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The genetic causes of mental retardation in Estonia: fragile X syndrome and creatine transporter defect

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to Karl, Janar and Siim,
their families

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals (I–VI), and some unpublished data:

I. Puusepp H, Kahre T, Sibul H, Soo V, Lind I, Raukas E, Õunap K. Prevalence of the Fragile X syndrome among Estonian mentally retarded and whole children's population. J Child Neurol 2008; 23: 1400–1405.

II. Puusepp H, Rein R, Zordania R, Kurvinen E, Jakovlev Ü, Õunap K. Fragiilse X sündroom Eestis: patsientide ja premutatsiooni kandjate kliinilised ja sotsiaalsed probleemid. Eesti Arst 2008; 87: 500–509 (in Estonian).

III. Puusepp H, Männik K, Zilina O, Parkel S, Kurg A, Õunap K. Vaimse arengu mahajäämuse geneetilised põhjused: X-liiteline vaimse arengu mahajäämus. Eesti Arst 2007; 86: 239–245 (in Estonian).

IV. Puusepp H, Kall K, Salomons GS, Talvik I, Männamaa M, Rein R, Jakobs C, Õunap K. The screening of *SLC6A8* deficiency among Estonian families with X-linked mental retardation. J Inher Metab Dis 2008; doi: 10.1007/s10545-008-1063-y.

V. Puusepp H, Zordania R, Paal M, Bartsch O, Õunap K. Girl with partial Turner syndrome and absence epilepsy. Pediatr Neurol 2008; 38: 289–92.

VI. Puusepp, H., Zilina, O., Teek, R., Männik, K., Parkel, S., Kruustük, K., Kuuse, K., Kurg, A. and Õunap, K.: 5.9 Mb microdeletion in chromosome band 17q22–q23.2 associated with tracheo-esophageal fistula and conductive hearing loss. Eur J Med Genet 2009; 52: 71–74.

ABBREVIATIONS

AAIDD	American Association on Intellectual and Developmental Disabilities
ADHD	Attention-Deficit Hyperactivity Disorder
AGAT	Arginine: Glycine Amidinotransferase
AS	Angelman syndrome
BMP	Bone Morphogenetic Protein
CGH	Comparative Genomic Hybridisation
CNV	Copy Number Variation
Cr	Creatine
Crn	Creatinine
CRTR	Cellular Creatine Transport
CT	Creatine Transporter
EEG	Electroencephalography
GAMT	Guanidinoacetate methyltransferase
FISH	Fluorescence In Situ Hybridization
FMRP	Fragile X Mental Retardation Protein
FMR1	Fragile X Mental Retardation gene-1
FoSTeS	Fork Stalling and Template Switching
FXS	Fragile X Syndrome
FXTAS	Fragile X-associated Tremor/Ataxia Syndrome
Gc	Crystallized Intelligence
Gf	Fluid Intelligence
ICD	International Classification of Disease
IQ	Intelligence Quotient
K-ABC	Kaufman Assessment Battery for Children
KAIT	Kaufman Adolescent and Adult Intelligence Test
LCR	Low Copy Repeats
LOH	Loss of Heterozygosity
MAPH	Multiplex Amplifiable Probe Hybridization
Mb	Megabases
MLPA	Multiplex Ligation-dependent Probe Amplification
MR	Mental Retardation
MRS	Proton Magnetic Resonance Spectroscopy
NAHR	Non-Allelic Homologous Recombination
NS-XLMR	Non-Syndromic X-Linked Mental Retardation
NHEJ	Non-Homologous End Joining
OA	Oesophageal Atresia
OFC	Occipitofrontal circumference
OMIM	Online Mendelian Inheritance in Man
PCr	Phosphorylcreatine
PCR	Polymerase Chain Reaction

PHOG	Pseudoautosomal Homeobox-containing Osteogenic Gene
POI	Premature Ovarian Insufficiency
PZA	Common vector-derived forward primer
PZB	Common vector-derived reverse primer
PWS	Prader-Willi syndrome
RPM	Raven Progressive Matrix
RT-qPCT	Real-Time quantitative Polymerase Chain Reaction
SD-SOE	Statistical Database of the Statistical Office of Estonia
SHOX	Short Stature Homeobox
SNP	Single Nucleotide Polymorphism
SpEd	Special Education
S-XLMR	Syndromic X-linked Mental Retardation
TEF	Tracheo-Esophageal Fistula
TGFβ	Transcription Growth Factor-beta
XLMR	X-linked Mental Retardation

1. INTRODUCTION

Mental retardation (MR) is one of the main causes of handicap among children and young adults. Despite extensive investigations, up to 80% of the people (Rauch *et al.*, 2006) do not have an accurate genetic diagnosis, leaving families without exact genetic counselling or reproductive options, such as prenatal diagnosis.

Intellectual disability is the currently preferred term for the disability historically referred to as mental retardation (AAIDD, 2002); however, mental retardation is a well-known, widely used term.

MR is one of the main causes for referral in paediatric, child-neurologic and clinical genetic practice. Although knowledge of the aetiology of MR usually does not allow for a treatment, it is helpful for disease management as well as for the family's acceptance of the disability and connection with other parents and support groups. Additionally, causal diagnosis provides a significant and long-lasting emotional relief for the parents (Lenhard *et al.*, 2005).

In Central Europe, healthcare expenditure on mental handicap as defined by the International Classification of Disease (ICD) accounts for about 8% of cost and by far exceeds the expenses that are related to any other ICD category (Ropers and Hamel, 2005). According to the data provided by Estonian Health Statistics (Egel *et al.*, 2007) the average length of stay in the hospital with a MR diagnosis was 18.6 (in 2002) to 36.7 days (in 2000). Furthermore, there has been in average 345 hospitalisations a year (2000–2004) with a MR diagnosis (by ICD-10 F70-F79). This data exemplifies that healthcare expenditure on individuals with MR are also very high in Estonia.

Without an accurate genetic diagnosis, it is difficult to give the exact recurrence risk to the family. Van Naarden Braun *et al.* (2005) found that the risk of having a second child with isolated mild MR if the first affected child had isolated mild MR was 7.1%, which was considerably higher than the recurrence risk for isolated severe MR (4.7%). Thus, in a standardised situation where the index patient is a boy with either mild or severe MR, published recurrence risks vary from 3.5 to 17.8% (Turner and Partington, 2000). Therefore, the main target for clinicians is to find out the exact aetiology for MR for providing the precise prenatal testing in next pregnancies.

The relatively high prevalence of MR, extensive investigations that still have not been able to give an accurate genetic diagnosis to a majority of families, high cost and burden to society and families – renders MR one of the most important unsolved problems in medicine.

Previously there has been only one study in Estonia which investigated the aetiology of MR (Mikelsaar, 1993). Main findings in this study were that in 8.4% of cases, the cause of MR was Mendelian disorder and in 2.7% of cases, syndromes with unknown genetic transmission were diagnosed. Furthermore, they found that chromosomal aberrations were detected more often among

children with MR (23.5%), compared to children with normal mental development (2.2%).

The present study was initiated to characterize two most common X-linked MR disorders: fragile X syndrome and creatine transporter deficiency in Estonia. In addition, our aim was to evaluate the correlation between clinical features and molecular finding in cases of rare submicroscopic chromosomal aberration, which causes MR in complicated dysmorphic patients.

2. LITERATURE REVIEW

2.1. Definition and classification of MR

MR is a lifelong human disability characterized by impairment of cognitive and adaptive skills. The definition of MR formulated by the American Association on Intellectual and Developmental Disabilities (2002) states that mental retardation 'is a disability characterized by significant limitations both in intellectual functioning and in adaptive behaviour as expressed in conceptual, social, and practical adaptive skills. This disability originates before the age of 18.'

General intellectual functioning is defined by the intelligence quotient (IQ). IQ across the population is normally distributed with the mean set at 100 and an IQ of less than 70 classified as MR. Mild MR is defined as an IQ of 50–55 to approximately 70. It is characterized by a few months delay of developmental milestones in childhood and MR obvious from age 1–2 years. In adulthood, there are very simple reading, writing, and math abilities, but good understanding including long sentences. Moderate MR is an IQ of 35–40 to 50–55. Several months delayed developmental milestones characterize it in childhood and MR is obvious from age 1 year. An adult person understands almost everything, but uses small sentences and many signs. Severe MR is described as an IQ of 20–25 to 35–40. It can be outlined with delayed developmental milestones from several months up to one year, and MR is obvious before age 1 year. An adult person understands simple, daily sentences and single words, but uses sentences of 2–3 words, and many signs. While severely retarded person is mostly able to walk, then profoundly MR person with IQ of less than 20–25, walks rarely and usually not before age 5 years without an aid. Additionally, a person with profound MR shows minor or no response to the surroundings. (Zhang *et al.*, 2005; Raymond, 2006)

2.2. Prevalence of MR

Estimates of MR prevalence vary widely between epidemiological studies. In Western countries, the prevalence of MR is 1.5–2%, and 0.3–0.5 are severely impaired (Leonard and Wen, 2002), but these numbers vary in different studies from one to ten per cent (Curry *et al.*, 1997; Battaglia *et al.*, 1999; Stevenson, 2000; Leonard and Wen, 2002; Flint and Knight, 2003; Ropers and Hamel, 2005). However, the prevalence of MR tends to be even higher in developed countries. By now it has been revealed that males are about 1.4 times more likely to have severe MR and 1.9 times more likely to have mild MR than females (Croen *et al.*, 2001). The overall proportion of severely retarded boys and girls has been found to be 3:2 (Laxova *et al.*, 1977) and at the same time the sex-specific population prevalence of MR is shown to be 1.13% for males and 0.87% for females (Stevenson and Schwartz, 2002).

According to Estonian Health Statistics (Egel *et al.*, 2007) in years 2000–2004 the number of all new cases of mental and behavioural disorders (in the ICD-10 with codes F70-F79) per 100,000 population increased by 32%. The largest number of mental and behavioural disorders is diagnosed in boys 5–14 years of age, after that age the number of new diagnosed cases decreases (Egel *et al.*, 2007). Also, in children (age 0–14) psychological development disorders make up to 40% of all new cases of mental and behavioural disorders (Egel *et al.*, 2007).

Furthermore, according to the Statistical Database of the Statistical Office of Estonia (SD-SOE, http://www.stat.ee) there were 65,728 live births in years 2000 to 2004. The incidence of MR among children within the same years was reported to be 2111 (Egel *et al.*, 2007). The estimated prevalence of MR children in years 2000 and 2004 is 3.21%. Compared to previously published data, where approximately 2–3% of the population in developed countries have mild to moderate intellectual disability and 0.5–1% of the population have moderate to severe MR (Raymond, 2006), we can assume that the prevalence of MR in Estonia is similar to MR prevalence in developed countries.

2.3. Etiological causes of MR

The underlying causes of MR are extremely heterogeneous, and they can be genetic and non-genetic factors. There are several non-genetic factors that act prenatally or during early infancy to cause brain injury, these include infectious diseases (such as cytomegalovirus infection during pregnancy and postnatal meningitis), very premature birth, perinatal asphyxia, and foetal alcohol syndrome, which is caused by excessive maternal alcohol consumption during pregnancy (Chelly and Mandel, 2001). The use of artificial reproductive technology, is another factor with possible implication for MR, being 2.6 times higher than in spontaneous pregnancies (Menezo *et al.*, 2000). Furthermore, prevalence of MR has been associated with low socioeconomic status, low maternal education, low birth weight and older maternal age at delivery (Croen *et al.*, 2001; Leonard and Wen, 2002). In addition, increased risk for mild MR has been found for multiple births, second or later-born children; and for severe MR has been observed for children with Hispanic, African or Asian mothers (Croen *et al.*, 2001).

Age can be one of the factors for having child with MR. For example, the germ-line mutation rate in human males, especially in older males, is generally much higher than in females, mainly because in males there are many more germ-cell divisions (Crow, 2000). There is no evidence for a paternal age effect on the *de novo* rate of genomic rearrangements (Lupski, 2007a). Nevertheless, it has been revealed that the rate of occurrence of deletions is higher in females than in males. To illustrate, in Duchenne muscular dystrophy the majority of deletions arise in oogenesis, and most point mutations stem from spermato-genesis (Grimm *et al.*, 1994). Moreover, the best-known example of a maternal age effect is Down syndrome (Hassold *et al.*, 1996).

A genetic aetiology has been identified in more than half of MR cases (Leonard and Wen, 2002). A large study was done by Baird *et al.* (1988), who found that the overall rate for cases with congenital anomalies and/or MR having a genetic aetiology of some kind was found to be 2.7% of live births; furthermore, total single-gene disorders accounted for 3.6/1,000, consisting of autosomal dominant (1.4/1,000), autosomal recessive (1.7/1,000), and X-linked recessive disorder (0.5/1,000). Although, the human X-chromosome carries only about 4% of the protein-coding genes in the human genome, X-linked gene defects are thought to be responsible for about 8–12% of the MR found in males (Ropers and Hamel, 2005). Previously, Zechner *et al.* (2001) examined entries in the Online Mendelian Inheritance in Man (OMIM) database that are associated with MR, and they found that compared with autosomes, the X chromosome contains a significantly higher number of genes that, when mutated, cause MR. In addition, chromosomal anomalies accounted for 1.8/1,000, and multifactorial disorders 46.4/1,000 (Baird *et al.*, 1988). To illustrate further the importance of genetic factors in human mental development, for IQ the proportion of genetic factors are found to increase from 30% at age 5, to 80% at age 12 years (Polderman *et al.*, 2006). The finding in OMIM of 1546 entries, searching for "mental retardation", also reflects the high frequency of the involvement of genes in MR aetiology (January 18, 2009).

Genomic rearrangements, being high-frequent events, play a major role in the pathogenesis of human genetic diseases with MR. Although, most of the mutations have a very small effect and to a large extent are compensated by environmental improvements; mild mutations are disproportionately frequent, and that disproportion increases as mutations without effect are investigated (Crow, 2000). Genomic rearrangements are thought to occur frequently enough that both inherited and *de novo* events can be observed in the same family (Lupski, 2007a). For example, in the diploid genome of a newborn there may be one segmental deletion in between 1 in 25 and 1 in 4 newborns (Lupski, 2007a). Furthermore, Bugge *et al.* (2000) found that 36% of translocations were *de novo*, 11% were of maternal, 25% were of paternal and the rest were unknown origin. Also, in study performed by Rauch *et al.* (2006), about 40% of apparently balanced translocations/insertions occurred *de novo* and were obviously all disease-causing by disruption of a single gene. Flint *et al.* (2003) summarized that unbalanced translocations account for 54% of cases, deletions 39% and duplications 6%. In almost all cases, unbalanced translocations occur because a parent carries the balanced form. In addition, chromosomal deletions are found to have more severe phenotypic consequences, are smaller in size and have been reported for less of the genome than chromosomal duplications (Brewer *et al.*, 1998, 1999).

Over 90% of all spontaneous mutations are single nucleotide substitutions; and these are about 25 times more common than all other mutations, deletions are about three times more common than insertions, and complex mutations are

very rare (Kondrashov, 2003). The total number of new mutations per diploid human genome per generation is found to be about 100 (Kondrashov, 2003).

However despite new developed methods, no definite aetiology was found in between 32% and 75% of children with MR (Leonard and Wen, 2002). For example, in more recent study the aetiology of MR was not found in 59.3% of cases (Rauch *et al.*, 2006). The underlying cause of MR remains unknown in up to 80% of patients with mild MR (Rauch *et al.*, 2006).

2.4. X-linked mental retardation

The concept of X-linked MR (XLMR) is based on the observation that MR is more common in males than in females, with an excess of 30% (Turner and Turner, 1974) and the large number of multigenerational pedigrees in which MR segregates in an X-linked pattern; that is with males predominantly affected, absence of male-to-male transmission, and carrier females normal or less severely affected than males (Stevenson and Schwartz, 2002). The excess is found among those with mild MR and those with severe MR (Stevenson and Schwartz, 2002) and this prevalence of males over females has been shown also previously in Estonia (Egel *et al.*, 2007). The major surveys of MR report 4.8% of cases resulting from XLMR (Laxova *et al.*, 1977); however, in more recent studies this number is estimated to be between 5% and 13% (Stevenson *et al.*, 2000; Stevenson and Schwartz, 2002).

XLMR is divided into "syndromic" and "non-syndromic" or "non-specific" forms. In syndromic forms (S-XLMR), MR is present in association with a specific pattern of physical, neurological, and/or metabolic abnormalities (Renieri *et al.*, 2005). In non-syndromic (NS-XLMR) forms, MR is the only phenotype. However, the clinical diagnosis of XLMR is still usually a diagnosis of exclusion of other causes of developmental delay in a male (Raymond, 2006). The main reason for this is that XLMR is very heterogeneous, and about two-third of patients have clinically indistinguishable NS-XLMR (Fishburn *et al.*, 1983).

In the past 18 years, great advances have been made in the identification of molecular bases of XLMR. Compared with autosomes, the X chromosome contains a significantly higher number of genes that, when mutated, cause MR. Approximately 4.6% (1,344) out of more than 28,900 protein-coding genes or sequences presently listed in ENSEMBL (http://www.ensembl.org/index.html) are located on chromosome X. The average X-linked disease has an incidence of ~1 in 30,000–100,000 males (an incidence about tenfold lower than that of the fragile X syndrome), and a similar frequency is likely for most of XLMR genes (Chelly and Mandel, 2001). Chiurazzi *et al.* (2008) presented a map of the 82 XLMR genes cloned by November 2007; furthermore, 215 XLMR conditions had been listed. These conditions were subdivided according to their clinical presentation: 149 with specific clinical findings, including 98 syndro-

mes and 51 neuromuscular conditions, and 66 NS-XLMR forms (Chiurazzi *et al.*, 2008). However, there are thought to be 931 genes in chromosome X (Skuse, 2005), approximately 3.75% of all genes; and 100–130 of those genes are probably contributing to monogenetic XLMR (Kleefstra and Hamel, 2005). In addition, cause is still unknown in about 100 S-XLMR conditions (Chelly and Mandel, 2001).

The most prevalent X-linked syndrome is fragile X syndrome (FXS), which may account for 25% of all families with XLMR (Fishburn *et al.*, 1983). *ARX* mutations are the second most prevalent, giving rise to non-syndromic XLMR and to a variety of syndromic forms in more than 5% of the families (Gecz *et al.*, 2006). *CUL4B, JARID1C* and *SLC6A8* mutations are also relatively frequent, each accounting for 2–3% of the XLMR families. Defects of all other known XLMR genes seem to be significantly less common, in the 1% range or below (Ropers, 2008).

Mutations in the following X-linked genes have been associated with a NS-XLMR phenotype: *FMR2, PAK3, GDI1, IL1RAPL1, RSK2, ATRX, ARHGEF6, MECP2, TM4SF2, SLC6A8, FACL4, FGD1, ARX, XNP, AGTR2, ZNF41, ZNF81, ZNF674, GRIA3, NLGN4, FTSJ1, DLG3, JARID1C,* and *PQBP1* (Ropers and Hamel, 2005; Ropers, 2006). Some of those genes are associated with S-XLMR too: *RSK2* (Merienne *et al.*, 1999), *MECP2* (Meloni *et al.*, 2000; Orrico *et al.*, 2000; Couvert *et al.*, 2001), *SLC6A8* (Salomons *et al.*, 2001), *ARX* (Bienvenu *et al.*, 2002; Stromme *et al.*, 2002), *ATRX* (Yntema *et al.*, 2002), *AGTR2* (Vervoort *et al.*, 2002), and *FGD1* (Lebel *et al.*, 2002). Moreover, Ropers *et al.* (2003) showed mutations that give rise to NS-XLMR are not evenly distributed along the length of the X-chromosome. They indicated that NS-XLMR mutations are clustered in three X-chromosomal regions, with the Xp11.2-p11.3 region carrying almost 30% of all gene defects. The other two loci that they found a very high concentration of NS-XLMR genes are Xq28, where *FMR2, GDI1, MECP2* and *SLC6A8* genes have been identified and Xp22.1-p21.3.1, distal to the gene for Duchenne muscular dystrophy, which includes *ARX* and *IL1RAPL1* gene.

Common additional features of XLMR are microcepahaly, seizures, behavioural problems, including autistic behaviour, speech delay or absent speech, and spastic paraplegia (Raymond, 2006). At a younger age when the MR has been usually diagnosed, the proportion of patients with apparently NS-XLMR is most probably even higher, because syndrome-specific symptoms (for example, characteristic facial features and large testicles in FXS) are not always recognizable in early childhood. Although, FXS is considered to be the most frequent example of S-XLMR and in MR testing for FXS is generally part of the diagnostic routine, it is only found in 2% of intellectually handicapped males (Biancalana *et al.*, 2004; Gronskov *et al.*, 2004). Furthermore, 46% of the mothers of MR males were of borderline or mildly retarded intelligence (Fishburn *et al.*, 1983). Interestingly, it has been revealed that recognized forms of XLMR do not account for the 20%–

40% excess of male patients who have been reported in the major surveys conducted over the past 20 years (Hahn *et al.*, 2002).

We have published an article (Publication III) that contains a short summary of the most frequent XLMR syndromes: FXS, Rett, Coffin-Lowry, Aarskog-Scott, ATR-X, West, and Partington syndrome, X-linked infantile spasms or other forms of epilepsy, X-linked cleft-lip-palate and *L1CAM* gene mutations in order to introduce them to family doctors and paediatricians (Puusepp *et al.*, 2007). In addition, we described already known autism correlations with XLMR. In this thesis, we focused on two of the most common forms of monogenic X-linked disorders that are causing MR, fragile X syndrome (OMIM 300624) and X-linked creatine transporter deficiency (OMIM 300352).

2.4.1. Fragile X syndrome

2.4.1.1. Genetic mechanisms of fragile X syndrome

In 1943, Martin and Bell (1943) described sex linked MR without dysmorphic features in a family in which both affected males and females were observed. Thirty-six years later Lubs (1969) reported a marker X chromosome (later to be known as the fragile X chromosome) as an inconsistent finding in cytogenetic studies in leucocytes of some MR males. The gene involved in the fragile X syndrome (FXS, OMIM 300624), the Fragile X Mental Retardation gene (*FMR1,* OMIM 309550) was identified in 1991 (Kremer *et al.*, 1991; Oberle *et al.*, 1991; Verkerk *et al.*, 1991; Yu *et al.*, 1991).

FXS is the most common X-linked monogenic diseases and also one of the most common monogenic causes of inherited MR. This syndrome is caused by an unstable CGG trinucleotide repeat in the *FMR1* gene at Xq27.3 (Kremer *et al.*, 1991; Oberle *et al.*, 1991; Verkerk *et al.*, 1991; Yu *et al.*, 1991). The FXS CGG repeat has four forms: common (6–40 repeats) – there is no risk of being a carrier and having FXS; intermediate (41–60 repeats) – unstable, causes very rarely FXS in the next generation; premutation (61–200 (230) repeats) – unstable, the larger is the number of CGG repeats the higher risk is having a child with FXS in the next generation; and full mutation (>200–230 repeats). Full mutation in *FMR1* causes methylation of the gene and production failure of FMRP (fragile X mental retardation protein) (Hinds *et al.*, 1993). *FMR1* gene is widely expressed in the brain, testes, placenta, lung and kidney (Hinds *et al.*, 1993).

The CGG repeat number varies in the normal population. For example, Brown *et al.* (1993) analyzed 570 pregnant women and found that the most common number of CGG repeats in Caucasians is 30. The same results were reported in Greece, where they found that the most common alleles in the Hellenic population in Greece for *FMR1* repeats in females were 29/30 (15.3% for heterozygous) and 30/30 (27.6% for homozygous) (Patsalis *et al.*, 1999). In

the populations of China (Zhong *et al.*, 1999), India (Sharma *et al.*, 2001) and the former Republic of Yugoslav (Major *et al.*, 2003) the most prevalent CGG repeat number has been shown to be 29.

As mentioned, premutation and intermediate alleles are also susceptible to disorder-causing expansion in the next generation. By now it is known, that 59 repeats is the smallest size known to expand to the FXS causing mutation in the next generations within a family (Nolin *et al.*, 1996; Maddalena *et al.*, 2001), and it was seen in 13.4% of children from premutation mothers. The highest risk (97.3%) of expansion to full mutation was reported to be in those families where the mother carries a premutation with 90–199 repeats (Nolin *et al.*, 1996). In the same study a majority (62%) of daughters of premutation fathers had a larger repeat number, while a few had either smaller (22%) or the same (16%) repeat number, compared to their father's sizes. The contraction size of CGG repeats in the daughters of premutation males was in a range of 2–20, with a mean of 10, and the range of expansion was found to be 2–54, with the mean of 18. An interesting finding from this study was that the contractions did not continue in the next generation, in which a full mutation was inherited from many of the daughters who carried a contraction. Furthermore, they suggest that the reversion rate of a premutation allele to a normal allele is low (0.76%) (Nolin *et al.*, 1996).

The genetics of FXS are unusual: approximately 35% of carrier females exhibit MR while 21% of males with the fragile X chromosome are non-penetrant, phenotypically normal individuals (Sherman *et al.*, 1985). These males will transmit the chromosome to their daughters, who are consistently unaffected, and they may have affected grandsons. This phenomenon of anticipation, with the risk of MR in fragile X pedigrees appearing to be contingent upon the position of individuals in the pedigrees, has commonly been referred to as the "Sherman paradox" (Fu *et al.*, 1991).

2.4.1.2. The incidence of FXS

The assumption, at the beginning of the 1990s was that FXS is the most common form of inherited MR associated with a wide range of developmental disabilities and behavioural and physical features, in both males and females (Hagerman *et al.*, 1991). The first estimates of prevalence of FXS, based on cytogenetic testing, were 1:1,000–1:2,600 for males (Turner *et al.*, 1986; Webb *et al.*, 1986b), but this has proved to be less specific than expected. Turner *et al.* (1996) realized in 1996 that a more realistic figure based on molecular analysis is 1:4,000. More recent large studies suggest that prevalence of the full mutation ranges from 1:3,717 to 1:8,918 Caucasian males in the general population (Crawford *et al.*, 2001) (Table 1). The carrier state for females is about 1 in 300 (Stevenson and Schwartz, 2002). According to recent estimates by Hagerman (2008) the expected frequency of FXS full mutation males and females is 1:2,355 and the expected frequency for premutation males is 1:282.

Table 1. Prevalence of the FXS among males determined by cytogenetic and DNA-based techniques. Adapted from Crawford *et al.* (2001).

Country	Method	Repeats/ homo	Years	Estimated character	Target population	No. Positive/ No. tested	Estimated prevalence	References
U.K. (Wessex)	Southern, PCR	65% – 29,30,31	1994–1999	Population based	Special Education (SpEd) population (ages 5–18) years, unknown aetiology	20/3,738	1/5,530	(Murray *et al.*, 1996; Youings *et al.*, 2000)
UK (Coventry)	Cytogenetic	–	–	Population incidence	Children identified as handicapped in the school population and in residential accommodation with a Coventry address, age 11–16 years	16/219	1/1,360 (boys)	(Webb *et al.*, 1986a)
U.S.A. (Atlanta, Georgia), Caucasian	Fluoresc. PCR, Southern	–	1993	Population prevalence	SpEd population (public school children, aged 7–10 years, in SpEd-needs classes)	4/1,979 (940 Caucasians)	1/3,460	(Meadows *et al.*, 1996; Crawford *et al.*, 1999)
USA, South Carolina	Cytogenetic	–	1982–1987	Incidence	MR institutions and community program	28/1,865 (Black and Caucasians)	MR:2,5–3,3%	(Schwartz *et al.*, 1988)
USA, New York	Cytogenetic	–	1991	Population screening	Males with MR of unknown aetiology	43/489	–	(Nolin *et al.*, 1992)
Hellenic population of Greece and Cyprus	PCR, Southern	30	–	Population prevalence	Referred clinical population of idiopathic MR, age 3–25 years	8/611	1/4,246	(Patsalis *et al.*, 1999)
Hellenic population	PCR, Southern	–	–	Population incidence	Unselected individuals, age 3–25 years, with nonspecific MR from various parts of Greece and Cyprus	4(full)+1(pre)/ 257	1.94% (boys)	(Syrrou *et al.*, 1998)
Australia (Sydney)	Cytogenetic	–	–	Population prevalence	Children with MR in SpEd Mild handicapped aged 8–12 years and moderate and severely handicapped aged 5–16 years	10/472	1/2610	(Turner *et al.*, 1986)
Australia (Sydney)	Southern	–	–	Prevalence in MR	Re-examined Turner *et al.* 1986 mildly retarded 8–12 years group	5/323	1/4,350 males	(Turner *et al.*, 1996)
Australia, NSW	Cytogenetic	–	1984–1992	Population based	Intellectually handicapped people	253/14,225 (214/8,671 males)		(Turner *et al.*, 1992)

Table 1. Continued

Country	Method	Repeats/ homo	Years	Estimated character	Target population	No. Positive/ No. tested	Estimated prevalence	References
Yugoslav	PCR	29	–	Population prevalence	Males with non-specific MR	2/97	MR:2,56%	(Major *et al.*, 2003)
Netherlands	PCR, Southern	–	1992–1996	Population prevalence	Schools and institutes for the MR	32 prev.+ 11 new (9 males)/2189	1/6,045 for males	(de Vries *et al.*, 1997; de Vries *et al.*, 1999)
India, New Delhi	Southern, PCR	29	–	Estimated frequency	MR individuals from SpEd schools, institutionalized MR individuals	9/93 (130 individuals)	MR:8%	(Sharma *et al.*, 2001)
Brazil	PCR, Southern	–	–	Prevalence among insti-tutionalized individuals	Institutionalized individuals with severe nonspecific MR, aged 1–68 years	0/85(47 fem/38 male)	–	(Mulatinho *et al.*, 2000)
Brazil	PCR	–	–	–	Patients with idiopathic MR	7/51	–	(Christofolini *et al.*, 2006)
Southern Häme, Finland	Southern	–	–	Population prevalence	Adult males (>16 years) registered in the Southern Häme Care Organization with MR, unknown aetiology	6/344	1/4,400	(Arvio *et al.*, 1997)
Denmark (Funen)	Cytogenetic	–	1980–1992	Population prevalence	Children born 1978–80 (age 8–10 years) and administratively classified as educationally subnormal.	0/67 boys	0,04/1000 – previously registered 9 males from that region	(Tranebjaerg *et al.*, 1994)
China	Southern, PCR	29	–	Population prevalence	MR individuals attending SpEd schools	32/1,127	MR:2,8%	(Zhong *et al.*, 1999)
Poland (Warsaw)	Southern, PCR	–	1995	Population prevalence	Males in institutions or SpEd with MR	6/201	1/2,857–1/5,882	(Mazurczak *et al.*, 1996)

Table 1. Continued

Country	Method	Repeats/ homo	Years	Estimated character	Target population	No. Positive/ No. tested	Estimated prevalence	References
Argentina	Southern, PCR	–	–	–	Patients with clinical diagnosis of fragile X syndrome were referred from various health-centres in Argentina	14/240	Clinical diagnosis of FXS:6%	(Florencia *et al.*, 2006)
Spain	PCR, Southern	–	–	Population prevalence	Children in SpEd or clinically referred with MR of unknown aetiology; no known family history of MR	5/180	1/6,200–1/8,200	(Millan *et al.*, 1999)
Canada	Mini-Southern, PCR	–	–	Population incidence	Mother-offspring pairs	1/24,446	1:24,446	(Rousseau *et al.*, 2007)

2.4.1.3. Clinical picture of males with FXS

The "classical" phenotype of males with FXS is that of a moderately retarded adult male with large testes and typical, long face (Fryns, 1984). However, the clinical phenotype of FXS is not as simple as thought at the very beginning when this syndrome was discovered. For example, a long narrow face, prominent jaw and prominent ears are often not present in the prepubertal children (Hagerman and Hagerman, 2002). In the facial phenotype of young boys with FXS, puffiness around the eyes, strabismus, narrow and elongated palpebral fissures, high-arched palate, a large head relative to body size, and prominent ears are more frequently found (Goldson and Hagerman, 1992) (see Table 5).

Macroorchidism has been described as one of the cardinal characteristics of males with FXS (Turner *et al.*, 1975; Fryns, 1984), and it is present in more than 80% of adult males with FXS (Merenstein *et al.*, 1996). However, it is not a good diagnostic marker for FXS in boys younger than 8 years, because it has been shown that macroorchidism did not occur until the age of 6, and usually did not occur until after age 8 years in boys with FXS (Lachiewicz and Dawson, 1994). Also this symptom is not specific to FXS (Hagerman *et al.*, 1988). Testicular biopsies have been taken to reveal the cause of the macroorchidism and they have shown the thickened peritubular basement membranes, slight increase in peritubular collagen fibers and diffuse hyperplasia of the interstitial cells (Turner *et al.*, 1975). Interestingly, the sperm of males with the full mutation contains the premutation even though the full mutation is present in all other tissues (Reyniers *et al.*, 1993). This means that all daughters of FXS males are premutation carriers and sons are healthy. But Gu *et al.* (2006) investigated five FXS subjects aged between 18–60 and found all of them to have erectile dysfunction. However, males with FXS carrying mosaic mutation have been documented to be fertile and capable of reproduction (Willems *et al.*, 1992; Rousseau *et al.*, 1994).

MR and behavioural problems dominate in the clinical presentation of FXS. For young males who do not demonstrate macroorchidism or long narrow face it may be one of the main indicators for testing for FXS (de Vries *et al.*, 1998). Typical behaviour in boys includes poor eye contact, tactile defensiveness, hand flapping, hand biting and perseverant speech; and it can be seen in 60–90% of FXS boys (Hagerman *et al.*, 1986). Although FXS individuals are generally friendly, some may show aggressive behaviour. At the same time, hyperactivity and short attention span are also very frequent findings in FXS boys, but actually these are common paediatric problems and not specific to FXS. Moreover tantrums, which may be caused by hypersensitivity to situations with excess auditory, visual, or tactile stimuli, are also quite frequent problems (Hagerman and Hagerman, 2002).

FXS has been associated with other developmental disabilities, such as autism. Previously, autistic-like features have been seen in almost all of FXS

patients. These include poor eye contact, shyness, hand flapping, hand biting and hand stereotypes (Hagerman *et al.*, 1986). However, the majority of patients with FXS do not demonstrate the core social deficits that are typical to autism (Hagerman and Hagerman, 2002); and only 5.4% of males with autism have been shown to test positive for FXS (Fisch, 1992). Furthermore language delays, not speaking in sentences by two to three years of age, with language peculiarities, such as echolalia, have been noticed in majority of children with FXS, and these are more often seen at age two to five years.

FXS males show different level of intellectual disability, being in most of the prepubertal boys moderate, and in adults moderate to severe (de Vries *et al.*, 1998). High functioning FXS males are more common in children aged 7 years or younger (Hagerman *et al.*, 1994) and the highest cognitive abilities (IQ score) is between ages 10–15 years (Dykens *et al.*, 1989). In addition, Fisch *et al.* (1996) has shown declines in IQ scores, verbal reasoning, visual/spatial reasoning, quantitative reasoning, short-term memory, daily living skills, communication and socialization (mean interest interval was 2.3 years). However, males with a full mutation have lower IQ scores than males with a mosaic pattern (Staley *et al.*, 1993).

The most common neurologic abnormality is seizures, they have been reported in 8–22% of FXS patients (Musumeci *et al.*, 1999; Lachiewicz *et al.*, 2000; Hagerman and Hagerman, 2002). However, previous studies have shown that seizures never started before the age of 2–4 years or after the age of 8–9 years (Kluger *et al.*, 1996; Musumeci *et al.*, 1999). The same authors reveal that seizures in FXS are frequently of the complex partial type and less frequently of the partial motor and generalized type and they are usually well controlled by anticonvulsants. In addition, Kluger *et al.* (1996) indicate that epilepsy in FXS is partially age dependent and this finding was supported by Musumeci *et al.* (1999), who found that spikes in electroencephalography (EEG) tended to disappear in adulthood but that when present they were usually non-specific, rare and limited to only one location.

Different additional clinical problems have been described in males with FXS. Firstly, orthopaedic problems and connective tissue dysplasia have been reported in males with FXS. For example, 50–70% of the males have flat feet and in 57–67% excessive laxity of the joints (hyperextensible metacarpophalangeal joints) has been found (Davids *et al.*, 1990; Hagerman and Hagerman, 2002). Scoliosis, *pectus excavatum* and *cubitus valgus* are also very common musculoskeletal problems. Furthermore, patients with FXS have also distinctive skin manifestations. The skin, especially on hands, is soft and smooth; the palms may occasionally appear wrinkled and in some cases, single palmar crease is present. A callus is also often seen on the hands from hand biting (Hagerman and Hagerman, 2002).

Moreover, connective tissue dysplasia can cause cardiac problems to patients with FXS. In previous studies, 55% of FXS patients have been reported to have

mitral valve prolapse (Loehr *et al.*, 1986). The same authors found that the frequency of mitral valve prolapse was equal in males and females with FXS, but in 80% of males older than 18 years, it was diagnosed. Ten per cent of the male patients with FXS less than age 18 years have been shown to have aortic root dilatation, whereas 33% of those older than 18 years have demonstrated this abnormality (Loehr *et al.*, 1986).

Equally important recurrent otitis media is found to occur in 63% of pre-pubertal boys with FXS compared to 15% of their normal siblings and 38% of developmentally disabled children without FXS (Hagerman *et al.*, 1987). Same authors concluded that males with FXS are at higher risk for recurrent ear disease, which may exacerbate the cognitive and behaviour problems. Additionally, recurrent otitis media has been associated with fluctuating conductive hearing loss and subsequent language and articulation deficits (Bennett and Haggard, 1999). The exact reason why children with FXS are predisposed to recurrent otitis media infections is unknown. It has been associated with the facial structure, including long face and a high-arched palate, that may affect the angle of the Eustachian tube and prevent appropriate drainage of the middle ear (Hagerman and Hagerman, 2002). The frequent otitis media and sinusitis in affected children require adequate intervention (antibiotics or polyethylene tubes or both) (Hagerman *et al.*, 1987).

In addition, ophthalmological problems have been found in approximately one third of the children with FXS. For example, in 17% hyperopia and astigmatism; and in 8% up to 36% strabismus has been described (Hatton *et al.*, 1998; Hagerman and Hagerman, 2002), showing much higher prevalence than in the normal population (0.5–1%) (Hatton *et al.*, 1998). To demonstrate this further, 22% of FXS males have been reported to wear glasses (Hagerman and Hagerman, 2002).

2.4.1.4. Clinical problems of females with FXS full mutation

Females with full mutation have a wide spectrum of phenotype ranging from severely affected as mild to very mild or undetectable, and it is because they have two X chromosomes. Clinical evaluation of females with FXS reveals similar phenotype to males with FXS. Females are found to have pre- and postnatal overgrowth, macrocephaly, large ears and long face (Borghgraef *et al.*, 1990). For behavioural characteristics a mild to moderate attention deficit disorder, hyperactivity, nervous body movements, anxiety and sudden, unexpected bursts of panic, inappropriate laughter or giggling, extreme shyness, avoided social interaction and short and fugitive eye-contact has been described previously in females with FXS (Borghgraef *et al.*, 1990).

2.4.1.5. Clinical problems of females with FXS premutation

For a long time it was presumed that carriers of the FXS premutation are healthy; however, premutation has been found to be a risk factor for diminished ovarian function at a relatively young age. Premature ovarian insufficiency (POI) is defined as a condition of spontaneous menopause, or secondary hypergonadotropic hypoestrogenic amenorrhoea that occurs before the age of 40 years (Hundscheid *et al.*, 2001), in other words these females are infertile. POI has been estimated to occur in approximately 1% of the general population, but about 16–24% of females with the premutation experience early menopause (Schwartz *et al.*, 1994; Allingham-Hawkins *et al.*, 1999; Murray *et al.*, 2000; Sherman *et al.*, 2007). Hundscheid *et al.* (2001) found a serum FSH of ≥ 15 IU/l in 25% of menstruating premutation carriers before age 40 years, which suggests that premutation carriers have a poorer prognosis for future pregnancy and refers to ovarian ageing. Females with premutation are also reported to have irregular menses and use of hormones to regulate menses more commonly than non-carriers (Schwartz *et al.*, 1994). In the same study both FXS and premutation carriers are shown to have similar frequency of spontaneous abortions (9% and 10%, respectively) and their frequencies were lower than in the control group (15%).

Even though, individuals with the premutation have been considered to be cognitively unaffected (Mazzocco *et al.*, 1993; Reiss *et al.*, 1993) there is plenty of evidence that premutation carriers have a cognitive phenotype similar to the full mutation. For example, in approximately 40–56% of premutation carriers who were mothers of FXS children, affective disorders have been diagnosed (Franke *et al.*, 1996; Franke *et al.*, 1998); and these include unipolar affective disorder that was diagnosed in 21.3%, major depression in 19.7% and bipolar affective disorder in 11.5% of subjects. Furthermore, anxiety disorder was found in 41% of premutation carriers who had son with FXS, and the most prevalent of those was social phobia that was diagnosed in 18% of females (Franke *et al.*, 1998).

2.4.1.6. Tremor/ataxia syndrome in adult FXS premutation carriers

Although, male carriers of the FXS are spared from the serious neurodevelopmental problems that the full mutation carriers are having, some adult premutation carriers develop a neurological syndrome called fragile X-associated tremor/ataxia syndrome (FXTAS) (Hagerman *et al.*, 2001). It has been predicted that as many as ~1:3,000 males aged >50 years in the general population may have the carrier-specific FXTAS (Hagerman, 2008). Interestingly, the pattern of deficits is similar in females, to that observed in males, but the X-inactivation ratio has an impact on the prevalence and degree of severity of symptoms in females (Hagerman *et al.*, 2004; Berry-Kravis *et al.*, 2005). Premutation carrier

can develop a progressive action tremor, cerebellar dysfunction, cognitive decline, loss of distal reflexes and reduction in vibratory sense (Hagerman *et al.*, 2001; Greco *et al.*, 2006). However, slow intention tremor and gait ataxia are reported to be the most obvious clinical features in FXTAS. Also progressive cognitive decline with memory loss, decline in executive functioning, and eventual dementia can be present. The patients also appear to experience psychological symptoms, including anxiety, reclusive behaviour, and irritability or mood lability (Hagerman and Hagerman, 2004). Problems related to autonomic dysfunction also appear to be associated with FXTAS; these include impotence, bowel incontinence, urinary incontinence, and hypertension (Hagerman *et al.*, 2001). Some individuals are reported to have atypical Parkinson disease – masked facies, resting tremor, or increased tone (Hagerman *et al.*, 2001). Magnetic resonance imaging of affected patients demonstrate mild to moderate brain atrophy, which includes the cerebellum, ventriculomegaly and scattered peri-ventricular white matter disease (Greco *et al.*, 2006).

2.4.1.7. Genetic testing for FXS

Testing for FXS is commonly done to cases with MR, with generally little clinical pre-selection (Chelly and Mandel, 2001). Although, severely MR children are more likely to be analysed, physicians should also think about high functioning FXS males, who are more common in children aged 7 years or younger (Hagerman *et al.*, 1994). Furthermore, declines in IQ have been shown in FXS males (Fisch *et al.*, 1996), therefore the diagnosis as early as possible is critically important, because special education and training in younger age could improve the language and motor development. CGG-repeat expansion mutations account for more than 99% of cases of FXS and DNA based tests that detect and measure the CGG-repeat region of the *FMR1* gene are more than 99% sensitive. Despite positive results are 100% specific (Maddalena *et al.*, 2001) this testing has a consequently low rate for positive diagnoses (~2–5%) (Chelly and Mandel, 2001). Still, this test miss point mutations and deletions in the *FMR1* gene that would result in the same clinical outcome (Chelly and Mandel, 2001).

2.4.2. X-linked creatine transporter deficiency

2.4.2.1. Creatine metabolism

Three inherited disorders of creatine metabolism have been described. Among those, two enzymatic defects of creatine biosynthesis are autosomal recessively inherited – guanidinoacetate methyltransferase (GAMT; OMIM 601240) (Stockler *et al.*, 1996) and arginine:glycine amidinotransferase deficiency (AGAT; OMIM

602360) (Item *et al.*, 2001). The third disease is X-linked creatine transporter deficiency (OMIM 300352) caused by mutations in the *SLC6A8* gene (OMIM 300036) (Salomons *et al.*, 2001; Bizzi *et al.*, 2002). The *SLC6A8* gene has been mapped to Xq28 and encodes the creatine transporter-1 (CT1) (Gregor *et al.*, 1995) for a protein that transports creatine into cells. This gene consists of 13 exons (GenBank accession number Z66539), spans ~8.4 kb, and encodes a protein of 635 amino acids with a predicted molecular weight of 70 kDa (Sandoval *et al.*, 1996).

Humans maintain their creatine pool by biosynthesis and nutritional uptake. Approximately 50% of the required creatine is derived from the diet (mainly animal protein). The highest levels of creatine (Cr) and phosphorylcreatine (PCr) are found in skeletal muscle, heart, and photoreceptor cells of the retina. Cr is synthesized mainly in liver and pancreas by the action of AGAT and GAMT. Cr reaches muscle and brain through an active transmembrane creatine transport system (CRTR), and then it is utilized in the cellular pool of creatine/ creatine-phosphate, which, together with creatine kinase and ATP/ADP, provides a high-energy-phosphate buffering system. Intracellular Cr and PCr are nonenzymatically converted to creatinine (Crn) at an almost steady rate (~2% of total Cr per day). Then Cr diffuses out of the cells and excretes in urine. Urinary creatine excretion is directly related to the intracellular creatine pool. (Wyss and Kaddurah-Daouk, 2000; Stromberger *et al.*, 2003)

Eichler *et al.* (1996) revealed identical Xq28 and 16p11.1 genomic structures for both the *SLC6A8* gene and five exons of the *SLC6A10* gene (*CT2*). Overall nucleotide similarity within the duplication was found to be 94.6%. The same authors detected that both of these genes are transcriptionally active, but predicted amino-acid composition of the *SLC6A10*, identifies the presence of a `premature` stop codon in exon 4. This suggests that the 16p11.1 creatine transporter is non-functional or that it encodes a truncated four trans-membrane domain protein. In addition, Iyer *et al.* (1996) found that *SLC6A10* gene is expressed in testis only. However, recently Bayou *et al.* (2008) described an autistic patient with a translocation and disruption of *SLC6A8* paralogous gene, and suggested that this gene may be expressed in human brain.

2.4.2.2. The prevalence of creatine transporter defect

As far as we know only six studies (Rosenberg *et al.*, 2004; Newmeyer *et al.*, 2005; Clark *et al.*, 2006; Lion-Francois *et al.*, 2006; Arias *et al.*, 2007; Betsalel *et al.*, 2008) have been performed investigating the prevalence of *SLC6A8* mutation, which causes X-linked creatine transporter (CT) defect. Mutations in the *SLC6A8* gene have been found in families with XLMR, as well as in males with sporadic MR (Clark *et al.*, 2006). The largest study in number was performed by Arias *et al.* (2007), who found the prevalence of *SLC6A8* deficiency to be 0.25% among males with MR and/or autism. However, in an

other study about 1% of males with MR of unknown aetiology were found to have a *SLC6A8* mutation (Clark *et al.*, 2006). The prevalence among XLMR patients has been in the range from 1.5% (Betsalel *et al.*, 2008) up to 5.4% (Lion-Francois *et al.*, 2006) making it one of the most frequent identifiable causes of XLMR. Rosenberg *et al.* (2004) identified 6 pathogenic mutations in 288 patients from the European XLMR Consortium making an average prevalence of *SLC6A8* of 2.1%.

2.4.2.3. Clinical features of creatine transporter defect

Data from affected adults suggest that brain creatine deficiency syndrome (GAMT and AGAT deficiency, and *SLC6A8* defect) is a systemic disease with a slow progressive course (Rosenberg *et al.*, 2004). The most common clinical characteristic of all creatine deficiency syndromes are MR and speech delay (Salomons *et al.*, 2003). Patients with AGAT and CT deficiency may additionally have epileptic seizures that respond satisfactorily to common antiepileptic drug (Schiaffino *et al.*, 2005). GAMT-deficient patients and CT deficient patients may show autistic behaviour; furthermore, in patients with severe phenotype, (extra) pyramidal symptoms are present (Salomons *et al.*, 2003; Stromberger *et al.*, 2003; Anselm *et al.*, 2006). In patients with CT deficiency at childhood age, the phenotype seems to be different from that seen in patients at older age (Kleefstra and Hamel, 2005). In childhood, the phenotype comprises low weight, low height, poor muscle build, hypotonia, seizures, expressive language delay, movement disorder, and behaviour problems (Hahn *et al.*, 2002; Mancini *et al.*, 2005; Clark *et al.*, 2006). However, at adult age short stature, ptosis, myopathic facies, midface hypoplasia, soft skin, hypotonia, intestinal problems, spastic/ dystonic gait, seizures, and mood/behaviour problems can be seen (Hahn *et al.*, 2002; Anselm *et al.*, 2006; Clark *et al.*, 2006). Also, large testicles have been described in one case (Clark *et al.*, 2006).

The behavioural phenotype of children with a CT defect is described as a developmental disability in which the domains of attention, impulse control, speech, oral movements and language are more affected than other cognitive and motor functions (deGrauw *et al.*, 2002; Mancini *et al.*, 2005). It has been assumed that this specific combination is unique to the CT deficiency syndrome (Mancini *et al.*, 2005). In addition, previously aggressive behaviour has been described (Clark *et al.*, 2006).

Approximately 50% of female carriers of *SLC6A8* mutation show learning disabilities and/or behavioural problems (Hahn *et al.*, 2002; Salomons *et al.*, 2003). For example, two of the female relatives of the patient described by deGrauw *et al.* (2003) demonstrated intact visual-perceptual and visual-constructional ability and fine motor skills. At the same time patient's mother and maternal grandmother had a history of severe learning disability (deGrauw *et al.*, 2003).

Table 2. Pathogenic mutations and sequence variations of the *SLC6A8* gene.

Pathogenic	Mutation	Deduced effect	Non-pathogenic	Sequence variation
(Anselm *et al.*, 2006)	Deletion of 1–13 Deletion of 8–13	Deletion of 1–13 Deletion of 8–13	(Rosenberg *et al.*, 2004; Clark *et al.*, 2006; Rosenberg *et al.*, 2007)	c.1678A>G
(Cecil *et al.*, 2001; Salomons *et al.*, 2001; deGrauw *et al.*, 2003; Rosenberg *et al.*, 2006)	p.Arg514X	c.1540C>T	(Rosenberg *et al.*, 2004; Rosenberg *et al.*, 2007)	c.1885G>A
(Schiaffino *et al.*, 2005)	c.263–2A>G	IVS1–2A>G	(Rosenberg *et al.*, 2007)	c.11A>G
(Kleefstra *et al.*, 2005)	C337W		(Rosenberg *et al.*, 2007)	c.76G>A
(Rosenberg *et al.*, 2004; Rosenberg *et al.*, 2007)	c.1661C>T	p.Pro554Leu		
(Rosenberg *et al.*, 2004; Rosenberg *et al.*, 2007)	c.259G>A	p.Gly87Arg	(Clark *et al.*, 2006)	c.544G>A
(Rosenberg *et al.*, 2007)	C321_323delCTT	p.Phe107del	(Clark *et al.*, 2006)	c.813C>T
(Rosenberg *et al.*, 2004; Rosenberg *et al.*, 2007)	c.950_951insA	p.Tyr317X	(Rosenberg *et al.*, 2004; Clark *et al.*, 2006)	c.1494C>T
(Rosenberg *et al.*, 2004; Rosenberg *et al.*, 2007)	c.1011C>G	p.Cys337Trp	(Clark *et al.*, 2006)	c.603C>T
(Clark *et al.*, 2006; Rosenberg *et al.*, 2007)	c.1040_1042delTCA	p.Ile347del	(Clark *et al.*, 2006)	c.780C>T
(Rosenberg *et al.*, 2004; Rosenberg *et al.*, 2007)	c.1169C>T	p.Pro390Leu	(Clark *et al.*, 2006)	c.1437C>T
(Clark *et al.*, 2006; Rosenberg *et al.*, 2007)	c.1171C>T	p.Arg391Trp	(Clark *et al.*, 2006)	c.1662G>A
(Clark *et al.*, 2006; Battini *et al.*, 2007; Rosenberg *et al.*, 2007)	c.1006_1008delAAC	p.Asn336del	(Clark *et al.*, 2006)	c.262+53G>C
(Clark *et al.*, 2006)	c.1016+2C>T		(Clark *et al.*, 2006)	c.1016+41_45dup TGCCC
(Clark *et al.*, 2006)	c.11A>G	p.Lys4Arg	(Clark *et al.*, 2006)	c.1017–38C>G
(Clark *et al.*, 2006)	c.76G>A	p.Gly26Arg	(Clark *et al.*, 2006)	c.1142–130C>T
(Poo-Arguelles *et al.*, 2006)	c.878–879delTC	p.Lys293fsX3	(Clark *et al.*, 2006)	c.1254+39G>T
(Bizzi *et al.*, 2002; Poo-Arguelles *et al.*, 2006)	c.1221_1223delCCT	p.Phe408del	(Clark *et al.*, 2006)	c.1255–59C>T
(Anselm *et al.*, 2008)	c.1690_1730del	p.Phe564fs	(Clark *et al.*, 2006)	c.1392+31T>C
(van der Knaap *et al.*, 2000)	c.327G>A		(Clark *et al.*, 2006)	c.1496–5C>T

Table 2. Continued

Pathogenic	Mutation	Deduced effect	Non-pathogenic	Sequence variation
(Hahn *et al.*, 2002)	c.114G>C	p.Gly381Arg Exon 7/intron 7 splice error	(Rosenberg *et al.*, 2004)	IVS7–99C>A
(deGrauw *et al.*, 2002; Pyne-Geithman *et al.*, 2004; Rosenberg *et al.*, 2004)	c.319_321delCTT	p.F107del	(Rosenberg *et al.*, 2004)	IVS8–35G>A
(Betsalel *et al.*, 2008)	c.1059_1061delCTT	p.Phe354del	(Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004)	IVS10–18C>T IVS11+21G>A
(Mancini *et al.*, 2005; Mancardi *et al.*, 2007)	c.1631C>T	p.Pro544Leu	(Rosenberg *et al.*, 2004)	IVS12+15C>T
(Mancini *et al.*, 2005)	c.1495+5G>C	IVS10+5G>10	(Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004) (Rosenberg *et al.*, 2004)	207G>C IVS12+32C>A IVS1+26G>A IVS7+37G>A IVS7+87A>G IVS7–35G>A IVS12–3C>T IVS2+88G>C IVS9–36G>A IVS12–82G>C IVS8+28C>T c.1416G>A IVS6+9C>T IVS7–151_152delGA

2.4.2.4. Treatment of creatine transporter defect

Many authors state that Cr supplementation of *SLC6A8* deficient patients is inefficient (Cecil *et al.*, 2001; Bizzi *et al.*, 2002; deGrauw *et al.*, 2002; Newmeyer *et al.*, 2005; Poo-Arguelles *et al.*, 2006; Anselm *et al.*, 2008). Moreover, creatine treatment in CT deficient patients have been shown not to improve the neurological symptoms and no observable increase in brain creatine with in vivo proton magnetic resonance spectroscopy (MRS) has been found (Cecil *et al.*, 2001). In addition, it has been associated with increase in body weight (Poo-Arguelles *et al.*, 2006). However, AGAT- and GAMT-deficient patients can be treated with oral Cr supplementation (Bianchi *et al.*, 2007; Braissant and Henry, 2008). Even though in AGAT- and GAMT-deficient patients very high doses of creatine are being used, the replenishment of cerebral creatine takes months and results only in the partial restoration of the cerebral creatine pool (Stockler *et al.*, 1996; Item *et al.*, 2001; Battini *et al.*, 2002). For example, Bianchi *et al.* (2007) showed creatine and phosphocreatine rise to 90% of normal when AGAT-deficient-children were supplemented with a Cr intake of 400 mg/kg/ body weight/day. At the same time, a male patient with GAMT treated with the same amount of Cr, had a corresponding brain total creatine of 71% and phosphocreatine of 65% of the mean normal values.

2.4.2.5. Diagnostics of creatine transporter defect

There are three worldwide acknowledged and recommended tests for initial testing (a schematic diagram for screening for *SLC6A8* deficiency in MR patients can be found from Rosenberg *et al.*, 2007). First analysing method is urine metabolite (guanidinoacetate, creatine and creatinine) analysis, more specifically an elevated urinary creatine:creatinine ratio is measured and it is found to be a strong indicator for *SLC6A8* deficiency (Almeida *et al.*, 2004). Variation of the compounds during the day was found to be not very significant, indicating that a random urine sample is sufficient for the diagnosis of creatine deficiency syndromes (Almeida *et al.*, 2004). However, an elevated urinary creatine as false positive finding have been found in 1.8% of the patients, since the creatinine ratio normalizes in repeat samples (Arias *et al.*, 2007). The same authors also showed that a high-protein intake – meal based on beef or oily fish – result in false positive results in 4 out of 13 non-affected individuals. Furthermore, in urine both guanidinoacetate concentration and Cr:Crn ratio decrease as age increases (Arias *et al.*, 2004).

Secondly, in vivo proton MRS of the brain, which shows a severe reduction or absence of creatine (Newmeyer *et al.*, 2005). It can be used for diagnosing two inborn errors in creatine biosynthesis and also CT defect (Verhoeven *et al.*, 2005), although this method alone cannot always distinguish between synthesis

and transport defects. Disadvantage of this method is that MRS is only available in specialized institutes and usually sedation is needed for patients with MR.

Thirdly, DNA sequence analysis of *SLC6A8* gene is used. The broad spectrum of sequence variants in the *SLC6A8* gene in (XL)MR patients causes problems with regard to classification of these changes as pathogenic mutations or non-pathogenic variants/polymorphisms (Rosenberg *et al.*, 2007). Throughout exons 1–13 of the coding region of the *SLC6A8* gene, almost 35 pathogenic mutations and more than 40 different sequence variations have been identified (Table 2). Clear pathogenic DNA variants are deletions, a frameshift mutation, splice errors, and nonsense mutations, but missense mutations are more difficult to interpret. Also, however, Rosenberg *et al.* (2004) were able to investigate only 93% of the coding sequence.

It has been suggested that all male patients with MR, autistic behaviour, epilepsy and/or expressive speech and language delay should be tested for creatine deficiency disorders (Stockler *et al.*, 2007).

2.5. Chromosomal anomalies

2.5.1. Chromosomal anomalies detectable by routine cytogenetic analysis

Chromosomal analysis is the most widespread routine genetic test in the world, offered by hundreds of diagnostic laboratories. Despite new and more accurate technologies that have been developed, karyotyping is still so widely used method because large chromosomal aberrations detectable by chromosomal analysis are most common known cause of MR (Chelly and Mandel, 2001; Rauch *et al.*, 2006). According to different authors, cytogenetically visible chromosomal aberrations are contributing in 4–28% of MR (Laxova *et al.*, 1977; Curry *et al.*, 1997; Leonard and Wen, 2002; Rauch *et al.*, 2006), and have been found in one out of seven individuals with severe cognitive impairment (Leonard and Wen, 2002).

Many different syndromes result from complete trisomies, partial trisomies, and deletions of the various chromosomes. The most common trisomy is Down syndrome with an incidence of 1 in 1,000 (Laxova *et al.*, 1977), similar results were also found in an Estonian study where overall livebirth prevalence of Down syndrome was found to be 1.17 per 1000 livebirths (Reimand *et al.*, 2006). The most prevalent chromosomal deletion is complete or partial monosomy X or Turner syndrome (Lippe, 1991), with the incidence of 1 in 2500 live female births (Hook and Warburton, 1983).

Moreover, it has been estimated that micro-rearrangements occur in about 6–8% of the MR population (Shaffer and Lupski, 2000; de Vries *et al.*, 2001). A study performed by Rauch *et al.* (2006) showed that after Down syndrome, the

most prevalent causes of MR were the common microdeletion 22q11.2 (2.4%), Williams-Beuren syndrome (1.3%), and monosomy 1p36.3 (0.6%). Furthermore, disease associated balanced chromosomal rearrangements are found to occur in an average of 0.3% (Bugge *et al.*, 2000), being also a considerable cause of developmental delay.

2.5.2. Subtelomeric investigations and chromosomal abnormalities detectable by array-based methods

Although large changes in DNA are usually detected with regular cytogenetic analysis (> 5 megabases – Mb), for small aberrations (<3 Mb), methods with higher resolution are needed. Approximately half of the chromosomal aberrations are cytogenetically visible, the other half is cryptic, and since the introduction of array-CGH, it is becoming even more clear that submicroscopic deletions and duplications are equally frequent (Ropers, 2008). Interstitial chromosomal duplications and deletions account for 4–5% of unexplained MR, implying that the total contribution of subtelomeric and interstitial chromosomal abnormalities is in the range of 10–15% (Vissers *et al.*, 2003; Koolen *et al.*, 2004).

Small chromosomal rearrangements involving the (sub)telomeres have been found in association with idiopathic MR. The frequency has been reported to be 5%, increasing to 7% in patients with moderate to severe developmental delay (Flint and Knight, 2003). Chromosomal rearrangements involving the ends of chromosomes (subtelomeres) are important cause of human genetic disease. Once recognisable syndromes have been excluded, abnormalities that include the ends of chromosomes are the commonest cause of MR in children with undiagnosed moderate to severe MR (Knight *et al.*, 1999). Knight *et al.* (1999) found by using multiple FISH assay that compared to 0% in controls, the prevalence of subtelomeric chromosomal rearrangements was 7.4% in children with moderate MR and 0.5% for children with mild MR. In another study a microdeletion syndrome was diagnosed in 5.3%, while subtelomeric screening revealed only 1.3% of causes (Rauch *et al.*, 2006). Although FISH has the advantage of high resolution (50–100 kb), it has an extremely low throughput if an unknown aberration has to be detected. In addition, multiprobe FISH-based approaches are used in the majority of diagnostic laboratories for detection of subtelomeric aberrations, even though it is a time consuming technique.

Other commonly used methods to detect subtelomeric rearrangements include GTG-banding, multiplex ligation-dependent probe amplification (MLPA), and microarray-based comparative genomic hybridisation (array-CGH) (Flint and Knight, 2003); previously multiplex amplifiable probe hybridisation (MAPH) method has also been utilised (Hollox *et al.*, 2002).

Microarray is an emerging molecular cytogenetic approach to genome-wide analysis of copy number changes of genomic DNA and gene expression (Xu and Chen, 2003). Array-based technologies have been used to detect chromosomal copy

number changes in the human genome across the whole genome. DNA arrays that detect changes in the copy number of specific parts of the genome have made it possible to detect many submicroscopic genomic deletions and duplications that cause complex MR syndromes. Several technologies, such as array-CGH (Solinas-Toldo *et al.*, 1997), MAPH (Armour *et al.*, 2000), array-MAPH (Patsalis *et al.*, 2005; Patsalis *et al.*, 2007; Kousoulidou *et al.*, 2008), MLPA (Schouten *et al.*, 2002), and array-based MLPA (Kooy, 2008), have been developed to search for genomic imbalances in undiscovered regions. According to Rauch *et al.* (2006) molecular karyotyping/ array CGH in every patient with MR would have the highest diagnostic yield (28.9%).

CGH is a technique, which screens the whole genome for imbalances. Most of the clinically available array-CGH platforms are designed to detect aneupoloidies, well-characterized microdeletion/microduplication syndromes and subtelomeric or other unbalanced chromosomal rearrangements (Shinawi and Cheung, 2008). Already Bauters *et al.* (2005) developed a full coverage X chromosome array-CGH with a theoretical resolution of 82kb, for the detection of copy number variations (CNV) in patients with suspected XLMR. Recently Lybaek *et al.* (2008) presented a study where 590 patients suspected of having a minor or cryptic genomic imbalances as the cause of MR with dysmorphic signs and/or malformations have been investigated with high-resolution CGH. They detected genomic imbalances in 40 (7.2%) of investigated patients.

An alternative methodology to array-CGH is the MAPH methodology (Patsalis *et al.*, 2007; Kousoulidou *et al.*, 2008), a DNA based method that allows the accurate and reliable determination of changes in copy number in complex genomes, certain chromosomes or loci. The array-MAPH is a further development of MAPH principle (Armour *et al.*, 2000), a relatively simple method based on hybridization and quantitative polymerase chain reaction (PCR). This method is based on quantitative recovery of probes, after their hybridisation to immobilized DNA. Array-MAPH is expected to significantly contribute to the detection of small-scale genomic imbalances in clinical practice, offering high flexibility, sensitivity and resolution. MAPH can provide extremely high resolution and enable the sensitive detection of loss and gain of genomic DNA sequences as small as 150 bp (Patsalis *et al.*, 2005). However, MLPA is the most widely used technique for targeted screening because of its accuracy, robustness, low reaction cost, and the relative ease with which probes can be generated. To increase the throughput of this method Kooy (2008) has recently developed an array-based MLPA method for analysing all recurrent microdeletion syndromes. In addition, Illumina BeadArray technology offers a complete solution to high throughput genomic analysis, including single nucleotide polymorphism (SNP) genotyping, loss of heterozygosity (LOH) and genome amplification detection, DNA methylation profiling, and gene expression profiling, including allele-specific expression profiling and mRNA isoform profiling (Fan *et al.*, 2006).

2.5.3. Mechanisms of chromosomal aberration formation

Regardless of mechanism, structural features of the genome can predispose a particular region to rearrangement. Genomic rearrangements are not random events, but a result from predisposition to rearrangement due to the existence of complex genomic architecture that may create instability in the genome (Shaw and Lupski, 2004). Any region of the genome may be subject to rearrangement, but certain parts of the genome are more susceptible than others (Brewer *et al.*, 1998, 1999); they predominate in the pericentromeric and subtelomeric regions, particularly in intervals containing complex genomic architecture, such as low-copy repeats (LCRs) or AT-rich palindromes (Shaw and Lupski, 2004). Still, some regions of the genome have never been observed to have deletions or duplications, and these regions are thought to contain critical dosage-sensitive gene(s), where copy number variations (CNV) would be lethal (Shaffer and Lupski, 2000).

Genomic disorders are defined as a group of diseases that result from genomic rearrangements associated with LCRs, which are genomic features that affect chromosome stability and can produce disease-associated rearrangement. They can contain one or multiple genes, pseudogenes, gene fragments, retroviral sequences, regulatory regions, or other paralogous segments (Lupski, 1998). Furthermore, the human genome sequence project reveals that LCRs may account for 5% of the genome (Inoue and Lupski, 2002). There are two primary recombination mechanisms for generating genomic deletions and duplications that cause CNVs that can be associated with genomic disorders: non-allelic homologous recombination (NAHR) and non-homologous end joining (NHEJ) (Stankiewicz and Lupski, 2002; Shaw and Lupski, 2004; Lupski and Stankiewicz, 2005) (Figure 1).

Intra-chromatid, intra- and inter-chromosomal genomic architectural features are favourable for rearrangements to occur via NAHR, which is also the most common mechanism underlying disease associated genome rearrangement (Shaw and Lupski, 2004). NAHR is found to cause gene CNV-s, that consist of LCRs, which are (1) bigger than 10 kb in size, (2) with more than 97% sequence identity, (3) directly oriented, (4) within 5 Mb of each other, and (5) located on the same chromosome (Lupski, 1998). Furthermore, these intra-chromosomal microdeletions have been hypothesized to be a result from one of three LCR/NAHR-based mechanisms. Firstly, unequal crossing over between direct LCRs on sister chromatids is predicted to result in deletion/duplication derivative chromosomes (Dorschner *et al.*, 2000; Shaffer and Lupski, 2000). Secondly, the intrachromatid fold-back loop mediated by directly oriented repeats, followed by a crossing-over event, leads to the loop excision and deletion (Dorschner *et al.*, 2000). Thirdly, intrachromatid recombination events between LCRs can lead to an inversion when the LCRs are in an inverted orientation and NAHR occurs within a single chromatid (Lupski, 1998). For example, in chromosome 22 band 22q11.2 is predisposed to rearrangements due to misalignments of LCRs (Ensenauer *et al.*, 2003).

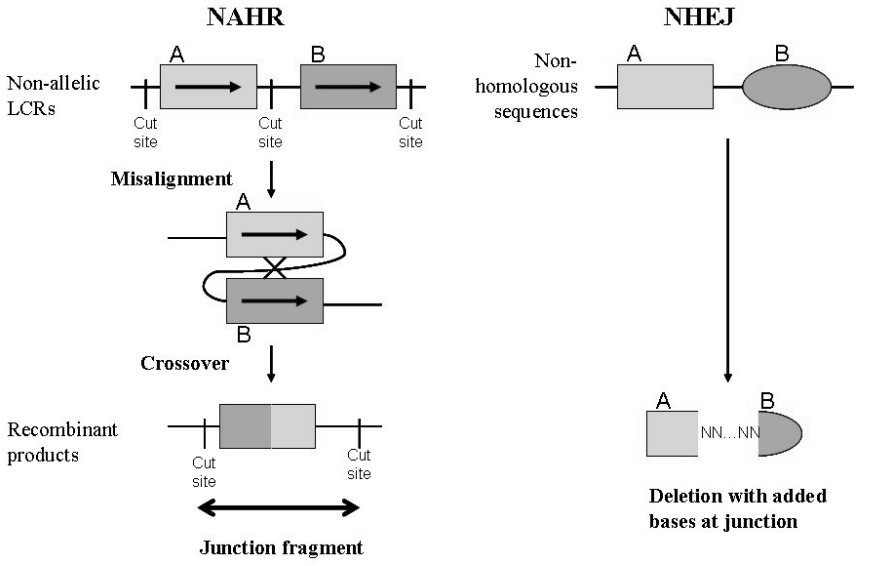

Figure 1. Origination of deletion rearrangement by NAHR and NHEJ, adapted from Shaw and Lupski (2004).

Previously, some other mechanisms for genomic rearrangements have been reported. For example, non-recurrent rearrangements, such as NHEJ and replication fork stalling and template switching (FoSTeS) have been implicated; and both of these mechanisms have been shown in *PLP1* gene, with aberrations resulting in Pelizaeus-Merzbacher disease (Inoue *et al.*, 2002; Lee *et al.*, 2007). Deletions in *PLP1* gene are shown to be caused by NHEJ (Inoue *et al.*, 2002); however, Lee *et al.* (2007) proposed complex duplication and deletion rearrangements associated with the same disease, and potentially other non-recurrent rearrangements, may be explained by FoSTeS.

Many variants in copy number are probably benign, but specific variations are associated with common Mendelian (single-locus) conditions such as colour blindness, Charcot-Marie-Tooth disease type 1A, and other disorders of the nervous system (Lupski, 1998). Furthermore, sporadic disease might be found to result from a new genomic alteration that causes variation in the copy number or from a combination of two or more variations in copy number inherited from two parents, in each of whom the un-combined variation did not provide a genetic burden that was great enough to cause disease (Lupski, 2007). A study performed by Kato *et al.* (2006) suggests that the frequency of *de novo* translocation depend on the size of the palindromic AT-rich repeats. For example, they found that virtually all of the *de novo* translocations appeared to originate from the ~450-base pair palindromic AT-rich repeats.

2.5.4. Chromosomal aberrations of chromosome X

In this thesis as a part of this study, we present two case reports. First report is about one female patient with complex aberration in chromosome X.

Turner syndrome is a common genetic disorder, resulting from the partial or complete absence of one X chromosomes, and occurring in approximately 50 per 100,000 live-born girls in a Caucasian population (Gravholt, 2005). The classic phenotype of Turner syndrome includes short stature, ovarian failure and variable somatic stigmata. It is associated with the chromosome X monosomy; although, the classical karyotype accounts only for half of the cases (Gravholt, 2005). Mosaic and partial deletions and/or duplications of the chromosome X have been shown to result in different degrees of Turner syndrome (Elsheikh *et al.*, 2002). Deletions of the whole short arm of the chromosome X in females are associated with short stature, gonadal dysgenesis and the classic stigmata of Turner syndrome (Ferguson-Smith, 1965).

The genetics of the Turner syndrome phenotype have been associated with haploinsufficiency of *SHOX* gene (short stature homeobox gene), also known as *PHOG* (pseudoautosomal homeobox-containing osteogenic gene), and cloned from the short arm pseudoautosomal region. It is thought to explain a large proportion of the described deficit in height, and also is believed to explain the short forth metacarpal, cubitus valgus, Madelung deformity, mesomelic growth, high arched palate, micrognathia, sensorinerual deafness and dysproprtionality of skeletal size (Ellison *et al.*, 1997; Rao *et al.*, 1997; Gravholt, 2005).

2.5.5. A partial deletion of 17q chromosome

The second case, about a male with a 17q partial deletion, was reported because interstitial deletions of the long arm of chromosome 17 are rare events. It is known that haploinsufficiency of dosage-sensitive genes in deleted interval might cause an abnormal phenotype. In chromosome 17 in the region q22–24, to our knowledge, only eight cases have been reported previously (see Table 3) (Park *et al.*, 1992; Dallapiccola *et al.*, 1993; Khalifa *et al.*, 1993; Levin *et al.*, 1995; Thomas *et al.*, 1996; Mickelson *et al.*, 1997; Marsh *et al.*, 2000; Shimizu *et al.*, 2008). Common findings in patients with this aberration include micro-cephaly, characteristic facies, hand anomalies including symphalangism and proximal placement of the thumbs, growth retardation, and moderate to severe developmental delay.

Table 3. Clinical features of previously published cases and patients with the mutation in *NOG* gene.

	Case 1 (Park *et al.*, 1992)	**Case 2** (Dallapiccola *et al.*, 1993)	**Case 3** (Khalifa *et al.*, 1993)	**Case 4** (Levin *et al.*, 1995)	**Case 5** (Thomas *et al.*, 1996)	**Case 6** (Mickelson *et al.*, 1997)	**Case 7** (Marsh *et al.*, 2000)	**Case 8** (Shimizu *et al.*, 2008)	***NOG* gene** (van den Ende *et al.*, 2005)
Birth information									
Gestation	at term	at term	at term	35 week	at term	at term	32 week	at term	–
Birth weight (g)	2370	2780	3425	1160	3180	2590	1420	2800	–
Birth length (cm)	47	47	52	36	41.5	49	–	–	–
Birth OFC (cm)	30.5	33	32	27	–	–	27	–	–
Sex	F	M	M	F	M	F	M	M	–
Craniofacial									
Microcephaly	+	+	+	+	–	+	–	n.a.	n.a.
Palpebral fissures slant up	+	+	+	–	–	+	+	n.a.	n.a.
Hypertelorism	+	+	–	+	+	–	+	+	n.a.
Posteriorly rotated/low set ears	+	–	+	+	+	–	–	+	n.a.
Broad nasal root	n.a.	n.a.	+	flat	+	–	–	n.a.	+
Thin lips	n.a.	n.a.	n.a.	+	+	n.a.	+	n.a.	+
Abnormal palate/cleft	+	+	–	+	–	high	–	n.a.	n.a.
Micrognathia	+	+/–	+	–	+	+	–	n.a.	n.a.
Skeletal									
Symphalangism	+	+	+	–	+	+	–	+	+
Proximal thumbs	+	+	+	+	+	+	+	n.a.	n.a.
Vertebral anomaly	–	–	–	+	n.a.	–	–	+	n.a.
Other									
TEF	+	+	–	–	–	–	+	n.a.	n.a.
Heart anomaly	–	+	–	+	–	–	+	n.a.	n.a.
Hearing impaired	–	n.a.	+	n.a.	–	–	n.a.	+	+
Impaired vision	–	n.a.	+	+	+	+	n.a.	+	+
Cryptorchidism		n.a.	+		+		–	+	n.a.
Age of last evaluation	6m	4m	3y	17d	8y	4.5y	3.5m	19y	–
Death	–	4m	–	17d	–	–	3.5m	–	–
Karyotype	del17 q21.3q23	del17 q21.3q24.2	del17 q21.3q23	del17 q23.2q24.3	del17 q23.1q24.2	del17 q23.1q23.3	del17 q22q23.3	t(15.3:q22) del17q22	c.615G>C in *NOG*

3. AIMS OF THE STUDY

The aims of the present study were:

1. To establish the incidence and live-birth prevalence of FXS in Estonia and to compare our results with data from other populations;

2. To characterize the clinical phenotype of the boys with FXS and female carriers in Estonia;

3. To evaluate the effectiveness of creatine transporter defect screening among XLMR families;

4. To establish the incidence of creatine transporter defect among Estonian XLMR families;

5. To characterize the clinical phenotype of the patients with creatine transporter defect;

6. To evaluate the correlation between clinical features and molecular findings in cases of rare submicroscopic chromosomal aberrations.

4. MATERIAL AND METHODS

4.1. Patients

4.1.1. Patients involved in FXS epidemiological and clinical study

There were 676 patients (516 children, 448 boys and 68 girls; and 160 adults, 27 males and 133 females) investigated for FXS within years 1997 to 2006 in the Department of Genetics of United Laboratories of Tartu University Hospital. The 516 children in the study were born from 1984 until 2005 and were investigated for FXS because of MR, behavioural problems (mainly hyperactivity), muscular hypotonia, speech delay, epilepsy, autistic behaviour and the characteristic features of FXS for example, large or prominent ears, large testicles, hyperextensible finger joints and a long narrow face. Only individuals with genetically proven diagnosis of FXS were included in the FXS epidemiological and clinical study.

The annual numbers of live births during 1984–2005 were obtained from the SD-SOE (http://www.stat.ee), with final update on January 2008.

4.1.2. Patients with suspicion of unspecific MR

In collaboration with Department of Genetics of United Laboratories of Tartu University Hospital, Children's Clinic of Tartu University Hospital, and Tallinn Children's Hospital we have collected DNA material and phenotype data from 83 Estonian families. This group was divided into 3 subgroups: families mainly with male probands and with suspicion of XLMR, families mainly with female probands and with suspicion of XLMR and single cases with unspecific MR.

4.1.2.1. Familial XLMR group where probands were mainly boys

The study group for familial XLMR group were 53 families (out of 83) where probands in the families were mainly boys. These boys were with normal phenotype or they had some micro-anomalies. Pedigree data showed at least two boys with MR, child's mother and no less than one of her brother or sister had MR and/or there were at least two MR individuals in the pedigree from mother's side.

We included 49 families out of the 53 to the X-linked creatine transporter defect study. Four families were excluded, as they did not want to collect the additional urine analysis for creatine transporter defect study.

4.1.2.2. Familial XLMR group where probands were girls

We have collected 19 families with MR were probands in the families were mainly girls. Some of those families were divided into this subgroup as the mother has had multiple spontaneous abortions and/or there were only girls living with the family – boys have been institutionalised or deceased.

4.1.2.3. Non-specific MR group (single cases)

We also collected the clinical data and DNA samples from 11 families with single cases of MR in the family. Those MR patients had a dysmorphic phenotype of unknown aetiology and all common dysmorphic syndromes were excluded. Some cases had clinical suspicion of syndromic XLMR or there was a consanguineous marriage.

4.2. Methods

4.2.1. DNA analysis for FXS

DNA was extracted from peripheral blood leukocytes using the standard salting out method. During 1997–2001 testing for the fragile X expansion mutation was carried out using the standard Southern blot method. A brief summary of this method is that DNA was simultaneously digested with EcoRI and the methylation-sensitive restriction nuclease EagI and electrophoresed in a 1% agarose gel. Blots were incubated with ^{32}P-labeled StB12.3 probe, which allows direct detection of affected males, carrier females, normal transmitting males, as well as prenatal diagnosis (Puissant *et al.*, 1994). It hybridizes to the region from base pairs 14461 to 15537 in *FMR1* gene. After the EcoRI/SacII digestion, normal male show 2,8kb fragment and normal female generate an additional 5.2-kb EcoRI/EcoRI fragment from the methylated allele. DNA patterns were analyzed in terms of expansion site and methylation status as previously reported (Rousseau *et al.*, 1991).

Since 2001 assessing the mutation was based on PCR amplification of the CGG repeat region of the *FMR1* gene. PCR conditions and amplification were performed according to the manufacturer's suggestions (Fragile X Size Polymorphism Assay Kit – PE Corporation). Electrophoresis of PCR products was carried out on an ABI PRISM® 377 DNA Sequencer. Fragment analysis was performed using GeneScan® 2.1 Analysis Software and FRAXA Genotyper® 2.0 Software (PE Corporation). To avoid false negatives, the PCR analysis was follow-up by Southern blot for any samples that fails to amplify by PCR and any females who appeared to be homozygous.

4.2.2. Diagnostic of creatine transporter defect (*SLC6A8* gene)

4.2.2.1. Urinary creatine/guanidinoacetate analysis

An analytical method published earlier (Arias *et al.*, 2004; Caldeira Araujo *et al.*, 2005) was used with some modifications for urinary creatine and guanidinoacetate analysis. In case of increased creatine/creatinine (Cr:Crn) ratio a repeat urine sample was collected. Sample preparation: In a 2 ml glass vial (equipped with solid cap with silicone/teflon septa and a magnetic stirring bar) 50 µl of 15% $NaHCO_3$, 100 µl of urine, 600 µl of toluene and 50 µl of hexafluoroacetylacetone were added and mixture was stirred for 8 h at 80ºC. To another 2 ml vial 50 µl of N-(tert-Butyldimethylsilyl)-N-methyl-trifluoro-acetamide and the upper (toluene) layer from the first vial was added. This mixture was held at 80 ºC for 0.5 hour. Solvent was evaporated using N_2 flow, 0.5 ml of hexane was added and sample was injected to GC/MS.

Chromatography: Hewlett-Packard 6890 gas-chromatograph and HP 5973 mass-spectrometer was used with capillary column HP 5-MS (5% phenyl methyl siloxane), length = 25 m, ID=250 mm, film thickness = 0.25 µm. Splitless injection at 250 ºC was used and sample volume was 1 µl. Oven temperature program was as follows: 70ºC (hold 2 min.), 20 ºC/min – 180 ºC (hold 1 min.), 30 ºC/min - 275 ºC (hold 20 min.). The MS was operated in the electron impact (EI) mode using selective ion monitoring (SIM). Ions selected for creatine were m/z = 239, 258 and 360 (of which 360 was the target ion, used for quantification) and for guanidinoacetate m/z = 220, 225 and 346 (target ion).

Quantification: A standard solution of creatine and guanidinoacetate was prepared in double destilled water and stored at – 20 ºC. A calibration curve was made using two different concentrations: 1000 and 100 µmol/l for creatine and 100 and 10 µmol/l for guanidinoacetate. The calibration curve was forced through zero. A certified reference material (ERNDIM, Control Special Assay in Urine) was used to estimate the accuracy of the analysis.

4.2.2.2. *SLC6A8* gene mutational analysis

All 13 exons and the adjacent splice sites of *SLC6A8* gene were amplified by polymerase chain reaction. The open reading frame of the *SLC6A8* gene and the adjacent splice sites have been analyzed by DNA sequence analysis using genomic DNA as described previously (Rosenberg *et al.*, 2004).

4.2.2.3. *SLC6A8* cDNA sequence analysis

Sequence analysis of amplified cDNA of *SLC6A8* was performed using standard technique as described previously (Salomons *et al.*, 2001).

4.2.2.4. Neuropsychological investigations of the patients with creatine transporter defect

General intelligence (IQ) was assessed with Raven Progressive Matrices (RPM) (Raven, 1981) and Kaufman's tests. The Kaufman Assessment Battery for Children (K-ABC) (Kaufman and Kaufman, 1983) was administered for younger (between 2.5 and 12.5 years old) family members and The Kaufman Adolescent and Adult Intelligence Test (KAIT) (Kaufman and Kaufman, 1993) for older ones. The RAVEN is a widely used test to measure nonverbal intelligence, abstract reasoning and fluid abilities (abilities to solve new and unfamiliar problems and tasks) (Lynn *et al.*, 2004). The total number of correct responses was used and transformed into percentile rank to measure general intelligence level. The K-ABC is an individually administered measure of intelligence and achievement designed for children between 2.5 and 12.5 years old. It measures intelligence as a problem solving ability on the basis of the information processing style – either sequential or simultaneous (Kaufman and Kaufman, 1983). The Sequential Scale measures problem solving in a sequential manner, focusing on serial or temporal order of stimuli to complete the tasks. The Simultaneous Scale measures problem solving and task completion by processing several stimuli at the same time in a gestalt-like manner or focusing on spatial integration of information. The combination of the Sequential and Simultaneous Scale is a measure of *fluid intelligence* (Gf). The Achievement Scale measures the factual knowledge and school-related skills and is a measure of *crystallized intelligence* (Gc). Gf refers to novel problem solving and the ability to learn, Gc involves acquired skills, knowledge, and judgments that have been systematically taught and learned (Lichtenberger *et al.*, 2000). The RPM is a widely used test to measure nonverbal intelligence, abstract reasoning and fluid abilities (abilities, to solve new and unfamiliar problems and tasks) (Lynn *et al.*, 2004). The total number of correct responses was used and transformed into percentile rank to measure general intelligence level.

The KAIT is a measure of intelligence of individuals in age group from 11 to 85 years. Intelligence is defined as a combination of fluid and crystallized abilities and is measured by Gf and Gc (Kaufman and Kaufman, 1993). The original norms of the tests were used. The raw score of the each subtest was transformed to the scaled score with a mean of 10 and standard deviation of three. The total number of subtests' scores yields different scales standard scores having a mean of 100 and standard deviation of 15.

4.2.3. Gene chip analysis

4.2.3.1. Array-MAPH

We have applied the microarray-based multiplex amplifiable probe hybridization (array-MAPH) method to analyze chromosome X for submicroscopic copy-number changes. Array-MAPH methodology (Figure 2) requires probe selection and preparation, microarray preparation, array-MAPH hybridization and data analysis. Probes were selected so that they are unique in the human genome; evenly spaced over the studied genomic region; similar in the size (preferably 400–600bp) and GC content (30–55%) to ensure similar hybridization conditions. From the human chromosome X 558 probes were selected on the basis of uniform spacing, maintaining a 150–350 kb between probes, resulting in median distance of 238 kb. Another 107 probes from human autosomal chromosomes were designed as normalization controls. The candidate probes were amplified by PCR from normal 46,XY source of genomic DNA. Probes were cloned into the pCR2.1 vector. The successfully cloned candidate probes were cultured in LB medium with 40 μg/ml ampicillin and cryopreserved with 10% glycerol at –80ºC. Array-MAPH amplifiable probes were developed by PCR amplification using PZA (common vector-derived forward primer) and PZB (common vector derived reverse primer) universal primers (Patsalis *et al.,* 2007).

Array-MAPH target sequences were amplified for spotting onto the array by PCR using specific unique primers for every clone probe. The array-MAPH target sequences were then dissolved in 25% DMSO at final concentration of 30 ng/ml and spotted onto Genorama SAL-1 microarray slides (Asper Biotech Ltd, Tartu, Estonia) in duplicates with the use of VersArray ChipWriter Pro arrayer (BioRad Laboratories, Hercules, CA, USA). All probes were arrayed in random order, to minimize the possibility that a spatial artefact during array hybridization will be incorrectly interpreted as an aberration. For array-MAPH hybridization, two micrograms of human test genomic DNA was immobilized on Hybond+ filters (GE Healthcare, Piscataway, NJ, USA) as described previously (Armour *et al.*, 2000). The filters were then hybridized with the array-MAPH amplifiable probes and washed to remove unbound and non-specifically bound probes. Each filter was placed into separate tubes and all bound probes were recovered from filters by denaturing and quantitative PCR amplification. The recovered probe mixture was purified and labelled by nick translation using aminoallyl-dUTP-s and later treatment with aminoreactive Cy3 dye (GE Healthcare). The recovered probe mixture was hybridized to the microarray for quantification using automated hybridization station HS-400 (Tecan Austria GmbH, Gro¨dig/Salzburg, Austria).

The microarrays were scanned using Affymetrix 428 microarray scanner (Affymetrix Inc., Santa Clara, CA, USA). Raw signal intensities were extracted

with BaseCaller module of Genorama Genotyping Software 4.2 Package (Asper Biotech Ltd). As each target sequence was presented in duplicate, their average pixel fluorescence intensity was used for further analysis with the specifically designed software called MAPH-Stat. For resorting probes into their genomic order and for normalizing microarray signals the MAPH-Stat program (between-slide normalization) with respect to the median of autosomal control probe-specific signals from the given microarray was used.

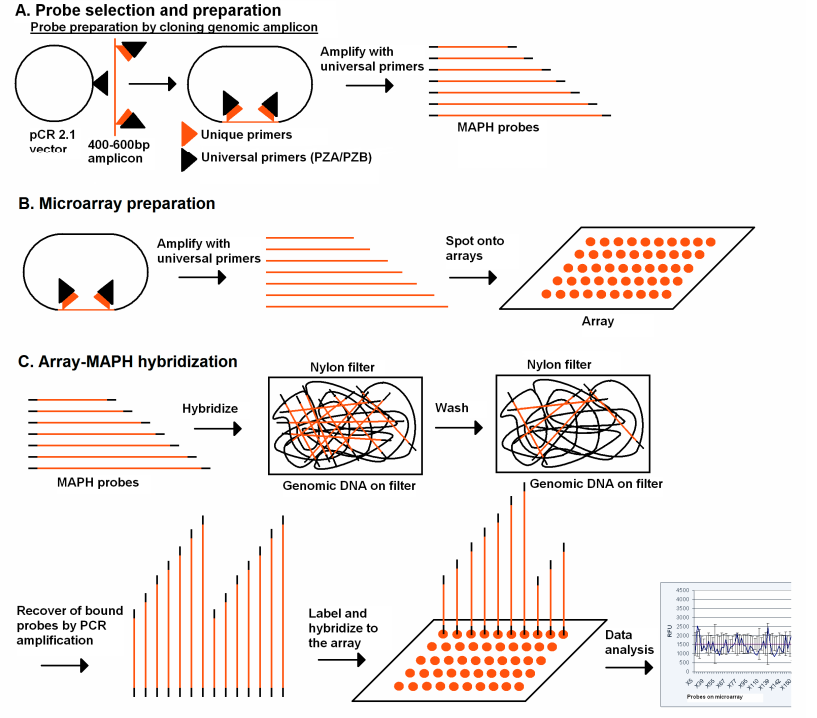

Figure 2. A flow diagram of array-MAPH methodology.

4.2.3.2. Illumina SNP arrays

We have applied Infinium II whole-genome genotyping assay with Human370CNV-Duo BeadChips (Illumina Inc., San Diego, CA, USA). The Infinium II Whole-Genome Genotyping Assay couples allele-specific hybridization with primer extension, which helps to achieve higher specificity. HumanCNV370-Duo has coverage of 52,167 markers from 14,000 CNV regions of the genome, with coverage of 90 Mb of DNA. More than 318,000 tag SNP markers have been selected using International HapMap Project Phase I and II data, so that each HumanCNV370-Duo BeadChip genotypes more than

370,000 loci. The median SNP spacing is 5.0 kb (7.9 kb mean). This BeadChip method enables an accurate whole-genome disease-association study and CNV studies, also identification of genome-wide SNP-s, detection of loss of heterozygosity and genotyping. (Illumina Inc.)

Assay protocol:

1. Whole-genome amplification step (input amount of genomic DNA is 750 ng)
2. Amplified DNA fragmentation
3. Amplified DNA precipitation and resuspension
4. BeadChip preparation
5. Sample hybridisation to BeadChip
6. Extension and staining of the samples on BeadChip
7. BeadChip washing and scanning with Illumina BeadArray Reader

Results were analysed with BeadStudio v3.1 Data Analysis Software Modules provided by Illumina Inc., and QuantiSNP software designed and optimized for Illumina SNP genotyping data at Oxford University (Colella *et al.*, 2007). BeadStudio v3.1 offers integrated copy number tools and a Genome Viewer, which allows visualization of the data on a chromosomal level. QuantiSNP provides copy number estimates and bookmarks with confidence scores. It is mostly useful for finding small-sized regions with copy number changes that cannot be detected by eye.

4.2.4. Real-time quantitative PCR

We used real-time quantitative PCR (RT-qPCT) for more accurate determination of chromosomal breakpoints and for confirmation of the findings detected by array-based methods. For the breakpoints detection, we designed unique sequence primer pairs located the proximal and distal to the breakpoints and primer pairs specific to chromosome 1, which was used for normalization. All qPCR products were initially analyzed on a 1.5% agarose gel to verify the expected product size. Real-time PCR reactions were performed on ABI 9700 Real Time system (Applied Biosystems, Foster City, CA), using the DNA-binding dye SYBR Green I (Wittwer *et al.*, 1997).

Individual real-time PCR reactions were carried out in 20 µl volumes in a 96-well plate (ABgene, Epsom, Surrey, UK) containing ABsolute™ QPCR SYBR® Green ROX (500nM) Mix (ABgene), 250 nM of each forward and reverse primer and 5 µl of DNA sample with different concentrations.

The cycling parameters were as follows: 10 minutes at 95°C for DNA polymerase activation, 40 cycles at 95°C for 15 seconds (denaturation) and 60°C for 60 seconds (annealing and extension). All reactions were run in triplicate. After PCR amplification, a melting curve was generated for every

PCR product to check the specificity of the PCR reaction (absence of primer-dimers or nonspecific amplification products) (Ririe *et al.*, 1997).

Each assay included a non-template control, a mix of five cytogenetically normal male's DNA, a mix of five cytogenetically normal female' DNA and the patient's DNA. Three different loci, one of which is the endogenous control from chromosome 1, can be studied simultaneously in one 96-well plate. The results obtained from different plates were analyzed separately. The experiments were performed using the absolute quantification by standard curves assay method. To generate the standard curves for each studied locus, we performed SYBR Green I amplifications on a series of samples with regularly decreasing DNA concentrations. The corresponding amounts per reaction were 10, 5, 2.5, 1.25 and 0.625 ng of total genomic DNA of phenotypically and cytogenetically normal female. Standard curves were used to determine the amount of input DNA in unknown samples (mix of five normal male, mix of five normal female and patient's DNA) using SDS 2.2.2. software (Applied Biosystems). The input amounts of unknown samples were equal (2.5 ng per reaction). The obtained results were transferred to MS Excel for further calculations. The endogenous control from chromosome 1 was used for normalization. To estimate the copy-number of each locus, the ratio between different samples was calculated. As we deal with chromosome X, the ratio male/female has to be 0.5; in case of deletion in the female patient the ratio has to remain 1 between normal male and patient, 2 between normal female and patient and so on.

4.2.5. Statistical methods

Statistical analysis for estimating the live-birth prevalence of FXS among boys and whole children's population, from the 1984–2005 annual live-birth data (SD-SOE, http://www.stat.ee), was done with the Generalized Linear Model Analysis using the GENMOD procedure of the SAS system, Release 8.2. (Sas Institute, 1999). Distribution of the prevalence cases was assumed to be Poisson distribution, which is a good model for rare events. Default logarithmic link function was used. The only factor in the model was the observation year. The mean (expected) prevalence rate for a given year, and the corresponding 95% confidence limits, were predicted with the OUTPUT statement of the GENMOD procedure.

4.2.6. Ethics

The Ethics review Committee on Human Research of the University of Tartu approved the study and informed consent was obtained from the parents or legal guardians of the child with MR and FXS for the participation of the study.

5. RESULTS AND DISCUSSION

5.1. Fragile X syndrome

5.1.1. The prevalence of fragile X syndrome among MR population in Estonia (publication I)

The full mutation in the *FMR1* gene was found in 14/516 children (2.7%) of the total MR individuals tested (14 boys). One adult male with full mutation was excluded from epidemiological study as he was born before 1984. Among them, we found that 3.1% (14/448) of boys with MR had FXS (Figure 3). Two (2/15, 13%) of the males with FXS were mosaic for a premutation and a full mutation in *FMR1* gene.

Screening studies of Caucasian males diagnosed with non-specific MR have yielded frequencies from 2.6 to 8.7% (Table 1) (Sherman, 1996; Crawford *et al.*, 2001). Usually 1% to 2% of samples referred for molecular testing for the FXS have actually been positive for the syndrome (Curry *et al.*, 1997; Hunter, 2000). Furthermore, it has been shown that about 15% of patients show a mosaic pattern consisting of both full mutation and premutation (Mandel and Biancalana, 2004). Results from previously published studies are in accordance with our study.

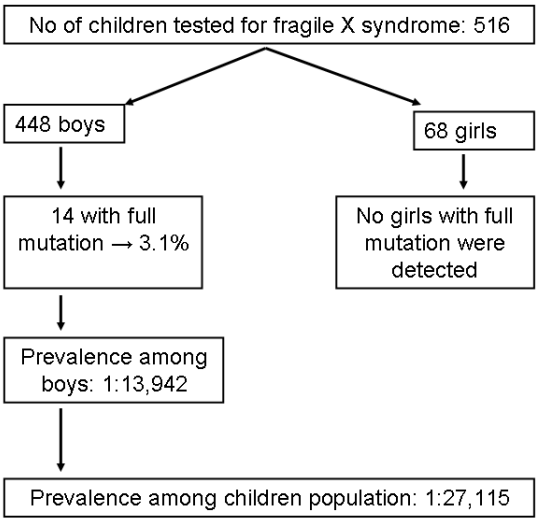

Figure 3. Study profile of FXS epidemiological study.

5.1.2. The live-birth prevalence of FXS (publication I)

There are 14 live-birth male FXS patients from 1984 to 2005 in our study group. The total number of live-births in the same period, according to the (SD-SOE, http://www.stat.ee), was 379,616 (195,184 boys and 184,432 girls). This indicates minimum live-birth prevalence for FXS among boys of 1 in 13,947 (95% confidence intervals 1:8,264–1:23,529). The live-birth prevalence showed a slight increase among boys (Figure 4), but was not statistically significant (P = 0.89). The prevalence of FXS was not calculated separately for girls, as we did not find any girls with the full mutation. The overall live-birth prevalence of FXS among Estonian children was 1:27,115 (95% confidence intervals 1:16,059–1:45,787).

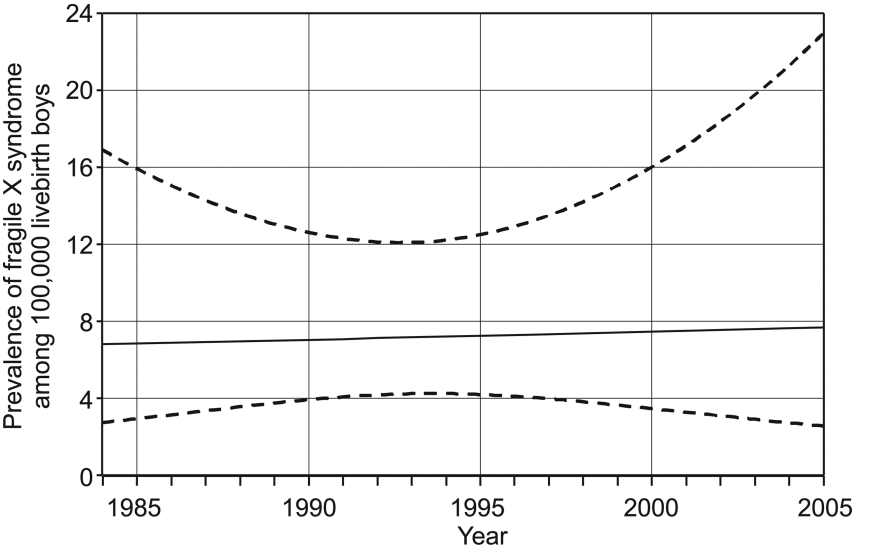

Figure 4. Prevalence of Fragile X Syndrome in Estonian boys born from 1984 to 2005; the steady line presents predicted, with the help of linear regression, the prevalence (number of cases among 100,000 livebirths). The dashed lines correspond to 95% confidence intervals for the prevalence.

Previously reported population-based studies suggest that the prevalence of the full mutation is about 1:4,000 in Caucasian males in the general population (Turner *et al.*, 1996), in the range of 1:3,717 up to 1:8,918 (Table 1) (Crawford *et al.*, 2001), which is much higher than live-birth prevalence found in Estonia. However, Crawford *et al.* (2001) indicated, that most of studies have screened target populations (as in this study) and later a few of them extrapolated these

results to the general population. Direct estimates of the frequency of full mutation alleles in a given population requires screening of samples of at least 50,000 individuals (Hagerman, 2008). Presently, there is only one work by Rousseau *et al.* (2007) who screened the *FMR1* alleles among 24,446 mother-newborn pairs from the general population. They reported the incidence of the full mutation causing FXS syndrome in the Canadian population is 1:24,446 (upper limit of 95% confidence interval 1:7,065), and those results are very similar to ours (1:27,115). We propose, that most FXS cases in Estonia should be included in our study as almost all males with the full mutation exhibit some clinical features of this syndrome (Crawford *et al.*, 2001) and they usually occur by the age of 3 years (Bailey *et al.*, 1998). However, Crawford *et al.* (2001) hypothesized that the syndrome is not being diagnosed through the referral system because identification of new milder cases of the FXS among school-aged children. Therefore, it is possible that we have missed the mild cases of FXS among children who are not reached school age yet, but on the other hand, half of our tested children were older than 7 years. Moreover, the results of the statistical analysis support the case that most FXS cases should be included, as the prevalence of FXS have been stable during 1984–2005 (Figure 4).

We could miss FXS patients because of the fact that we did not visit all the long-term care institutions and special educational facilities for disabled children in Estonia. However, our Health Insurance system has been built up so that all children with developmental delay have to be referred to two main children's hospitals every two to three years in order to get disability support. Our assumption is also based on the observations by Õiglane-Shlik *et al.* (2006) (epidemiological study for AS and PWS) – who did not find any additional cases in this process. Their study proved that the regular evaluation according to consensus documents of children below 18 years of age with developmental problems is very effective in Estonia. In addition, this study included patients with FXS who were institutionalised at the time of investigation, but diagnosed during regular evaluations in one of our tertiary hospitals. It is also important that the Department of Genetics of United Laboratories of Tartu University Hospital is the only diagnostic laboratory for the whole Estonia and is available for all hospitals and institutions. This department therefore, has tested all patients with a suspicion of FXS and consequently the whole population of Estonia is covered. The molecular study for the diagnosis of FXS in Estonia was performed by two different techniques: Southern blot analysis and PCR with fluorescent marked primers followed by CGG repeat length detection at ABI PRISM 377. These are worldwide accepted screening methods (Crawford *et al.*, 2001).

5.1.3. The age of diagnosis of FXS (publication I)

In our study, the age of patients at the moment of FXS analysis was possible to evaluate retrospectively in most cases (509/516 patients, 98.6%). In 286/509 (56.2%) the analysis was performed before or at 7 years of age, and in 223/509 (43.8%) of cases at school age. The median age at diagnosis of males with a full mutation in Estonia was 4.5 years (ranging from 10 months to 17 years).

In France the median age of FXS diagnosis for male probands was 4.9 years (Biancalana *et al.*, 2004), and at the same time in US the average FXS diagnosis occurred at 2.7 years (Bailey *et al.*, 2003). This is considered to be a good indicator of the degree of awareness of FXS within the medical profession (Mandel and Biancalana, 2004). Based on this data we can conclude that the consciousness of FXS in Estonia is similar to other European countries.

5.1.4. CGG repeats of *FMR1* gene in investigated individuals (publication I)

In our study, we also calculated the most common allele size in Estonia. There were 323 males and 43 females analyzed in the period 2001–2006 with the PCR-based method, which enabled the detection of the exact number of repeats. The highest incidences of CGG repeats in the *FMR1* gene in Estonia were 30 and 29 repeats, 29.3% and 16.4% respectively of all detected repeats. The distribution of all investigated *FMR1* alleles is seen in Figure 5.

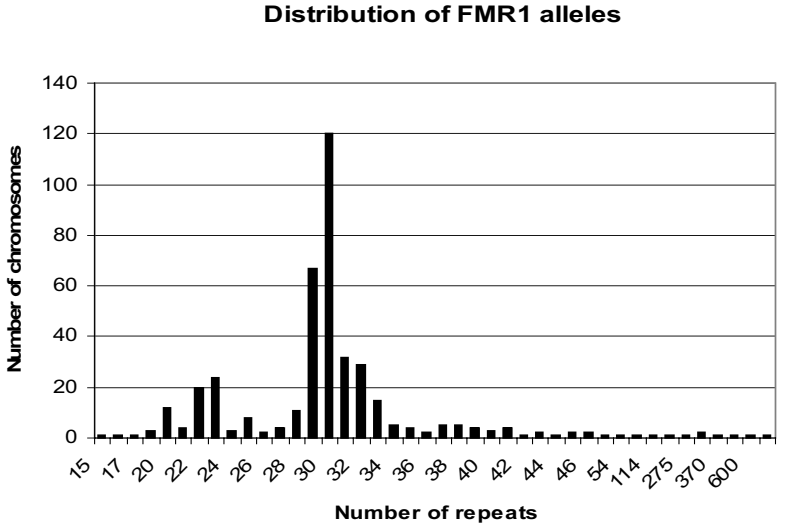

Figure 5. Distribution of CGG repeats of *FMR1* in the X chromosomes from all analysed patients.

Our results are similar to study performed by Brown *et al.* (1993) who analyzed 570 pregnant women and found that the most common number of CGG repeats in Caucasians is 30. Similar results were also reported in Greece, where they found that the most common alleles in the Hellenic population for *FMR1* repeats in females were 29/30 (15.3% heterozygous) and 30/30 (27.6% homozygous) (Patsalis *et al.*, 1999). However, in the populations of China (Zhong *et al.*, 1999), India (Sharma *et al.*, 2001) and the former Republic of Yugoslav (Major *et al.*, 2003) the most prevalent CGG repeat number has been shown to be 29.

5.1.5. Clinical phenotype of FXS (publications I and II)

All patients with pre- and full mutation were recalled for a genetics consultation, where we were able to re-evaluate 78% of patients. Data of one adult male with full mutation was additionally included to clinical study to the previous group of 14 boys. Although clinical description was possible for all of the patients with a full mutation (15 males and 1 female), in three cases we had to rely on clinical history only. The reasons for that were – one patient was deceased at the age of 6 years due to epileptic status and obesity, the family of the second patient did not want further investigations, and we were not able to find the third patient. Two boys with FXS were institutionalised and we visited them in those institutions. Furthermore, we were able to re-evaluate 14 females with a premutation.

5.1.5.1. The clinical problems in patients with full mutation in *FMR1* gene (15 males and 1 female)

All of the clinical data has been summarized in Tables 4 and 5. In Estonia the majority of males with FXS full mutation were born from first (8/15, 53%) or second (5/15, 33%) pregnancies, and first deliveries (12/15, 80%). Sixty-four percent (9/14) of males were born at term, 29% (4/14) of pregnancies were two weeks past due dates one pregnancy terminated prematurely at 36 weeks.

Risk of miscarriage occurred in 23% of patients (31%), breech presentation 13% (2/15) and caesarean section was performed in 27% (4/15) of pregnancies. Median birth weight was 4024g (+1.5 SD) (patient who was born on 36 weeks was excluded); median height was 52 cm (+0.5 SD). According to Lachiewicz *et al.* (2000) there was no significant difference in mean birth weight and length in FXS patients.

Among Estonian FXS, patient's speech delay was present in all of the cases, and echolalia was seen in 47% of cases (Table 5). Furthermore, developmental delay at early childhood was noticed in most of the FXS cases in Estonia. To

demonstrate, 57% (8/14) of children acquired head control after the age of 3 months; only 25% (3/12) of children ambulated by the age of 14 months; 75% (9/12) of children with FXS began walking after the age 15 months; moreover, one third of them started walking after the age 2 years. It is known that almost all children with FXS are not speaking in sentences by two to three years of age (Hagerman and Hagerman, 2002). According to Bailey *et al.* (1998) overall development in FXS progresses at approximately half the rate expected for typically developing children and similar prevalence was seen in Estonian FXS patients.

Two third of boys were in normal height (71%), 21% were tall (over +2 SD) and one 19-year-old male was relatively short (–1.5 SD). In our study, only boys under the age of 15 years were taller than the normal population (over +2 SD). Furthermore, only one-third (36%) of the FXS patients were of normal weight, more than half were overweight (64%), and half of them had severe obesity (over +5 SD). Medium body mass index was 25.3 kg/m^3; moreover, mean body mass index among older than 15-year-olds was 31.1 kg/m^3. Three patients (21%) had macrocephaly (+2 SD). Previously, it has been shown that majority of younger than 15 year-old boys with FXS have growth percentiles above the fiftieth percentile and one third are at or above the ninety-fifth percentile, and the head circumference growth is mildly increased during childhood and persisted into adult life (Sutherland and Hecht, 1985). Although, we did not follow the patient's growth pattern within a long period, we could see similar growth pattern in our patients.

The majority of males with FXS had the characteristic facial phenotype of FXS (Figure 6a, 6b and 7). The following facial characteristics of FXS were seen most often among Estonian FXS patients: elongated face (87%), high and wide forehead (80%), long (≥ 70 mm in an adult, 75%) and/or prominent (93%) ears, dental crowding (60%) and prominent jaw (53%). In Figures 6a and 6b a facial phenotype is shown at different ages, in Figure 6a the patient is age 3 years and in Figure 6b the same patient is age 19 years.

MR was present in all of the cases with FXS. Severe MR was seen in 27%, moderate MR in 40% and unspecified MR in 33% of cases. Due to severe MR and behavioural problems, two of the patients were institutionalised. Many of the patients had difficulties in coming to the genetic consultation, so we visited them at their homes. According to Hagerman and Hagerman (2002) males with FXS function within the mild to moderate ranges on standardized assessment of intelligence and adaptive behaviour. Majority of the Estonian patients had moderate MR.

Table 4. Perinatal and postnatal history of the patients with full mutation.

Clinical picture of patients with full mutation	**LT**	**LL**	**PJ**	**PS***	**RR**	**KK**	**KM**	**MG ***	**TK**	**DS**	**KSv**	**DK**	**BD**	**KSi**	**AV**	**Positive findings/ number of investigated patients (%)**
Pregnancy	1	1	2	4	1	1	2	2	1	2	5	1	2	1	1	1st - 8/15 (53%) 2nd - 5/15 (33%) 3rd - 0/15 (0%) 4th - 1/15 (7%) 5th - 1/15 (7%)
Delivery	1	1	1	3	1	1	1	1	1	2	3	1	1	1	1	1st - 12/15 (80%) 2nd - 1/15 (7%) 3rd - 2/15 (13%)
Spontaneous abortion risk	–	–	–	–	–	+	–	+	–	–	+	–	–	–	+	4/15 (27%)
Breach position	–	–	–	–	–	+	–	–	–	–	–	–	+	–	–	2/15 (13%)
Caesarean section	+	–	–	–	–	+	–	–	+	–	–	+	–	–	–	4/15 (27%)
At term	+	+	2w over	2w over	+	2w over	+	At 36w	+	+	+	2w over	+	+	ND	At term 9/14 (64%) 2 weeks over 4/14 (29%)
Birth weight (g)	4150	3200	4300	4720	3466	4254	4300	2940	3750	4746	3800	4500	3620	3500	–	Mean 4024 g
Birth length (cm)	54	–	51	54	49	54	52	46	49	–	49	–	51	57	–	Mean 52 cm (10)
OFC at birth (cm)	37	–	–	–	34	–	36	–	36	37.5	–	–	–	–	–	Mean 36.1 cm (5)
Neck holding	7m	later	later	>6m	1m	In time	<7m	<7m	<7m	3m	In time	8m	–	4m	1m	Later 8/14 (57%)
Walking	24m	–	31m	12m	14m	19m	15m	14m	18m	25m	17m	29m	–	21m	ND	Later 9/12 (75%)

ND=not detected; w=week; m=month

Among Estonian patients, most prevalent behavioural problems were "autistic-like" behaviour (87%), poor eye contact (87%), stereotypes (80%), inadequate laughter (73%), adaptation problems (67%), shyness (60%), moodiness (67%), and hyperactivity (67%). We saw different stereotypic movements in FXS patients – rotating movements of hands, striding on one spot, swinging the body or jumping, also hand clapping, clapping on the chest, swinging hand up and down, biting and beating the head on the wall or on the table. Obvious marks from biting were seen only in boys under age 6 years. More than half of the boys were biting themselves at an earlier age. The behaviour of males with FXS represents a phenotype that may often be diagnostically helpful (Hagerman and Hagerman, 2002). Although, FXS individuals are generally friendly, some may show aggressive behaviour in adulthood (de Vries *et al.*, 1999), which was also seen in our patients. Furthermore, about 4% of cases of autism are caused by FXS (Fisch, 1992); however, autistic-like features, such as poor eye contact, hand flapping, and hand biting, are often seen by two to five years of age (Hagerman and Hagerman, 2002). Importantly, children with autism and FXS together were found to be significantly more delayed in social skills and exhibited a greater degree of impairment in cognitive, communication, and social skills, than children with autism or FXS alone (Bailey *et al.*, 2000).

Table 5. Clinical features of patients with pre- and full mutation.

Clinical symptom	Male patients with full mutation n=15 (%)	Full-mutation patients in literature	Females with premutation n=14 (%)
Measurements			
Height over +2 SD or over	3/14* (21)		0 (0)
Weight over +2 SD or over	9/14* (64)		3 (21)
Head circumference +2 SD or over	3/14* (21)	81% (Lachiewicz *et al.*, 2000)	1 (7)
Facial phenotype			
Long narrow face	(13) 87	50–83% (Merenstein *et al.*, 1996; Lachiewicz *et al.*, 2000; Pandey *et al.*, 2004)	3 (21)
High and wide forehead	12 (80)		3 (21)
Long ears (≥7cm)	9/12* (75)	72% (Lachiewicz *et al.*, 2000)	3 (21)
Prominent ears	14 (93)	62–84% (Merenstein *et al.*, 1996; Arvio *et al.*, 1997; Lachiewicz *et al.*, 2000)	2 (14)
Strabismus	6 (40)	36% (Hagerman and Hagerman, 2002)	1 (7)
Epicanthic folds	7 (47)		0 (0)
Mild ptosis	6 (40)		0 (0)
High arched palate	7/13* (54)	62–94% (Merenstein *et al.*, 1996; Lachiewicz *et al.*, 2000)	5 (36)

Table 5. Continued

Clinical symptom	Male patients with full mutation n=15 (%)	Full-mutation patients in literature	Females with premutation n=14 (%)
History of cleft lip/palate	1 (7)	2.8% (Lachiewicz *et al.*, 2000)	0 (0)
Dental crowding	9 (60)		4 (29)
Prominent jaw	8 (53)		7 (50)
Muscle and connective tissue			
Muscular hypotonia	13 (87)	72.2% (Lachiewicz *et al.*, 2000)	2 (14)
Hyperextensible finger joints	15 (100)	57–100% (Merenstein *et al.*, 1996; Arvio *et al.*, 1997; Lachiewicz *et al.*, 2000)	6 (43)
Cubitus valgus	3 (20)		9 (64)
Pectus excavatum	7 (47)	50% (Lachiewicz *et al.*, 2000)	0 (0)
Kyphoscoliosis	2 (13)	5.6% (Lachiewicz *et al.*, 2000)	3 (21)
Hand calluses	2 (13)	13–27.8% (Merenstein *et al.*, 1996; Lachiewicz *et al.*, 2000)	0 (0)
Flat feet	12/13* (92)	69–72% (Merenstein *et al.*, 1996; Lachiewicz *et al.*, 2000)	4 (29)
Soft skin	5 (33)	100% (Lachiewicz *et al.*, 2000)	1 (7)
Functional systolic murmur	2 (13)	1% (Merenstein *et al.*, 1996)	1/11* (9)
Inguinal hernia	0 (0)	8.3–15% (Lachiewicz *et al.*, 2000; Hagerman and Hagerman, 2002)	–
Psychomotor development and behavioural problems			
MR	15 (100)	70% (Hagerman and Hagerman, 2002)	1 (7)
Speech delay	15 (100)		1 (7)
Echolalia	7 (47)	10%, 85–86% perseverant speech (Sudhalter *et al.*, 1990; Merenstein *et al.*, 1996)	1 (7)
Autistic-like features	13 (87)		1 (7)
Poor eye contact	13 (87)	57–86% (Merenstein *et al.*, 1996; Arvio *et al.*, 1997; Lachiewicz *et al.*, 2000)	3 (21)
Concentration problems	13 (87)	68% (Merenstein *et al.*, 1996)	1 (7)
Stereotypes	12 (80)		1 (7)
Inadequate laughter	11 (73)		1 (7)
Adaptation disorders	10 (67)		1 (7)

Table 5. Continued

Clinical symptom	Male patients with full mutation n=15 (%)	Full-mutation patients in literature	Females with premutation n=14 (%)
Hyperactivity	10 (67)	57–70% (Merenstein *et al.*, 1996; Arvio *et al.*, 1997)	0 (0)
Moodiness	10 (67)		0 (0)
Shyness	9 (60)	66–73% (Merenstein *et al.*, 1996; Arvio *et al.*, 1997)	0 (0)
Biting	8 (53)	16–56% (Merenstein *et al.*, 1996; Arvio *et al.*, 1997)	0 (0)
(Auto)aggressive behaviour	5 (33)	42% (Merenstein *et al.*, 1996)	0 (0)
Tactual defensiveness	4 (27)	61–81% (Merenstein *et al.*, 1996; Lachiewicz *et al.*, 2000)	0 (0)
Hand-flapping	3 (20)	31–83% (Merenstein *et al.*, 1996; Arvio *et al.*, 1997)	0 (0)
Other symptoms			
Seizures	4 (27)	8.6–22% (Lachiewicz *et al.*, 2000; Hagerman and Hagerman, 2002)	1 (7)
Otitis at least once	6 (40)	85% (Hagerman and Hagerman, 2002)	7/13* (54)
Gynecomastia	6 (40)		–
MR in siblings	5 (33)	30% (Arvio *et al.*, 1997)	11 (79)
MR in mother	4 (27)	15% (Arvio *et al.*, 1997)	0 (0)

* In few cases, it was not possible to perform measurements because of the patient's opposition or MR.

During infancy, connective tissue abnormalities, such as congenital hip dislocations and inguinal hernia may be present in FXS patients. Although, connective tissue abnormalities such as kyphoscoliosis was seen in only 13% of Estonian patients, flat feet were seen in almost all of patients (92%). Additionally, all of the males had hypermobility of the fingers; and in 87% muscular hypotonia was seen. Two of the patients were using orthopaedic shoes (13%). According to the information that we received from families, there were no cases of congenital hip dislocations, inguinal hernia and heart problems, but heart problems had only been excluded in 46% of the patients. According to other studies in later life the connective tissue dysplasia may lead to scoliosis, flat feet, and mitral valve prolapse (Loehr *et al.*, 1986). Flat feet were already present in majority of our investigated patients, being more prevalent than in previously reported studies (Merenstein *et al.*, 1996; Lachiewicz *et al.*, 2000).

Figure 6a and 6b. Patient with FXS full mutation: (a) at the age 3 years, facial phenotype contains high and wide forehead, large ears; (b) at the age 19 years, in addition to previous findings long and narrow face and prominent chin.

Among Estonian patients strabismus was quite frequent finding (40%); although, additional ophthalmologic investigations were not performed in the present study. It has been reported that almost half of the FXS cases need ophthalmologic help for strabismus, myopia, or hyperopia (Boucher *et al.*, 1995), and approximately 25% of the children had clinically significant ocular findings that included refractive errors (17%, primarily hyperopia and astigmatism) and strabismus (8%–36%) (Hatton *et al.*, 1998; Hagerman and Hagerman, 2002).

In Estonia 40% of FXS patients had experienced otitis at least once in their lifetime, but recurrent ear infections had not been documented in any of the cases. However, recurrent otitis media is a frequent complaint in children with FXS in early childhood, with a frequency of about 63% of FXS boys (Hagerman *et al.*, 1987). We can conclude that ear infections were not a major problem among Estonian FXS patients.

Unlike Down syndrome, FXS is more difficult to diagnose clinically. Especially challenging is diagnosing FXS soon after birth when there is no suspicion of FXS in the family history. It has been found, that with the use of clinical checklist it is possible to eliminate 60 to 86 percent of testing leading to negative results (Giangreco *et al.*, 1996; de Vries *et al.*, 1999). Firstly, Giangreco *et al.* (1996) described a six-variable checklist with clinical characteristics of

moderate to severe MR, maternal female with psychiatric disorder or maternal history of X-linked MR, elongated face, large or prominent ears, attention-deficit hyperactivity disorder (ADHD) and autistic-like behaviour. Secondly, de Vries *et al.* (1999) showed the exclusion rate to be 83% for the seven item checklist that compromised family history of MR, elongate face, large and prominent ears, hyperextensible joints, soft/smooth skin, macroorchidism and personality. Lastly, our study support using this kind of criteria, as MR was present in all of patients with FXS, and the majority of males with a full mutation had the characteristic facial phenotype of FXS.

Figure 7. 23-year-old male with FXS full mutation; note high forehead, long and narrow face, large ears, prominent chin; He has gaze avoidant and inadequate laughter.

We found one female with a full mutation from our study group. The patient was a 36-year-old female, who had a son with FXS. She had normal growth and the specific FXS facial phenotype – elongated face, deep-set eyes, prominent ears, and a prominent jaw. Our patient was unable to give adequate information because of moderate MR. Moreover, the menstrual cycle in the Estonian patient was regular, and this finding is supported by the estimate for POI among full

mutation carriers being similar to the general population risk of 1% (Allingham-Hawkins *et al.*, 1999). Furthermore, our female patient's other clinical findings were similar to the ones that have been previously reported to be associated to FXS phenotype in females (Borghgraef *et al.*, 1990; Hagerman *et al.*, 1992). According to Hagerman *et al.* (1992) 53% of girls with FXS had a lowered IQ in the borderline or MR range and 21% had significant attention difficulties. This Estonian female patient has a similar phenotype to the previously reported cases.

5.1.5.2. The clinical problems of the female premutation carriers (14 females)

Clinical description was possible for 14 females (out of 20) with a premutation. The median age at diagnosis was 29.4 years and median age of delivery of a child with FXS was 25.7 years. However, according to Franke *et al.* (1998) the median age when the diagnosis was made among premutation females was 32.3 years and the median age when an affected child was born was 27.3 years. This reveals that in Estonia the age when premutation diagnosis is made and age of the delivery is two to three years earlier.

The majority of patients with a premutation had normal anthropometrical indices, only two of the patents were short (less than –2 SD) and three of the patients were overweight (over +2 SD). Macrocephaly (over +2 SD) was present in one patient with a premutation. Single microanomalies were seen in the facial phenotype of females with a premutation: long narrow face (21%), prominent forehead (21%), prominent ears (14%), high arched palate (36%), dental crowding (29%) and prominent jaw (50%) (Table 5).

Among Estonian premutation carriers, we saw poor eye contact (21%); other behaviour problems were less expressed (Table 5), but additional psychological studies were not performed on females with a premutation in the present study. In one (7%) female patient with a premutation MR was present. The same patient had also behavioural problems, such as concentration problems, autistic-like features, adaptation problems, inadequate laughter, and stereotypes. Steyaert *et al.* (1992) concluded that normal intelligent female FXS carriers have similar cognitive profiles as affected FXS boys, although it has been mildly equivocated. Furthermore, Franke *et al.* (1998) revealed that mothers (of FXS children) with a premutation, obtained anxiety disorder diagnoses more often compared to the control groups. Besides they indicated that mothers with a premutation reported a major depressive episode more often than their permutated siblings without affected children (Franke *et al.*, 1998). In addition, FXS premutation carriers have also been found to have poor attention skills and an impulsive way of completing tests (Steyaert *et al.*, 1992).

In Estonia, in 29% of premutation carriers, POI had been diagnosed. Nevertheless, by the time of the re-evaluation, 43% of patients had irregularity of the menstrual cycle or POI. Furthermore, in two patients the menstrual cycle terminated between the ages of 23 and 24 years. Previously it has been shown that about 16–25% of premutation carriers experienced POI compared with 0% of full mutation carriers, and 0.4–6% of non-carriers (Schwartz *et al.*, 1994; Allingham-Hawkins *et al.*, 1999; Murray *et al.*, 2000). Schwartz *et al.* (1994) demonstrated irregular menses in 38% of premutation carriers; however, in the same study, 24% of negative non-carriers were reported to have irregular menses. Among Estonian patients, primary ovarian failure and hypoplastic uterus was present in one patient with a premutation, in other patients, menstruation initiated at age 12 to 15 years. According to previously published data, the average age of menarche was 13 years (Schwartz *et al.*, 1994).

In our study hormonal therapy was ordained to 21% of the females with a premutation at the time of the consultation and 19% of females with a premutation had been using contraceptive pills in the past. Schwartz *et al.* (1994) showed that premutation carriers reported the use of hormones to regulate menses more often than non-carrier women.

Additionally, some signs of connective tissue impairment were seen in females with a premutation; for example, *cubitus valgus* (64%), mild kyphoscoliosis in the thoracic region (21%), hyperextensible finger joints (43%), flat feet (29%), and muscular hypotonia (14%). In half of the patients with a premutation, the heart had been evaluated before our investigation. One patient had a congenital valve defect and ventricle defect; in other patients, no abnormality was found. Surprisingly, otitis media had been diagnosed in 54% of the Estonian patients. As far as we know, the clinical phenotype of females with a premutation has been reported to be normal.

Among Estonian patients, epilepsy was present in one (7%) patient with a premutation. Previously Kluger *et al.* (1996) reported two patients with a premutation association with epilepsy. First patient was a boy who had Rolandic epilepsy. The second patient was a 5-year-old girl who had groups of generalized spike wave complexes on EEG.

There were two main causes why females with a premutation were sent for FXS testing. The majority (79%) of patients were evaluated because of FXS in the family; and an additional 21% were investigated because of the infertility or POI.

5.1.6. Practical problems in genetic counselling of FXS

In article II we described one familial case, where a healthy grandfather with a premutation (79–81 CGG repeats) passed on his chromosome X with the premutation to his two daughters (77–79 CGG repeats). Because of the premutation, one of the daughters had POI at the age of 24 years and due to that she was infertile. At the age of 28 years, she was fertilised with her sisters' germ cell and she gave birth to a boy with FXS. The cause of the infertility had been undiagnosed by doctors and FXS had not been excluded; at the same time, the FXS premutation was not excluded in the donor of the germ cell, who was the mother's sister.

One of the many problems for premutation carriers is fear of having children. Bailey *et al.* (2003) found that only 55% of families who found out about the diagnosis of FXS in their first child were willing to have another child. In our study, we did not ask specifically if women were willing to have a second child after knowing about the diagnosis, but we saw two types of reaction. Firstly, after families were informed about POI, there were many women who turned to a genetic counsellor in their pregnancy. Secondly, we also noticed a fear of pregnancy among younger females with a premutation. Therefore, we realized how important it is to consult the families about prenatal diagnosis and about the possibilities of *in vitro* fertilization.

FXS is a very complex inherited disease causing problems to carriers of premutation and individuals with a full mutation. Moreover, FXS is a complicated disease because patients go to different specialists, for example to paediatricians, neurologists, psychiatrists, orthopaedics, gynaecologists, otorhinolaryngologists, and hopefully finally also to a geneticists. Performing this study we saw patients with full and premutation, intellectually normal and disabled, and both males and females. There were situations where it was difficult for the family to come to the consultation, because of the MR and/or behavioural problems. Moreover, there were situations where it was impossible to get adequate information from the family because the mothers had MR. It is essential to understand that although FXS has not always manifested in the family, the FXS premutation is one of the causes of POI. Therefore it is necessary to exclude the FXS premutation in all infertile females. The single occurrence of POI in a female should be an indication for fragile X syndrome DNA analysis.

5.2. Creatine transporter defect (*SLC6A8* gene defect) (publication IV)

5.2.1. Urinary creatine and creatinine analysis

An elevated urinary creatine:creatinine (Cr:Crn) ratio is a strong indication for *SLC6A8* deficiency, and it is considered to be an important marker for screening. In our study we found, among 49 families with XLMR, in 11 boys from 9 families, increased urinary Cr:Crn ratio (in 18% of investigated families, cases 1–11) (Table 6). As previously also shown, false positive urinary Cr:Crn ratios were found in 1.8–10% of investigated patients, which may normalize in repeat samples (Rosenberg *et al.*, 2007). Almeida *et al.* (2004) revealed that variation of the creatine compounds in urine sample during the day was not significant, indicating that a random urine sample is sufficient for the diagnosis of creatine deficiency syndromes. Still, creatine excretion can be increased after eating oily fish and beef, nutrition with a high creatine content. This could be the reason why 18% of our patients (case 4–11) showed transitory increased urinary Cr:Crn ratio. Therefore, we suggest that a repeated biochemical test should be performed before *SLC6A8* gene sequencing analysis.

Table 6. Results of the urinary creatine:creatinine ratio and *SLC6A8* gene DNA sequencing analysis.

Case	**Creatine/Creatinine ratio** mmol/mol (normal reference value depending on age of the patient)	**Repeated urinary analysis**	***SLC6A8* gene DNA analysis**
1 (VV)*	0.75 (<0.24)	n.d.	c.1271G>A
2 (RV)*	0.95 (<0.24)	0.82	c.1271G>A
3 (TV)*	1.9 (<0.72)	n.d.	c.1271G>A
4 (ML)	1.9 (<0.72)	0.29	c.1596+24–45dup
5 (RA)	1.63 (<1.2)	0.17	ND
6 (NL)	1.8 (<0.72)	0.14	ND
7 (TO)	0.29 (<0.24)	0.06	ND
8 (RR)	1.7 (<1.2)	0.055	ND
9 (RP)	0.241 (<0.240)	0.026	ND
10 (KM)	0.76 (<0.72)	0.063	ND
11 (MP)	0.466 (<0.24)	0.977; 0.018	ND

* from the same family; n.d. – not done; ND – no mutation detected

5.2.2. *SLC6A8* gene sequencing

To confirm false positive findings, we performed the DNA sequencing analysis of the *SLC6A8* gene in all patients with increased urinary Cr:Crn ratio.

In three brothers (patient 1–3) the diagnosis of CT defect was confirmed. Mutational analysis by DNA sequencing revealed a hemizygous missense mutation in the *SLC6A8* gene. Their mother was a carrier of the mutation. The mutation consists of a G to A transition (c.1271G>A) in exon 9 that results in the substitution of glycine by aspartic acid at position 424 (p.Gly424Asp). Although, many pathogenic missense mutations have been described, this mutation identified has not been previously reported (Table 2). This mutation is considered to be disease causing since it segregates with the phenotype, the glycine 424 is highly conserved across evolution and this mutation has not been identified in 276 control male chromosomes (Rosenberg *et al.*, 2004).

DNA sequence analysis in patient 4 showed a hemizygous variant in intron 11 (c.1596+24_45dup) of the *SLC6A8* gene. DNA studies in this patient did not identify any aberrant spliced *SLC6A8* products. From these results, we assume this intronic variation is non-pathogenic.

In patient 11 we additionally performed MRS, which showed a normal brain creatine level, because in this patient two repeated samples showed increased urinary Cr:Crn ratio, but no mutation was found in the *SLC6A8* gene. A creatine transport defect was excluded by normal creatine level in a brain MRS.

5.2.3. Epidemiology of creatine transporter defect

In the present study, among the investigated 49 families with suspicion of XLMR, a pathogenic mutation in the *SLC6A8* gene was found in one family. It constitutes a prevalence of 2% (95% confidence limits: 0.05–11.1%) of the total investigation group.

Previously, the prevalence of the creatine transporter defect has been assessed in six published studies (Rosenberg *et al.*, 2004; Newmeyer *et al.*, 2005; Clark *et al.*, 2006; Lion-Francois *et al.*, 2006; Arias *et al.*, 2007; Betsalel *et al.*, 2008). Among males with MR and/or autism the prevalence varies from 0.25% (Arias *et al.*, 2007) to 3.5% (Lion-Francois *et al.*, 2006). Studies focusing on the investigation of males from families with XLMR have found a prevalence of *SLC6A8* deficiency between 1.5% (95% confidence interval: 0 to 4.46) (Betsalel *et al.*, 2008) to 5.4% (95% confidence interval: 0 to 12.7) (Lion-Francois *et al.*, 2006). The same authors also suggested that *SLC6A8* mutations have a frequency about 3–5 times greater than most other XLMR genes and our work supports their data. All males with MR, autistic behaviour, epilepsy and/or expressive speech and language delay should be tested for creatine transporter defect.

5.2.4. Clinical phenotype of three brothers with creatine transporter defect

Previously it has been reported that patients with less-severe mutations (i.e., missense mutations) may have milder clinical signs (Salomons *et al.*, 2001). However, the clinical expression varied widely among affected males of this family.

5.2.4.1. Patient 1 (V.V.)

The oldest of the three brothers was a 21-year-old male, who was born normally at term with a birth weight of 3,400 g and with a length of 50 cm. In patient 1, bilateral varus clubfoot was evaluated after birth and operated on at the age of 1.5 years. He had speech delay – single words from the age of 14 months, sentences at the age 2 to 3 years. He started to walk independently at 14 months. His preschool development was considered age appropriate. At school, learning difficulties were noticed. At the age of 9 years and 11 months, he was assessed as having mild MR. At the age of 12 he was diagnosed with a myopathic syndrome of unknown aetiology. ENMG showed a motosensory axonal neuropathy. Muscle biopsy showed predominance of T1 fibres (70%). A brain CT scan was normal. At the age of 13.5 years a heart ultrasound showed a mild left sided dilated cardiomyopathy.

At the age of 22 years 6 months he was re-evaluated – his height was 181 cm (+0.3 SD), weight 61.7 kg (–1 SD) and head circumference 58 cm (+0.5 SD). He was noted to have muscle weakness, myopathic facies, broad forehead, midface hypoplasia, flat malar region, hyperextensible finger joints, soft skin, multiple nevi on his back and mild scoliosis. His behaviour was mildly autistic; additionally, he had social anxiety and attention deficit. Brain MRI was normal, however, and MRS demonstrated a marked reduction of brain creatine (Figure 8).

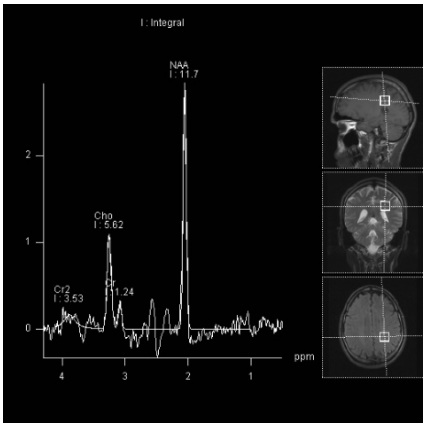

Figure 8. MRS image showing marked reduction of brain creatine in patient 1.

A neuropsychological study showed that his general intelligence as a combination of fluid and crystallized intelligence was significantly below average (68) and crystallized intelligence was slightly better than fluid intelligence (subtests mean scaled score was 5) respectively Gc73 versus Gf65 (Table 7). The lowest results were in subtests, where tasks require a comprehension of auditory sequentially presented information and listening comprehension (crystallized ability subtest Auditory Comprehension) and in planning speed and associative learning (fluid ability subtests Mystery Codes and Rebus learning). The test results of the RPM also indicated a mild deficit of the fluid abilities. Both tests indicate that according to the Diagnostic and Statistical manual of Mental Disorders – IV (DSM-IV) (American Psychiatric Association, 1994) he has mild MR.

5.2.4.2. Patient 2 (R.V.)

The second patient was the 15-year-old son of the family, who was born normally at term with a birth weight of 3,090 g and with a length of 50 cm. Similarly, to his older brother mild clubfoot was noticed soon after birth. His early motor development was normal – walking at the age of 13 months. However, his speech and language development were delayed: first words came from the age of 12 months, but by the age of two years there were 10 words in his vocabulary. The first time he was admitted to the hospital was at the age 13 months, for management of generalized seizures in addition to motor problems and MR. The EEG showed slow background activity with multi-focal spike waves in both hemispheres, which normalised by the age of 5 years. Seizures were treated successfully with sodium valproate until the age of 13 years. At the age of 3 years, his MRI showed symmetrical enlarged ventricles and wide subarachnoial spaces with cerebral atrophy. At the same time, a hypoplastic left testis was noticed, and it has not been described in any other patient with creatine transporter deficiency before. Neurological examination revealed a mild myopathic syndrome at the age of 6 years 6 months, with mildly increased tendon reflexes, dystonia (mentioned at age 8 years), ataxia, and dysarthria. He had a normal ENMG at the age of 6 years. Heart ultrasound showed minimal features of cardiomyopathy. Biopsy of the muscles showed predominance of T1 fibres (70%), similar as that seen in his brother.

At age 16.5 years, he was re-evaluated. His height was 165.7 cm (–1.5 SD), weight 51 kg (–1.5 SD) and head circumference 56 cm (–0.5S D). At that age, some dysmorphic features were noticed: muscle weakness, myopathic facies, flat malar region, high palate, hyperextensible finger joints, and soft skin. It was not possible to perform MRI and MRS in this patient due to his anxiety. He had moderate MR, expressive speech, and language delay. He was autistic with poor eye contact, social anxiety, stereotypic behaviour, attention deficit, aggressi-

veness, self-injurious, and obsessive-compulsive behaviour. He had been educated in a special school. Unfortunately, the assessment of general intellectual ability was not successful by an age appropriate test (KAIT), because he was not able to follow test instructions. The results of the RPM indicated moderate deficit of the fluid abilities.

Table 7. Kaufman tests scaled scores and RPM test percentile rank of investigated patients.

Tests and scaled score	**Patient 1** 22 years	**Patient 2** 16 years	**Patient 3** 8 years	**Mother** 44 years
Fluid intelligence				
K-ABC Sequential Scale subtests		*	56	
K-ABC Simultaneous Scale subtest		*	79	
KAIT subtests	65			73
Crystallized intelligence (Gc)				
K-ABC Achievement Scale subtests		*	88	
KAIT subtests	73			93
General intelligence				
K-ABC or KAIT	68	*	58	81
RPM	10–25%	<5%	10–25%	25–50%

* not administered

5.2.4.3. Patient 3 (T.V.)

The 8-year-old son was also born normally at term with a birth weight 3345 g and with a length of 50 cm. At the age of 2 months, he was for the first time investigated and spastic diplegia was diagnosed. Hepatitis of unknown origin was diagnosed at the age of 1 year. A brain scan was normal. Early development was age appropriate, walking at 14 months; single words from age 9 months and sentences at age 2 years. At the age of 4 years speech delay was noticed. He has mild pyramidal tract syndrome – spastic gait and increased tendon reflexes. Additionally, behavioural problems were noticed – attention deficit disorder, aggressiveness, impulsiveness, mood disorder and hyperactivity.

At the age of 8 years and 4 months his height was 127.5 cm (–0.5 SD); weight 25.6 kg (–0.5 SD) and head circumference 53.3 cm (0 SD). Moreover, similarly to his older brothers poorly developed muscle mass, myopathic facies, with unfolded superior helices, hyperextensible finger joints and soft skin were noted. A MRI at age 8 years 4 months was normal; but unfortunately, brain MR spectroscopy was not possible due to hyperactivity.

He attended the First Grade in a normal school. He had learning difficulties in reading and math and some attention and concentration problems. His general intelligence as fluid intelligence was significantly below average

(Gf58). Although, achievement of crystallized intelligence was found to be significantly better (Gc88), the general intelligence level stays only slightly below average. It was diagnosed as a mixed disorder of specific learning disabilities and not as MR (Table 6). The results of the boy were lower in Simultaneous Scale subtests (mean scaled score was 3), e.g. in tasks of visual closeness and simultaneous processing (subtest Gestalt Closure – scaled score 1) and inductive and general sequential reasoning (subtest Photo Series – scaled score 2). The results in Sequential Scales subtests (mean scaled score was 6) were slightly better in tasks of working memory, memory span and short-term auditory memory (subtest Number Recall and Word Sequence – both scaled scores 7). The test results of the RPM also indicate a weakness of fluid abilities.

5.2.4.4. Discussion of clinical features of all male patients

The phenotype of described patients is somewhat different from published cases. Myopathic syndrome and mild cardiomyopathy was diagnosed in our patient 1 and 2; they had also a congenital foot deformity, which may be secondary to muscular weakness (Hahn *et al.*, 2002). All three brothers had facial myopathy. Kleefstra *et al.* (2005) pointed out myopathic faces in adult patients and suggested that this condition may be progressive. The presence of a mutation in *SLC6A8* may result in muscle weakness or other symptoms of muscle impairment due to an inability to generate ATP from phosphocreatine (Hahn *et al.*, 2002). The poorly developed muscular system was described earlier and in some cases even muscular or mitochondrial disorders were first suspected (Mancini *et al.*, 2005). However, previous studies have shown normal creatine concentration in muscle (deGrauw *et al.*, 2003). The evidence of cardiomyopathy in two of them could mean that the heart is creatine deficient and it should be studied further.

Mancini *et al.* (2005) observed severe language delay in combination with social problems as a semantic-pragmatic language disorder such as dysarthria, oral dyspraxia and an attention deficit hyperactivity disorder. They also noticed a pattern of MR, in which the domains of attention, impulse control, speech, oral movements and language, are more affected than other cognitive and motor functions. The same authors considered this cognitive pattern to be unique to the creatine transporter defect. Among our patients, we also saw significant problems in speech and language development combined with attention and behavioural difficulties. In addition to poor verbal abilities, all three patients had weaker fluid intelligence compared with crystallized intelligence, which refers to novel problem solving and ability to learn. According to a large body of research (Kaufman and Kaufman, 1983, 1993), crystallized intelligence remains throughout the adult lifespan, while fluid intelligence rises in late teens

and starts to decline in the ageing process. Among our patients, the deficit of fluid abilities seems to be affected throughout life.

5.2.4.5. Female carrier of creatine transporter defect

The 45 year old mother of patients 1–3 had a head circumference of 55 cm (0 SD), height 162.4 cm (–1 SD) and weight 54.3 kg (–1 SD); and down-slanting eyelids were noticed. Her main clinical problems were headaches and heat intolerance. Additionally, she had attention deficit and impulsiveness. MRS of the brain demonstrated mild reduction of creatine (Figure 9).

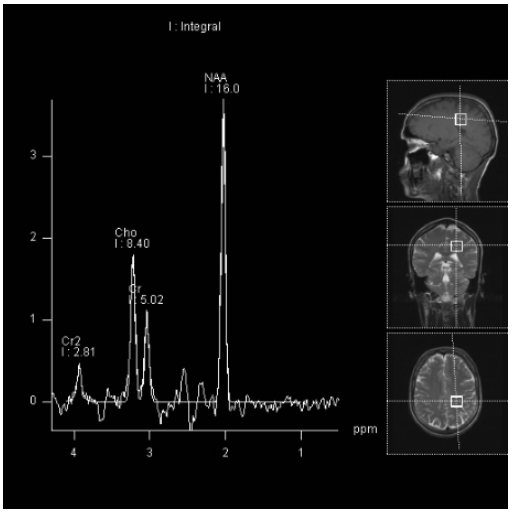

Figure 9. MRS image showing marked mild reduction of brain creatine in the mother.

Previously, approximately 50% of female carriers show behavioural and learning disabilities, which are ascribed to skewed chromosome inactivation (Mancini *et al.*, 2005). Some other authors have also shown that female carriers may have a mild cognitive impairment, behavioural disturbances and/or learning disability (Salomons *et al.*, 2001; Hahn *et al.*, 2002; Kleefstra *et al.*, 2005; Mancini *et al.*, 2005). Our patient has graduated from secondary school (12 grades). Her general intelligence as a combination of fluid and crystallized intelligence was below average (81) and crystallized intelligence was slightly better than fluid intelligence (subtests mean scaled score was 7) respectively Gc93 versus Gf73 (Table 7). The lowest results were in subtests, where adequate solution of the tasks needs a comprehension of auditory sequentially

presented information and listening comprehension (crystallized ability subtest Auditory Comprehension) and in planning speed and associative learning (fluid ability subtests Mystery Codes and Rebus learning). She gained better scores in verbal tasks, which need semantic flexibility (subtest Double Meaning), and visual closure and inferring part-whole relationships (subtests Definition) – both tests refer to the crystallized abilities. The test results of the RPM indicate the average level of fluid abilities. The intellectual functioning of the mother is at the average level by both tests results.

5.3. Single cases detected with gene chip technologies (array-MAPH, Illumina SNP array)

In 83 patients with unspecific MR and their family members, we performed gene chip analysis. Array-MAPH analysis was introduced first in 2006, and in 2008 Illumina analysis. Among investigated patients, we present two case reports as a part of this thesis.

5.3.1. A female with partial deletion Xp22.33 and duplication Xp22.12–22.32 (publication V)

The distal part of the short arm of the chromosome X is particularly complaisant for studies because of the high frequency of deletions occurring in this region (Ballabio *et al.*, 1989). Females with Xp deletions seldom manifest any abnormalities due to the presence of a normal chromosome X and preferential inactivation of the abnormal chromosome X. We reported a 16-year-old female with short stature (–5 SD), normal puberty, panic attacks, absence epilepsy, some stigmata of Turner syndrome and a Madelung deformity; she carries a complex aberration in Xp22.

The patient was the first child of healthy Estonian non-consanguineous parents. Family history was negative for congenital anomalies. The patient was born by normal vaginal delivery at term. First problems occurred at the age 10 years, when epilepsy was first noticed. At the age of 11 years, symptomatic typical absence epilepsy was diagnosed and treated at the beginning with *valproic acid* (29mg/kg/day). *Lamotrigine* (50mg/day) was added because seizures were not fully controlled. EEG investigations have been performed every year since age 11 years. The EEG investigations show always normal background activity, that corresponds abrupt with high voltage symmetrical and synchronous single, double and occasionally triple spike wave complexes around three times per second. These paroxysms are never provoked by photic stimulation. They occur either occasionally or mostly provoked by hyperventilation. Duration of these

paroxysms is 4–10 seconds. On parallel video-monitoring mild eyelid myoclonia was mentioned during electrical discharges. Despite of treatment absence type electrical discharges on EEG still perceive. The treatment was changed at age 12 years 3 months; since then she is on *topiramate* monotherapy (100mg/day). An MRI of the brain at age 13 years was normal. Moreover, clinical absences are seldom observed by the patient's mother.

Laboratory testing at age 11 showed mildly increased thyroid-stimulating hormone 8.97 mIU/L (N: 0.4–4.0); insulin-like growth factor level-1 (231.2 μg/L, N: 102–664 μg/L), triiodothyroxine (1.22 nmol/L, N: 1.2–4.5 nmol/L), and thyroxine (124.1 nmol/L, N: 75–160 nmol/L) levels were all normal; anti-thyroglobulin was negative. Her thyroid gland was small (volume 2.1 mL). X-ray examination for bone age was normal, but densitometry showed moderate osteoporosis. US of abdominal organs were normal. Her psychomotor development was normal. At the age of 13 years she was treated with *levothyroxine*, *calcium carbonate* and *somatotropine* (4 IU/day). Later, at the age of 14 years, the treatment was changed to *gonadotropine* (1.8 mg/day). Treatment was discontinued at the age of 14 years 10 months when her growth stopped.

Routine chromosomal analysis indicated an abnormal chromosome X with the suspicion of additional material on the short arm. FISH analysis showed metaphases with a normal chromosome X and an abnormal X chromosome that carried a deletion of the distal part of Xp22.33 (including short stature homeobox gene), and a duplication of Xp22.3 proximal to the deleted segment. Array-MAPH methodology (Patsalis *et al.*, 2007) was used to identify the size of the duplication and data showed the same duplication found by fluorescent *in situ* hybridization analysis. Moreover, the duplicated area spanned a greater region than expected, extending to chromosomal bands Xp22.12-p22.32. Her parents were not investigated as they refused additional investigations.

There are three publications with similar combined subtelomeric deletion/ duplication events in the X chromosome. In all of these cases, the distal deletion of the short arm of the X chromosome was similar to our patient, but duplication was on the long arm of the X chromosome (Mohandas *et al.*, 1987; Kokalj-Vokac *et al.*, 2004; Orellana *et al.*, 2006).

Sex chromosome aberrations are frequently associated with stature changes (Ogata and Matsuo, 1993); and the growth pattern in Turner syndrome is characterized by intrauterine growth retardation, progressive decline in growth velocity in childhood, lack of pubertal growth spurt, and delayed growth cessation (Ogata and Matsuo, 1995). Our patient's birth weight 2,300 g (–2.5 SD) and length 47 cm (–2 SD) were small. Similarly, at the age of 15 years her height was 140.2 cm (–5 SD), weight 43.5 kg (–2 SD), and OFC 54 cm (–0.5 SD). However, her mother's height was 158 cm and father's height 170 cm.

The likelihood of ovarian failure in patients with terminal Xp deletions is directly related to the size of the deletion (Zinn *et al.*, 1998; Ogata *et al.*, 2001; Zinn *et al.*, 2007). In our patient, pubertal development at the age of 11 years was

normal: breasts at stage of puberty 2–3 and pubic hair at stage of puberty 2. Ultrasonography of genitals showed small ovaries. Furthermore, at the age of 14 years 10 months she started her menstrual cycle, which was irregular at the beginning, but later became regular. At the age of 16 years, laboratory testing of hormones showed normal pubertal development: follicle-stimulating hormone was 5.30 mIU/mL (prepubertal value 0.5–3.7); luteinizing hormone 4.60 IU/L (prepubertal value <3.8), and oestradiol 413 pmol/L (prepubertal value <15pg/mL).

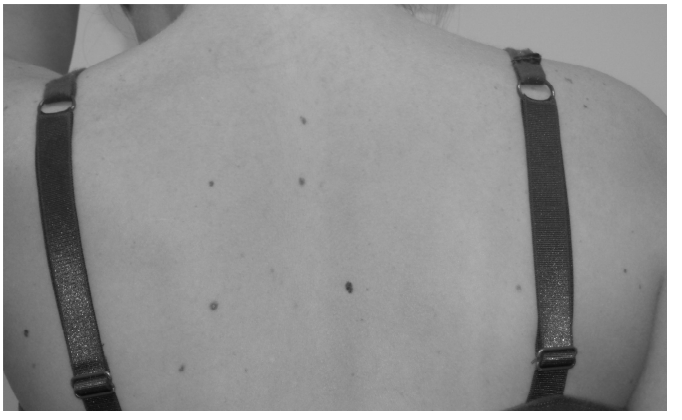

Figure 10. The back of the patient note numerous pigmented nevi.

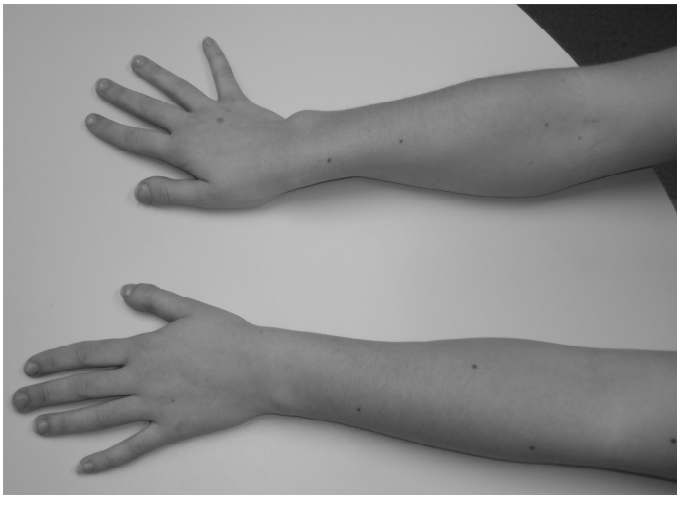

Figure 11. The hands of the patient note Madelung deformity.

The deletion in our patient involves the Xp22.33 region, which according to the OMIM database contains 11 genes, six of them without known function. There are two genes amongst these which may contribute to the patient's phenotype: the *SHOX* gene (OMIM 604271) which is responsible for short stature (Rao *et al.*, 1997) and the visuospatial/perceptual abilities gene (OMIM 313000) gene which causes the neurocognitive phenotype in Turner syndrome patients.

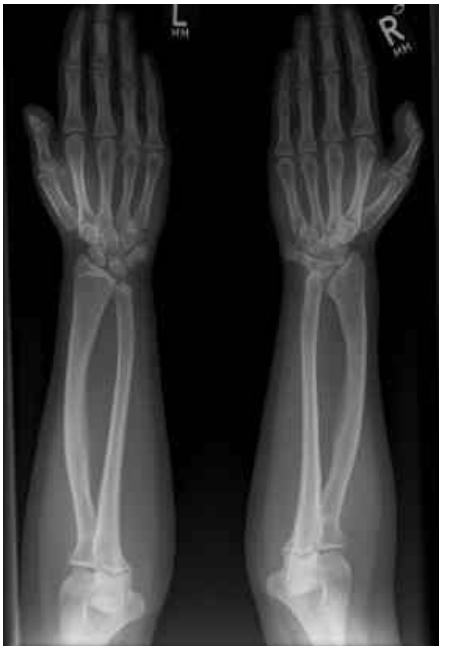

Figure 12. X-ray of the hand and elbows.

Haploinsufficiency of the *SHOX* gene has been shown to cause Turner skeletal features such as short metacarpals, *cubitus valgus*, and Lèri-Weill dyschondrosteosis. In our patient there were present symptoms that may contribute to the deletion of the *SHOX* gene – she had a low occipital hairline, high arched palate, numerous pigmented nevi (Figure 10), *cubitus valgus*, shortening of 4th and 5th metacarpals, and mild hyper-extensible knees and Madelung deformity (Figure 11), but not a typical Lèri-Weill dyschondrosteosis. However, loss of the Xp22.3 region is not always associated with the classic presentations of Lèri-Weill syndrome, or condrodysplasia punctata (Boycott *et al.*, 2003). Madelung deformity was also confirmed in our patient with an X-ray investigation of the hands and elbows, that showed Madelung deformity, hypoplasia of the distal part of the ulna and *processus styloideus*, short distal phalange of both thumbs, and the distal part of ulna dislocated dorsally (Figure 12). Prevalence of the *SHOX* gene mutation in children with unexplained short stature is at least 2.4% (Rappold *et al.*, 2002). However,

Ogata and Matsuo (1993) have shown that *SHOX* gene haploinsufficiency alone is unlikely to explain the growth failure in Turner syndrome. Moreover, Madelung deformity is seen in only about 7% of Turner syndrome females, despite the fact that the vast majority of these individuals have only one copy of the *SHOX* gene (Zinn and Ross, 2001). However, the phenotypic expression in differential patients may be the result of different X-inactivation. Unfortunately, it was not possible to investigate X-inactivation in our patient.

Already Boycott *et al.* (2003) indicated that one or more genes involved in learning and attention may reside in Xp22.3, and this opinion has found support by Zinn *et al.* (2007) who proved that the distal 8.3 Mb of the X chromosome, is responsible for a Turner syndrome neurocognitive phenotype. The same authors suggest that absence of a specific gene that codes for specific cognitive dysfunction within Xp22.3 most likely affects a biological activity that is intimately involved in the development of the phenotype, manifesting as a complex and nonfocal neurocognitive phenotype. They also assume that the Turner syndrome cognitive phenotype is due to multiple cognitive determinants and multiple genes. Our patient's clinical phenotype supports this hypothesis, as she had at the age 15 years shyness, easy excitability, unexplained fear of medical investigations and incline to panic. Furthermore, mild MR was revealed during neurological examination. One isoform, *SHOXa*, is expressed in many embryonic tissues, including the brain (Rao *et al.*, 1997), and it has been speculated that the gene affects functions in nervous system development (Ross *et al.*, 2000). However, intellectual impairment is not observed in patients with Langer mesomelic dwarfism (Jones, 2006), in whom there are homozygous *SHOX* gene mutations. Ross *et al.* (2000) also concluded that the defined Turner syndrome neurocognitive phenotype is genetic in aetiology and the responsible gene is visuospatial/perceptual abilities gene, which maps to distal Xp22.3. The smallest deletion associated with the Turner syndrome neurocognitive phenotype was reported in a mother and daughter missing <2 Mb of terminal Xp (Ross *et al.*, 2000).

The duplication in our patient involves the Xp22.12–p22.32 region, which according to the OMIM database contains at least 93 genes, 49 of them with unknown function. It is difficult to speculate which gene over-expression in this region may contribute to the phenotype of our patient. However, at least two of them, MR, epileptic seizures, hypogonadism/hypogenitalism, microcephaly and obesity gene, and aristaless-related homeobox gene, are associated with epileptic seizures, which occurred in our patient. There are not, to our knowledge, descriptions of any other patients with similar Xp22.12-p22.32 duplication.

5.3.2. A boy with partial 17q deletion (publication VI)

We reported an additional case with partial 17q deletion, a 7-year-old boy with multiple congenital anomalies and developmental problems. The data of previous published cases are summarized in Table 3.

The patient was born by normal vaginal delivery at term. His birth weight and length were normal. He had microcephaly (–2 SD). His umbilical cord was very short. He was the first child in this Estonian couple; the mother had two healthy daughters from a previous marriage. The family history was unremarkable.

First evaluation by a clinical geneticist was performed at the age of 2 weeks in intensive care unit. He had coarse facial features, hypertelorism, epicanthic folds, a broad nasal bridge, dysplastic ears, short neck with prominent skin folds, hirsutism, genital anomaly (micropenis), broad gap between I-II toes and partial syndactyly of II-III toes. Chromosomal analysis was performed, which showed an apparently balanced karyotype 46,XY,inv(9)(p12q13). Parental karyotypes were normal; his father carried the same inversion of chromosome 9. At the age of two months, he was placed in institutional care.

At the age of 20 months Feingold syndrome (microcephaly-oculo-digital-esophageal-duodenal syndrome, OMIM 164280) or a still unidentified small chromosomal aberration were considered as differential diagnoses. Since, his clinical findings had some overlap with this syndrome, where tracheo-esophageal fistula (TEF)/esophageal atresia (OA) or duodenal atresia is commonly reported together with microcephaly and mesobrachyphalangy (Celli *et al.*, 2000). FISH analysis was done using DAPI staining and DNA probe YAC 953g11, which maps to 2p23–24. YAC clone 953g11 had previously been used for detecting the deletion of the chromosome region for Feingold syndrome (Celli *et al.*, 2000). In all 9 analyzed metaphases of this patient, YAC 953g11 showed normal hybridization signals on both chromosome 2 homologues.

At the age of 7 years clinical evaluation revealed normal stature, poor weight gain (–2.5 SD), severe microcephaly (–5 SD) and trigonocephaly. The patient had profound MR with stereotypic movements. Additional, features included a high forehead, upslanting palpebral fissures, blepharophimosis, ptosis, hypertelorism, epicanthal folds, strabismus, thin upper lip, broad nasal tip, high glabella, high palate, low set and dysplastic ears with one ear tag on the right side, prognathia, and mild hypoplasia of maxilla (Figure 13a-b). His all fingers were long and fixed in extension and symphalangism (proximal and distal interphalangeal joints). There was partial syndactyly of III-V fingers and II-IV toes, and sandal gap of I-II toes (Figure 14a–b). He also had kyphoscoliosis, and contractures of large joints (Figure 13c). In addition, X-ray investigation showed spina bifida occulta in the region of 1st and 6–9th thoracic vertebrae; bone osteoporosis and abnormally formed metacarpal and phalangeal bones. He had genital anomaly with a micropenis, and cryptorchidism. Brain MRI was

normal. During paediatric evaluations, consecutive clinical problems such as asthma, iron deficient anaemia, and mild hypothyroidism were diagnosed.

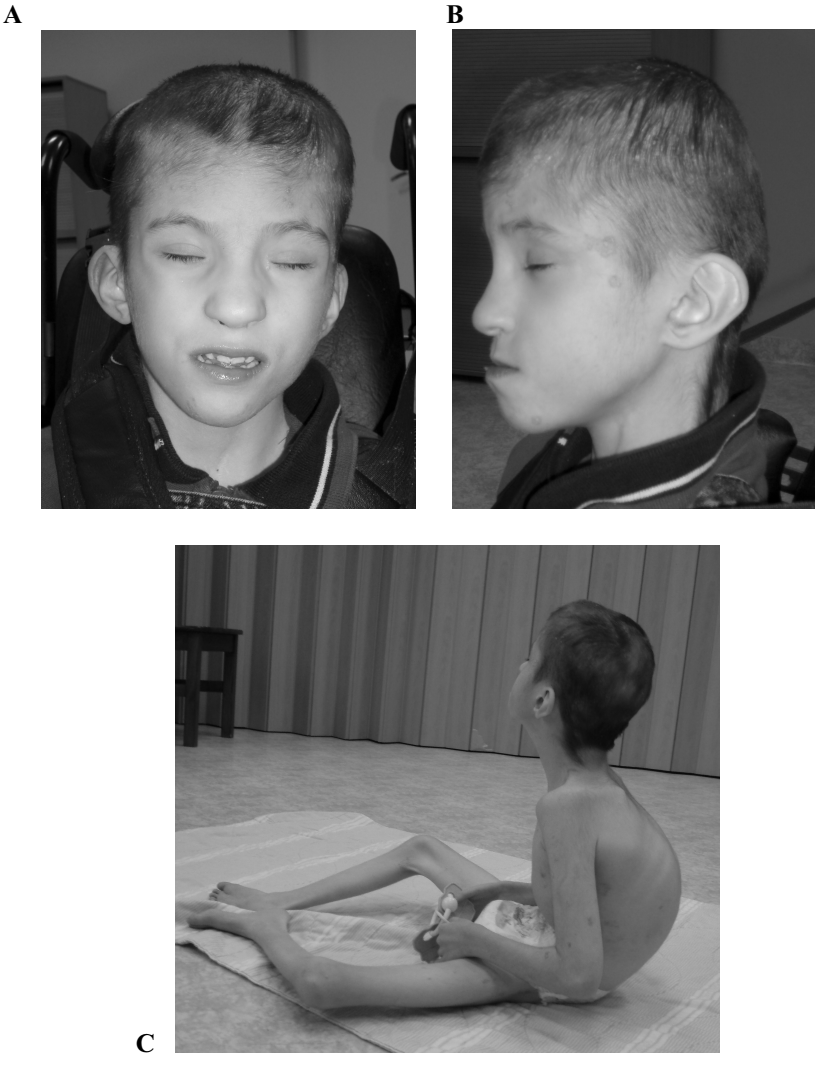

Figure 13. (a) The facies of the patient; note high forehead, upslanting palpebral fissures, blepharophimosis, ptosis, hypertelorism, epicanthal folds, thin upper lip and broad nasal tip. (b) The facies of the patient from the side; note high glabella, low set and dysplastic ears, prognathia, and mild hypoplasia of maxilla. (c) Patient in sitting position; note kyphosis, scoliosis, and contractures of large joints.

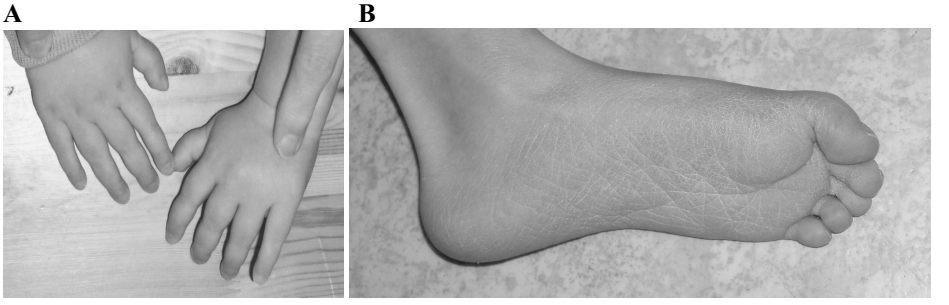

Figure 14. (a) Hands of the patient; note partial symphalangism of II–V fingers. (b) Left feet of the patient; note sandal gap of I-II toes.

We applied Infinium-2 whole-genome genotyping assay with Human370CNV-Duo BeadChips (Illumina Inc.), which showed a 5.9 Mb deletion in chromosome band 17q22-q23.2 with breakpoints between 48,200,000–48,300,000bp and 54,200,000–54,300,000bp (according to NCBI 36). The aberration was confirmed by real-time quantitative PCR analysis. The investigation of his parents was not possible; he was institutionalized.

The deletion in our patient involves the region 17q22–q23.2, where according to the Ensembl (http://www.ensembl.org) database more than 40 genes are located. Already, Marsh *et al.* (2000) suggested that locus for tracheo-oesophageal fistula (TEF)/oesophageal atresia (OA) may be located in the region 17q22–q23 and our findings support this hypothesis, because soon after birth also in our patient a tracheo-oesophageal fistula was diagnosed and corrected. Moreover, TEF/OA has been previously described in two other cases (Park *et al.*, 1992). The aberrations in these cases overlap with the deleted region in our case (Figure 15). Felix *et al.* (2007) have found in animal studies that three genes in this region (*NOG, RARα* and *TBX4*) play a role in the development of TEF/OA. Animal studies demonstrated the involvement of *NOG* gene in foregut morphogenesis. Null mutant mice develop TEF/OA in approximately 70% of cases (Felix *et al.*, 2007). However, so far mutations in *NOG* have not been associated with TEF/OA in humans (van den Ende *et al.*, 2005; Hirshoren *et al.*, 2008). Mutations in retinoid acid receptor alfa (*RARα*) display abnormalities in the separation of trachea and oesophagus (Luo *et al.*, 1996), but in our case *RARα* is located outside the deleted region. The third candidate gene for the development of TEF/OA was the *TBX4* gene (Felix *et al.*, 2007), but in our patient this gene is also not deleted. However, there may be other candidate genes in this deleted region, which function is presently unknown for us. Therefore, it still remains unknown, which gene in this region precisely contributes to the development of TEF/OA, but we suggest that it may

be the *NOG* gene. Of the proposed candidate genes haploinsufficiency of the *NOG* gene remains the most likely candidate.

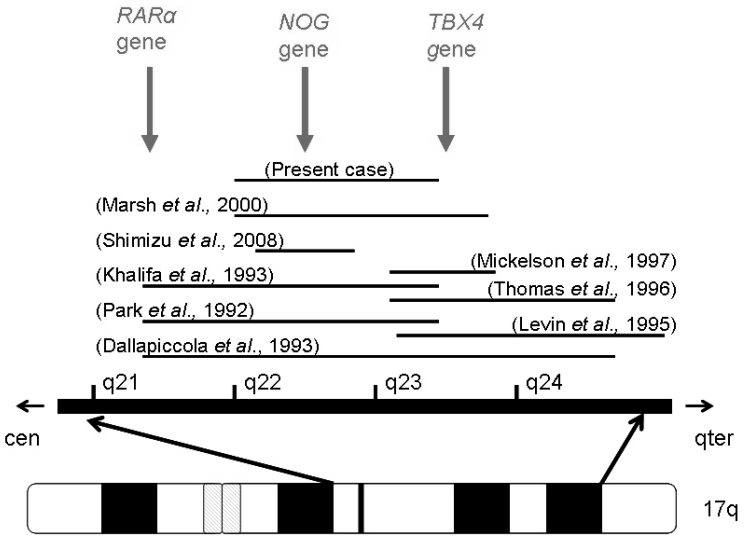

Figure 15. Location of genes and deleted regions of our patient and previously reported cases in region 17q21-q24, see also Table 3.

For exclusion of 201 mutations in eight genes that are known to cause bilateral hearing loss in this patient, APEX (arrayed primer extension) gene chip analysis was performed. This gene chip covers mutations in several connexin genes (*Connexin-26, Connexin-30, Connexin-31, Connexin-43*), in two genes of the SLC26 family (*Prestin* and *Pendrin* gene), and in two mitochondrial genes (12S-ribonuclear-RNA and the transfer RNA for the serine gene) (Gardner *et al.*, 2006). This patient did not carry any of 201 investigated mutations.

NOG gene encodes the polypeptide Noggin, which is a bone morphogenetic protein (BMP) antagonist, and belongs to the Transcription Growth Factor-beta superfamily (TGFβ) (Balemans and Van Hul, 2002). Noggin is expressed in multiple sites such as lung, skeletal, muscle and skin (Valenzuela *et al.*, 1995); and it has been shown to bind and inactivate members of the TGFβ, such as BMP2 and BMP4 (Zimmerman *et al.*, 1996). Furthermore, heterozygous nonsense and missense mutations in *NOG* gene located in 17q21–22 (MIM 602991), have been shown to inflict conductive hearing loss caused by congenital stapes ankylosis (van den Ende *et al.*, 2005; Hirshoren *et al.*, 2008). In our patient bilateral conductive hearing loss (60–70dB) was also diagnosed, but unfortunately the presence of stapes ankylosis was not possible to specify in

this case. Bilateral hearing loss has been revealed previously only in one case with deletion 17q22-q23.2 (Khalifa *et al.*, 1993). In other reported cases no hearing loss has been documented (Dallapiccola *et al.*, 1993; Levin *et al.*, 1995; Marsh *et al.*, 2000), however some case have been reported at an age where a milder hearing loss may not have been clinically apparent. However, Noggin is shown to be essential for proper skeletal development (Brunet *et al.*, 1998). The excess BMP activity in the Noggin null mutant mice was revealed to cause excess cartilage and failure to initiate joint formation (Brunet *et al.*, 1998). In addition, mutations in *NOG* gene cause in humans proximal symphalangism (SYM1 [OMIM 185800]) and multiple-synostoses syndrome (OMIM 186500) (van den Ende *et al.*, 2005; Hirshoren *et al.*, 2008). Features of SYM1 include failure of joint formation at the proximal interphalangeal joints of the hands, but distal interphalangeal joints are rarely affected. In our patient we observed symphalangism and ankylosis in proximal and distal interphalangeal joints of the hands. Moreover, symphalangism (Park *et al.*, 1992; Dallapiccola *et al.*, 1993; Khalifa *et al.*, 1993; Thomas *et al.*, 1996; Mickelson *et al.*, 1997) and joint contractures (Park *et al.*, 1992; Khalifa *et al.*, 1993; Thomas *et al.*, 1996; Marsh *et al.*, 2000) were seen in most of the previously described cases.

Hyperopia and strabismus has also been described in patients with mutations in *NOG* gene (van den Ende *et al.*, 2005; Hirshoren *et al.*, 2008). Furthermore, vision impairment has been described in four patients (Khalifa *et al.*, 1993; Levin *et al.*, 1995; Thomas *et al.*, 1996; Mickelson *et al.*, 1997) with partial deletion of 17q, causing haploinsufficiency of the *NOG* gene. In more detail, hyperopia was described in two cases (Khalifa *et al.*, 1993; Mickelson *et al.*, 1997) as well as in our patient, who had profound visual impairment (hyperopia, right eye +7 diopters, left eye +4 diopters). Furthermore, esotropia was seen in two cases (Thomas *et al.*, 1996; Mickelson *et al.*, 1997), and abnormal optic nerves was seen in one patient (Table 3) (Levin *et al.*, 1995). This last patient died on day 17 of life, and it was too early for diagnosing hyperopia. However, one patient with a deletion of 17q, who was reported not to have vision impairment, was evaluated at the age of 6 months (Park *et al.*, 1992), but vision impairment can develop at a later age.

One additional genetically heterogeneous clinical problem in our patient – psoriasis, which had since 4 years of age, could be also related to partial deletion of 17q. Many groups have demonstrated linkage or association of familial psoriasis to a locus on 17q25 (Hwu *et al.*, 2005). However, all genes in the region 17q25 that have been suggested to contribute to psoriasis susceptibility, are located more distally and outside the deleted region in our patient. Psoriasis has not been reported in any other cases with partial deletion of 17q.

In conclusion, we hypothesize that conductive hearing loss, skeletal anomalies including symphalangism, contractures of joints, and hyperopia in our patient is probably caused by haploinsufficiency of the *NOG* gene.

6. CONCLUSIONS

1. The prevalence of fragile X syndrome among the MR population in Estonia was 2.7%; and the prevalence among boys with MR was 3.1%. The prevalence of FXS among the MR population is similar to the results of previously reported studies that were performed on Caucasian males.

2. The overall live-birth prevalence rate of FXS from 1984 to 2005 was 1:27,115 (95% confidence intervals 1:16,059–1:45,787). Moreover, the minimum live-birth prevalence for FXS among boys was 1:13,947 (95% confidence intervals 1:8,264–1:23,529). The live-birth prevalence among boys in Estonia is lower than the prevalence in other countries.

3. The median age at diagnosis in males with FXS in Estonia was 4.5 years. This shows that the awareness of FXS among Estonian doctors is similar to other European countries.

4. The clinical phenotype of the boys with FXS was similar to previously reported cases. All males with full mutation were mentally retarded (moderate to severe). A majority of males (87%) had typical facial features: long narrow face, high and wide forehead, long and/or prominent ears, prominent jaw and dental crowding. Behavioural problems were seen among males with FXS in 87% of cases, most frequently autistic features, poor eye contact, concentration problems and stereotypes. Obesity was a problem in 64% of male patients with FXS.

5. The main clinical feature of the females with FXS premutation was premature ovarian insufficiency or irregular menstruation (43%). A minority of females had some features of the FXS facial phenotype (21%). Only one patient with a premutation had mental retardation and behavioural problems. The single occurrence of premature ovarian insufficiency in a female should be an indication for FXS DNA analysis.

6. Among 49 families with XLMR, in 11 boys from 9 families we found increased urinary Cr:Crn ratio (in 18% of investigated families). The number of false positive results was higher than in previous studies. Therefore, we suggest that a repeated biochemical test should be performed before *SLC6A8* gene sequencing analysis.

7. In three related brothers and their mother a missense mutation – c.1271G>A (p.Gly424Asp) was identified. This mutation has not been previously reported.

8. The prevalence of creatine transporter deficiency among investigated families with suspicion of XLMR was 2 % (95% confidence limits: 0.05–11.1%). All males with MR, autistic behaviour, epilepsy and/or expressive speech and language delay should be tested for creatine transporter defect.

9. The phenotype of patients with creatine transporter defect was somewhat different from published cases and the clinical expression varied widely among affected brothers. Patient 1 and 3 had relatively mild clinical expression (mild MR and attention deficit disorder), but patient 2 had all the typical clinical signs such as moderate MR, autistic features, expressive dysphasia and epilepsy. Myopathic syndrome and mild cardiomyopathy were diagnosed in patients 1 and 2; they also had a congenital foot deformity, which may be secondary to muscular weakness. All three brothers had facial myopathy. We saw significant problems in speech and language development combined with attention and behavioural difficulties.

10. A phenotype of a 16-year-old female (short stature, normal puberty, mild mental retardation, absence epilepsy, some stigmata of Turner syndrome and a Madelung deformity) is caused by the deletion of the Xp22.33 region. Two genes in this region may contribute to the patient's phenotype: short stature homeobox and visuospatial/perceptual abilities. She had additionally the duplication of the Xp22.12-p22.32 region, whose contribution to her phenotype remained unknown to us. This small complex chromosome X aberration has not been described before.

11. In a 7-year-old boy profound MR, severe microcephaly, facial dysmorphism, symphalangism, contractures of large joints, hyperopia, strabismus, bilateral conductive hearing loss, genital abnormality, psoriasis vulgaris, and tracheo-esophageal fistula were caused by a 5.9 Mb deletion in chromosome band 17q22-q23.2. Haploinsufficiency of *NOG* gene was implicated in the development of conductive hearing loss, skeletal anomalies including symphalangism, contractures of joints, and hyperopia in our patient and may contribute to the development of tracheo-oesophageal fistula and/or oesophageal atresia.

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SUMMARY IN ESTONIAN

VAIMSE ARENGU MAHAJÄÄMUSE GENEETILISED PÕHJUSED EESTIS: FRAGIILNE X SÜNDROOM JA KREATIINI TRANSPORTERI DEFEKT

Fragiilne X sündroom

Fragiilne X sündroom (OMIM 309550) (FXS) on kõige sagedasem inimesel esinev monogeenne haigus, mis põhjustab pärilikku vaimse arengu mahajäämust. Seda sündroomi determineerib lookuses Xq27.3 asuva *FMR1* (*fragile X mental retardation 1*) geeni inaktivatsioon, mis on tingitud *FMR1* geenis paiknevate trinukleotiidsete (CGG) korduste arvu suurenemisest (Kremer *et al.*, 1991; Oberle *et al.*, 1991; Verkerk *et al.*, 1991; Yu *et al.*, 1991). FXS CGG kordustel on neli vormi: normaalne (6–50 kordust), vahepealne (50–58 kordust), premutatsioon (59–200 kordust) ja haiguslik (>200 korduse). Populatsiooniuuringutega on näidatud, et täismutatsiooni esinemissagedus valge rassi meeste seas on 1:3717 kuni 1:8918 (Crawford *et al.*, 2001). Vastavalt hiljutistele Hagermani (2008) arvutustele eeldatav täismutatsiooni esinemissagedus meeste ja naiste seas on 1:2355 ning eeldatav premutatsiooni esinemissagedus meeste seas on 1:282.

FXS puhul esineb meeste fenotüübis iseloomulik prominentne otsmik ja lõug, suured, düsmorfsed ja peast eemale hoidvad kõrvad, makroorhidism ja kõrge suulagi. Vaimse arengu mahajäämus võib olla 19%-l juhtudel kerge, 32%-l mõõdukas ja 38–47%-l raske (Hagerman and Hagerman, 2002). Suureks probleemiks selle sündroomiga meeste puhul on käitumishäired: umbes pooltel juhtudel esineb hüperaktiivsust, rahutust, kontsentreerumise häireid, arglikkust, puudulikku silmside kontakti; kolmandikul juhtudest esineb autistlikke käitumisjooni, (auto)agressiivsust, tujukust ja hammustamist. Samuti esineb FXS meestel tavapopulatsioonist sagedamini sidekoe nõrkust, sellest tulenevalt liigeste hüpermobiilsust, mitraalklapi prolapsi, alaneva aordi laienemist, lampjalgsust, kubemesonga, õhukest sametist striiadega nahka, tortikollist, suulaelõhet ja küfoskolioosi.

FMR1 premutatsiooni esinemist on seostatud õpiraskuste, enneaegse ovariaalse puudulikkuse ja fragiilse X seotud treemor/ataksia sündroomiga (FXTAS) (Hagerman and Hagerman, 2004; Sherman *et al.*, 2007).

Kreatiini transporteri defekt

Praeguseks on kirjeldatud kolme kreatiini metabolismi häiret. Esimesed kaks on autosomaalsed retsessiivsed haigused, nimelt guanidinoatsetaat metüültransfe-

raasi (GAMT; OMIM 601240) (Stockler *et al.*, 1996) ja arginiin : glütsiin amidinotransferaasi defitsiitsus (AGAT; OMIM 602360) (Item *et al.*, 2001). Kolmas haigus on X-liiteline kreatiini transporteri defekt (OMIM 300352), mis on põhjustatud Xq28 lookuses asuva *SLC6A8* geeni mutatsioonist (OMIM 300036) (Salomons *et al.*, 2001; Bizzi *et al.*, 2002). Meie teada on praeguseks teostatud vaid kuus uuringut (Rosenberg *et al.*, 2004; Newmeyer *et al.*, 2005; Clark *et al.*, 2006; Lion-Francois *et al.*, 2006; Arias *et al.*, 2007b; Betsalel *et al.*, 2008) kreatiini transporteri defekti esinemissageduse kindlaks tegemiseks X-liitelise vaimse arengu peetuse või ebaselge etioloogiaga vaimse arengu mahajäämusega patsientide populatsioonis. Kreatiini transporteri defekti esinemissagedus perekondliku vaimse arengu mahajäämusega patsientide seas on leitud vahemikus 1.5%-st (Betsalel *et al.*, 2008) kuni 5.4%-ni (Lion-Francois *et al.*, 2006). *SLC6A8* geeni mutatsiooni esinemissagedus Euroopa XLMR Konsortsiumi patsientide hulgas on 2.1% (Rosenberg *et al.*, 2004).

Kreatiini transporteri defektiga patsientide fenotüübis esineb vaimse arengu mahajäämus, autistlikud jooned, epilepsia ja/või ekspressiivse kõne ja keele mahajäämus (Bizzi *et al.*, 2002). Sellise fenotüübiga patsiente on soovitatud mitmetes uuringutes (Salomons *et al.*, 2001; Rosenberg *et al.*, 2004) uurida selle ainevahetushaiguse suhtes, mida ka meie tegime.

Töö eesmärgid

1. Määrata fragiilse X sündroomi esinemissagedus elussündide kohta ning vaimse arengu mahajäämusega patsientide seas Eestis.
2. Kirjeldada fragiilse X sündroomi kliinilist pilti täismutatsiooniga poistel ning premutatsiooni kandjatel naistel Eestis.
3. Uurida kreatiini transporteri defekti skriiningu efektiivsust X-liitelise vaimse arengu mahajäämusega perekondade seas;
4. Määrata kreatiini transporteri defekti esinemissagedus Eesti X-liitelise vaimse arengu mahajäämusega patisentide seas.
5. Kirjeldada kreatiini transporteri defektiga patsientide kliinilist pilti.
6. Uurida kliinilise fenotüübi ja molekulaarsete tulemuste seoseid harva-esinevate submikroskoopiliste kromosomaalsete aberratsioonide korral.

Uurimisgrupid ja uurimismeetodid

Fragiilne X sündroomi uuring

Uuringu teostamisel võeti aluseks 1997–2006 aastal SA Tartu Ülikooli Kliinikumi Ühendlabori geneetikakeskuses teostatud FXS-i positiivsete DNA analüüside tulemused. Ajavahemikul 1997–2006 oli Eestis tehtud 676 patsiendil

(516 last ja 160 täiskasvanut) FXS DNA analüüs. Patsientidel, kellel enne 1997 aastat FXS diagnoos oli pandud kromosoomanalüüsi või ainult Southern Blot analüüsi tulemuste põhjal, korrati uuringut. Kõik täis- ja premutatsiooniga patsiendid kutsuti välja korduskonsultatsiooniks. Neilt küsiti täpne perekonna ja raseduse anamnees ning tehti kliinilise fenotüübi uuring. Täiendavalt pakuti FXS uuringu võimalust teistele perekonnaliikmetele, kellel sugupuu alusel oli risk FXS premutatsiooni kandluseks. Kõigile meie poolt välja kutsutud pereliikmetele tehti FXS DNA analüüs.

Kreatiini transporteri defekti uuring

Koostöös SA Tartu Ülikooli Kliinikumi Ühendlabori geneetikakeskuse, Tartu Ülikooli Lastekliinikumi ja Tallinna Lastehaigla Geeneetikateenistusega koguti DNA materjal ja kliinised andmed 83-st Eesti perest. Sellest grupist valiti 49 peret kelle perekonna anamnees viitas pärilikule X-liitelisele vaimse arengu mahajäämusele. Selleks, et välja selgitada X-liitelise kreatiini transporteri defekti (*SLC6A8*) esinemissagedus Eesti populatsioonis teostati uriini kreatiini ja guanidinoatsetaadi analüüs 49-st Eesti perekonnast 51-l meessoost indiviidile. Kõigil patsientidel kellel leiti ühekordne uriini kreatiini : kreatiniini suhte tõus teostati *SLC6A8* geeni sekveneerimine.

Submikroskoopiliste koopiaarvu variatsioonide uurimine

Submikroskoopiliste koopiaarvu muutuste välja selgitamiseks eelnevalt kirjeldatud vaimse arengu probleemidega Eesti perekondades kasutasime kiibil põhinevat MAPH (*array multiplex amplifiable probe hybridisation*) ja Infinium II kogu genoomi genotüpiseerimise analüüsi koos Human370CNV-Duo kiipidega (Illumina Inc., San Diego, CA, USA). Muutuste kinnitamiseks kasutasime FISH analüüsi ja reaal-aja kvantitatiivset PCR-i.

Uuringu peamised tulemused

1. FXS esinemissagedus Eesti vaimse arengu mahajäämusega patsientide hulgas oli 2,7% ning esinemissagedus vaimse arengu mahajäämusega poiste hulgas oli 3,1%. Esinemissagedus vaimse arengu mahajäämusega patsientide populatsioonis on sarnane eelnevalt publitseeritud tulemustega Kaukaasia rassist meeste kohta.

2. FXS üldlevimus Eestis aastatel 1984–2005 oli 1:27,115 (95% usalduspiiriga 1:16,059–1:45,787) elusalt sündinud vastsündinu kohta. Samas FXS esinemis-

sageduse alumine piir oli 1:13,947 (95% usalduspiiriga 1:8,264–1:23,529) elusalt sündinud poiste kohta. FXS esinemissagedus poiste hulgas elussündide kohta on Eestis madalam kirjanduses publitseeritud andmetest.

3. FXS-ga meessoost patsientide hulgas oli keskmine vanus diagnoosimisel 4,5 aastat. See näitab, et Eesti arstide teadlikkus sellest sündroomist on sarnane teiste Euroopa riikide arstide teadlikkusega.

4. FXS-ga poiste kliiniline pilt oli sarnane eelnevalt kirjeldatud juhtudele. Kõik täismutatsiooniga meesoost patsiendid olid (mõõduka kuni raske) vaimse arengu mahajäämusega. Enamusel patsientidest (87%) esines FXS-le iseloomulik nägu: pikliku kujuga kitsas nägu, kõrge ja lai otsmik, pikad ja/või peast eemalehoidvad kõrvad, hammaste reastumishäire ja prominentne lõug. Käitumishäireid esines 87% juhtudest, kõige sagedamini esines autistlikke jooni, puudulikku silmside kontakti, stereotüüpiaid ja keskendumishäireid. Ülekaalulisus oli väga suureks probleemiks, mis esines 64%-l FXS-ga patsientidel.

5. FXS premutatsiooniga naispatsientide peamiseks kliiniliseks avaldumiseks oli enneaegne ovariaalne puudulikkus või ebaregulaarsed menstruatsioonid (43%). Vähestel patsientidel esines FXS-le iseloomulikke näo fenotüübi tunnuseid (21%). Üks premutatsiooniga patsient oli vaimse arengu mahaäämuse ja käitumisprobleemidega. Ainuüksi enneaegse ovariaalse puudulikkuse esinemine peaks olema FXS-i DNA analüüsi näidustuseks.

6. Me leidsime tõusnud uriini kreatiini erituse 11-l poisil (9 perekonnast) 49 X-liitelise vaimse arengu mahajäämuse kahtlusega perekonna hulgast (18%-s uuritud perekondadest). Meie uurimistöös leitud vale-positiivsete tulemuste arv oli suurem kui eelnevalt publitseeritud töödes. Seetõttu me soovitame, et enne *SLC6A8* geeni sekveneerimist peaks kordama biokeemilist analüüsi.

7. Hemisügootne punktmutatsioon – c.1271G>A (p.Gly424Asp) leiti ühe perekonna kolmel vennal ja nende emal. Seda mutatsiooni ei ole varasemalt kirjeldatud.

8. Kreatiini transporteri defitsiiti leiti X-liitelise vaimse arengu mahajäämuse kahtlusega perekondade seas 2%-l (95% usalduspiiriga 0,05–11,1%). Kõikidel meessoost indiviididel, kellel esineb vaimse arengu mahajäämus, autistlikud jooned, epilepsia ja/või ekspressiivse kõne ja keele arengu häire, peaks olema testitud kreatiini transporteri defekti suhtes.

9. Kreatiini transporteri defektiga patsientide fenotüüp oli erinev eelnevalt publitseeritud juhtudest. Haiguse kliiniline väljendumine oli väga erinev ühe

perekonna haigestunud vendade seas: patsientidel 1 ja 3 oli haigus suhteliselt kergelt väljendunud (kerge vaimse arengu mahajäämus ja tähelepanuhäire), kuid patsiendil 2 esinesid tüüpilised kliinilised tunnused, nimelt mõõdukas vaimse arengu mahajäämus, autistlikud jooned, ekpressiivne düsfaasia ja epilepsia. Müopaatiline sündroom ja kerge kardiomüopaatia oli diagnoositud patisentidel 1 ja 2; lisaks esines neil ka kaasasündinud komppöiad, mis võib olla tingitud lihaste nõrkusest. Kõigil kolmel vennal esines müopaatiline nägu. Lisaks esines märkimisväärseid probleeme kõne ja keele arengus kombineerituna tähelepanu ja käitumishäiretega.

10. 16-aastase naispatsiendi fenotüüp (lühike kasv, normaalne puberteet, kerge vaimse arengu mahajäämus, absansi tüüpi epilepsia, mõned Turneri sündroomile iseloomulikud stigmad ja Madelungi deformatsioon) on põhjustatud deletsioonist regioonis Xp22.33. Selles piirkonnas võib patsiendi fenotüübiga olla seotud kaks geeni: *SHOX* ja *VSPA*. Lisaks esines sellel patsiendil duplikatsioon Xp22.12-p22.32 regioonis, mille seos fenotüübiga jäi meile ebaselgeks. Sellist komplekssет X-kromosoomi aberratsiooni ei ole varasemalt kirjeldatud.

11. 7-aastasel poisil esinev sügav vaimse arengu mahajäämus, raske mikrotsefaalia, näo düsmorfsus, sümfalangism, suurte liigeste kontraktuurid, hüperoopia, strabism, bilateraalne konduktiivne kuulmislangus, genitaalide anomaalia, äge psoriaas ja trahheo-ösofageaalne fistul, mis olid tingitud 5.9 Mb suurusest deletsioonist regioonis 17q22-q23. Konduktiivne kuulmishäire, skeleti anomaaliad (sümfalangism, suurte liigeste kontraktuurid) ja hüperoopia võivad olla tingitud selles regioonis asuva *NOG* geeni haploinsuffitsientsusest. Samuti võib seostada selle geeniga trahheo-ösofageaalse fistuli arengut ja/või söögitoru atreesiat.

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Kokku on ilmunud 9 publikatsiooni ja 4 ettekannet rahvusvahelistel konverentsidel.

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