophagy pathway [Cullup et al. 2013]. Its deficiency is associated with Vici syndrome (OMIM 242840).
10	M	9y	After birth	6	Unsolved	
11	M	27y	2m	6	Unsolved	
12	M	11y	1.5m	6	Unsolved	
13	M	29y	Early infancy	6	Unsolved	
14	M	1y2m	At birth	6	Unsolved	
15	F	Died at 5y	2m	5	*LMNA* (NM_170707.2)	Encodes the structural protein prelamin A/C, which underlie the inner nuclear membrane and determine nuclear shape and size. Its defects are associated with various diseases, including Emery-Dreifuss muscular dystrophy 2 (OMIM 181350), Emery-Dreifuss muscular dystrophy 3 3 (OMIM 616516), congenital muscular dystrophy (OMIM 613205), limb-girdle muscular dystrophy type 1B (OMIM 159001), cardiomyopathies, lipodystrophies, peripheral neuropathies and progeria syndromes [Fisher et al. 1986].
16	M	5y	4m	5	*CACNA1A* (NM_023035.2)	Encodes the voltage-dependent P/Q-type calcium channel subunit alpha-1A acting as an ion pore and a voltage sensor. Impairment of this calcium channel leads to synaptic dysfunction and profound neuronal loss throughout the cerebellum. Its defects are associated with early infantile epileptic encephalopathy (OMIM 617106), episodic ataxia type 2 (OMIM 108500), familial hemiplegic migrane (OMIM 141500) and spinocerebellar ataxia 6 (OMIM 183086).[Reinson et al. 2016b]
17	M	20y	7y	5	*TSPOAP1 (BZRAP1)* (NM_004758.3)	Encodes the peripheral-type benzodiazepine receptor-associated protein 1. RIMs and RIM-BPs are multidomain scaffolding proteins that bind directly or indirectly to nearly all other presynaptic active zone proteins and Ca^{2+} channels and are essential to all active zone functions. (Acuna *et al*, 2016). This protein has so far not been associated with any Mendelian disorders, but we have described a novel AR dystonia syndrome [Pajusalu et al. 2017]
18	M	8y	At birth	5	*NKX2-1* (NM_003317.3)	Encodes the homeobox protein Nkx-2.1 a transcription factor that is expressed during early development of thyroid, lung, and forebrain regions, particularly the basal ganglia and hypothalamus. Defects in this protein are associated with choreoathethosis, congenital hypothyroidism with or without lung dysfunction syndrome (OMIM 610978) and benign hereditary chorea (OMIM 118700)[Thorwarth et al. 2014].

Pt no	Sex	Current age	Onset	MDC	Gene	Function of the gene and protein, disease causality
19	M	18y	Early infancy	5	Unsolved	
20	F	2y	At birth	5	Unsolved	
21	M	Died at 16y	Early infancy	4	*ALS2* (NM_020919.3)	Encodes alsin protein which is a member of the guanine nucleotide exchange factors for the small GTPase RAB5 and plays a role in intracellular endosomal trafficking [Hadano et al. 2006]. Its defects are associated with juvenile amyotrophic lateral sclerosis (OMIM 205100), juvenile primary lateral sclerosis (OMIM 606353) and infantile onset ascending spastic paralysis (OMIM 607225).
22	M	6y	At birth	4	*ATRX* (NM_000489.3)	Encodes the transcriptional regulator ATRX an ATP-dependent chromatin remodelling factor, which is thought to play a variety of key roles at tandem repeat sequences within the genome. Its defects are associated with X-linked alpha-thalassemia/ mental retardation syndrome (OMIM 301040), mental retardation – hypotonic facies syndrome (OMIM 309580) and tumorigenesis [Clynes and Gibbons 2013].
23	F	Died at 1y3m	At birth	4	*SCN2A* (NM_021007.2)	Encodes the alpha-subunit of the voltage-sensitive sodium channel NaV1.2, which is responsible for the generation and propagation of action potentials early in development in neurons in hippocampus and cortex. Its defects are associated with early infantile epileptic encephalopathy (OMIM 613721) and benign familial infantile seizures (OMIM 607745) [Liao et al. 2010].
24	F	Died at 7m	Prenatal	4	Unsolved	
25	F	Died at 1y5m	3m	3	Unsolved	
26	M	Died at 26y	1y	3	Unsolved	
27	M	2y	1.5m	2	*ABCC6* (NM_001171.5)	Encodes the multidrug resistance-associated protein 6. Its defects are associated with *pseudoxanthoma elasticum* (OMIM 264800) [Germain 2017].
28	F	7y	6y	1	*MYBPC3* (NM_000256.3)	Encodes cardiac myosin-binding protein C, which is arrayed transversely in sarcomere A-bands and binds myosin heavy chain in thick filaments and titin in elastic filaments. Phosphorylation of this protein appears to modulate contraction. Its defects are associated with dilated cardiomyopathy (OMIM 615396), hypertrophic cardiomyopathy (OMIM 115197) and left ventricular noncompaction (OMIM 615396) [Previs et al. 2012].

From this study, we have published five articles reporting cases, which provided new information on the specific disease/gene. We have described a new inheritance pattern for *CACNA1A* associated disorders (Table 7, Pt. No 16) in cooperation with the scientists from the Radboud University Medical Center, Nijmegen, The Netherlands. The details are described and discussed in Chapter 5.4. In collaboration with the Wellcome Trust Centre for Mitochondrial Research, Institute of Neuroscience, Newcastle University, United Kingdom, we have shown that autosomal dominant *SLC25A4* variants can cause early-onset severe MD (Table 7, Pt. No 1), which is described and discussed in detail in Chapter 5.5. We have also expanded the phenotype of patients with mutation in the *NDUFB11* gene and presented a broad phenotypic divergence between these patients with scientists from Institute of Human Genetics, Technical University of Munich, Germany. Further information about the *NDUFB11*-related phenotype (Table 7, Pt. No 7) can be found in Chapter 5.6. Additionally we have carried out functional studies to elucidate the pathomechanisms of *SPATA5* gene deficiency (Table 7, Pt. No 5) in cooperation with the Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia; and have concluded *SPATA5*-related diseases can be indirectly categorized under MDs [Puusepp et al. 2018]. In addition, we have described a novel genotype phenotype association of biallelic mutations in the *BZRAP1* gene (Table 7, Pt. No 17), causing an autosomal recessive dystonia syndrome. The delineation of the *BZRAP1*-associated syndrome and the results of functional studies are shown elsewhere [Pajusalu et al. 2017]. As written above, international and local cooperation between research institutions becomes more and more important. The networking is required to optimize identification of molecular causes of rare disorders (including MDs), in both research and clinical settings. Such networks will help advance the genetic diagnosis for patients with rare disorders, and hopefully will be followed by an era of development of effective treatments for them. However, as stated above, WES is not the magic tool leading to an effortless diagnosis in all cases, especially in clinical work. The ordering physician should be aware of its pitfalls and limitations. In Estonia, clinical geneticists work in the same department with the research laboratory and their cooperation is close, it is highly likely that the details related to the patient are thoroughly discussed before and after ordering the analysis, including secondary findings and rare finding, which may need additional (international) investigation.

One part of this study was also to observe the association between the MDC score (12–8 points were definite, 5–7 points were probable, 2–4 points were possible and <2 points were unlikely) and effectiveness of WES. The outcome was expected – the higher the MDC score, the greater the probability of MDs (Table 7). Three out of four cases with a definite MDC scoring were confirmed as having MD and one patient with spinal muscular atrophy type I (SMA I).

In case of patients with a probable MD scoring, the results vary widely. Two patients were confirmed to have MD; two patients had variants in myopathy/ muscular dystrophy-causing genes, two in synaptic dysfunction-causing genes,

one in an autophagy-related gene, and one in a transcription factor encoding gene, with the latter two causing a multisystemic disease. In the group with possible MD scoring, no patients were confirmed to have MD. Four confirmed cases had variants in genes with different functions and heterogeneous phenotypes.

In our group, six patients died before establishing a diagnosis; only one of them before the age of one year and she had mutation in *SMN1* gene. Unfortunately, in our group there were also three deceased patients, whose cases remain unresolved.

All these details show that we cannot clearly differentiate MDs from other diseases on clinical grounds and metabolic/imaging studies alone. Verity et al. have also shown that children do not have many of the symptoms that are characteristic of MDs when they first present, and the typical features may only appear later in life [Verity et al. 2010]. Therefore, using WES as a first-tier genetic analysis for patients with neuromuscular, neurological, and/or multisystem signs and symptoms should be a standard protocol. This is especially true in children because regarding MDs, nuclear gene defects are overrepresented in pediatric cases [Alston et al. 2017; Wortmann et al. 2017].

A muscle biopsy is an excellent choice for detecting mtDNA mutations, deletions, and depletion [Wortmann et al. 2015], but it is an invasive and quite traumatizing procedure for the child and often, parents would like to postpone it whenever possible. There is currently a scientific debate on whether the WES analysis should precede the muscle biopsy [Narayanaswami et al. 2014; Phadke 2017; Schuelke et al. 2017; Soden et al. 2014]. Based on our current study, we suggest that WES is performed prior to the muscle biopsy; however, the muscle analysis might be needed afterwards for confirmation of the variants found, or to obtain additional information about the patient's phenotype.

A less invasive procedure to study respiratory chain enzyme activity is the analysis of cultured fibroblast cells derived from the skin biopsy. However, there might be cases with decreased activity in muscle, but not in fibroblasts [Phadke 2017], as was the case in our patient no. 1 (Table 7).

For patients with no findings with WES, the next step should be the mtDNA analysis. Presently, laboratories are already working on methods to analyze nuclear DNA (nDNA) and mtDNA concomitantly from blood lymphocytes using commercially available exome sequencing kits [Picardi and Pesole 2012]. Dinwiddie et al. have shown that using exome sequencing without specifically enriching for mitochondrial sequences also provides sufficient coverage for mtDNA, and both homoplasmic and heteroplasmic mitochondrial variants may be detected [Dinwiddie et al. 2013]. We have also developed a bioinformatics pipeline for analyzing mtDNA from the data of WES. We experienced large differences between samples, depending on which enrichment kit was used for the WES library preparation. The SureSelect kits (Agilent) had poor coverage, whereas the Nextera and TruSeq (Illumina) had quite good coverage. However, the SureSelect enrichment kit was used in the patient with a known mtDNA mutation, which we also detected using WES, despite the low coverage. There-

fore, this is a promising technique to analyze mtDNA, without the invasive procedure of the muscle or skin biopsy. However, we should bear in mind that with this technique we analyze blood lymphocytes, which may not contain the mtDNA variants, especially in patients with mitochondrial myopathy. Another bottleneck is mitochondrial heteroplasmy – a low level of the variant may not be detected [Phadke 2017] and in the leukocytes, the level of heteroplasmy of mtDNA mutations tends to decrease during life, whereas it can increase in postmitotic tissues like in muscle [Wortmann et al. 2017]. In addition, mtDNA deletions and mtDNA depletion may be missed. Therefore, a negative mtDNA analysis from the WES data does not exclude the possibility of an mtDNA mutation, which can only be achieved with certainty by examining DNA from clinically affected tissue. On the other hand, if a mutation is detected by WES, this likely prevents the need for further (invasive) procedures. The simplified diagnostic algorithm for patients with neuromuscular, neurological, and/or multisystem symptoms is shown in Figure 1.

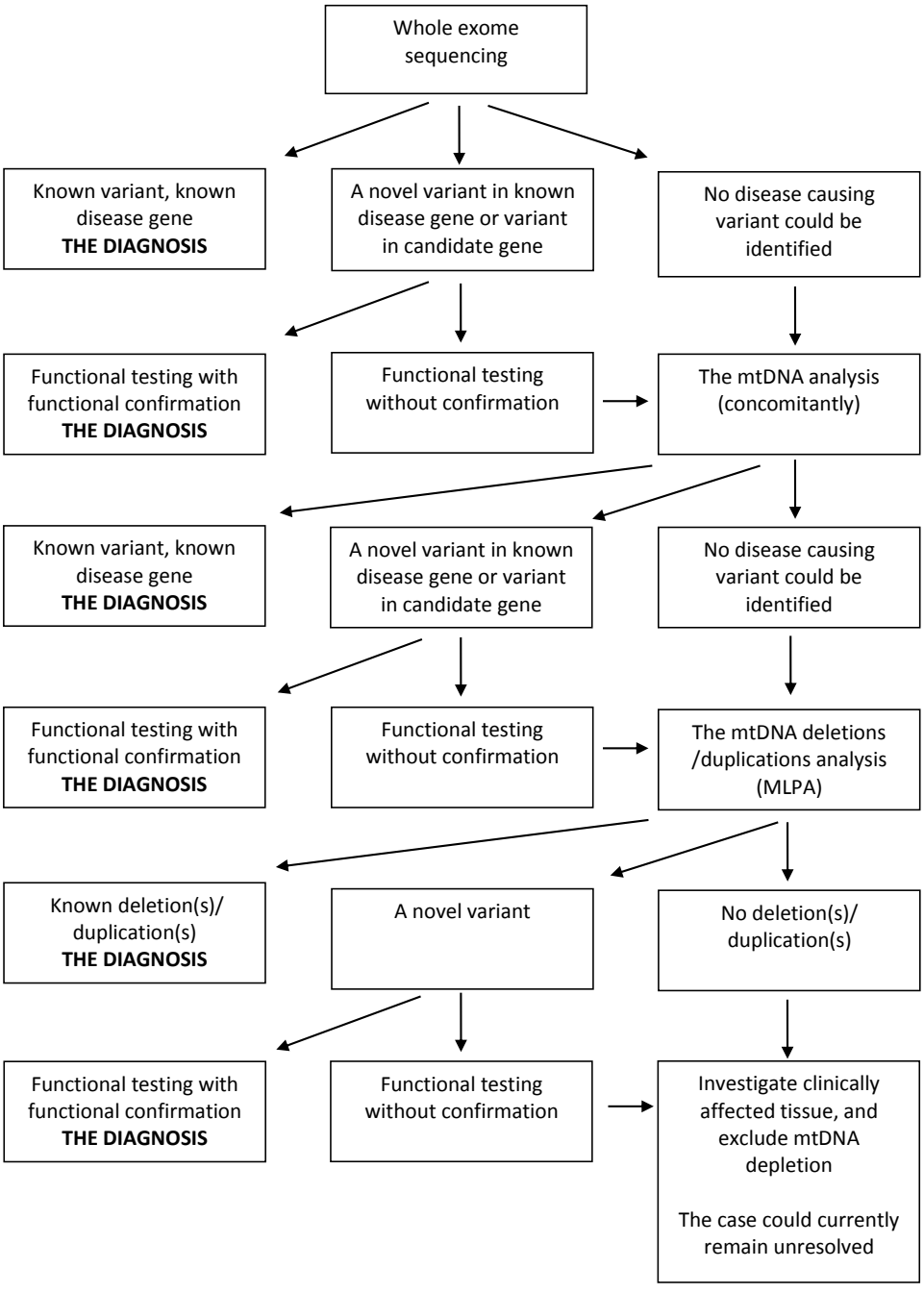

Figure 1. Diagnostic algorithm for children with any multisystem, neurological and/or neuromuscular problem.

In conclusion, the effectiveness of WES in Estonia for patients with a clinical suspicion of MDs is 61% and therefore, we suggest the WES analysis as the first-tier method in clinical genetic practice for children with any multisystem, neurological, and/or neuromuscular problems. This recommendation is also supported by the fact that many patients in our study group have reached the age of adulthood before the molecular diagnosis was confirmed. As nDNA variants are more common in children with MDs, a large number of patients harbor disease-causing variants in genes other than mitochondria-related ones, and the clinical presentation might not always point towards MDs. Based on our experience, the higher the MDC score, the more probable it is to find not only MDs, but an overall genetic diagnosis by the WES analysis. Therefore, we believe that the MDC scoring is useful, but it only serves its purpose, when the patients are clinically, biochemically and instrumentally thoroughly investigated.

5.4 Early onset epileptic encephalopathy with progressive cerebral, cerebellar and optic nerve atrophy caused by biallelic mutations in the *CACNA1A* gene (Paper III)

This patient (Table 7, Pt. No 16) was born from uneventful labor as the sixth child of non-consanguineous Estonian parents (Figure 2a, II:7). The pregnancy was complicated by a polyhydramnios.

At 4 months of age, he was hospitalized due to tremulous movements in his arms and legs. His occipitofrontal circumference was 44 cm (+3 SD), weight 6.4 kg (+1 SD), and length 62 cm (+0.5 SD) with mild dysmorphic features (Figure 2b). Eye movements were dysconjugated with occasional episodes of nystagmus and no eye contact. He was markedly hypotonic, lacked deep tendon reflexes and a negative Babinski sign; had no head control and almost no spontaneous movements. The electroencephalogram (EEG) was abnormal with a high-voltaged up to 300 microV asymmetrical slow 2–3 Hz background activity accompanied by multifocal sharp waves and spike-wave complexes; there was no difference between the awake and sleep state. The brain MRI showed an optic nerve atrophy, small corpus callosum, dilatation of the frontal horns of the lateral ventricles and diffuse hypomyelination. The magnetic resonance spectroscopy (MRS) was normal.

At 3 year of age, his EEG showed frontal diphasic waves and the brain MRS indicated a decreased N-acetylaspartic acid to creatine ratio (1.53) and slightly increased myo-inositol (1.1). The last MRI at the age of 4 years showed cerebellar atrophy and moderate cerebral atrophy (Figure 2c). In the occipital and parietal regions, a hyperintense white matter signal on T2 images with concomitant decreased volume was present (Figure 2d).

At the age of 5 years, he was blind, bedridden and had global developmental delay with marked muscular atrophy and rigidity as well as additional Friedreich-like foot deformity on his left foot. He had some overt seizures (tonic

spasms) with high voltage continuous irregular epileptiform discharges and did not display an age-appropriate sleep pattern on EEG. He was able to recognize the voices of his family members; he could roll himself onto his side; also, he had to be fed, but he was able to swallow.

Family history: the third child in this family, a girl (Figure 2a, II:4) died at the age of 5 years due to epileptic encephalopathy. The first hospitalization took place at the age of 4 month due to seizures and at that point, her development corresponded to the age of 1 month with no emotional contact and extreme muscular hypotonia. Her phenotype, clinical course and MRI findings including optic nerve atrophy, were very similar to our patient. Her EEG was abnormal with multifocal interictal epileptiform discharges and ictal electric discharges.

The oldest daughter (Figure 2a, II:3) in the family had mild ID diagnosed by the Wechsler Intelligence Scale for Children III (WISC-III) testing. Occasionally, she complained about the migraine and since the age of 13, she had exercise-induced vertigo attacks. About a year later, she experienced the first episode of aggression and self-injury necessitating isolation to the acute unit of psychiatric department. The most recent brain MRI study was done at the age of 14 and showed no abnormalities.

The third daughter (Figure 2a, II:5) had also mild ID (full scale intelligence quotient (IQ) was 62 by WISC-III) with impaired fine motor skills. She was seizure-free, but had generalized epileptiform (spike and slow wave complexes) discharges on EEG during photostimulation and in shallow sleep. Brain MRI, done at the age of 11 years, was normal.

The youngest daughter (Figure 2a, II:6) had normal intellectual development. There was no clinical information of the mother's two elder sons (Figure 2a, II:1 and II:2).

Both parents had mild ID (performance IQ 73 in the father and 63 in the mother by the Wechsler Adult Intelligence Scale III) and poor social adjustment. Two years before our patient was born, his mother (Figure 2a, I:2,) was diagnosed with probable alcohol-induced cerebellar ataxia: intention tremor of the hands, poor balance, and wide-based gate with horizontal nystagmus. Her brain MRI images showed cerebellar atrophy predominately affecting the vermis. The father (Figure 2a, I:3,) had no ataxia, migraines, or seizures. He had only modest complaints of rare headaches (without hemiplegia) and impairment of short-term and working memory. Brain MRI images were normal.

WES identified two previously unreported heterozygous mutations in our patient's *CACNA1A* gene: c.4315T>A p. (Trp1439Arg) in exon 27, and c.472_478delGCCTTCC p. (Ala158Thrfs_6) in exon 3 (RefSeq NM_023035.2). Confirmation of the *CACNA1A* mutations was performed by Sanger sequencing.

The missense mutation c.4315T>A is predicted pathogenic by all tested software tools such as PolyPhen-2 [Adzhubei et al.2010] (HumDiv score 1), SIFT [Kumar et al., 2009] (score 0), and CADD [Kircher et al., 2014] (phred score 27.1). The amino acid position Trp1439 is conserved across species with tryptophan residue being present to C. elegans (PhyloP score 4.48), and is located in ion transport domain (InterPro IPR005821). The frameshift deletion

is predicted to cause a truncation of the protein in the exon three and therefore is apparently a loss-of-function mutation. Neither variant was present in ExAC (Exome Aggregation Consortium) database of 60,706 individuals, our in-house next generation sequencing database of 339 individuals, HGMD professional, or ClinVar. The missense mutation can be classified as likely pathogenic (class 4) and the frameshift mutation as pathogenic (class 5) according to guidelines by the American College of Medical Genetics and Genomics [Richards et al., 2015].

Both mutations were discovered also in the deceased sister (Figure 2a, II:4). Their mother (Figure 2a, I:2) and the eldest sister (Figure 2a, II:3) were heterozygous for c.4315T>A, and the father (Figure 2a, I:3) together with the second living sister (Figure 2a, II:5) were heterozygous for c.472_478delGCCTTCC. The youngest healthy sister (Figure 2a, II:6) has neither of the mutations.

Expression of the *CACNA1A* is particularly high in Purkinje and granule cells [Pietrobon 2010; Volsen et al. 1995]. Mice that completely lack this neuronal calcium channel are born alive but have a severe phenotype of chronic dystonia and die soon after birth [Hoffman 2001]. Studies with knockout (KO) mice showed that the loss of $Ca_v2.1$ channel upregulates other voltage-gated calcium channels like $Ca_v2.2$ and $Ca_v1.2$ at most central synapses, although with different efficiency, resulting in synaptic dysfunction [Damaj et al. 2015], which may explain very variable phenotypes caused by the same mutation. Additionally, studies with KO mice demonstrate profound neuronal loss throughout the cerebellum [Rose et al. 2014], because in cerebellar networks, $Ca_v2.1$ channels regulate the whole-cell calcium current density and the intrinsic excitability of Purkinje cells and granule cells. This leads to altered cerebellar output by respectively decreasing the excitatory drive on Purkinje cells or their ability to release neurotransmitters, causing ataxia and dyskinesia in mice [Damaj et al. 2015]. Therefore, we can assume that this phenomenon has taken place in our described patients too. The most remarkable is cerebellar atrophy in our cases (Figure 2c).

Additionally, from a clinical aspect, there is no previous highlight on progressive optic nerve atrophy, which was observed in the children carrying compound heterozygous mutations in the *CACNA1A* gene. To our knowledge, optic nerve has not investigated in previously published studies with mice [Damaj et al. 2015; Jen 1999; Mallmann et al. 2013; Pietrobon 2010; Rose et al. 2014] also.

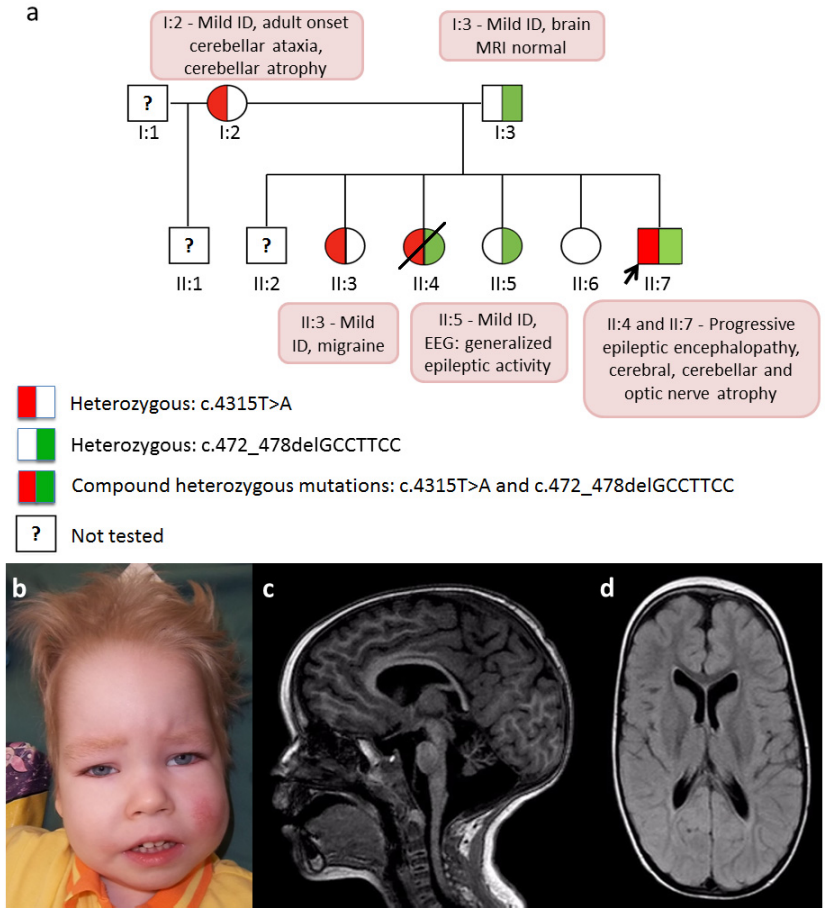

Figure 2. (a) Pedigree. The family with seven affected individuals from two generations. (b) Picture of our patient at the age of 5 years. Dysmorphic features include dolichocephaly, a high and narrow forehead, widely spaced eyes, broad eyebrows, long eyelashes, a wide nasal bridge, a short nose, anteverted nares, a smooth philtrum, and a tented vermillion of the upper lip. An MRI at the age of 4 years: (c) a sagittal section of a T1-weighted image shows pronounced atrophy of cerebellum and a small corpus callosum; (d) an axial section of a T2-weighted image showing mild cerebral atrophy with enlargement of ventricles.

Because of striking muscular hypotonia, a muscle biopsy was done to our patient. The study exhibited lipid deposits in otherwise normal muscle fibres (Figure 3a, b). It is difficult to explain this finding as there is very little known about changes taking place in the muscles of patients with biallelic mutations in the *CACNA1A* gene. Considering the fact that the patient was on treatment with a steroid and valproic acid, we cannot declare it as a disease-specific change. The muscle biopsy was not performed on the deceased sister (Figure 2a, II:4), so further studies of other patients with biallelic *CACNA1A* mutations are necessary, to make reliable conclusions.

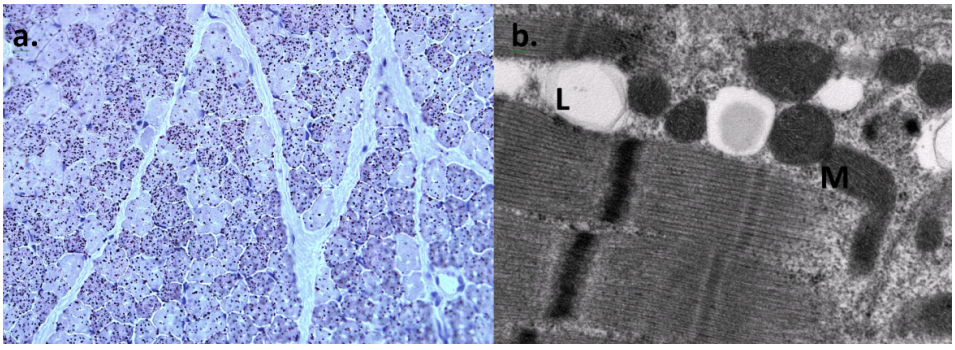

Figure 3. Muscle histopathology and electron microscopy in patient with two previously unreported heterozygous mutations in the *CACNA1A* gene. (a) Oil Red O staining of lipids. 40x objective (Ob); (b) Electron micrograph of muscle biopsy cells. Lipid (L) deposits were in close connection with mitochondria (M) membranes. Ob.27000x

It is previously known that mutations in the *CACNA1A* gene cause three allelic autosomal dominant conditions – EA2, SCA6, and FHM1. Damaj et al., [Damaj et al. 2015] recently stated that *CACNA1A* haploinsufficency may also cause cognitive impairment, autism and epileptic encephalopathy with mild cerebellar symptoms. These conditions occasionally overlap, since approximately 20% of the patients with FHM1 have cerebellar signs ranging from nystagmus to progressive, usually late-onset, mild ataxia [Ducros et al. 2001; Garcia-Baro-Huarte et al. 2014; Terwindt et al. 1996] and 33% of patients with SCA6 display episodic features which are characteristic for EA2 [Mantuano et al. 2010]. About half of the EA2 patients report migraine headaches during the attacks [Jen et al. 2004], and a minority has episodes of hemiplegia [Jen 1999]. Only rare cases of the *CACNA1A* gene mutations have been associated with epileptic encephalopathy [Epi et al. 2013; Hino-Fukuyo et al. 2015; Ohmori et al. 2013].

When comparing the clinical picture between our other family members, we can see some similarities but also differences. The mother (Figure 2a, I:2) carries a novel mutation in the *CACNA1A* gene – c.4315T>A that causes an adult onset cerebellar ataxia and atrophy. Her eldest daughter (Figure 2a, II:3) has the exact same mutation but the clinical picture varies in many ways. The mother's problems with ataxia started in adulthood but the daughter (Figure 2a, II:3) experienced first signs of clumsiness and ataxic movements at the age of thirteen with no visual changes in brain MRI. At the age of sixteen, the main problem for her (Figure 2a, II:3) is uncontrollable episodes of aggression and self-injury requiring psychiatric admission. Psychiatric disorders with social phobia and anxiety are frequent findings in EA2 cohorts, these symptoms have been noticed especially when the disease started early in life [Nachbauer et al. 2014]. The mother (Figure 2a, I:2) has never had psychiatric problems although she has a history of chronic alcohol abuse. It is stated that families including individuals with both early and late disease onset show a tendency to decrease

in age of onset in next generations [Choi et al. 2013; Cleves et al. 2010; Krishnan et al. 2008].

The father (Figure 2a, I:3) has also a novel mutation in the *CACNA1A* gene – c.472_478delGCCTTCC presumably causing a translational frameshift. He shares the same mutation with one of his living daughters (Figure 2a, II:5), they both have mild ID and no pathologic findings on brain MRI. Surprisingly, this daughter (Figure 2a, II:5) has generalized epileptic activity seen in an EEG at the age of eleven, but no overt seizures. Unfortunately, we do not have information about the father's EEG study.

None of our described family members has experienced episodic coma with or without cerebral oedema like described by Blumkin et al.[Blumkin et al. 2010]. Furthermore, none of our family members has received specific treatment for etiological disease.

This is the first description of patients with biallelic mutations in the *CACNA1A* gene, which we believe to cause a unique clinical presentation – early onset epileptic encephalopathy, progressive cerebral, cerebellar and optic nerve atrophy with global developmental delay, severe muscular hypotonia and markedly reduced lifespan. Therefore, we are convinced that our case gives a novel insight into the mutations in the *CACNA1A* gene and expands the *CACNA1A*-related phenotype.

5.5 Clinical description of child with a novel mutation, causing a third distinct phenotypic group associated with *SLC25A4* gene (Paper IV)

This female (Table 7, Pt. No 1) infant was born at term weighing 3,520g (0 SD), and she was the second child of healthy non-consanguineous Estonian parents. She was floppy at birth with paucity of limb movements and hyporeflexia. She required low-level supplemental oxygen in the immediate postnatal period, but was breathing air from 5 to 48 hour of age, at which point she deteriorated with a decrease in conscious level, feeding difficulties, and respiratory impairment. She was intubated and mechanically ventilated. Elevated lactate was noted in blood (9.7 mmol/L, normal < 2.2 mmol/L) as well as CSF (4.8 mmol/L, normal range 1.1–2.4 mmol/L). The urine analysis revealed increased excretion of lactate, 2-hydroxybutyric acid, fumarate, ketoglutarate, ethylmalonate, and 4-hydroxyphenyl lactate. Echocardiography (echoCG) showed a mildly enlarged apex of the left ventricle. At day 7, despite treatment with coenzyme Q10 (20 mg/kg/day), riboflavin (100 mg/kg/day), creatine (200 mg/kg/day), and L-carnitine (100 mg/kg/day), she developed persistent generalized clonic seizures without EEG abnormalities. Serial MRIs initially demonstrated communicating hydrocephalus and subsequently progressive cerebral white matter atrophy. Presently, at 6 years, she has a tracheostomy, is unable to speak, and can breathe independently for less than one hour. She is reliant on a

wheelchair due to proximal muscle weakness, but can walk briefly with a frame. Communication is by pointing or pictogram.

Our patient had the diagnostic muscle biopsy done for the evaluation of suspected mitochondrial disease using a range of histopathological and biochemical assays. The assessment of mitochondrial respiratory chain complex activities showed low complex I and IV activities in muscle, while the combined measurement of complexes II+III (succinate: cytochrome c reductase activity) was within the normal range (Table 8).

Table 8. Result of the biochemical examination of the patient's frozen muscle tissue. These data were previously published as a part of a diagnostic algorithm for mitochondrial disorders in Estonian children (Case 2) [Joost et al. 2012b].
mU/UCS = milliunits per unit of citrate synthase

Enzyme activities	Activity	Control range
Complex I	28 units/UCS	84–559
Complex II + III	51 mU/UCS	37–285
Complex IV	58 mU/UCS	520–2080
Citrate synthase	118 mU/mg protein	45–187

Histopathological assessment of the muscle biopsy revealed small and rounded muscle fibers with little variation in fiber size and without evidence of atrophy, necrosis, fibrosis, or inflammatory changes; Oil Red O staining showed lipid deposition and modified Gomori trichrome staining disclosed abnormal staining around the subsarcolemmal region of some fibers; unfortunately, histochemical assessment of oxidative enzyme activities was not undertaken. Electron microscopy investigation confirmed the accumulation of both lipid and glycogen, revealing numerous mitochondria with disorganized cristae, although paracrystalline inclusions were not detected (Figure 4).

Despite the serious clinical problems, this girl was for a long time without etiologic diagnosis. Her thorough investigation began already at the age of newborn, when she was suspected to have MD, which was also indicated by the muscular biopsy; therefore, the mutations in *POLG* and *TK2* were excluded after the chromosomal microarray analysis revealed no abnormalities. At that time, a predominantly traditional approach was used and details about this are described elsewhere [Joost et al. 2012b].

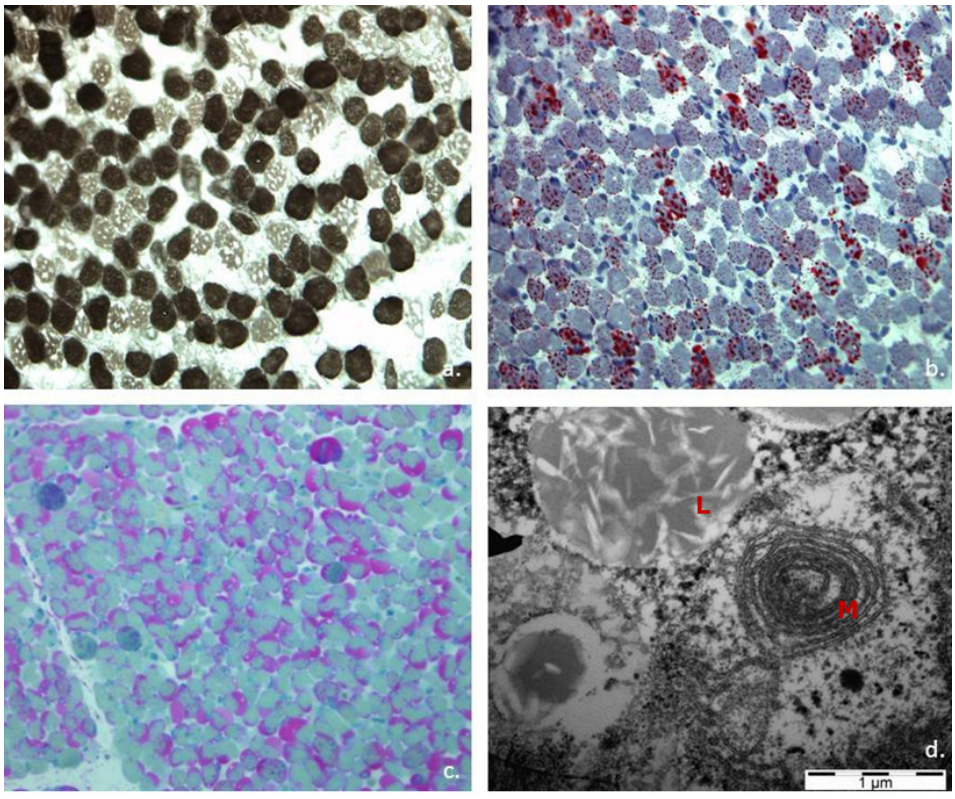

Figure 4. Muscle histopathology and electron microscopy in patient with *de novo* heterozygous c.239G>A, p.(Arg80His) *SLC25A4* mutation
(a) ATPase at pH 10.2 shows numerous vacuoles in type 1 fibers; (b) Oil Red O staining highlights the lipid content of the vacuoles; (c) 1 µm sections stained with methylene blue-azure II-basic fuchsin staining reveals glycogen in the periphery of fibers; (d) An electron micrograph shows abnormal mitochondria (M) with circularly arranged cristae and lipid droplets (L).

Eventually, we had the chance to perform WES and DNA was extracted by standard methods from the muscle biopsy. The genomic DNA fragments were enriched for exome sequences using the Agilent SureSelectXT Human All Exon 50Mb Kit (Agilent Technologies, Santa Clara, CA, USA). WES was carried out on an Illumina HiSeq 2000TM platform. A novel heterozygous NM_001151.3:c.239G>A p.(Arg80His) mutation was detected in the *SLC25A4* gene. Software tools PolyPhen-2 (HumDiv score 0.966) [Adzhubei et al. 2010], SIFT (score 0) [Kumar et al. 2009], and CADD (phred score 34) [Kircher et al. 2014] predicted the variant to be pathogenic. The amino acid position is highly conserved across different species (PhyloP score 6.072). The variant was not present in ExAC database of 60,706 individuals and this finding was confirmed by Sanger sequencing. Both of her parents were also tested for this mutation by Sanger sequencing and found out not to carry this mutation suggesting a *de novo* origin.

In addition, the mtDNA/nDNA ratio was determined in the frozen muscle biopsy using the real-time quantitative PCR to perform an mtDNA depletion test. The details of this method have been described previously [Dimmock et al. 2010]. This revealed an mtDNA/nDNA ration of 436 (the average mtDNA/ nDNA ratio in the age-matched reference group is 1288). The percentage of the mtDNA/nDNA ratio compared to the average on the reference group is 34%. The lowest reference value corresponds to 60% of the average mtDNA/nDNA ration, as observed in muscle tissue of healthy controls [Dimmock et al. 2010]. This result confirms the mitochondrial depletion syndrome in our patient.

Our patient was one of the seven patients with *de novo*, heterozygous, single-nucleotide substitutions in *SLC25A4*. Four of the seven patients presented an identical c.239G>A, p.(Arg80His) mutation and the other 3 cases harbored c.703C>G, p.(Arg235Gly) mutation. All these affected individuals presented with severe congenital hypotonia and profound muscle weakness necessitating artificial ventilation, and several died in early infancy. Our patient is the oldest living patient in this group. Available muscle biopsies from affected individuals revealed histopathological evidence of mitochondrial myopathy and a severe combined respiratory chain defect as a consequence of marked depletion of mtDNA copy number. These two variants affect highly conserved and functionally important amino acid residues, associated with the loss of steady-state AAC1 levels in patients' skeletal muscle and diminished ATP transport activity in vitro.

Mutations in *SLC25A4* are well-recognized causes of mitochondrial disease; dominantly inherited *SLC25A4* mutations have been documented in cases of adPEO, a adulthood-onset MD characterized by ptosis, restriction of eye movements, and the accumulation of clonally expanded mtDNA deletions in post-mitotic tissues on account of disordered mtDNA maintenance [Deschauer et al. 2005; Kaukonen et al. 2000; Siciliano et al. 2003]. Additionally, recessively inherited *SLC25A4* mutations are known to cause childhood or early adulthood-onset mitochondrial myopathy and cardiomyopathy phenotype with lactic acidosis and proximal muscle weakness [Echaniz-Laguna et al. 2012; Korver-Keularts et al. 2015; Palmieri et al. 2005; Strauss et al. 2013]. The cases we present here, with *de novo* dominant mutations, can be considered to show a third distinct clinical phenotype associated with mutations in *SLC25A4*, which is more severe in its clinical presentation than those previously described.

Despite the findings of *SLC25A4* mutations in adPEO with multiple mtDNA deletions [Kaukonen et al. 2000], the mechanism of how an ADP/ATP carrier causes mtDNA instability is unknown. The current finding of mtDNA depletion in early-onset cases with *SLC25A4* mutations links this gene to the causes of typical mtDNA maintenance disorders and strongly suggests that the transporter dysfunction causes insufficient adenine nucleotide availability for dATP synthesis and consequent imbalanced dNTP pools, leading to mtDNA depletion.

Reports of recessive *SLC25A4* mutations do not fit with correlation of AAC1 transport ability and clinical severity, since the mutations, including nonsense [Korver-Keularts et al. 2015; Strauss et al. 2013], splicing [Echaniz-Laguna et

al. 2012], and missense [Korver-Keularts et al. 2015; Palmieri et al. 2005]) are completely null but have a much milder clinical phenotype than the case presented here. Knockout mouse studies have also shown that loss of AAC1 produces a similar myopathy and cardiomyopathy phenotype to individuals with recessive *SLC25A4* mutations [Esposito et al. 1999; Graham et al. 1997]. We, therefore, postulate that the complete lack of functional AAC1 triggers a compensatory mechanism to upregulate expression of other isoforms of the ADP/ATP carrier in cases of recessive disease. There are four different AAC isoforms and at least another three isoforms of the ATP-Mg/Pi carrier, which can also transport ADP and ATP [Fiermonte et al. 2004].

The detailed explanation of possible causes of mtDNA depletion with an attempt to explain the differences in clinical presentation by analyzing the positions of affected amino acid residues within the structure of AAC1 are described by Thompson et al.[Thompson et al. 2016] in cooperation with us.

Reaching the results described above and confirming the pathogenicity of this *de novo* mutation has immense implications for the application of WES and international collaboration by analyzing the data of a larger group of patients. This is an ideal example of how the previous approach is time consuming and sometimes does not end up with conclusive result, whereas WES along with a biochemical and functional studies can provide excellent final solution.

5.6 Diverse phenotype in patients with complex I deficiency due to mutations in *NDUFB11* (Paper V)

This male infant (Table 7, Pt. No 7 and Table 9, Pt. No 1) was born from healthy non-consanguineous Estonian Russian parents at full term (Figure 5). During the delivery, fetal bradycardia was noticed and childbirth was ended by vacuum-assisted vaginal delivery. His birthweight was 3218g (–1 SD) and Apgar scores were 8^{1} and 9^{5}. Soon after birth, he became hypotonic and bradycardia was noticed. The first blood gases analysis revealed profound lactic acidosis – 9.2 mmol/L (normal <2.2 mmol/L), peak value was 20mmol/L. At the age of 11 hours, he required intubation and mechanical ventilation with dopamine infusion for hypotension treatment.

The first echoCG was done on the first day of life and it revealed pulmonary hypertension greater than 69 mmHg at rest, with significant tricuspid valve insufficiency, maximum velocity >4 m/s. Right ventricle was moderately trabecular and left ventricular contractile function was impaired. Abdominal ultrasound showed mild renal pelvis dilatation.

EEG was also abnormal, revealing constant background activity depression, but no epileptic activity recognized. Periventricular cystic lesions were noticed on cranial MRI but no basal ganglia hyperintensity.

The clinical picture and the phenomenon where the lactic acidosis worsened during the high glucose infusion led us to suspect a high energy demanding IEM.

The measurement of serum acylcarnitines profile by tandem MS revealed no pathology. The lactate and pyruvate ratio in blood was mildly elevated – 25 (normal <20). Within the days after the vitamin-based and cofactor-based mitochondrial therapy was applied – thiamine, L-carnitine, coenzyme Q10 and riboflavin – lactic acidosis declined and lactate remained 2.5–5 mmol/L.

He had mild normocytic anemia at the age of two months, during the stay in the intensive care unit; he recovered by additional iron administration and after that episode, there has been no indication for anemia and the peripheral smear, done twice, showed no abnormalities.

At the age of 3.5 years, his psychomotor development is age appropriate, the length is 98cm (0 SD), weight 14kg (–1 SD) and head circumference 51cm (0 SD). He continues to have treatment with riboflavin, L-carnitine and coenzyme Q10 that keeps the blood lactate persistently below 3mmol/L. His thyroid function is normal, the brain MRI revealed no pathology, but echoCG shows hypertrophic cardiomyopathy (CM) with slightly decreased contractile function and mild pulmonary hypertension. This cardiac condition is achieved by sildenafil and digoxin treatment.

Mitochondrial respiratory chain complex activities were measured spectrophotometrically in cultured fibroblasts and revealed a reduced activity of the mitochondrial enzyme-complex I, while the activity of the other respiratory chain enzymes and complex V were normal (Table 9).

Table 9. Spectrophotometrically measured mitochondrial respiratory chain complex activities in cultured fibroblasts.
*The German patient, published with us in paper V.
mU/UCS = milliunits per unit of citrate synthase

	Patient 1 (our case)	Patient 2*	Ref. range	
Complex I	**60**	**83**	163–599	mU/UCS
Complex II	445	515	335–888	mU/UCS
Complex III	784	793	570–1383	mU/UCS
Complex IV	532	560	288–954	mU/UCS
Complex V	651	643	193–819	mU/UCS
Citrate synthase	363	416	151–449	U/mg protein

WES was carried out with a search for rare compound heterozygous, homozygous or hemizygous variants in genes that have been previously associated with mitochondrial dysfunction prioritized the following *NDUFB11* (RefSeq NM_019056.6) mutation in a hemizygous state: c.328C>T, p.(Pro110Ser).

Our study also included one German patient (Table 9, Patient 2), to whom WES was also performed and c.286T>C, p.(Ser96Pro) mutation in a hemizygous state was found in the *NDUFB11* gene. Both these missense mutations were

located in the second exon and within the NADH domain (Figure 5B). These variants were validated by Sanger sequencing and carrier testing confirmed their *de novo* origin. These mutations had not been previously reported in public (dbSNP, ExAC) and in-house databases.

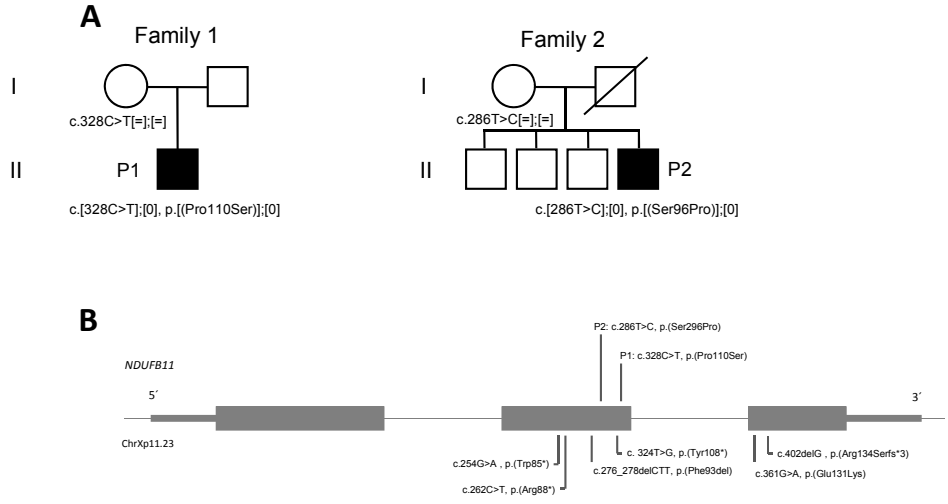

Figure 5. A. Pedigrees; B. *NDUFB11* gene. Two new variants from this study are shown above the gene and are located in the second exon, within the NADH domain. Previously published variants are written below it.

Western blotting of mitochondrial extracts from fibroblasts showed that the expression levels of the NDUFB11 protein was decreased in both patients' cells (Figure 6A and D). In addition, we observed clearly reduced levels of intact CI by BN-PAGE Western blot experiments (Figure 6B), which are compatible with the results of reduced CI enzyme activities (Table 9).

To demonstrate that the *NDUFB11* defect was responsible for the observed CI deficiency in both patients (our case and the German patient), we performed a lentiviral complementation experiment in which we introduced the wild type *NDUFB11* gene in the patient cells and in two control cell lines. As a control experiment, we introduced the green fluorescent protein (GFP). Western blots confirmed the expression of the transgenes (Figure 6C). The respiratory chain enzyme activity measurements in different transduced cell lines showed that the introduction of wild type *NDUFB11* restored the CI enzyme activity in both patient cell lines, while the control experiment with GFP-transduction had little or no effect in comparison to the activities in non-transduced cells, as shown in Table 9. In control cells, there was no significant change in CI activity. The effects on CI in the patient cells are specific for this enzyme, as there was little or no effect on the activities of the other respiratory chain enzymes (Figure 7B). In conclusion, the results of the functional data obtained in this experiment support the notion that the CI deficiency in the two patients is caused by *NDUFB11* genetic defects.

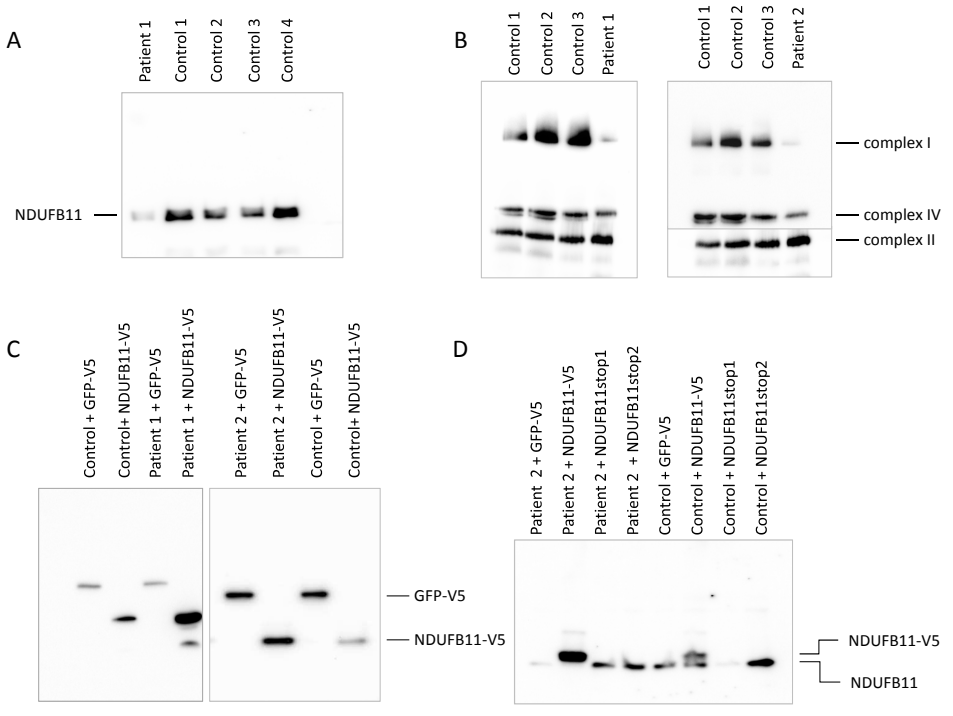

Figure 6. Analysis of NDUFB11 protein expression in patient fibroblasts.

(A) A western blot analysis using anti-NDUFB11 antibodies (Ab183716, Abcam) showed that NDUFB11 expression was clearly reduced in fibroblasts of patient 1 in comparison to fibroblasts from four different control subjects.

(B) A blue native PAGE/Western blot experiment shows a reduced amount of holo-complex I in both patients' fibroblasts in comparison to three controls, as detected by NDUFB11 and NDUFA9 (ab14713, Abcam) antibodies (left and right panel, respectively).

(C) Lentiviral transduction of fibroblasts from both patients with NDUFB11-V5 and GFP-V5 fusion constructs shows expression of the transgenic proteins in both patients and in controls, as detected by an anti-V5 antibody (R960-25, Invitrogen) using SDS-PAGE/Western blotting.

(D) SDS-PAGE/western blot analysis showing that patient 2 has a reduced expression level of endogenous NDUFB11 protein, whereas transduction of the cells by either NDUFB11-V5 or NDUFB11 without a tag (NDUFB11-stop; 1 and 2 indicate two different lentiviral preparations) rescues NDUFB11 expression. Note that the NDUFB11-V5 fusion protein has a slightly slower mobility than the endogenous NDUFB11 in the control fibroblasts and the transgenic NDUFB11 protein (without tag) in both control and patient fibroblasts

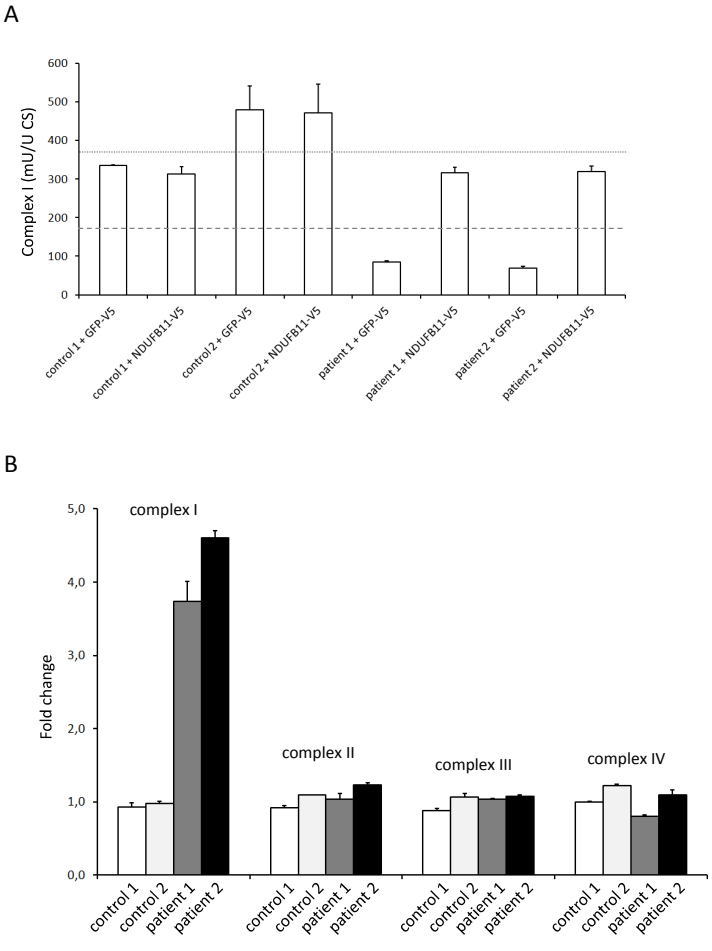

Figure 7. Transgenic expression of wild type *NDUFB11* (Refseq NM_019056) in fibroblasts of the two patients specifically rescues complex I activity. The NDUFB11 cDNA was cloned into the pLenti6.2/V5-DEST vector by Gateway cloning (Invitrogen). HEK293FT cells were transfected to produce lentivirus according to the manufacturer's protocol (Invitrogen). Viral particles were harvested and used to transfect patient and control fibroblasts. Blasticidin selection was used to obtain stably transfected cells expressing the transgene. In this way, stable cell lines expressing either a NDUFB11-V5 fusion or a GFP-V5 fusion (as negative control) were obtained.

(A) The respiratory chain enzyme activities, as measured spectrophotometrically, showed a specific and significant rescue of complex I activity by NDUFB11 in the both patients' cells to levels that were near the mean control level, indicated by a dotted line in the bottom graph (p-value <0.01). The dashed line indicates the lowest reference value for complex I.

(B) The effect was specific for complex I, as the other respiratory chain enzymes showed little or no response to the NDUFB11-V5 transduction, compared to the activities measured in the control transduction experiments with GFP-V5 (top figure). The results shown for NDUFB11 are those obtained with a NDUFB11-V5 fusion construct. For patient 2, we also performed a rescue experiment with NDUFB11 without a V5 tag, which gave very similar results (data not shown).

Therefore, biochemical, functional and molecular studies confirmed that mutations found in the *NDUFB11* gene gave rise to the above described phenotypic features in our two new cases.

In addition, over the past few years 13 affected patients with four asymptomatic family members have previously been described, and we can conclude that different mutations in the *NDUFB11* gene can cause broad phenotypic divergence. Nevertheless, even the genotypic homogeneity in some previously described patients (Table 10) give a great deal of phenotypic heterogeneity. However, our findings indicate also a clinical distinction of *NDUFB11*-related manifestation from other CI deficiencies.

Most reported cases harboring mutations in the *NDUFB11* gene tend to have some kind of cardiac muscle impairment (10/15 cases, 67%), while in patients who are CI deficient, it occurs in 20% of cases [Koene et al. 2012]. Every affected female with a *NDUFB11* mutation had cardiomyopathy – four with histiocytoid cardiomyopathy (HCM) [Rea et al. 2017; Shehata et al. 2015; van Rahden et al. 2015], one with dilated CM and one, who was investigated as a fetus, had thickened myocardium and pericardial effusion [van Rahden et al. 2015]. There were also four male patients with cardiac involvement and it is noteworthy that one boy's fetal cardiac ventricular hypertrophy was spontaneously resolved (Table 10, Pt. No 4) [Torraco et al. 2016]. However, it is important to point out that it is often difficult to diagnose HCM and the vast majority of cases of HCM are identified postmortem or on examination of the native explanted heart during cardiac transplantation [Bird et al. 1994]; therefore, it is possible that some cases of HCM remain undetected, and are considered as some other type of CM.

Hematological manifestations in mitochondrial disorders, including CI deficiency, are aplastic, macrocytic, or sideroblastic anemia, leukopenia, neutropenia, thrombocytopenia or pancytopenia [Finsterer and Frank 2015]. In *NDUFB11* defects, it is essential to highlight that almost half of the previously described patients (all boys) had congenital sideroblastic anemia (7/15, 47%) [Lichtenstein et al. 2016; Torraco et al. 2016] with no other hematological manifestation. To determine the potential role of the *NDUFB11* gene in erythropoiesis, Lichtenstein et al. examined the phenotype of zebrafish *ndufb11* morphant embryos and found that partial knockdown of *ndufb11* resulted in a decrease in the number of erythroid cells and diminished hemoglobin, which indicates that the *NDUFB11* could have a phylogenetically conserved role in erythropoiesis [Lichtenstein et al. 2016]. This explains seven male patients' phenotype. Concurrently, there are no other abnormalities in erythropoiesis described in affected patients and no female patient has had congenital sideroblastic anemia.

Table 10. Detailed comparison of the clinical features associated with mutations in the *NDUFB11* gene.
CSF – cerebrospinal fluid; congen. – congenital; EEG – electroencephalogram; h – hour; hem – hemizygous; het – heterozygous; mo – month; N – normal; n.a. – not available; y – year; † – patients 7 and 8 are siblings; ††– two cases are from a single pedigree and they also have a heterozygous deletion of at least 70 kb at 2p16.3, containing part of the *NRXN1 gene*, which may have influenced the phenotype. Intragenic mutations and deletions in the *NRXN1* are enriched in cohorts with neurodevelopmental and autistic spectrum disorders

No.	Sex	cDNA (NM_019056.6)	Protein change	Inheri-tance	Age at last exami-nation	Age of onset	Skeletal muscle	Skin	MRT	Epileps	Develop-mental delay/intel-lectual disability	Eyes	Growth	Congen. sidero-blastic anemia	Lactic acidosis	Cardiac abnormality	Other anomalies	
1.	F	c.254G>A (het)	p.(Trp85*)	*de novo*	n.a.	n.a	n.a	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	histiocytoid cardio-myopathy		Shehata et al.
2.	F	c.262C>T (het)	p.(Arg88*)	*de novo*	6 mo	after birth	axial hypotonia	linear skin defect	n.a.	n.a.	n.a.	lacrimal-duct atresia	failure to thrive	no	no	histiocytoid cardio-myopathy	died at the age of 6 mo. Autopsy revealed thyroid abnormalities (large and multiple sites of oncocytic metaplasia)	van Rahden et al.
3.	F	c.262C>T (het)	p.(Arg88*)	*de novo*	13 mo	fetal	mild-to-moderate bulbar palsy	N	focal histiocytoid change in the choroid plexus of the brain	no	n.a.	Intermittent squint	n.a.	no	n.a.	histiocytoid cardio-myopathy	focal histiocytoid change in the thyroid and lungs.	Rea et al.
4.	M	c.276_278delCTT (hem)	p.(Phe93del)	*de novo*	9 y	fetal	N	N	N	no	no	n.a.	N	yes	4.2–8.2 mmol/L	fetal tachycardia and hypertrophy of the ventricular walls with moderate trabeculature of the right ventricle	dysmorphic features: hypertelorism, saddle nose, low set ears. Hepatospleno-megaly and hydrocele.	Torraco et al.
5.	M	c.276_278delCTT (hem)	p.(Phe93del)	*de novo*	2 y	after birth	n.a	N	n.a.	no	no	N	short stature	yes	no	no		Lichtenstein et al.
6.	M	c.276_278delCTT (hem)	p.(Phe93del)	from healthy mother	23 y	n.a.	myopathy	N	n.a.	no	no	N	N	yes	yes	no		Lichtenstein et al.
7.	M	c.276_278delCTT (hem)	p.(Phe93del)	from healthy mother †	20 y	fetal	n.a.	N	n.a.	no	yes	congen. optic atrophy	short stature	yes	no	no		Lichtenstein et al.
8.	M	c.276_278delCTT (hem)	p.(Phe93del)	from healthy mother †	16 y	fetal	n.a.	N	n.a.	no	yes	congen. optic atrophy	short stature	yes	no	no		Lichtenstein et al.

No.	Sex	cDNA (NM_019056.6)	Protein change	Inheri-tance	Age at last exami-nation	Age of onset	Skeletal muscle	Skin	MRT	Epileps	Develop-mental delay/intel-lectual disability	Eyes	Growth	Congen. sidero-blastic anemia	Lactic acidosis	Cardiac abnormality	Other anomalies	
9.	M	c.276_278delCTT (hem)	p.(Phe93del)	un-known	8 y	fetal	myopathy	N	n.a.	yes	no	N	N	yes	no	no	single kidney, pulmonary stenosis, congenial inguinal hernia	Lichtenstein et al.
10.	M	c.286T>C (hem)	p.(Ser96Pro)	*de novo*	16 y	3-4 y	N	N	mild cerebral atrophy	no	yes	optic atrophy	short stature	yes	CSF lactate 3.1 mmol/L (normal <2.1 mmol/L)	hypertrophic cardio-myopathy	joint contractures, non-progressive mild hearing impairment.	Patient 2
11.	F	c.324T>G (het)	p.(Tyr108*)	*de novo*	child	n.a	n.a	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	histiocytoid cardio-myopathy		Shehata et al.
12.	M	c.328C>T (hem)	p.(Pro110Ser)	*de novo*	3,5 y	after birth	N	N	N	no; EEG – mild back-ground activity depres-sion	no	N	N	no	Serum lactate 2-20 mmol/L	hypertrophic cardio-myopathy		Patient 1
13.	M	c.361G>A (hem)	p.(Glu131Lys)	*de novo*	55 h	fetal	n.a.	redun-dant skin	n.a.	n.a.	n.a.	n.a.	intraute rine growth restriction	n.a.	n.a.	heart failure	died at the age of 55 hours	Kohda et al.
14.	F	c.402delG (het)	p.(Arg134Serfs*3)	from healthy mother ††	4,5 y	n.a.	n.a.	linear skin defect	corpus-callosum agenesis and dilated lateral ventricles	yes	severe global development al delay	myopia, nystagmus, strabismus	failure to thrive	no	no	dilated cardio-myopathy		van Rahden et al.
15.	F	c.402delG (het)	p.(Arg134Serfs*3)	from healthy mother ††	pregnancy was terminated at the 24th week	fetal	n.a.	n.a.	corpus-callosum dysgenesis, connected lateral ventricles, a small cerebellum and the cavum septum pellucidum	n.a.	n.a.	n.a.	intrauterine growth restriction	n.a.	n.a.	thickened myocardium and pericardial effusion		van Rahden et al.

The neurological symptoms in patients with CI deficiency are observed to be variable, whereas muscle hypotonia is seen in 60% of patients and no other single sign is present in more than 35% of patients [Koene et al. 2012]. In those with *NDUFB11* mutations, only two previously described patients (2/15, 13%) have myopathy (one adult and one 8-year-old boy [Lichtenstein et al. 2016]); one infant had axial hypotonia (died at the age of six months [van Rahden et al. 2015]) and one with mild to moderate bulbar palsy [Rea et al. 2017]. Importantly, there are some less specific phenotypical features shared by most CI deficient patients such as brain MRI abnormalities, particularly signs of Leigh syndrome, which have been noted for more than 80% of CI-deficient patients [Fassone and Rahman 2012; Koene et al. 2012]. None of the previously described affected patients or carriers of mutation in the *NDUFB11* gene has been described with Leigh syndrome. MRI findings were described only in 4/15 cases with *NDUFB11* defects, and are rather rare containing histiocytoid change in the choroid plexus of the brain, mild cerebral atrophy and developmental malformation (including corpus callosum) [Rea et al. 2017; van Rahden et al. 2015].

The failure to thrive or short stature was described in 7/15 (47%) affected patients with *NDUFB11* defect (three females and four males), which is also a quite commonly (34%) reported clinical symptom in patients with nuclear-encoded CI deficiency [Koene et al. 2012]. Developmental delay or ID was described in analyzed cohort in three boys and one girl (4/15, 27%); epilepsy was even less common – one male and one female (2/15, 13%).

In the group of *NDUFB11* patients, only two patients died at an early age: one female (age six months [van Rahden et al. 2015]) and one male (age 55 hours [Kohda et al. 2016]); however, one pregnancy was also terminated at the 24th gestational week, due to multiple developmental malformations [van Rahden et al. 2015]. This data do not correspond with the CI deficiency, which often results in a fatal phenotype: approximately 75% of the patients die before the age of ten years, and nearly 50% of them before the age of two years [Koene et al. 2012].

It is widely known that mitochondrial diseases show a broad spectrum of clinical symptoms and we still cannot explain why identical mutations in the same gene can cause such phenotypic divergence (Table 10). Most of the affected *NDUFB11* males with sideroblastic anemia had several coexisting complaints such as myopathy, lactic acidosis, or short stature, with other neuro-developmental or organ dysgenesis. Similar considerable clinical overlap is seen in patients with HCM. Therefore, it should be recognized that patients with *NDUFB11* mutations can give rise to a variable clinical phenotype.

Gurok et al. noted that the *NDUFB11* gene is highly expressed in mice heart tissue [Gurok et al. 2007]. A higher expression level was also in kidney and skeletal muscle, which were rarely impaired in affected humans. Medium expression was found in the brain, whereas in all other organs it was weakly expressed. During mouse development, the transcript was detected at all stages, including embryonic development, with the highest expression at early age

[Gurok et al. 2007]. These results demonstrate serious disturbance in fetal development [van Rahden et al. 2015] and may also give a partial explanation of disappearance of some clinical manifestations (Table 10, Pt. No 4 [Torraco et al. 2016]).

There are different potential explanations for the wide phenotypic manifestation and one is somatic mosaicism [Rea et al. 2017; van Rahden et al. 2015]. Several studies have demonstrated X-inactivation in peripheral blood leukocytes, demonstrating the skewing toward the unaffected X chromosome [Lichtenstein et al. 2016; van Rahden et al. 2015]. This phenomenon could help to explain why healthy mothers and sister carry the same mutation as affected family members. Furthermore, phenotypic variability may reflect different abilities of developing tissues and organs in embryonic cells to handle a defective mitochondrial respiratory chain system; the phenotype can be influenced by the presence of additional variants, and environmental or epigenetic factors [van Rahden et al. 2015].

In conclusion, the mutations in the *NDUFB11* are the cause of isolated CI deficiency and similar to various other genes, which are affecting mitochondrial CI, there is a relative wide variation in clinical symptoms of affected patients. However, our findings present clinical distinctions of *NDUFB11*-related manifestation from other CI deficiencies, and highlight that histiocytoid cardiomyopathy and/or congenital sideroblastic anemia could be indicative of *NDUFB11* gene defects.

6. CONCLUSION

1. Based on our four-year study period, we can conclude that the choice of screening methodology and structure of our expanded newborn screening program has been justified in Estonia (Paper I and VI).
   a. We have screened 54,899 newborns and confirmed the diagnosis of 29 children, therefore the overall prevalence of congenital metabolic disorders identified with newborn screening in Estonia is 1:1,893 newborns.
   b. Unexpectedly, the most common disorder, which was identified with NBS, was congenital acquired vitamin B12 deficiency. The prevalence of this diagnosis, calculated for three years, is 1:2,959.
   c. The second most common detected group of disorders were HPA, as was assumed earlier.
   d. Based on the four-year NBS screening experience, we can affirm that the incidence of MCADD in Estonia remains rare, as was previously presumed. During that period, we did not diagnose any newborn with this condition.
   e. Over the four years, the FPR has remained to 0.1% and the PPV has risen to 36.7%. In the last (fourth) year, the FPR was 0.07% and the PPV was 52.6%. The overall specificity is 99.9% and since we still have not diagnosed any false negative case, the overall sensitivity of tandem MS screening remains 100%.

2. Based on our study results and previously published literature, we can conclude that, the WES analysis in clinical practice in patients with suspicion of childhood-onset MDs is providing very high effectiveness (Paper II).
   a. We found disease-causing gene variants in 17 out of 28 patients, therefore, the effectiveness of WES in patients with suspected MD in an onset of early childhood was high – 61%.
   b. An MD was found in only 14% (4/28) of the patients in nuclear DNA. Other variants found were associated with a neuromuscular disease, a neurodegenerative disorder, a multisystemic disease, and in one case with an isolated cardiomyopathy-causing gene.
   c. We have also successfully developed and conducted the analysis of mtDNA from standard WES reads and diagnosed one patient with mtDNA mutation (a point mutation in the *MT-ATP6* gene). This provides evidence that this method can be routinely used in clinical practice.
   d. Based on our results, we recommend the WES analysis as a first-tier method in clinical practice for children with any multisystem, neurological and/or neuromuscular problems as nuclear DNA variants are more common in children with MD.

3. Clinical and molecular investigations of two severely affected siblings revealed biallelic mutations in the *CACNA1A* gene, expanding the genotypic and phenotypic spectra of *CACNA1A*-related disorders (Paper III).
   a. Our study group presented for the first time two siblings with compound heterozygous mutations in the *CACNA1A* gene. All other variants, reported before, were only heterozygous state.
   b. Whole exome sequencing identified two previously unreported heterozygous mutations in our patient's *CACNA1A* gene: c.4315T>A p.(Trp1439Arg) in exon 27, and c.472_478delGCCTTCC p.(Ala158Thrfs_6) in exon 3.
   c. These semidominantly inherited mutations in the *CACNA1A* gene are likely to cause early onset epileptic encephalopathy, progressive cerebral, cerebellar, and optic nerve atrophy with severe muscular hypotonia and reduced lifespan.

4. Clinical, molecular and functional investigations revealed a novel *de novo* mutation in the *SLC25A4* gene, which causes a third distinct clinical phenotype associated with this gene (Paper IV).
   a. As a result of international cooperation, we evaluated the functional and clinical effect of a novel *de novo* heterozygous, single-nucleotide substitutions in the *SLC25A4* gene in seven patients.
   b. Four patients harbored a c.239G>A (p.Arg80His) mutation and three patients had a c.703C>G (p.Arg235Gly) mutation. Both mutations affected highly conserved and functionally important amino acid residues, associated with the loss of steady-state AAC1 levels in patients' skeletal muscle and diminished ATP transport activity in vitro.
   c. The phenotypes of all the affected individuals were very similar, presenting severe congenital hypotonia and profound muscle weakness, necessitating artificial ventilation, and causing frequent deaths in early infancy.
   d. Available muscle biopsies from the affected individuals revealed histopathological evidence of mitochondrial myopathy and a severe combined respiratory chain defect as a consequence of marked depletion of the mtDNA copy number.

5. Clinical, molecular, and functional investigations from two patients revealed two novel *de novo* mutations in the *NDUFB11* gene that cause mitochondrial CI deficiency. Our findings together with the review of the 13 previously described patients demonstrate a wide spectrum of clinical features associated with the *NDUFB11*-related CI deficiency (Paper V).
   a. Both patients had lactic acidosis, hypertrophic cardiomyopathy and isolated CI deficiency due to *de novo* hemizygous mutations (c.286C>T and c.328C>T) in the *NDUFB11* gene.
   b. The expression levels of the NDUFB11 protein was decreased in both patients' cells and a lentiviral complementation experiment demonstrated

that the CI deficiency in the two patients was caused by *NDUFB11* genetic defects.

c. All patients with the mutation in the *NDUFB11* gene (including previously described patients) present broad phenotypic divergence, which distinguishes it from other CI deficiencies. However, histiocytoid cardiomyopathy and/or congenital sideroblastic anemia could be indicative for mutation in the *NDUFB11* gene.

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WEB RESOURCES

CALIPER. Canadian Laboratory Initiative on Paediatric Reference Intervals. This project is a multi-centre, nation-wide initiative aimed at developing a comprehensive database of healthy reference values (reference intervals) for blood tests in children: http://www.sickkids.ca/caliperproject/index.html

Chromsystems. Chromsystems Instruments & Chemicals GmbH is a company, offering technologies for clinical analysis by HPLC and mass spectrometry, including validated and ready-to-use assays for newborn screening: https://www.chromsystems.com/products/newborn-screening.html

ClinVar. Database of genomic variation and its relationship to human health: https://www.ncbi.nlm.nih.gov/clinvar/

ERNDIM. The External Quality Assurance Programme for Amino Acids, Quantitative Organic Acids, Purines and Pyrimidines, Special Assays in Serum and Urine, Cystine in White Blood Cells and Lysosomal Enzymes: http://www.erndimqa.nl/

ExAC. The Exome Aggregation Consortium (ExAC) is a coalition of investigators seeking to aggregate and harmonize exome sequencing data from a variety of large-scale sequencing projects, and to make summary data available for the wider scientific community: http://exac.broadinstitute.org/

GATK. Variant discovery toolkit for high-throughput sequencing data: https://software.broadinstitute.org/gatk/

OMIM. Online Mendelian Inheritance in Man. An Online Catalog of Human Genes and Genetic Disorders: http://omim.org

Picard. A set of command line tools for manipulating high-throughput sequencing data: http://broadinstitute.github.io/picard/

R4S. Region 4 Stork is a collaborative laboratory performance improvement project focused on newborn screening: https://www.clir-r4s.org/

Statistics Estonia is a government agency in the area of administration of the Ministry of Finance. The main task of Statistics Estonia is to provide reliable and objective information on the economic, demographic, social and environmental situation and trends in Estonia: https://www.stat.ee/

SUMMARY IN ESTONIAN

Uued diagnoosimeetodid kaasasündinud ainevahetushaiguste varajaseks avastamiseks Eestis

Inimese tervisliku seisundi hindamisel peeti tema kehaeritisi väärtuslikuks infoallikaks juba Vana-Hiina kultuuris (1500–2000eKr), kuid tõeline revolutsioon organismi ainevahetuse (AV) toimimise osas saabus 20. sajandi alguses, kui saksa keemik Eduard Buchner avastas ensüümid ja nende funktsiooni. See teadmine nihutas edasiste uuringute fookuse rakusisestele protsessidele (sh ensüümaatilised ja biokeemilised reaktsioonid) ning peatselt avastas inglise arst Sir Archibald Garrod, et metaboolsete radade muutused on põhjustatud monogeensetest geenidefektidest [van der Greef et al. 2013]. Need teadmised panid aluse kaasaegsele teadusele pärilikest AV-haigustest.

Kuigi tegemist on kaasasündinud haigustega, ei ole enamikel vastsündinutel sünnihetkel mingeid viiteid sellele, kuna raseduse ajal kompenseerib ema organism transplatsentaarselt loote AV puudujäägid ning alles sünni järgselt võivad järk-järgult ilmneda haigusele iseloomulikud sümptomid, mis sageli on pöördumatud ja põhjustavad olulist eluea lühenemist. Kuid on AV-haigusi, nagu fenüülketonuuria (PKU), mis varajase diagnoosimisega on hästi ravitavad/kontrolli all hoitavad. 1960. aastate alguses leidiski aset põhimõtteline suunamuutus selliste haiguste diagnostikas, kui dr Robert Guthrie kirjeldas esmakordselt vastsündinutel PKU avastamise meetodit [Guthrie and Susi 1963]. See teadmine pani aluse vastsündinute sõeltestimisele (VS).

Järgmine oluline samm pärilike AV-haiguste diagnoosimisel toimus 1990-ndatel, kui VS-l võeti kasutusele tandem mass-spektromeetria (MS), millega oli võimalik ühest proovist ühe analüüsitsükli jooksul määrata >50 erinevat metaboliiti. See muutis VS senist kontseptsiooni, kus iga sõeltestitava haiguse määramiseks oli vaja üht meetodit, millega määrata üht metaboliiti [Chace D.H. 2005]. Tandem MS võimaldas sõeltestida ka väga haruldasi haigusi, mida eelnevalt ei oleks otstarbekuse kaalutlusel uuritavate haiguste loetellu lisatudki. Lisaks andis uus meetod võimaluse eristada iatrogeenseid muutusi VS-i tulemustes ning kirjeldada täpsemalt metaboolseid muutusi, mida üks või teine haigus endaga kaasa toob.

Eestis algas VS 1993. aastal ja seda vaid ühe haiguse osas, milleks oli PKU ning selleks kasutati fenüülalaniini määramist fluoromeetrilisel meetodil [Ounap et al. 1998] ehk siis üks meetod ühe haiguse sõeltestimiseks. Esimese süstemaatilise üle-eestilise andmeanalüüsi põhjal selgus, et PKU esinemissagedus Eestis on varasemast hinnangust oluliselt kõrgem – 1:6010 elussünni kohta [Ounap 1999]. Kolm aastat hiljem lisandus skriiningprogrammi kaasasündinud hüpotüreoos [Mikelsaar et al. 1998] ning sellisena toimus Eestis VS enam kui 20 aastat.

Tänapäeval on enamikes Lääne-Euroopa riikides ja Ameerika Ühendriikides kasutusel tandem MS ning ka sõeltestitavate haiguste arv küündib riigiti 29-ni

nagu Austrias [Burgard et al. 2012]. Seega vajas Eestis VS täiustamist, seda enam, et meil puudus ülevaade enamike sõeltestitavate haiguste esinemissageduste kohta siin regioonis ning tandem MS oli kliinilises töös (atsüülkarnitiinide profiili määramine seerumist) kasutusel juba aastast 2008 [Joost et al. 2012a]. Kahe rasvhapete beeta-oksüdatsioonidefektide gruppi kuuluva haiguse: keskmise ahelaga atsüül-CoA dehüdrogenaasi puudulikkuse (ingl k *medium-chain acyl-CoA dehydrogenase deficiency* ehk MCADD) ja pika ahelaga 3-hüdroksüatsüül-CoA dehüdrogenaasi puudulikkuse (*long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency* ehk LCHADD) esinemissagedused on meile arvutuslikult teada, kuna juba mõnda aega on meil olnud võimalik määrata mõlema haigusseoselise geeni (MCADD-i kahtlusel *ACADM* geenis ja LCHADD-i kahtlusel *HADHA* geenis) põhimutatsioone. Saadud andmete põhjal ilmnes, et *ACADM* geeni põhimutatsiooni c.985A>G (p.K329E) kandlus on Eestis 1:220, seega hinnanguliselt võiks MCADD-i esinemissagedus olla 1:193 000 [Lillevali 2000] ja *HADHA* geeni põhimutatsiooni c.1528G> C (p.E510Q) kandlust 1:173 ning arvestades, et Eesti LCHADD-i patsientidel moodustab põhimutatsioon 87,5% haiguse alleelidest, on LCHADD hinnanguline levimus Eestis 1:91 700 [Joost et al. 2012a].

Kahjuks ei ole kõik kaasasündinud AV-haigused sõeltestitavad ja ravitavad asümptoomses perioodis. Suure grupi selliseid kaasasündinud haigusi moodustavad mitokondriaalsed haigused (MH), mille esinemisel on molekulaarselt kinnitatud diagnoosini jõudmine vahel väga keeruline, kuna kliinilised probleemid ja nende algusaeg on äärmiselt varieeruv ka sama pere liikmete seas. Sestap on mitokondriaalsete haiguste diagnoosimiseks välja töötatud mitmeid juhiseid ja algoritme [Morava et al. 2006b; Nissenkorn et al. 1999; Wolf and Smeitink 2002]. Aastal 2012 töötati ka Eestis välja neuroloogiliste probleemidega laste hulgast MH tuvastamiseks algoritm, mis baseerus patsiendi kliinilise seisundi ning instrumentaalsete ja biokeemiliste uuringute tulemuste analüüsil, jõudmaks ühe geeni sekveneerimiseni [Joost et al. 2012b]. Selle uuringutöö raames (2003–2009) tuvastati Eestis lapseea-algusega MH esinemissagedus 1:20 764 [Joost 2012; Joost et al. 2012b]. Eelkirjeldatud meetod on enamjaolt ajamahukas, eeldab invasiivseid uuringuid ning tulemuslikkus jääb sageli tagasihoidlikuks [Neveling et al. 2013]. Sestap on viimastel aastatel järjest enam MH kahtlusega patsientide uurimiseks kasutama hakatud kogu eksoomi sekveneerimist (ingl k *whole exome sequencing* ehk WES) ning aastast 2013 on WES ka Eestis kasutusel.

Käesoleva uuringu eesmärgid

1. Hinnata vastsündinute laiendatud sõeltestimise programmi metoodikat ja efektiivsust Eestis (I ja VI artikkel).
2. Hinnata kogu eksoomi sekveneerimise efektiivsust kliinilises praktikas lapseeas alanud mitokondriaalse haiguse kahtlusega patsientide hulgas (II artikkel).
3. Hinnata ja kirjeldada esmakordselt *CACNA1A* geeni liitheterosügootse mutatsiooni kliinilist efekti (III artikkel).

4. Kirjeldada esmakordselt *SLC25A4* geeni uustekkese (*de novo*) mutatsiooni põhjustatud molekulaarseid muutusi ning sellest põhjustatud kliinilise fenotüübi kolmandat alavormi (IV artikkel).
5. Kirjeldada *NDUFB11* geeni funktsiooni, mutatsioonidest põhjustatud fenotüüpe ning võrrelda neid eelnevalt kirjeldatud fenotüüpidega (V artikkel).

Patsientide ja meetodite lühikirjeldus

Vastsündinute laiendatud sõeltestimine (VLS) Eestis algas 1. jaanuaril 2014a. ning esimesed 12 kuud toimus see pilootprojektina, mille jooksul sõeltestisime 13 643 vastsündinut. Sel perioodil loobusid 49 (0,36%) lapse vanemad sõeltestimisest. Alates 2015a. kuulub VLS Eesti Haigekassa poolt tasustatavate teenuste nimekirja ning nelja aasta (2014–2017) jooksul oleme sõeltestinud 54 899 vastsündinut. Selleks on kõigilt vastsündinutelt soovituslikult 3.–5. elupäeval kogutud kannast kapillaarveri Whatman 903® filterpaberile ning kuivatatud vereplekid on saadetud posti teel või kulleriga Eesti ainsasse VLS läbiviivasse laborisse – SA Tartu Ülikooli Kliinikumi ühendlabori kliinilise geneetika keskusesse (TÜK ÜL KGK). Analüüsimiseks kasutame Xevo TQD Triple Quadrupole tandem MS-i ning kommertsiaalset kitti – *MassChrom Kit,* millega määrame 13 aminohapet, 30 atsüülkarnitiini ning suktsinüülatsetooni. Tulemused on derivatiseeritud. Otsustuspiirid võtsime esialgu kirjandusest, kuid peale 1000 vastsündinu tulemuste laekumist kalkuleerisime esimest korda laborisisesed otsustuspiirid: 1. protsentiil vastab alumisele ja 99. protsentiil ülemisele otsustuspiirile. Andmete lisandumisel oleme neid korduvalt ümber arvutanud ning hetkel on kasutusel ligikaudu 30 000 vastsündinu tulemuste põhjal arvutatud otsustuspiirid.

Mitokondriaalse haiguse kahtlusega patsientide uuringugrupp koosneb kahest osast. Esimese osa moodustavad retrospektiivselt kliinilises töös uuritud patsiendid, keda on 2003–2013 aasta vahemikus TÜK ÜL KGK-s uuritud päriliku AV-haiguse suhtes ning kellel on jäänud kahtlus MH-le, kuid see pole molekulaarselt kinnitunud. Tavapraktika taoliste patsientide puhul on säilitada fibroblastide kultuur, mida oleks võimalik kasutada edasisteks uuringuteks, nagu ensüümi(-de) aktiivsuse määramine. Selliselt uuritud patsiente oli 181, kelle kliinilised andmed ja tehtud uuringud sai uuesti üle vaadatud/hinnatud ning ilmnes, et 21 patsiendi puhul püsis raviarstil jätkuvalt tugev kahtlus lapseea-algusega MH-le, kuid puudus molekulaarselt kinnitunud diagnoos. Neli väljavalitud patsienti jäid uuringugrupist välja kuna patsient loobus selles osalemast, puudus kontakt perekonnaga või olid kaebused taandunud. Teise grupi moodustavad 11 prospektiivselt (jaanuar 2014 – märts 2016) uuritud lapseea-algusega MH kahtlusega patsienti, kes kõik lisati uuringusse. Mõlema uuringugrupi patsiendid järjestasime kliiniliste andmete (sh instrumentaalsed ja biokeemilised uuringud) alusel kasutades Morava et al. poolt välja töötatud MH kliinilist skooringut [Morava et al. 2006b].

Kõikidele patsientidele teostati WES, DNA selleks eraldati vere lümfotsüütidest. Haigusseoselised leiud kinnitati ja perekondlik segregatsioonianalüüs tehti

Sanger sekveneerimisega. Mitokondriaalse DNA analüüs teostati samuti kõikidele patsientidele kasutades standardset WES-i.

Mitokondriaalse hingamisahela ensüümide aktiivsust mõõdeti fibroblastide kultuurist spektrofotomeetrilisel meetodil.

Peamised tulemused ja järeldused

1. Nelja-aastase uuringuperioodi tulemuste põhjal saame järeldada, et väljatöötatud vastsündinute laiendatud sõeltestimise meetodi valik ja ülesehitus on end Eestis õigustanud (I ja VI artikkel).
   a. VLS raames oleme uurinud 54 899 vastsündinut ning kinnitanud 29 lapsel kaasasündinud AV-haiguse, seega antud tulemuste põhjal hinnatuna on uuritavate haiguste üldine esinemissagedus Eestis 1:1893 vastsündinule.
   b. Meile ootamatult diagnoosisime kõige sagedamini kaasasündinud omandatud vitamiin B12 puudulikkust, mille esinemissagedus kolme aasta põhjal hinnatuna on 1:2959.
   c. Esinemissageduselt järgmise grupi moodustasid hüperfenüülalaninemiaga patsiendid. Antud tulemus on ootuspärane arvestades eelnevaid Eesti populatsiooni haaravaid uuringud.
   d. Tuginedes nelja-aastasele VLS-i tulemustele, võime kinnitada, et MCADD esinemine Eestis on haruldane. Antud ajaperioodil ei tuvastanud me ühtki selle diagnoosiga last.
   e. Nelja aasta jooksul on valepositiivsuse määr olnud 0,1% ja positiivne ennustusväärtus on tõusnud 36,7%-ni. Viimase (neljanda) aasta kokkuvõttes oli valepositiivsuse määr 0,07% ja positiivne ennustusväärtus 52,6%. VLS üldine spetsiifilisus on 99,9% ja kuna me ei ole ikka veel valenegatiivset juhtumit diagnoosinud, püsib tandem MS-ga sõeltestimise tundlikkus 100%.

2. Tuginedes meie uuringutulemustele ja varasemale sellekohasele kirjandusele, saame järeldada, et kliinilises töös on WES-i kasutamine lapseeaalgusega MH kahtlusega patsientidel väga hea efektiivsusega (II artikkel).
   a. Antud uuringugrupp koosnes 28 patsiendist, kellest 17-l tuvastasime WES-ga haigusseoselised geenimutatsioonid, seega on diagnostiline saagis väga kõrge – 61%.
   b. Vaid 14%-l (4/28) uuritavatest tuvastasime rakutuuma DNA-s mutatsioonid, mis põhjustavad MH-d. Ülejäänud tuumageenides tuvastatud mutatsioonid on seostatavad neuromuskulaarsete, neurodegeneratiivsete, multisüsteemsete või isoleeritud kardiomüopaatiat põhjustavate geenidega.
   c. Oma töös oleme edukalt välja töötanud ja kasutusele võtnud ka mtDNA analüüsimeetodi standardsel WES-l. Antud meetodiga tuvastasime ühel juhul mtDNA-s *MT-ATP6* geenis punktmutatsiooni, mis kinnitab meetodi tõhusust rutiinses kliinilises töös.

d. Tuginedes uuringutulemustele, oleme veendunud, et WES peaks olema esmane analüüs, mis kliinilises töös teostatakse lapsele, kellel esineb multisüsteemne, neuroloogiline ja/või neuromuskulaarne haigus. Seda enam, et lapseeas avalduva MH korral prevaleeruvad tuuma DNA-s esinevad mutatsioonid ning antud meetod võimaldab tuvastada punktmutatsioone ka mtDNA-s.

3. Kahe sama pere liikme (õe ja venna) kliiniline ja molekulaarne uurimine tuvastas *CACNA1A* geenis liitheterosügootsena mutatsioonid, mis laiendavad antud geeniga seotud genotüübi ja fenotüübi spektrit (artikkel III).
   a. Meie uuringugrupp kirjeldas esmakordselt kaht patsienti (õde ja vend), kellel esinevad liitheterosügootsena mutatsioonid *CACNA1A* geenis. Eelnevalt kirjeldatud patsientidel on tuvastatud *CACNA1A* geenis vaid heterosügootsed mutatsioonid.
   b. WES-ga tuvastati õel ja vennal *CACNA1A* geenis varem kirjeldamata heterosügootsed mutatsioonid: c.4315T>A p. (Trp1439Arg) 27. eksonis ja c.472_478delGCCTTCC p. (Ala158Thrfs_6) 3. eksonis.
   c. Eelkirjeldatud semidominantselt pärandunud mutatsioonid põhjustavad suure tõenäosusega varajase algusega epileptilist entsefalopaatiat, progresseeruvat suuraju, väikeaju ja *N. opticuse* atroofiat koos väljendunud lihashüpotoonia ja oluliselt lühenenud elueaga.

4. Kliinilised, molekulaarsed ja funktsionaalsed uuringud tuvastasid varem kirjeldamata uustekkese (*de novo*) mutatsioon *SLC25A4* geenis, mis põhjustab kolmanda, selgelt eelnevatest eristuva kliinilise fenotüübi (IV artikkel).
   a. Rahvusvahelises kootöös oleme hinnanud seitsmel patsiendil (sh üks patsient on Eestist) funktsionaalset ja kliinilist efekti varem kirjeldamata uustekkesest (*de novo*) ühenukleotiidse vahetusega mutatsioonist *SLC25A4* geenis.
   b. Neljal patsiendil selles grupis oli c.239G>A (p.Arg80His) mutatsioon ja kolmel c.703C>G (p.Arg235Gly) mutatsioon. Mõlemad mutatsioonid mõjutavad kõrgelt konserveerunud ja funktsionaalselt olulist aminohapete järjestust ning muutused antud piirkonnas on seotud stabiilse AAC1 valgu taseme langusega patsientide skeletilihastes ja seega on vähenenud ATP aktiivne transport *in vitro*.
   c. Kõigil uuringugrupis olevatel patsientidel esines sarnane fenotüüp: neil oli kaasasündinud väljendunud lihashüpotoonia ning lihasnõrkus, mistõttu vajasid nad mehhaanilist ventilatsiooni ja enamik suri varajases imikueas.
   d. Eelkirjeldatud geenimutatsioonidega patsientide lihasbiopsia materjali histopatoloogilisel uurimisel ilmnesid viited mitokondriaalsele müopaatiale. Edasisel uurimisel ilmnes hingamisahela ensüümide kombineeritud defekt, mis põhjustab olulise mtDNA koopiaarvu vähenemise.

5. Kliinilised, molekulaarsed ja funktsionaalsed uuringud tuvastasid kahel patsiendil varem kirjeldamata mutatsioonid *NDUFB11* geenis, mis põhjustavad isoleeritud mitokondriaalse hingamisahela esimese kompleksi puudulikkust. Meie poolt uuritud patsientide ja 13 eelnevalt kirjeldatud *NDUFB11* geenimutatsiooniga patsientide kliiniliste probleemide analüüsi põhjal saame öelda, et eelkirjeldatud geeni mutatsioonide korral esineb väga varieeruv kliiniline fenotüüp.
   a. Mõlemal meie poolt kirjeldatud patsiendil esines laktaatatsideemia, hüpertroofiline kardiomüopaatia ja isoleeritud mitokondriaalse hingamisahela esimese kompleksi puudulikkus, mis oli põhjustatud uustekkesest (*de novo*) hemisügootsest mutatsioonist (c.286C>T ja c.328C>T) *NDUFB11* geenis.
   b. Eksperimentaalsete uuringutega tõestasime, et mõlema patsiendi rakkudes oli NDUFB11 valgu ekspressioonitase langenud ning lentiviirus-vektoriga metsiktüüpi (*wild type*) alleeli tutvustamise järgselt mitokondriaalse hingamisahela esimese kompleksi aktiivsus taastus.
   c. *NDUFB11* geenimutatsioonidega patsientide fenotüübid on äärmiselt varieeruvad ja erinevad paljuski teistest isoleeritud mitokondriaalse hingamisahela esimese kompleksi puudulikkusega patsientidest. Samas histiotsütoidne kardiomüopaatia ja kaasasündinud sideroblastne aneemia võivad viidata *NDUFB11* geenimutatsioonile.

ACKNOWLEDGMENTS

The studies conducted in this thesis have been based on teamwork, with the contribution of many great people to whom I wish to express my deepest gratitude. In particular, I would like to thank my supervisor Professor Katrin Õunap for setting my focus and sharing her immense knowledge and insights to the world of science along with actuality. I am especially thankful for her everlasting enthusiasm which kept me going when times were tough. I can truly not imagine a better supervisor for my doctoral studies.

I would like to thank the reviewers of this thesis Professor Allen Kaasik and Professor Vallo Tillmann for finding the time to make many valuable comments and suggestions in order to improve this thesis.

This study would never have been possible without the help and contribution of my co-authors: Kadi Künnapas, Annika Kriisa, Ülle Murumets, Mari-Anne Vals, Kai Muru, Sanna Puusepp, Sander Pajusalu, Eva Õiglane-Šlik, Reet Rein, Inga Talvik, Richard J. Rodenburg, Ulvi Vaher, Anne Õunapuu, Margus Einok, Rita Teek, Tiiu Tomberg, Andres Piirsoo, Tiia Reimand, Reka Kovacs-Nagy, Margit Nõukas, Liesbeth T. Wintjes, Frans C.A. van den Brandt, Maaike Brink, Till Acker, Uwe Ahting, Andreas Hahn, Anne Schänzer, Tobias B Haack, Ursula Ilo, Kyle Thompson, and Robert W. Taylor. I am truly grateful to all the patients and their parents who participated in the studies and thus made this work possible.

I appreciate the cooperation with Dr Richard J. Rodenburg, whose feedback was always fast, honest and constructive. In addition, I would like to thank Prof Robert W. Taylor and Dr Kyle Thompson who introduced me the power of international cooperation. Furthermore, I received an unforgettable experience by visiting the Broad Institute of MIT and Harvard in Cambridge, MA, USA. The continuing collaboration with this team (especially Dr Monica Wojcik and Prof Daniel MacArthur) has instilled me even deeper interest in science without boundaries.

My true gratitude goes to my colleagues from the Department of Clinical Genetics in Tallinn at Tartu University Hospital who have shown sincere interest in my research, and supported me during my studies by encouraging me as well as enabling time for it. I am also grateful to my colleagues in Tartu, specially, Kadi Künnapas and Annika Kriisa for those alarming phone calls and the help you gave me at every turn; also to Tiina Kahre, Ülle Murumets and Hanno Roomere, who always happily accepted my confusing phone calls and did their utmost to understand me and find the best solution. I am grateful to Tiia Reimand, who repeatedly demonstrated that she deserves the middle name "CITO!" and to Pille Tammur for her delicacy and ability to make me feel calm; and Kati Kuuse, who took the trouble to correct my Estonian texts.

I highly appreciate the help and support from my colleagues who have become my friends: Reet Moor, Mari-Anne Vals, Kai Muru, Klari Noormets, Siret Saarsalu, Laura Roht and Sander Pajusalu. Those long motivating talks

have helped me to see a clearer and broader picture of what is really important in general and at the moment being.

I am very lucky to have such a patient, helpful and supportive family. I am deeply grateful to my parents Merle and Toivo for encouraging curiosity in me and guiding me instead of forcing. I would also like to thank my sister Kerli and her family, who constantly reminded me the real value of the family. I am glad that in my childhood, I had lots of interesting conversations with my paternal grandfather, I have just started to understand their wisdom and secrets. Finally, the highest appreciation, thankfulness and love belongs to my closest family – husband Raiko to whom I can always rely on and our little daughter Adeele, who did her best to keep me focused on my doctoral thesis.

This study was supported by the Estonian Research Council grant PUT355. During my PhD studies, I received support from the University of Tartu ASTRA Project PER ASPERA (European Union, European Regional Development Fund) and the national scholarship programs Kristjan Jaak and DoRa (Archimedes Foundation & Ministry of Education and Research of Estonia).

PUBLICATIONS

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List of publications (the first or shared first author in all articles)

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3. Thompson, K.; Majd, H.; Dallabona, C.; **Reinson, K.**; King, M.S.; Alston, C.L.; He, L.; Lodi, T.; Jones, S.A.; Fattal-Valevski, A.; Fraenkel, N.D.; Saada, A.; Chaham, A.; Isohanni, P.; Vara, R.; Barbosa, I.A.; Simpson, M.A.; Deshpande, C.; Puusepp, S.; Bonnen, P.E.; Rodenburg, R.J; Suomalainen, A.; Õunap, K.; Elpeleg, O.; Ferrero, I.; McFarland, R.; Kunji, E.R.S.; Taylor, R.W. (2016). *Recurrent de novo dominant mutations in SLC25A4 cause severe early-onset mitochondrial disease and loss of mitochondrial DNA copy number*. American Journal of Human Genetics, 99 (4), 860–876.10.1016/j.ajhg.2016.08.014.
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