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THE FRAGILE X SYNDROME IN MENTALLY RETARDED PATIENTS FROM LATVIA

Doctoral Thesis

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ANNOTATION

Mental retardation (MR) is a complex phenotype, affecting 2 - 3% of the general population. A quarter of cases are caused by genetic disorders. Mental retardation is the most frequent cause of severe handicap in children. We focussed our study on fragile X syndrome, which is both well known and a common cause of X-linked mental retardation.

One of principle tasks in our study was to estimate the prevalence of fragile X syndrome (FXS) in the entire Latvian male population. In the prevalence study we analysed retrospective data of the male individuals with mental retardation and developmental disabilities, diagnosed in ten years time. The prevalence of fragile X syndrome in the Latvian male population was estimated to be 1/6428 (95% CI 5538-7552) or 15.55/100 000 males (95% CI 13.24 – 18.05).

Fragile X syndrome is caused by an expanded CGG repeat (> 200 units, full mutation) at the 5' end of the *FMR1* gene. In our study we characterised the distribution and structure of CGG repeats among X chromosomes with normal CGG repeat alleles and chromosomes with full mutation. We analysed elsewhere described *FMR1* gene linked STR based markers FRAXAC1, FRAXAC2 and DXS548, and one SNP based marker ATL1, found within 150 kb of the *FMR1* CGG repeat. STR and SNP marker haplotypes were combined as follows: DXS548-FRAXAC1-ATL1-FRAXAC2.

DNA studies of X chromosomes with normal CGG repeats revealed high incidences of allele 30 (29.95%), allele 31 (13.10%) and allele 29 (12.83%). A statistically significant association with ATL1 SNP was found in following cases: allele 29 and G ($p = 0.001$); allele 30 and A ($p < 0.0001$) and allele 31 with A ($p = 0.0013$). Polymorphism G was found to be associated with grey-zone CGG alleles ($p = 0.0271$) and exclusively associated with all FXS alleles.

A structure analysis of grey-zone alleles suggest haplotype 7-4-A-5+ as a “protective” haplotype for CGG tract stability. The case-control study results also imply that in the Latvian population, haplotype 2-2-G-4 is a marker of CGG tract instability. Results of AMOVA for haplotypes revealed distinct genetic background for FXS chromosomes.

This is the first study regarding *FMR1* linked haplotypes in the Baltic States region. Our results provide evidence of different mutational pathways of CGG repeat expansion in North-Eastern Europe.

ANOTĀCIJA

Garīgā atpalicība (GA) ir komplekss fenotips, kurš skar vidēji 2 - 3% populācijas. Ceturtajai daļai gadījumu pamatā ir ģenētiska saslimšana. Garīgā atpalicība ir biežākais iemesls smagai bērna invaliditātei. Mūsu pētījums tika vērsts uz trauslās X hromosomas sindromu (FXS), labi zināmu un biežu ar X hromosomu saistītas garīgas atpalicības iemeslu.

Trauslās X hromosomas sindroma prevalences noteikšana kopējā Latvijas vīriešu populācijā bija viens no galvenajiem mūsu pētījuma uzdevumiem. Prevalence noteikta retrospektīvā pētījumā vīriešiem ar garīgo atpalicību un attīstības aizturi, diagnosticētiem desmit gadu periodā. Trauslās X hromosomas sindroma prevalence kopējā Latvijas vīriešu populācijā noteikta 1/6428 (95% CI 5538-7552) vai 15,55/100 000 vīriešu (95% CI 13,24 – 18,05).

Trauslās X hromosomas sindroma iemesls ir palielināts CGG atkārtojumu skaits (pilna mutācija > 200 atkārtojumiem) *FMR1* gēna 5` galā. Šajā pētījumā mēs raksturojām CGG atkārtojumu sadalījumu un struktūru X hromosomām ar normālu CGG atkārtojumu skaitu un hromosomām ar pilnu mutāciju. Mēs analizējām jau iepriekš aprakstītus, ar *FMR1* gēnu saistītus mikrosatelītu marķierus FRAXAC1, FRAXAC2 un DXS548, kā arī vienu SNP - ATL1, kuri atrodas 150 kb attālumā ap *FMR1* gēna CGG atkārtojumu rajonu. Mikrosatelītu un viena nukleotīda polimorfisma marķieri secīgi apvienoti haplotipos: DXS548-FRAXAC1-ATL1-FRAXAC2.

Balstoties uz DNS izpēti X hromosomām ar normālu CGG atkārtojumu skaitu noteicām, ka biežāk sastopamās alēles ir: 30 (29,95%), 31 (13,10%) un 29 (12,83%). Statistiski ticama saistība ar ATL1 SNP polimorfismu konstatēta: 29 CGG atkārtojumiem ar G (p = 0,001); 30 CGG atkārtojumiem ar A (p < 0,0001); 31 CGG atkārtojumam ar A (p = 0,0013). Konstatēta polimorfisma G asociācija ar pelākās zonas CGG alēlēm (p = 0,0271) un saistība ar visām FXS alēlēm.

Pelēkās zonas alēļu struktūras analīze liecina, ka haplotips 7-4-A-5+ iespējams ir "aizsargājošais" haplotips CGG atkārtojumu skaita stabilitātei. Ar *FMR1* gēna saistīto haplotipu gadījuma-kontroles pētījuma rezultāti ticami norāda uz haplotipa 2-2-G-4 saistību ar CGG atkārtojumu nestabilitāti Latvijas populācijā. AMOVA rezultāti pierādīja atšķirīgu ģenētisko izcelsmi FXS hromosomām.

Šis ir pirmais pētījums Baltijas valstīs veltīts ar *FMR1* gēnu saistītiem haplotipiem. Mūsu pētījuma rezultāti atklāj pierādījumus atšķirīgam CGG atkārtojumu skaita mutācijas ceļam Ziemeļaustrumeiropā.

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ABBREVIATION

ADD - Attention deficit disorder

ADHD - attention deficit hyperactivity disorder

AGG – Adenine – guanine – guanine

AMOVA - Analysis of molecular variance

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ARI – Acute respiratory infection

ARX - X-linked *Aristaless*-related homeobox

ATR-X - alpha thalassaemia, mental retardation syndrome X linked

AUTSX2 – Syndromic XLMR autism

ApoE - Apolipoproteine E

Bp – Base pairs

CAMP - Cyclic adenosine monophosphate

c7GdTP - 7-deaza-2`-deoxyguanosine triphosphate

CDGs - Congenital disorders of glycosylation

CGG – Cytosine – guanine – guanine

CGH - Comparative genomic hybridization

CI - Confidence interval

CNS - Central nervous system

CpG – Cytosine - phosphate – guanine

CREB - cAMP response element-binding

DD - Developmental disabilities

DMSO - Dimethyl sulfoxide

DNA - Deoxyribonucleic acid

EEG - Electroencephalography

DNTP - Deoxynucleotide Triphosphate

FISH - Fluorescence in Situ Hybridization

FMR1 - Fragile Site Mental Retardation 1 gene

FMRP - Familial Mental Retardation Protein

FSH - Follicle-stimulating hormone

FXS - Fragile X Syndrome

FXTAS - Fragile X-Associated Tremor/Ataxia Syndrome

IQ - Intelligence Quotient

Kb – Kilo base

KDa – Kilo Dalton

MECP2 - Methyl CpG binding protein 2

MLPA - Multiplex Ligation-dependent Probe Amplification

MR – Mental retardation

MRI - Magnetic resonance imaging

MRNA - Messenger Ribonucleic Acid

MRXS - Mental retardation, X-linked, syndromic form

mGluR5 - Metabotropic glutamate receptor 5

NGP – Normal-Grey-Premutation range

NLGN3 - neuroligin 3

NLGN4 - neuroligin 4

MIM - Online Mendelian Inheritance in Man

PCR - Polymerase Chain Reaction

PE – Premutation-Expansion range

PGD - Preimplantation genetic diagnosis

Pst1 - Restriction endonuclease from *Providencia stuartii*

PKU - phenylketonuria

POF - Premature ovarian failure

PWS – Prader-Willy syndrome

Rpm - revolutions per minute

RPS6KA3 - ribosomal protein S6 kinase, 90 kDa polypeptide 3

RTT - Rett syndrome

SLC6A8 - solute carrier family 6, neurotransmitter transporter, creatine, member 8

SNP - Single-Nucleotide Polymorphism

S.D. – Standard Deviation

Taq - Taq polymerase isolated from the thermophilic bacterium

TBE - Buffer solution that consists of a mixture of tris base, boric acid, EDTA, and water

TE - Tris-EDTA (Ethylenediamine Tetraacetic Acid; buffered solution)

TG – Timine – guanine

TSC1 - Tuberous sclerosis protein 1 or hamartin

TSC2 - Tuberous sclerosis protein 2 or tuberin

Vent – DNA polymerase isolated from the Archaean *Thermococcus litoralis*

XLMR - X-linked mental retardation

1. INTRODUCTON

Mental retardation (MR) is a complex phenotype, characterized by suboptimal functioning of the central nervous system (CNS) resulting in significant limitations both in intellectual functioning and in adaptive behaviour. Mental retardation affects about 2–3% of people and about a quarter of cases are caused by genetic disorders. Mental retardation is the most frequent cause of severe handicap in children. Therefore ascertainment of mental retardation aetiology is an important task in paediatrics.

Fragile X syndrome (FXS; MIM #300624; FRAXA, Xq27.3) is well known and a common cause of X-linked mental retardation. The fragile X syndrome is caused by an expanded CGG repeat (> 200 units, full mutation) at the 5' end of the *FMR1* gene, which is associated with methylation of a CpG island upstream of the *FMR1* gene and down regulation of the transcription (Oberle et al., 1991; Poustka et al., 1991; Rousseau et al., 1991).

Amongst individuals from the general population, the polymorphic CGG repeat ranges from 6 to 50 repeats and is usually interspersed every 9–10 repeats with an AGG (Eichler et al., 1996; Fu et al., 1991). Premutation alleles have a moderate expansion of the repeat (from 50 to ~200 units), they are unmethylated on an active X chromosome and do not affect *FMR1* expression. CGG repeat expansion over 200 is the basis for CpG island methylation, leading to silencing of the *FMR1* gene (de Vries et al., 1998). Intermediate or grey zone alleles are poorly defined. Boundaries for the grey zone range vary among studies, from 34 or 35 CGG repeats for the lower boundary to 58/60 repeats for the upper boundary (Moutou et al., 1997; Rife et al., 2004; Sherman et al., 2005). These alleles usually have stable transmission, but are more likely to exhibit unstable transmission with increasing size within this range.

The underlying mutational mechanism is not fully understood and remains a topic of debate. The gender of the parent carrying an expanded repeat (maternal imprinting), the number of repeats (dynamic mutation) and the absence of AGG interruptions in long tracts of CGG repeats have been described as the tree main factors related to this instability (Dombrowski et al., 2002; Eichler et al., 1996; Rife et al., 2004). The microsatellite markers DXS548-FRAXAC1-FRAXAC2 and the ATL1 SNP have previously been reported as markers associated with *FMR1* CGG repeat instability (Eichler et al., 1996; Gunter et al., 1998; Kunst et al., 1996; Macpherson et al., 1994; Murray et al., 1997; Oudet et al., 1993; Richards et al., 1991).

Haplotypes linked to FXS are widely described across Western European and Scandinavian populations; however, less is known regarding populations from North-Eastern Europe, including the Baltic States. This is the first study in the Baltic States region regarding *FMR1* linked haplotypes.

The first clinical indication of FXS is usually delay in child`s developmental milestones and mental retardation. In addition to mental retardation, speech and language skills are severely affected. Most speech is poorly articulated and expressive language is often limited to three- or four-word sentences. FXS patients often repeat words or phrases, an attribute typically associated with autism. Indeed many FXS males present autistic type behaviour – gaze aversion, shyness, hand biting, hand flapping and rocking (Bardoni et al., 2000; Garber et al., 2008; Hernandez et al., 2009).

The phenotype is subtle in young children and evolves with age. Hyperextensibility of finger joints, *pectus excavatum*, mitral valve prolapse and strabismus are other possible prevalent features (de Vries et al., 1996; Larbrisseau et al., 1982; Phadke 2005). The clinical manifestations of this syndrome in adult males include an elongated and narrow face with a large forehead and prominent chin, large and anteverted ears, joints with increased mobility, and uni-or bilaterally large testes. Macroorchidism is an important feature in post-pubertal age. However, it is not presented in all FXS males, but it is specific for FXS. Between 25-30% of all patients with FXS do not have the typical faces of the syndrome. The secondary characteristics of FXS in turn include tallness, a soft and silky skin, widened fingertips and flat feet (Ridaura-Ruiz et al., 2009).

Early diagnosis of fragile X syndrome is crucial in order to inform other members of the family of their risk of having affected offspring. Therefore it is recommended that most fragile X diagnostic tests will be carried out on a very broad range of patients regardless of a consequently low detection rate.

Ten years of experience with molecular diagnostic of the fragile X syndrome in Latvia and number of diagnosed patients in this time period, revealed a low pickup rate of patients and insufficient clinical recognition of symptoms.

1.1. Aim of the Study

Ascertain the prevalence of fragile X syndrome in Latvia, characterise genetic and clinical variability of the FRAXA locus *FMR1* gene in patients with unclear aetiology of mental retardation.

1.2. Tasks of the Study

1. Estimate the prevalence of the fragile X syndrome in the entire Latvian male population.
2. Perform a distribution and structure study of CGG repeats among X chromosomes with normal CGG repeat alleles.
3. Characterise the ATL1 SNP/CGG repeat number correlation within chromosomes with a normal CGG repeat number and chromosomes with a full mutation.
4. Perform a case-control study of *FMR1* gene linked haplotypes based on STR and SNP markers, to identify specific haplotypes among Latvian FXS patients and control group mentally retarded patients with a normal number of CGG repeats with respect to allelic stability.
5. Identify the association of grey-zone allele structure and *FMR1* linked haplotypes.
6. Evaluate genotype-phenotype correlation in patients with full mutation and/or repeat size/methylation mosaic.

1.3. Scientific Novelty of the Study

This study is the first study in the Baltic States region regarding *FMR1* linked haplotypes. Described haplotypes of Latvian fragile X syndrome patients differ from published studies in populations of Western European descent. Therefore this data provide evidence of different mutational pathways of CGG repeat expansion in the North-Eastern European region.

The estimated prevalence of fragile X syndrome in the Latvian male population contributes to the ascertainment of this disease distribution in our geographical region.

1.4. Practical Novelty of the Study

The estimated prevalence of fragile X syndrome in the Latvian male population is in line with the prevalence of this syndrome in several other European populations. The low number of confirmed patients with fragile X syndrome in ten years, point to a low detection rate of patients in paediatrician, child psychiatric and child neurology practices.

Haplotypes linked to unstable CGG repeat alleles in the Latvian FXS male population are very useful in practical family cascade testing and for consultation of families at risk.

The newly adapted clinical questionnaire form shall contribute to an increase in the detection rate of patients with suspected fragile X syndrome by paediatricians, child psychiatric and child neurology practices.

1.5. Elaboration of the Study

The current study was carried out in the Medical Genetics Clinic, University Children`s Hospital, Riga, Latvia in collaboration with Children`s Psychiatric Department, University Children`s Hospital, Riga, Latvia.

Conformation of FXS diagnosis by Southern blot analysis in was done in the DNA Laboratory, Department of Medical Genetics, Ullevål University Hospital, Oslo, Norway and in the DNA Diagnostic Laboratory, University Medical Center Nijmegen, The Netherlands.

The Latvian Central Committee of Medical Ethics and the Riga Stradins University Committee of Medical Ethics approved the study.

The financial support of the study:

- Project “Genomic studies of the Latvian population, their application for diagnosis and prevention of human pathology”. Supported by the Latvian Council of Science, "Elaboration of Phenylketonuria prenatal and fragile X Syndrome prenatal, postnatal DNA-based testing and quality control system in Latvia" (2000-2004).

- ESF project No. 2004/0005/VPD1/ESF/PIAA/04/NP/3.2.3.1./0001/0004 /0066. “Enhancement of competencies, qualification and skills of health care and health promotion professionals”, (2005-2008).

- Latvian National Research Programme in Medicine, "Multi-disciplinary research consortium on major pathologies threatening the life expectancy and quality of life of the Latvian population". Project No. 6, “Development of early diagnostics, prevention and treatment in children diseases”, (2006–2009).

- ESF project No. 2009/0147/1DP/1.1.2.1.2/09/IPIA/VIAA/009 “Enhancement of competencies, qualification and skills of health care and health promotion professionals” Sub-activity No 1.1.2.1.2 “Support to doctor’s studies”, (2011).

1.6. Author`s Contribution to the Work

This PhD project was initiated in 2005, based on scientific elaboration forerun of project “Genomic studies of the Latvian population, their application for diagnosis and prevention of human pathology”.

The author of this thesis performed the following laboratory investigations: DNA extraction (partly); routine screening PCR amplification; fluorescent PCR; Southern blotting (partly); ATL1 SNP analysis; AGG interspersion pattern analysis; fluorescent PCR of microsatellite markers and haplotype analysis. Author performed retrospective data collection and data analysis for prevalence study. All statistical data analysis and AMOVA were done by the author of this thesis.

Clinical evaluation of patients was done by clinical geneticists and child psychiatrists.

1.7. Outline of the Thesis

The thesis is composed on 124 pages in English, following classical scheme. The work is structured in ten chapters: Introduction; Literature review; Subjects and Methods; Results; Discussion; Conclusions; Publications; Acknowledgements; References and Appendixes. Text of thesis is supplemented by 19 Tables; 19 Figures and 14 Appendixes. Reference list consist of 131 cited references.

2. LITERATURE REVIEW

2.1. Mental Retardation

Mental retardation (MR) is a complex phenotype, characterized by suboptimal functioning of the central nervous system (CNS) resulting in significant limitations both in intellectual functioning and in adaptive behaviour. This is shown by the afflicted person's lack of conceptual, social and practical adaptive skills originating before 18 years of age (Chiurazzi et al., 2008; Luckasson et al., 2002). MR is the most frequent cause of severe handicap in children. On the basis of the Intelligence Quotient (IQ) value, mental retardation may be classified in four categories of severity according to the World Health Organization classification and terminology: mild (IQ 50–70), moderate (IQ 35–50), severe (IQ 20–35), and profound (IQ<20) (Pescucci, et al., 2007; Ropers and Hamel, 2005). With incidence estimates of 0.3–0.5% for moderate to severe MR (IQ ≤ 20) and variable estimates of 1–3% for mild MR (IQ 50 to 70) is included (Mandel et al., 2004).

There is also wide variation in the category of reported cause of mental retardation: 18.6% to 44.5% of cases have exogenous causes, such as teratogen exposure or infection, perinatal brain ischemia and foetal alcohol syndrome. Genetic causes - chromosomal (aneuploidies, microdeletion syndromes) and monogenic is thought to be of the order 17.4% - 47.1% (Moeschler et al., 2006). A precise cause is found only in about 50% of cases with moderate to severe MR, and an even lower proportion for individuals with mild MR. For those cases where no clear reason is found, one may invoke sporadic occurrence of an unknown single gene defect, multifactorial inheritance, or culturo-familial mental retardation (Mandel et al., 2004). In addition to cognitive impairment, MR patients always present adaptive functioning impairment. This manifests itself as a failure to cope with the demands of everyday life and a failure to meet the standards of personal independence that are expected from someone of that socio-economic and cultural background (Renieri et al., 2005). The high frequency of involvement of genes in MR aetiology is reflected by the findings in OMIM (Online Mendelian Inheritance in Man) of 1740 entries, searching for ‘‘mental retardation’’ (March 19, 2011).

MR may be present in association with other clinical manifestations (syndromic MR) or may be isolated (nonsyndromic MR) (Pescucci et al., 2007).

2.1.1. Chromosomal Causes of Mental Retardation

It is of prime importance to recognize chromosomal disorders amongst non-Mendelian genetic causes of MR. The most common chromosomal cause of MR is chromosome aneuploidies: Down syndrome (trisomy 21), Patau syndrome (trisomy 13) and Edwards syndrome (trisomy 18). Down syndrome is the single most frequent cause of mental retardation, affecting about 1/800 –1000 live births. Chromosome aneuploidies of the sex chromosomes are common, but not always associated with mental retardation. Turner syndrome (female possessing only one X) and Kleinfelter syndrome (XXY males) may be intellectually normal. However in cases of triple X syndrome, mental retardation is always evident. The above are all very well documented chromosomal aneuploidies. Chromosomal aberrations including deletions, translocations and inversions can also be a cause of mental retardation. The reported frequency of chromosomal anomalies detected by high-resolution karyotyping (≥650 bands) in patients evaluated for MR varies between 9% and 36% (Moeschler et al., 2006; Van Karnebeek et al., 2005).

A specific subcategory of cytogenetically invisible deletions includes deletions at the end of chromosomes. Chromosomal rearrangements involving the ends of chromosomes (telomeres) are a significant cause of idiopathic as well as familial mental retardation. Telomeres are composed of a TG rich repeat (TTAGGG)n which is similar in all vertebrates. This simple sequence is repeated several hundred to several thousand times and the number of repeats is variable between individuals and with age (Winnepenninckx, Rooms and Kooy et al., 2003). Approximately half of all structural chromosomal abnormalities include the telomere of the chromosome. The telomeric regions are extremely gene-rich which explains why the relatively small deletions of subtelomeric sequences frequently cause mental retardation (Winnepenninckx, Rooms and Kooy , et al., 2003).

A variety of methods have been successfully adapted for subtelomeric rearrangement screening and at least seven different methods have been applied. Multiprobe FISH and MLPA are the most widely used. Deletions of most, but not all, individual chromosome ends have been reported in patients with MR. Loss of specific chromosome ends may cause recognizable syndromes: Wolf–Hirschorn syndrome (MIM #194190), caused by the deletion of the tip of chromosome 4p; ATR-16

syndrome (MIM #141750), caused by deletion of the tip of chromosome 16; or Miller–Dieker syndrome (MIM #247200), caused by deletion of the tip of chromosome 17p. However, in many cases and because of the low number of patients with deletions, the definition of a specific phenotype associated with deletions of a particular chromosome end is sometimes not possible (Chelly et al., 2006; Moeschler et al., 2006). In addition to subtelomeric rearrangements, interstitial rearrangements have been implicated in a number of MR syndromes, including DiGorges (22q11 deletion; MIM #188400), Williams–Beuren (7q11.2 deletion; MIM #194050) and Smith–Magenis (17p11.2 deletion; MIM #182290), and are diagnosed mainly by molecular cytogenetic approaches. The median additional benefit of subtelomeric studies is 4.4% (Van Karnebeek et al., 2005).

Moreover, recent diagnostic studies using chromosome specific, or genome wide microarray-CGH (about 3500 clones at 1Mb resolution), have shown that interstitial chromosomal deletions or duplications may account for a significant proportion of unexplained MR (Chell, et al., 2006).

Genomic imprinting describes the preferential or exclusive expression of a gene from only one of the two parental alleles. Deregulation of imprinted genes has been observed in numerous human diseases such as Angelman syndrome (MIM #105830) and Prader-Willy syndrome (MIM #176270). Both syndromes are associated with cognitive impairment and the microdeletion of genomic region 15q11.2–15q13. Deletions of the paternally derived chromosome 15 caused Prader-Willy syndrome, and ones on the maternally derived chromosome 15 caused Angelman syndrome (Chelly et al., 2006).

2.1.2. Autosomal Monogenic Cause of Mental Retardation

Patients with an autosomal dominant form of mental retardation may arise as a consequence of novel mutation. For instance, Rubinstein-Taybi syndrome (MIM #180849). This disorder is caused by mutation in the CRBE protein gene located on chromosome 16p13.3 (Winnepenninckx, Rooms and Kooy et al., 2003). In addition, a familiar structure of mental retardation disorder is sometimes observed in families affected with disorders exhibiting a variable phenotype such as tuberous sclerosis complex (MIM #191100). This is caused by mutation in *TSC1* (MIM *605284) and *TSC2* (MIM *191092) genes.

Autosomal recessive MR mostly falls within a category of metabolic disorders. Most common and better known examples are Smith-Lemy-Opitz syndrome (MIM #270400), phenylketonuria (MIM +261600), galactosemia (MIM #230400), homocystinuria (MIM +236200), nonketotic hyperglycinemia (MIM #605899), lysosomal disorders and congenital disorders of glycosylation (CDGs). For metabolic investigations, the mean yield of all studies is ~1% (Van Karnebeek et al., 2005). Even in disorders where the fundamental biochemical defect is known, such as phenylketonuria (PKU) and other enzyme defects, the exact basis for brain dysfunction is uncertain (Garcia-Cazorla et al., 2009; Kahler and Fahey et al., 2003).

Eleven of 282 human MR genes are encoded by mitochondrial genome (Inlow et al., 2004).

2.2. X Linked Mental Retardation (XLMR)

X-linked mental retardation (XLMR) is a common cause of monogenic intellectual disability affecting mostly males, partly accounting for the higher prevalence of MR among males relative to females. However, female carriers may manifest (usually milder) symptoms, possibly because of skewed X-inactivation.

In 1938 Lionel Penrose first observed that more males than females in the population are mentally retarded in a survey and by classification of those in institutional care and their relatives (Turner and Turner, 1974; Raymond, 2006). The ratio of males to females was 1.25:1. This figure has been substantiated by numerous subsequent studies in the USA, Canada, Australia, and Europe and all agree with the observation of an approximately 30% excess of males being affected with mental retardation (Pescucci et al., 2007; Raymond, 2006). A prevalence of 1.83/1000 males with XLMR had been estimated in 1980 by Herbst and Miller, with the fragile X syndrome being by far the most prevalent condition (~20% of all XLMR cases). However, the finding of a much smaller contribution of individual genes, other than *FMR1*, to XLMR has recently led to a reduced prevalence estimate of 10–12% of all MR cases in males (Chiurazzi et al., 2008).

It has been suggested that the concentration of genes causing MR (number of MR genes per megabase) may be twice as high on the X chromosome compared to autosomes. These estimates will be confirmed or disproved only when all MR genes will have been cloned. The identification of X-linked conditions is easier due to the

hemizygosity of males, who inevitably manifest a phenotype when harbouring a mutant allele. There is only one X-linked condition known that contradicts this inheritance pattern, that is, EFMR - epilepsy and MR limited to females (MIM #300088). In this condition, heterozygous females are affected, while hemizygous males are apparently unaffected. The gene responsible for this condition and the mechanism leading to sparing of mutant males are still unknown (Chiurazzi et al., 2008).

X-linked mental retardation is usually divided into ‘‘syndromic’’ and ‘‘non-syndromic’’ or ‘‘nonspecific’’ forms. In syndromic forms (MRXS), MR is present in association with a specific pattern of physical, neurological, and/or metabolic abnormalities (Renieri et al., 2005). MRXS conditions have been somewhat arbitrarily subdivided into four classes: malformation syndromes, neuromuscular disorders, metabolic and dominant conditions. By “Malformation syndrome” is a condition characterised by MR and multiple congenital anomalies. A “Neuromuscular disorder” is one with a major involvement of the nervous system and/or muscles. “Metabolic” conditions are considered separately because their patho-physiology is known and due to the abnormal functioning of specific enzymes. “Dominant” conditions have been set apart because of their peculiar inheritance, with near absence of affected males (males die before birth, with the notable exception of EFMR) and presence of affected females (Chiurazzi et al., 2001; Chiurazzi et al., 2008). Summary of recently updated 215 XLMR conditions are shown in Table 2.1.

Table 2.1.

XLMR clinical conditions count by clinical presentation based on Chiurazzi et al., (2008)

	Total count	Mapped	Cloned
Syndromes	98	31	38
Neuromuscular	51	16	28
Nonspecific/MRX	66	50	16
Total conditions	215	97	82

2.2.1. Diagnosis of XLMR

The clinical diagnosis of XLMR is usually a diagnosis of exclusion of other causes of developmental delay in a male. Based on Shevell et al. (2003) the following investigation of a male child with suspected XLMR has been advised:

● Obtain three generation pedigree and details of development of all possibly affected individuals - the family history can help in suggesting a diagnosis, particularly when other family members are affected similarly. This is important especially in the case of male patients who have male relatives with DD/MR, related through females who are not mentally retarded (Moeschler et al., 2006).

● Refer to detailed clinical history of maternal health pre-pregnancy, pregnancy history, birth history and birth height, weight and head circumference.

● Developmental milestones and growth rates, educational history and IQ, examination for dysmorphic features and neurological signs - several studies of aetiology of mental retardation suggest that the dysmorphologic examination and syndrome recognition by an experienced clinical geneticist is the critical diagnostic modality (Moeschler et al., 2006).

● Karyotype analysis (550 banded resolution) - all patients where XLMR is suspected should have the benefit of contemporary karyotype analysis at >550 banded resolution, as unbalanced autosomal translocations from balanced carriers can be misclassified as X linked if no male-to-male transmission is observed (Raymond, 2006).

● Fragile X testing - fragile X syndrome is the most common genetic cause of DD/MR. Reviews suggest that only approximately 2.0% of patients with mental retardation (both genders) will be found to have a mutation in this gene (Moeschler et al., 2006).

● Subelomere screening – with introduction of subtelomeric analysis approximately 3–4% of familial mental retardation will be found to be due to submicroscopic telomeric deletions (Raymond 2006).

● Brain MRI can be performed if there are abnormal neurological findings or if head circumference indicates microcephaly or macrocephaly. Use EEG to assist in defining of epilepsy phenotype – a physical examination focused on detecting neurologic abnormalities is considered essential in the evaluation of every child with DD/MR (Raymond 2006).

● Metabolic screening can be performed if clinically indicated. In this case, consider a urine and plasma screen of creatine/creatinine ratio where possible. Consider a free T3 thyroid function tests if spastic paraplegin is present.

2.2.2. Genes and Conditions of XLMR

Figure 2.1. contains an ideogram of the X chromosome with the position of the 45 known XLMR genes. All these genes carry mutations in at least a single family with multiple affected individuals.

AUTSX2

For syndromic XLMR autism – AUTSX2 (MIM #300495) – a mutation was identified in *NLGN3* (neuroligin 3; MIM *300336) and *NLGN4* (neuroligin 4, X linked; MIM *300427) in two brother pairs with severe mental retardation and autism. Since then a further family has been described, but mutations have not been identified in any large cohort of autistic children to date, suggesting that abnormalities in this gene are a rare cause of autism. In addition, all cases have been associated with severe mental retardation (Raymond, 2006).

Coffin-Lowry syndrome

For Coffin-Lowry syndrome (MIM #303600) mutations in *RPS6KA3* (ribosomal protein S6 kinase, 90 kDa polypeptide 3; MIM *300075) (Xp22.2-p22.1), previously known as *RSK2*, has been described. Short stature, distinctive and coarse face with a prominent forehead, hypertelorism, prominent lips, large soft hands with thickened tapering fingers, hypotonia, hyperextensibility, and skeletal changes are characteristic for this syndrome. It includes MRX 19 (Chiurazzi et al., 2001; Raymond, 2006).

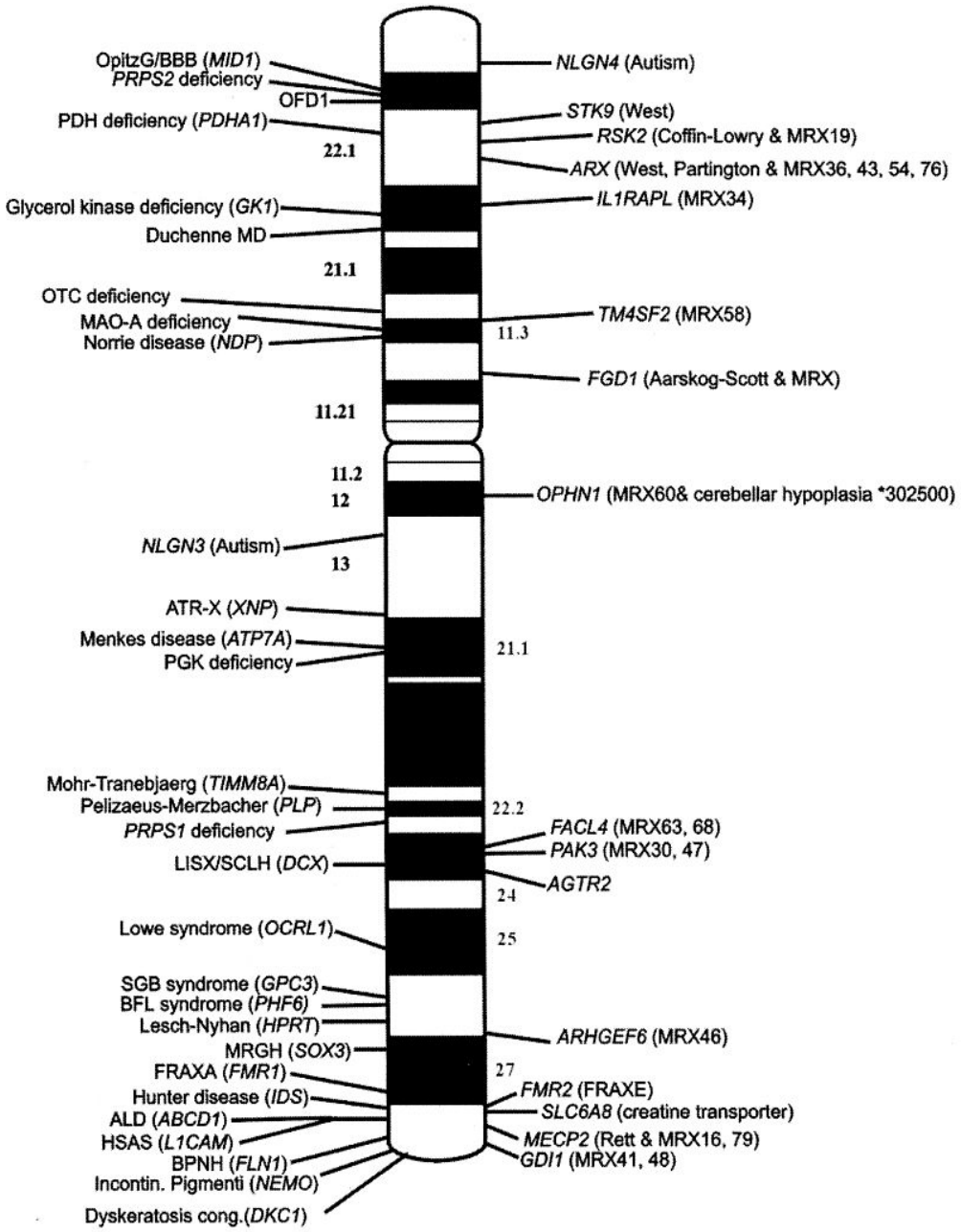

Fig. 2.1. G-banded ideogram of the X chromosome with 45 cloned XLMR genes (addapted from Chiurazzi et al., 2004)

ARX gene

The *ARX* gene (X-linked *Aristaless*-related homeobox; MIM *300382) was identified as the causative gene in several allelic brain diseases with MR. These include X-linked lissencephaly with abnormal genitalia (MIM #300215); Proud syndrome or mental retardation with agenesis of the *corpus callosum*, microcephaly featuring limb contractures, scoliosis, coarse faces, tapered digits and urogenital abnormalities (MIM #30004); myoclonic epilepsy syndrome (MIM #300432); West syndrome or X-linked infantile spasm syndrome with hypsarrhythmia and mental retardation (MIM #308350);

Partington dystonic syndrome (MIM #309510); non-syndromic X linked mental retardation (MIM #300382) (Chiurazzi et al., 2004; Raymond, 2006; Laperuta et al., 2007).

The gene is expressed in foetal and adult brains and in skeletal muscle. Homeobox-containing genes usually play a critical role during development (Chiurazzi et al., 2004). The *ARX* gene represents a hot spot for mutations in families with cognition disorders because its mutations account for 9.5% of X-linked MR families (Laperuta et al., 2007). The recent findings of different publications show that, *ARX* is a pleiotropic gene that, in a diverse genetic context and/or under the influence of modifier genes, controls different aspects of human brain morphogenesis and function. *ARX* mutations have been suggested to be more frequent in XLMR families than mutations in other known XLMR genes, apart from FMR1 (Chiurazzi et al., 2004; Gronskov et al., 2004; Laperuta et al., 2007; Raymond, 2006).

ATR-X syndrome

X linked alpha thalassaemia was initially thought to be clinically homogenous, but mutation analysis of ATR-X (alpha thalassaemia, mental retardation syndrome X linked; MIM #300040) has found that the following conditions are all allelic: Juberg-Marsidi, Chudley-Lowry, Smith-Fineman-Myers, Carpenter-Waziri, Holmes-Gang, and Martinez. Mutations in *XNP* gene (X-linked nuclear protein gene; MIM *300032) has been identified as cause for ATR-X syndrome (Chiurazzi et al., 2004; Raymond, 2006).

ATR-X patients have a characteristic “coarse” face, genital anomalies, and MR. Facial anomalies include: upswept frontal hairline, telecanthus, epicanthic folds, flat nasal bridge, midface hypoplasia, a small triangular upturned nose with the alae nasi extending below the columella and septum, a flat philtrum where the upper lip is tented and the lower lip is full and everted giving the mouth a “carplike” appearance, and incisors that are frequently widely spaced with the tongue protruding. Genital abnormalities are seen in 80% of these children. A characteristic observation in the ATR-X patients is a mild form of α-thalassemia (Chiurazzi, et al., 2004).

About half of the mutations found in typical ATR-X patients are located within exons 7–9 of the *XNP* gene, while it was hypothesized that mutations in other regions of the gene could lead to other, possibly less severe, MR phenotypes. It appears that *XNP* mutations are more likely to be found when female carriers have skewed X chromosome (Chiurazzi et al., 2004).

Rett syndrome

Rett syndrome (RTT; MIM #312750) including MRX 16, 73 is an X-linked dominant neuro-developmental disorder and a significant genetic cause of mental retardation, affecting 1/10 000–15 000 girls. The RTT gene on Xq28 was identified as *MECP2* (MIM *312750), which encodes the methyl-CpG–binding protein 2 that is normally involved in transcriptional silencing. In approximately 95% of patients, these mutations occurs *de novo*, and it has been shown that in most cases they are of paternal origin. Numerous studies have found various mutations (missense, nonsense, and frameshifts) in the coding region of *MECP2* in patients with RTT, identifying mutations for as many as 80% of patients. The remaining 20% may have mutations in other regions of this gene, such as regulatory elements and noncoding regions, but this remains to be determined (Buyse et al., 2000; Chiurazzi et al., 2004; Kleefstra et al., 2004).

Patients with classic RTT appear to develop normally until age 6–18 months, at which time they enter a period of neuro-developmental regression. Symptoms include gradual loss of speech and purposeful hand use, and development of microcephaly, seizures, ataxia, autistic features, intermittent hyperventilation and stereotypic hand movements. Recent studies indicate that females with RTT appear to represent a more heterogeneous phenotype than was first realized. There are cases with a very mild phenotype, with preserved speech variant or with a congenital, early seizure onset. It is hypothesized that these differences are mainly due to the genotype, variation in X-inactivation patterns and probably other polygenic modifiers (Buyse et al., 2000; Kleefstra et al., 2004). Unlike previous thoughts, *MECP2* mutations are not necessarily prenatally lethal in males, and are the cause of a variable phenotype, ranging from lethal congenital encephalopathy to Angelman-like phenotype and mild nonspecific X-linked mental retardation (Chiurazzi et al., 2004; Kleefstra et al., 2004).

SLC6A8 gene

Mental retardation in combination with epilepsy is relatively common, which means that the list of differential diagnoses remains long in cases that present these two features. However, mutations in *SLC6A8* (solute carrier family 6 (neurotransmitter transporter, creatine), member 8; MIM *300036) are usually associated with epilepsy, severe mental retardation, and autistic spectrum behavioural problems with particular

deficits in expressive speech and language often resulting in absent speech (Raymond, 2006).

Recently, a systematic screen of 288 families of the European XLMR Consortium (European XLMR Consortium, http://www.euromrx.com/index.htm) with both mental retardation and either proven X linked inheritance or two or more affected male family members, revealed mutations in 6/288 (2.1%) families. This suggests that mutations in this gene are a relatively common cause of mental retardation, although still 10 times less frequent than fragile X syndrome in familial cases (Raymond, 2006; Rosenberg et al., 2004).

2.3. Fragile X Syndrome (FXS)

2.3.1. Early Findings of FXS

In 1943, Martin and Bell described sex linked mental retardation without dysmorphic features in a family in which both affected males and females were observed. The original "Martin-Bell family" was restudied in 1981 and the typical cytogenetic and clinical features were found. The "Martin-Bell phenotype" was proposed as an eponym for the phenotype of affected males (Martin and Bell, 1943; de Vries et al., 1998).

In 1969, Lubs 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 mentally retarded males. The folic acid and thymidine depleted cell culture medium was identified as the essential factor for the induction of this heritable fragile site at Xq27.3 (Lubs, 1969; de Vries et al., 1998). This was confirmed in several families studied by Sutherland (1977). He also developed the cytogenetic methods for detection of the Fra(X) chromosome. The name of the syndrome – fragile X mental retardation syndrome (MIM # 300624) is derived from the characteristic chromosomal folate-sensitive fragile site at Xq27.3 (FRAXA), which is observed cytogenetically as non-staining constriction or a gap near the distal end of the X chromosome (Fig. 2.2.) (McKinlay, Gardner and Sutherland, 2004).

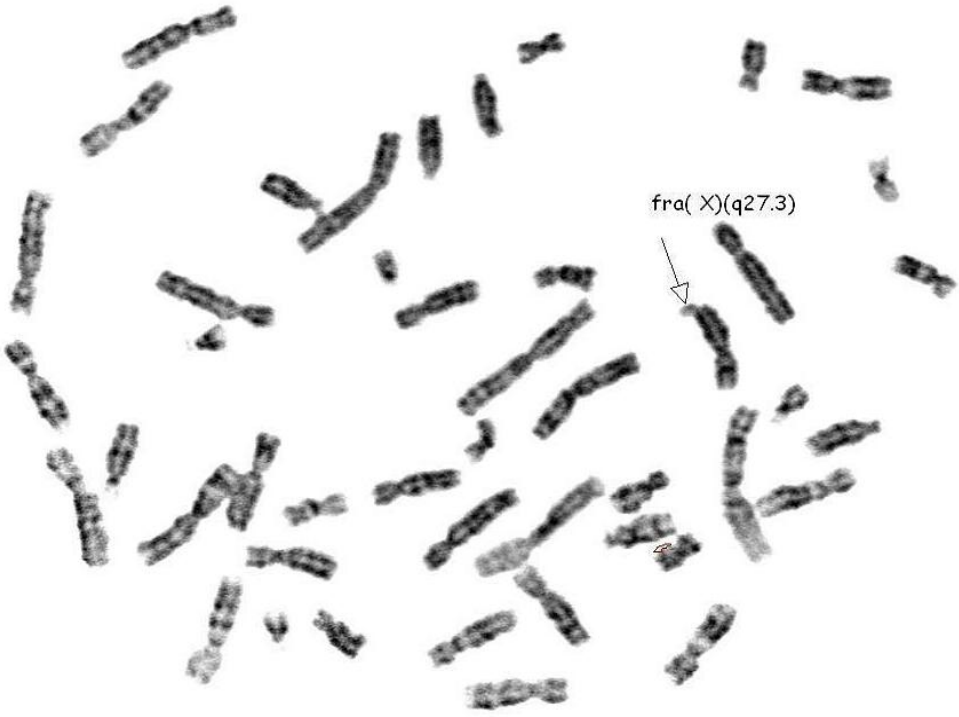

Fig. 2.2. Partial metaphase spread of G-b and stained chromosomes from a female expressing the fragile X site Xq27.3, indicated by the arrow, near the distal end of chromosome X long arm.

Several hypotheses have been proposed to account for the cytogenetic expression of the fragile site and the peculiarities in the inheritance of the syndrome, in particular the apparent necessity for the mutation to be passed through a female for phenotypic expression in later generations. The gene involved in the fragile X syndrome, the Fragile X Mental Retardation (*FMRl*) gene (MIM *309550), was identified in 1991. Abnormal DNA methylation at a single CpG island has been found to be associated with phenotypic expression of the fragile X syndrome. Cloning of sequences around this CpG island generated probes that detect both the fragile X mutations, as a size increase of a small target DNA fragment, and the abnormal methylation pattern. The gene defect was the first expansion of a trinucleotide repeat to be discovered and a whole class of disorders is now known to be associated with this type of mutation (de Vries et al., 1998; Oberle et al., 1991; Rousseau et al., 1991; Rousseau et al., 1994; Poustka et al., 1991).

2.3.2. Clinical Features of FXS

The fragile X syndrome is the most common single cause of inherited mental retardation. As originally described by Lubs (1969), the “marker X chromosome”

became a central trait of fragile X syndrome and was not associated initially with a specific phenotype other than mental retardation. After established cytogenetic methods and identified more FXS families, the phenotype was clarified (Bardoni et al., 2000). Specific clinical findings for males according to patient age are summarized in Table 2.2.

Table 2.2.

Clinical Features in Males with FXS (adapted from Tarleton and Saul, 1993)

Period	Clinical features
Delayed developmental milestones	• Sit alone (10 month) • Walk (20.6 month) • First clear words (20 months)
Pre-pubertal	• Developmental delay, especially speech • Abnormal temperament tantrums, hyperactivity, autism • Mental retardation: IQ 30-50 • Abnormal craniofacial findings: narrow and long face, prominent forehead, large ears, prominent lower jaw
Post-pubertal	• Macroorchidism (testicular size more than 30 ml) • Abnormal behaviour: shyness, avoiding eye contact • Ophthalmologic: strabismus • Orthopaedic: joint hyperextensibility, pes planus
Other	• Cardiac: mitral valve prolapsed • Dermatological: unusually soft and smooth skin

First clinical indication for FXS is usually a delayed developmental milestone (Table 2.2). The phenotype is subtle in young children and evolves with age. Macroorchidism is an important feature in post-pubertal age. However, it is not presented in all FXS males, but it is specific for FXS. Hyperextensibility of finger joints, pectus excavatum, mitral valve prolapse and strabismus are other possible prevalent features (de Vries et al., 1996; Larbrisseau et al., 1982; Phadke 2005). The clinical manifestations of this syndrome in adult males include an elongated and narrow face with a large forehead and prominent chin, large and anteverted ears, joints with increased mobility, and uni-or bilaterally large testes. Between 25-30% of all patients with FXS do not have the typical faces of the syndrome. The secondary characteristics of FXS in turn include tallness, a soft and silky skin, widened fingertips and flat feet (Ridaura-Ruiz et al., 2009).

In addition to mental retardation, speech and language skills are severely affected. Most speech is poorly articulated, expressive language is often limited to three- or four-word sentences. FXS patients often repeat words or phrases, an attribute typically associated with autism. Indeed many FXS males present autistic type behaviour – gaze aversion, shyness, hand biting, hand flapping and rocking (Bardoni et al., 2000; Garber et al., 2008; Hernandez et al., 2009).

Decreases in cognitive ability for FXS males manifest in all areas: verbal reasoning, abstract/visual ability, quantitative skills and short terms memory. Patients also display decreases in overall adaptive behaviour scores – communication, daily living skills, socialization (Bardoni et al., 2000). Anxiety and mood disorders, hyperactivity, impulsivity and aggressive behaviour can also be present (Garber et al., 2008). Investigation by Ke et al. (2005) showed that children with FXS had better personal social functions than children with autism, although both groups demonstrated delays in personal social functions.

In 1993 de Vries et al. described eight FXS patients patients who showed a "Prader-Willi-like" phenotype. The patients had features resembling the Prader-Willi syndrome (PWS), such as truncal obesity, hypogenitalism, and small hands and feet. Consequently, these fragile X patients might be erroneously diagnosed as having Prader-Willi syndrome. However, some major differences are observed between the classical Prader-Willi syndrome and the PW-like subphenotype in these fragile X patients. Unlike PWS patients, PW-like FXS patients have a normal birth weight and show no hypotonia with feeding problems during infancy. Furthermore, seven patients developed a sudden gain of weight at the age of 5 to 10 years without any change in

diet. This is not observed in PWS patients who become obese because of a change in eating pattern which often occurs at a younger age. Another diagnostic difference is the typical fragile X behaviour, including poor eye contact, hyperactivity, short attention span, and preservative speech, which is expressed by the fragile X patients with the PW-like sub-phenotype.

Several case reports described in literature lead to the proposition that a "Sotos-like" phenotype of the fragile X syndrome might exist. Originally, Sotos syndrome (MIM #117550) was characterised by large body size and early accelerated growth in combination with acromegaloid features, advanced bone age, developmental delay, and a nonprogressive neurological disorder. Developmental delay was observed in all presented cases (mental retardation is the major feature of the fragile X syndrome) and other features of Sotos syndrome are also apparent in the presented cases, including large body size and acromegaloid features (de Vries et al., 1995).

Because the disorder is X-linked, females are generally much more mildly affected than males, particularly in terms of cognitive functioning, but they tend to have a higher risk of emotional problems compared to the general population. Females with the full mutation usually have normal or borderline IQ, and most will have associated learning disabilities and/or emotional problems. Approximately 30-50% of all females with full mutation have a normal IQ score, though with learning difficulties, deficient executive function, attention disorders, difficulties with mathematics, and language deficiencies. The emotional and behavioural characteristics in females with FXS are usually variable. Females with the full mutation are prone to social anxiety, shyness, social avoidance, withdrawal, mood lability, and depression. Furthermore, females with the permutation have also been described to have social anxiety (Garber et al., 2008; Hessl et al., 2001; Loesch and Hay, 1988; Ridaura-Ruiz et al., 2009).

Seizures and EEG findings consistent with epilepsy are another common feature of FXS during childhood, with an incidence between 13 and 18% in boys and 5% in girls. Complex partial seizures are the major seizure type in FXS. Centrotemporal spikes are a frequent EEG finding in children with FXS (about 70% - 80% of patients), and seizures in FXS are generally easily controlled with anticonvulsants and mostly limited to childhood. Seizures are much less frequently encountered in girls with FXS than in boys (Berry-Kravis, 2002; Jacquemont et al., 2007). A key neurological feature of individuals with FXS is that, in certain areas of the brain, their neurons have immature and dense dendritic spines. The spines are the site at which the majority of excitatory synapses

occur, and, although it is not known whether they are a cause or an effect, similar abnormalities have been associated with other forms of mental retardation. It is believed that these differences represent a defect in dendritic spine development and maturation (Garber et al., 2008). The epileptogenesis of FXS was studied in the knockout mice model. The heightened circuit activity in neocortical circuits, coupled with a less synchronous network inhibition, is proposed as the underlying mechanism that leads to the EEG abnormalities and epilepsy associated with fragile X syndrome. Thereby, the failure to properly modulate the mGluR5 response in the absence of FMRP results in neuronal hyperexcitability (Hagerman and Stafstrom, 2009).

In order to investigate long-term effects of deficiency of FMRP, Smit and colleagues (2008) made examination of the acquisition, savings and extinction of delay eye blink conditions in male individuals with FRAXA. Subjects with FXS showed significantly poor performance in acquisition experiments compared to control subjects. In saving experiments FXS males showed similar levels of savings of conditioned responses compared to control male subjects. Extinction was faster in FXS patients. This study revealed that different mechanisms underlie acquisition, savings and extinction of cerebellar motor learning.

2.3.3. Molecular Basis of Disease

In 1991, the molecular basis of FXS was revealed using positioning cloning and it was shown to be associated with massive trinucleotide repeat (CGG) expansion within a gene *Fragile X Mental Retardation 1*(*FMR1*) at the chromosomal folate-sensitive fragile site Xq27.3 (FRAXA) (de Vries et al., 1998; Jin and Warren et al., 2000; Oberle et al., 1991; Poustka et al., 1991; Rousseau et al., 1991; Rousseau et al., 1994).

The *FMR1* gene is a highly conserved gene consisting of 17 exons and span ~ 38 kb, encoding mRNA of 3.9 kb. The cytosine-guanine-guanine (CGG) repeat is located in the first exon, corresponding to the 5` untranslated region. It is a part of CpG island that extends upstream of the transcription initiation site and plays an important role in gene expression. Among most individuals in the general population, the polymorphic CGG repeat ranges from 5 to 50 repeats and is usually interspersed every 9–10 repeats with an adenine-guanine-guanine (AGG) (Eichler, et al., 1996; Fu et al., 1991). These alleles tend to be inherited in a stable manner from parent to offspring. The allele of a

larger repeat size (51–200 repeats) can become unstable and has a risk of expanding in the next generation (Crawford et al., 2000).

The *FMR1* gene transcript is subject to alternative splicing that affects the presence of exons 12 and 14 and the choice acceptor site in exons 15 and 17. The *FMR1* gene product is the fragile X mental retardation protein (FMRP) - a cytoplasmic RNA-binding protein that negatively regulates local protein synthesis in neuronal dendrites. The expansion and methylation of CGG repeat region lead to the absence of FMRP. The transcripts normally regulated by FMRP are over translated. The resulting over abundance of certain proteins results in reduced synaptic strength due to α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking abnormalities that lead, at least in part, to the fragile X phenotype (Bardoni et al., 2000; Bear et al., 2004; Garber et al. 2008).

FMR1 mRNA and FMRP are particularly abundant in neurons, especially in the hippocampus and the cerebellum. FMRP is also present in several epithelia and in testis, primarily in spermatogonia. The presence of FMRP in lymphocytes, hair roots, amniocytes and chorionic villi is useful for diagnostic application (Bardoni, Mandel and Fish, 2000). Except for different research to clarify FMRP exact functions, it is not yet fully understood. FMRP properties suggest a role in mRNA transport, translatability or stability. It is hypothesised that FMRP normally functions as a repressor of translation of specific mRNAs. FMRP is essential for neuronal development and the production of connective tissue in the foetus. (Bardoni, Mandel and Fish, 2000; Bear et al., 2004; Eberhart et al., 1996; Oostra and Willemsen, 2003; Ridaura-Ruiz et al., 2009).

Mutations in *FMR1* Gene

The fragile X syndrome is caused by an unstable expansion of a CGG repeat located in the 5` untranslated region of the *FMR1* gene. Three major types of alleles can be distinguished. Normal alleles have between six and ~50 CGG repeats, generally with one or two AGG interruptions. Premutation alleles have moderate expansion of the repeat (from 50 to ~200 units), and are unmethylated on an active X chromosome and do not affect *FMR1* expression. They are thus found in clinically normal male or female carriers. Full mutation alleles are methylated larger expansions (>200 CGG repeats) that prevent transcription of the *FMR1* gene and result in mental deficit in ~100% of males and a milder form for heterozygous carrier females. The molecular mechanism of FXS

is shown in Fig. 2.3. (Crawford et al., 2000; Eichler et al., 1996; Giliberto, Szijan and Fereirro, 2006; Moutou et al., 1997).

The full mutation often shows somatic heterogeneity. In ~15% of carriers of a full mutation a mixture of premutation and full mutation can be detected by Southern blot analysis of leucocytes DNA. These have been called mutation mosaics. With more sensitive methods of detection, minor premutation sized fragments can be detected in as many as 40% of the full mutation carriers (Crawford et al., 2000; Eichler et al., 1996; Moutou et al., 1997). However, full mutation with a 90% unmethylated gene in male leucocytes (methylation mosaic) was reported by de Vries et al. (1996). For this case FMRP in 75% of leucocytes was detectable.

Intermediate or grey zone alleles is poorly defined, with the range of 41–60 CGG repeats recommended in the guidelines from the American College of Medical Genetics (Sherman et al., 2005), which is based on the claim that alleles below this size showed ‘‘no meiotic or mitotic instability’’. These alleles are perhaps the biggest single challenge to fragile X molecular diagnosis in terms of interpretation, reporting and genetic counselling, as they represent the overlap zone between stable normal alleles and unstable premutations. Boundaries for the grey zone range vary among studies, from 34 or 35 CGG repeats for the lower boundary to 58/60 repeats for the upper boundary (Moutou et al., 1997).

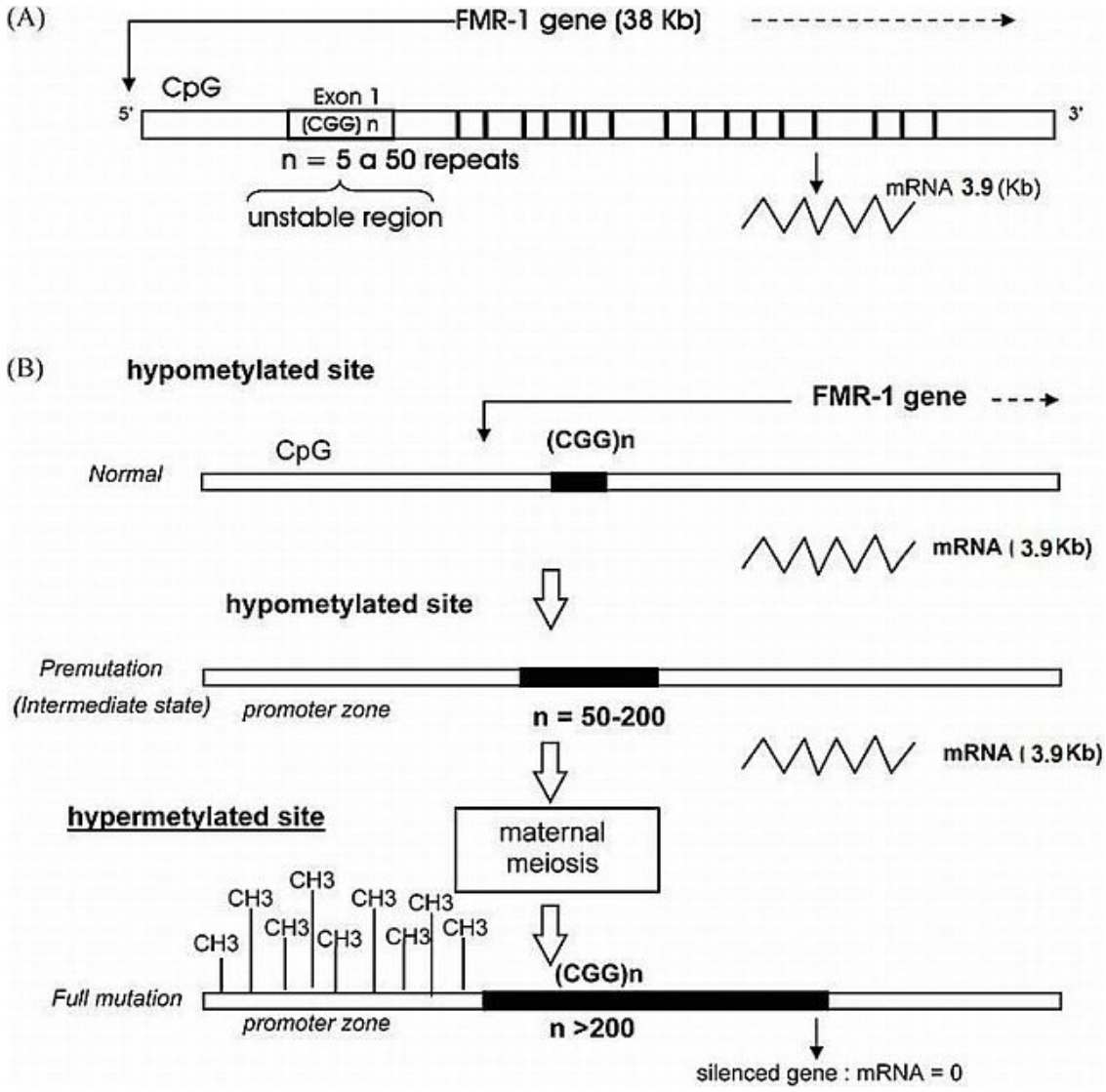

Fig. 2.3. *FMR1* gene and the molecular mechanism of fragile X syndrome (Giliberto, Szijan and Fereirro, 2006).

(A) schematic representation of the *FMR1* gene; CpG = CG-repeat island within the promoter; (CGG)n = trinucleotide-repeat sequence in exon 1. (B) Expansion of (CGG)n in different degrees and its effect on the functionality of the gene. Normal and expanded alleles (premutation and full mutation) and also the location of hypo and hypermethylated CpG islands are indicated.

These "intermediate" alleles are often transmitted stably, but are more likely to show unstable transmission with increasing size in this range. Most unstable transmissions of intermediate alleles are small increments of only one or two repeats;

frequently, an intermediate allele may show both stable and unstable transmissions within the same family.

The unusual inheritance pattern of FXS is now well understood, however there is little information about the factors that influence the instability of the CGG tract. The gender of the parent carrying an expanded repeat (maternal imprinting), the number of repeats (dynamic mutation) or the absence of AGG interruptions in long tracts of CGG repeats have been described as the main factors related to this instability (Rife et al., 2004). The replication-based model is one of hypothesised mutational mechanisms. This model assumes that slippage of perfect repeat Okazaki fragments leads to CGG repeat expansion. However this model cannot explain how slippage could generate the huge expansion during transition from premutation to full mutation (Jin and Warren et al., 2000). This transition occurs exclusively due to maternal transmission. It was shown that risk of expansion is highly correlated with the size of the maternal premutation, being very low for premutation of ~60 repeats, and close to 100% for premutation larger than 90–100 repeats. Even in the absence of such transition to full mutation, there is a tendency for an increased length of premutation upon maternal transmission, but not upon paternal transmission. These features of the transmission of the mutated *FMR1* alleles account for the characteristics of the segregation of the fragile X syndrome (Moutou et al., 1997).

The exact timing of the repeat expansion is still unclear but it must occur during meiosis or early embryonic development. The most accepted model assumes that full mutations are already presented in oocyte and thus all cells in the resulting embryo will also have a full mutation. Mitotic instability could explain the mosaic pattern which is quite often observed in FXS patients (Bontekoe et al., 2001).

In addition, rare cases of fragile X syndrome associated with point mutations have been reported. Only four point mutations in *FMR1* have been reported:

- a missense mutation p.Ile304Asn, a 1-bp deletion c.373delA in exon 5 resulting in a frameshift and premature truncation of the protein,
- a 2-bp change g.23714GG4TA spanning the intron/exon boundary of exon 2,
- a missense mutation p.Arg138Gln.

The CGG repeat and flanking sequences deletions have been reported several times (Gronskov et al., 2011; Hirst et al., 1995; Oostra and Willemsen, 2003). A 2-bp

deletion in intron 1 at position 14100 (14100delCT) was described as silent mutation (Gronskov, Hallberg and Brondum-Nielsen, 1998).

2.3.4. Other Clinical Conditions Related to the *FMR1* Gene

Early menopause and premature ovarian failure 1 (POF1) (MIM # 311360)

Premature ovarian failure is defined as secondary hypergonadotropic (FSH ≥ 40 IU/l) amenorrhoea occurring before the age of 40. It affects approximately 1% of females and it`s aetiology is still unknown in most cases. Among women who carry the premutation in the *FMR1* gene, approximately 21% have POF compared to only 1% in the general population. Furthermore, approximately 2% of women with isolated POF and 14% with familial POF, respectively, carry the premutation allele. The study carried out in a POF population by Bodega et al. (2006) shown similar data. The range of CGG repeat found in POF patients in this study was between 43 and 163 repeats, including grey-zone alleles and premutation alleles. Inactivation of X chromosome with increased CGG repeat number was found to be in correlation with POF manifestation. In other studies POF association with grey-zone alleles did not meet statistical significance (Jacquemont et al., 2007).

The CGG repeat size within the premutation range correlates with both the penetrance featuring an earlier onset of POF, and the increase of follicle-stimulating hormone concentrations. The incidence of premature ovarian failure becomes significant above 70–80 CGG repeats and seems to plateau or even decrease after 100 repeats (Jacquemont et al., 2007).

The aetiology of the ovarian failure and the risk factors associated with the FMR1 gene are under investigation (Bodega et al., 2006; Sherman et al., 2005). The data of study by Hundscheid and colleagues (2000) strongly suggest that in POF considerable proportion of the premutations are inherited paternally. It is hypothesised that this may own to a paternal genomic imprinting effect.

Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) (MIM #300623)

FXTAS is a late-onset neurodegenerative disorder caused by the presence of a premutation (55-200 CGG repeats) in the *FMR1* gene in affected individuals. Clinical signs in male subjects carrying the premutation include cerebellar ataxia, intention tremor, and cognitive decline, dementia, apathy, dysinhibition, irritability,

parkinsonism, depression occasionally associated with other symptoms such as peripheral neuropathy, lower limb proximal muscle weakness, and autonomic dysfunction, with age at onset between 50 and 70 years. The clinical presentation of patients, specific magnetic resonance imaging and neuropathological findings distinguishes FXTAS from other movement disorders (Berry-Kravis et al., 2007; Cellini et al., 2006; Jacquemont et al., 2003; Jacquemont et al., 2007).

The penetrance of disease and its severity is associated with number of CGG repeats and patient age. In most cases, premutation allele exceeds 70 CGG repeats. Although CGG repeat size correlates with some features of FXTAS, CGG repeat size alone does not predict who will develop FXTAS. Fragile X-associated tremor/ataxia syndrome predominantly affects males, although individual female carriers do occasionally have clinical and neurological symptoms. The neurological symptoms are almost always much milder in females than males, presumably due to a variable degree of protection provided by the expression of *FMR1* from the normal allele on the active X chromosome in a percentage of cells. Indeed, females with FXTAS symptoms tend to have skewed X-inactivation, with a greater fraction of cells expressing an active premutation. Unknown genetic, familial, or environmental factors likely modify the risk of individual carriers (Berry-Kravis et al., 2007; Hagerman and Hagerman, 2004; Jacquemont et al., 2003; Jacquemont et al., 2007).

Molecular mechanisms leading to neurological symptoms are still unclear. Recent studies on this field revealed that FMR1 mRNA level is elevated 5 – 10 times in premutation carriers, while the FMR protein (FMRP) level is about normal. This finding can lead to the hypothesis that the excess of *FMR1* mRNA with expanded CGG repeats can be toxic to human neural cells (Shan, Xu and Jin, 2008).

2.3.5. Screening for FXS and Genetic Counselling

Testing and screening recommendations from The American College of Medical Genetics (McConkie-Rosell et al., 2005) and The Clinical Molecular Genetics Society (CMGS) UK (Macpherson and Sawyer, 2005):

1) Individuals of either sex with mental retardation, developmental delay, learning/behavioural difficulties, speech delay, autistic features, Asperger syndrome, social dysfunction, poor eye contact and challenging behaviour as well as physical features such as a large head, large ears, macroorchidism, hand

flapping/biting and dysmorphic faces. A family history of fragile X syndrome, or a relative with undiagnosed mental retardation. Although the physical fragile X phenotype is usually well established in post-pubertal males, this is not true of females and young children where the full mutation phenotype is variable and often subtle.

2) Individuals with a family history of fragile X syndrome or a family history of undiagnosed mental retardation who are seeking reproductive counselling. When there is no established diagnosis of fragile X syndrome, testing the affected proband is preferable to screening an unaffected relative. However, this is not always feasible, especially in the prenatal setting.
3) Prenatal testing offered to individuals who are known FMR1 mutation carriers.
4) Individuals tested previously by cytogenetics who have results inconsistent with phenotype.
5) Women with reproductive or fertility problems associated with elevated FSH levels, especially if there is a family history of premature ovarian failure, fragile X syndrome, or undiagnosed mental retardation.
6) Individuals with late onset tremor or cerebellar ataxia of unknown origin, particularly when there is a family history of movement disorders, fragile X syndrome, or undiagnosed mental retardation.

Since early diagnosis of fragile X syndrome is crucial, to enable other family members to be informed of their risk of having affected offspring, most fragile X diagnostic tests will be carried out on a very broad range of patients with a consequently inevitable low pickup rate.

For families with suspected fragile X syndrome, or with a confirmed diagnosis, or for families at risk or with a known carrier, the following questions on family member's medical and psychosocial history are advised (McConkie-Rosell et al., 2005; Saul and Tarleton, 1998):

• Cognitive defects: mental retardation, developmental delay, learning disabilities, specific problems with maths.

• Speech delay or unusual speech pattern.

• Autistic spectrum disorders or autistic-like behaviour (gaze avoidance, repetitive behaviour, hand-flapping, hand biting, touch avoidance, etc.).

• Attention deficit disorder (ADD) or attention deficit hyperactivity disorder (ADHD).

• Dysmorphic features—macrocephaly, large ears, long face, broad forehead, prominent jaw, strabismus.

• Features of loose connective tissue: hyperextensible joints, flat feet, hypotonia, mitral valve prolapse, large testicles, hernias, recurrent ear infections.

• Neurologic symptoms: seizures, late-onset progressive tremor, ataxia, difficulty walking, balance problems, short-term memory loss, loss of sensation in limbs.

• Mental illness/personality disorders: depression, schizophrenia, bipolar disorder, obsessive-compulsive disorder, schizoaffective disorder, schizoid personality, etc.

• Behavioural problems: impulsiveness, anger outbursts, violent behaviour, solitary behaviour, counselling or medication for behavioural difficulties.

• Shyness, social anxiety, excessive worrying, counselling or medication for emotional difficulties.

• Premature menopause, fertility problems.

Consultation after confirmed diagnosis should include (McConkie-Rosell et al., 2005) :

1) Educational and health promotion – discuss clinical presentation of disease, inheritance pattern and genetics, treatment and therapy, follow-up recommendations.
2) Risk assessment.
3) Informing family members - because of the difficulty frequently encountered when informing relatives of genetic risk, a genetic counsellor should work with clients to develop a strategy to inform relatives as part of initial as well as follow-up genetic counselling sessions.

Individuals at risk for passing on fragile X mutation have a variety of options for reproductive choice (McConkie-Rosell et al., 2005; Platteau et al., 2002):

1) Consider adoption to bypass genetic risk.
2) Achieve pregnancy using donors eggs or sperm for mutation carriers.
3) Preimplantation genetic diagnosis (PGD) is possible but should be approached with caution.
4) Prenatal diagnosis.

2.3.6. Population genetics of FXS

The prevalence of the full mutation.

After cloning *FRM1* gene and introduction in clinical practice molecular diagnostics of FXS, the prevalence of the full mutation was estimated to be 1/1200 to 1/1500 males and 1/2000 to 1/2500 females (Gustavson et al., 1988; Webb et al., 1986a; Webb et al., 1986b). In recent studies the prevalence of FXS full mutation is approximately 1/4000 males to 1/6000 males and 1/8000 to 1/10000 females (Crawford, 2001).

The prevalence of FXS has been reported from different countries, including the European geographical region and Western European descent populations (Table 2.3). Prevalence of fragile X syndrome in overall European population was estimated to be 14.25/100 000 (Orphanet, 2010).

Table 2.3.

The Prevalence of Fragile X Syndrome among Males

Country	Prevalence (males)	Reference
North Finland	1/2500	Vaisanen, 1999
England	1/5530	Younings et al., 2000
Spain (Catalonia)	1/8333	Rife et al., 2003
Poland	1/2857-1/5882	Mazurczak et al., 1996
Estonia	1/13947 life-birth boys	Puusepp et al., 2008
The Netherlands	1/6045	de Vries et al., 1997
USA (different races)	1/5161	Coffee et al., 2009
Egypt	1/1079	Meguid et al., 2007
Australia	1/4300 (3550 – 5680)	Brown, 2010

Most of the published studies are based on selected population consisting of mentally retarded persons. The results from this type of approach could artificially give higher prevalence of the disease than actually exists. This effect was clearly demonstrated in the studies published in the next few years after cloning of the *FMR1* gene. The prevalence of full mutation causing FXS was estimated to be 1/1200 to

1/1500 males and 1/2000 to 1/2500 females (Gustavson et al., 1988; Webb et al., 1986a; Webb et al., 1986b). In later publications the prevalence of FXS full mutation was set twice lower, to 1/4000 - 6000 males and 1/8000 - 10000 females (Crawford, 2001). Most the published estimates are based on the ratio of confirmed/investigated patients and thus estimate the prevalence in the target population as a percentage. According to a review by Crawford at al. (2001), the lowest published prevalence (except for reports of absence of FXS in a population) was 0.3%, found in USA Caucasians. The highest prevalence of 17.3% was reported from Croatia. If we compare clinical symptoms based on a chosen target population, it becomes evident that prevalence correlate with the spectrum of clinical symptoms. The lowest reported prevalence was obtained from a population with a broad variation of symptoms – a special education needs population with an unknown aetiology of disorder. In contrast, the highest prevalence was found in patients clinically pre-selected for fragile X DNA analysis on the basis of MR of unknown aetiology, a positive family history, or at least on physical and/or behavioural characteristic of the fragile X syndrome. Similar tendencies are found in recent published literature. A study by a group in India reported a prevalence of 7.8% for FXS in a mentally retarded population (Chowdhury et al., 2006). The prevalence of FXS in the Egyptian mentally subnormal male population was given as 6.4% (Meguid et al., 2007). Interesting results from a study were published by an Estonian research group. Prevalence of FXS in the mentally retarded male population there was 2.7% but the prevalence of this syndrome in the entire children`s population was found to be 1/13 947 in live-birth boys (Puusepp et al., 2008) which is significantly lower than in other populations.

Very rare disorder fragile X syndrome was reported by Beresford et al. (2000) in the population of Nova Scotia (Canada). According to Crawford, Acuna and Sherman`s review of the fragile X syndrome in 2001, the prevalence of full mutation may differ across other populations. The majority of FXS in the population of Israel was reported to be of Tunisian Jewish descent. Conversely the lack of CGG expansion was reported in Native American populations.

CGG repeat stability and linked haplotypes in different populations.

The distribution of normal CGG repeat alleles are described in different populations. In Western European descent populations allele 30 is the prevalent allele (Arrieta et al., 2003; Chiurrazi et al., 1999; Kunst et al., 1996). In Asia descent populations allele 29 have been reported as common (Faradz et al., 2001). Diverse results are reported by two groups of researchers from Japan. Arinami and colleagues (1993) reported prevalence of alleles 28; 29 and 35 in contradistinction to Otsuka et al. (2010) who reported prevalence of alleles 27; 26 and 28. It is possible that discrepancy of data in one single repeat unit rose from genotyping errors.

The underlying mutational mechanism is not fully understood and remains a topic of debate. The gender of the parent carrying an expanded repeat (maternal imprinting), the number of repeats (dynamic mutation) and the absence of AGG interruptions in long tracts of CGG repeats have been described as the main factors related to this instability (Dombrowski et al., 2002; Eichler et al., 1996; Rife et al., 2004).

Sequence of CGG repeats is not pure but interspersed with an AGG (adenine-guanine-guanine). The pattern of CGG repeats show interspersion in every 9 – 10 repeats. Normal CGG alleles usually contain two or three AGG`s. Research of premutation range alleles revealed one or two interspersions on 5`- end of tract or even pure sequence. This finding led to a hypothesis that long, uninterrupted 3`- end sequence is an important factor for CGG repeat instability. Studies from different authors and populations have suggested that alleles with even > 24 pure repeats at the 3`- end of sequence may be inherited unstable with a possible increase to the premutation (Crawford et al., 2000). The i*n vitro* studies and studies in bacteria and yeast confirmed that length of repeats and the number of the AGG interruptions determine sequence stability (Bontekoe et al., 2001).

A number of previous studies of microsatellite markers within 150 kb of the *FMR1* CGG repeat have indicated that a part of FXS chromosomes shows linkage disequilibrium with DXS548-FRAXAC1-FRAXAC2 haplotype and the ATL1 SNP. Among Western European descended populations of the USA haplotypes which showed linkage disequilibrium with FXS chromosomes were identified. The haplotype 2-1-3 was presented in 14% of FXS chromosomes, haplotypes 6-4-4 and 6-4-5 in total accounted for 30% of FXS chromosomes. (Eichler et al., 1996; Gunter et al., 1998;

Kunst et al., 1996; Macpherson et al., 1994; Murray et al., 1997; Oudet et al., 1993; Richards et al., 1991).

Haplotypes linked to FXS are widely described across Western European and Scandinavian populations. However, less is known regarding populations from Eastern Europe, including the Baltic States. There are reports from Asia as well. Although several studies have identified specific haplotypes associated with FXS patients and normal CGG repeat alleles across European populations, not all studies used all three microsatellite markers for constructing haplotypes (Table 2.4).

As the analysed microsatellite loci and nomenclature assigned to alleles in the literature are different, confusion arises, which may lead to bias in the interpretation of literature data that compare haplotypic results from different populations.

Table 2.4.

Distribution of *FMR1* linked haplotypes among different population

Country/ Population	Haplotype FXS				Haplotype Normal				Reference
	DXS548	AC1	AC2	RF	DXS548	AC1	AC2	RF	
Finland	6	-	3	0.80	7	-	3	0.46	Haataja et al. 1994
Norway	6	4	-	0.50	7	3	-	0.68	Larsen et al. 2001
Spain/ Basque	-	-	-	-	7	4	-	0.68	Arrieta et al. 2003
Czech	2	2		0.21	7	4	-	0.67	Pekarik et al. 1999
Czech	2		5	0.27	7		3+	0.55	Malmgren et al. 1994
Sweden	6 7	- -	5+ 6	0.36 0.29	7 -	- -	3+ -	0.43 -	Malmgren et al. 1994
Croatia	7	4	-	0.44	7	4	-	0.66	Dokic et al. 2008
Portugal	6	5	-	0.21	7	4	-	0.69	Peixoto et al. 1998
Multiple European descent nationalities	7	-	3	0.44	7	-	3	0.16	Oudet et al. 1993
Caucasian /England + US	6 6 2	4 4 1	4 5 3	0.16 0.14 0.14	7 7 -	3 3 -	4+ 4 -	0.45 0.12 -	Eichler et al. 1996
Poland	2 -	4 -	7 7+	- -	7 -	3 4	7 -	- -	Rajkiewicz, 2008
Argentina	7 7	3 1	- -	0.28 0.26	7 7	3 4	- -	0.54 0.20	Bonaventure et al. 1998
India	7	3	4+	0.17	7	3	4+	0.18	Sharma et al. 2003
Singapore/ Chinese	-	-	-	-	7	4	7+	0.36	Zhou et al. 2006
Singapore/ Malays	-	-	-	-	7	4	7+	0.31	Zhou et al. 2006
Singapore/ Indians	-	-	-	-	7	3	5+	0.23	Zhou et al. 2006

AC1 = FRAXAC1; AC2 = FRAXAC2; RF = relative frequency

3. SUBJECTS AND METHODS

3.1. Subjects

3.1.1. Prevalence of the Fragile X Syndrome

The retrospective data of patients genotypes, analyzed in the Medical Genetic Clinic, University Children`s Hospital between 1998 and 2007, were summarized to assess the prevalence of FXS.

All patients were referred for exclusion/confirmation of fragile X syndrome by clinical geneticist at the Medical Genetic Clinic, University Children`s Hospital, by child psychiatrist for the hospitalized persons at the Children`s Psychiatric Department, University Children`s Hospital and by clinical geneticist at the children`s attending Social Care Centre Riga, Latvia.

Inclusion criteria for selecting patients' data were as follows:

- patients with mental retardation in various degrees with or without association with dysmorphic features
- MR patients with autism, autistic spectrum disorders and any type of behavioural disturbances
- genotype data with exact number of CGG repeats

Exclusion criteria for selecting patients' data were as follows:

- patient gender (female)
- consanguinity
- monogenic, chromosomal and metabolic diseases

The clinical features of the patients were assessed and family history obtained by clinical geneticist. The ethnical background of patients was not considered.

Based on inclusion/exclusion criteria 374 anonymous, unrelated male patient data were selected for prevalence study. The age of patients at the moment of DNA diagnostic test varied between two and seventeen years.

3.1.2. Variation of CGG Trinucleotide Repeats

To assess distribution of normal CGG repeat alleles retrospective data of patients genotypes, analyzed in the Medical Genetic Clinic, University Children`s Hospital between 1998 and 2007, were used. Based on inclusion/exclusion criteria (see chapter 3.1.1.) 374 anonymous, unrelated male patient data were used. We considered selected data

comparable, because for all 374 samples, both routine screening with PCR and fluorescent PCR following Applied Biosystems protocol for exact CGG repeat number detection, were performed (chapter 3.2.2. and 3.2.3.).

3.1.3. The Case-Control Study

For case-control study of *FMR1* linked haplotypes the control group of 122 unrelated male patients with normal number of CGG repeats were selected based on inclusion/exclusion criteria.

Inclusion criteria for selecting control group were as follows:

- parents or legal representatives of minors signed informed consent according regulations issued by Ethics Committee for participation in this study
- genotype data within a normal range of CGG repeats
- patients with mental retardation in various degrees with or without association with dysmorphic features
- MR patients with autism, autistic spectrum disorders and any type of behavioural disturbances

Exclusion criteria for selecting control group were as follows:

- patient gender (female)
- consanguinity
- monogenic, chromosomal and metabolic diseases

The case group consisted of 11 unrelated male patients with confirmed diagnosis (full mutation). Parents or legal representatives of minors signed informed consent according regulations issued by Ethics Committee for participation in this study. For FXS patients diagnosis was confirmed by Southern blotting (chapter 3.2.4.). For haplotype analysis of the FXS patient group and the control group DXS548; FRAXAC1; FRAXAC2 microsatellite markers and ATL1 SNP were used (chapter 3.2.6. and 3.2.7.).

3.1.4. Genotype-Phenotype Correlation

Genotype-phenotype correlation was assessed for 12 male patients with confirmed diagnosis of FXS in time period from 1998 to 2010. In this group of study siblings were included. The age of patients at the moment of diagnosis varied between two and sixteen years (average = 7.33 ± 4.46). Clinical information was obtained from case-records of patients by clinical geneticist or child psychiatrist. Anthropometric data were measured according to the "Smith recognizable patterns" and methodology described by Krūmiņa, Kokare and Biķis (2007). IQ tests were performed based on the Woodcock – Johnson test and Wechsler Intelligence Scale for Children. Autistic spectrum disorders were evaluated according to the Autism Diagnostic Observation Schedule (ADOS).

3.2. Molecular Studies

3.2.1. DNA Extraction

Five millilitres of peripheral blood were collected in EDTA-coated tubes. Before DNA extraction, blood samples were kept frozen at -20°C. DNA was extracted using "Genomic DNA Purification Kit" (Fermentas, Lithuania) according to the manufacturer protocol. The following procedure was performed:

- Lysis of the cells – 500 µl of blood was mixed with 1 ml of milliQ H_2O. The sample was centrifuged at 10 000 rpm for 2 min, supernatant was removed and 200 µl of 1x TE buffer added. Diluted residue was mixed with 400 µl of lysis solution and incubated for 10 min at 60°C.
- Extraction with chloroform and subsequent DNA precipitation using detergent – after lysis 600 µl of chloroform was immediately added to the sample and gently emulsified by inversion (3 -5 times), centrifuged at 10 000 rpm for 2 min. In advance prepared precipitation solution (720 µl of milliQ H_2O mixed with 80 µl of supplied 10 x concentrated solution) was transferred to a new tube. The upper aqueous phase containing DNA was transferred to the tube containing the freshly prepared precipitation solution, mixed and centrifuged at 14 000 rpm for 6 min. Supernatant was removed and DNA pellets dissolved in 100 µl of 1.2 M NaCl solution.
- Genomic DNA concentration and desalting by ethanol precipitation – 300 µl of cold ethanol (96°) was added to the dissolved DNA pellets and incubated for 10 min at -

20°C. Subsequently sample was centrifuged at 14 000 rpm for 6 min, the supernatant removed, the sample washed with ice cold 70° ethanol and centrifuged at 14 000 rpm for 6 min, forming supernatant was removed.

The residue was dissolved in 200 µl MilliQ water and kept at room temperature overnight. The DNA concentration was checked visually after electrophoresis on 1% agarose gel. Since 2009, the DNA concentration of samples was measured with the Thermo Scientific NanoDrop 1000 Fluorospectrometer.

3.2.2. Routine screening PCR amplification

For the amplification of normal CGG repeat allele within *FMR1* gene, primers sequence corresponding to position 212-241 and 599-571 at the 1kb *Pst1* fragment plasmid Ep5.1, containing CpG island and CGG repeat region, were used (Appendix 1).

The reaction was performed according to Chong et al. (1994) in a final reaction volume of 25 µl, using 1x *Pfu* DNA polymerase reaction buffer (20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1% Triton X-100, 0.1 mg/ml BSA, 2 mM MgSO4), 0.4 µM of each primer, 12,5% DMSO, 0.2 mM of each dNTP, 0,2 mM of c7GdTP, 150 ng of genomic template and 1.25 U of *Pfu* DNA polymerase (Fermentas, Lithuania).

The primers sequence was as follows:

Forward primer A: 5`- GGA ACA GCG TTG ATC ACG TGA CGT GGT TTC - 3`

Reverse primer 571R: 5`- GGG GCC TGC CCT AGA GCC AAG TAC CTT GT - 3`

Amplification conditions consisted of an initial denaturation of 5 min at 98°C, followed by 35 cycles of 1 min at 98°C, 1 min at 65°C and 2 min at 75°C. A final extension for 10 min was performed at 75°C. Reaction was carried out on PCR Mastercycler (Eppendorf, Germany). As an internal control for amplification of different CGG repeat alleles, synthetic oligonucleotides from Fragile X Genemer™ Control DNA (Genelink, USA), with 29 and 40 CGG repeats were used. As an internal control for reagents contamination, blank sample (template substituted by milliQ water) was used.

10 µl of PCR products were separated on 2.5% agarose gel in 0.5 X TBE at 5.5 V/cm for 60 min. Size standard GeneRuller™ 100 bp (Fermentas, Lithuania) was used. The gel was stained with ethidium bromide (0.1 µg/ml) and visualised with UV. PCR using described primers would be expected to generate a product of 430 bp with a 30

CGG repeat allele. A PCR product band larger than 500 bp was interpreted as the permutation range. For alleles larger than 55 CGG repeat, smears (asymmetric heteroduplexes formed by annealing of truncated single strand products) were found on agarose gel. For full mutation alleles, a lack of PCR products on agarose gel was observed.

Banding patterns of different *FMR1* gene CGG repeat size alleles is shown in Fig.3.1.

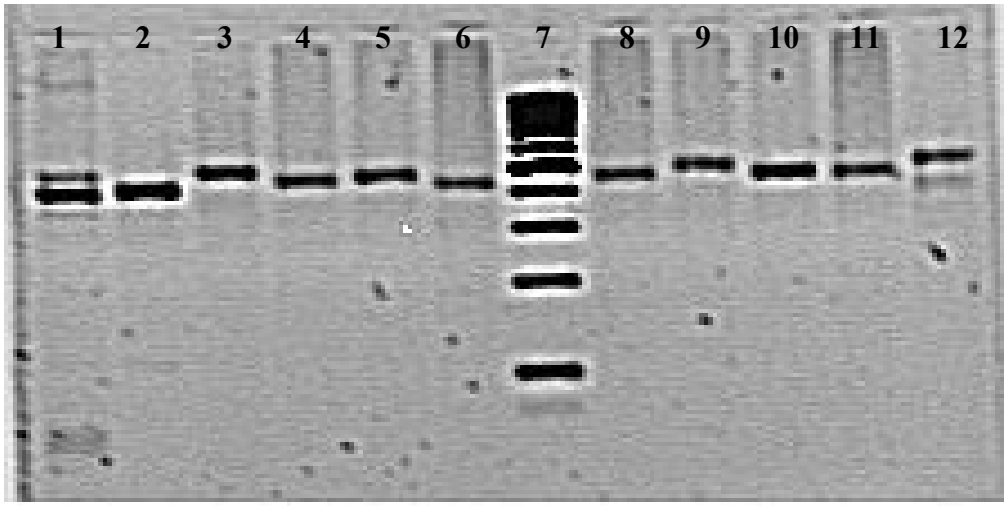

Fig.3.1. Ethidium bromide stained 2% agarose gel showing PCR products from several alleles with normal repeat number (lanes 4;5;6;8;9;10;11) and control samples: lane 1- 24/41 repeats; lane 2-29 repeats; lane 3-50 repeats; lane 12-40 repeats; line 7 – size standard GeneRuller™ 100 bp.

3.2.3. Fluorescent PCR

For a precise determination of CGG repeat number, fluorescent PCR was carried out according to the Applied Biosystems (USA) protocol. In advance the following reagent solutions were prepared:

- 1.0 M Tris sulphate, pH 9.0 – in total volume of 10 ml: 10.8g of Tris Base (Sigma T-1503, USA) + 1.90g TRIZMA Sulfate (Sigma T-8379, USA). MilliQ H_2O was added up to 10 ml of total volume.
- 1.0 M Ammoniuma sulphate – in total volume of 10 ml: 1.32 g of ammonium sulphate (Sigma, USA) was mixed with milliQ H_2O up to 10 ml of total volume.

- 5.0 M betaine – in total volume of 10 ml: 58.6g of betaine (Sigma B-2754, USA) (must be dry!) was mixed with milliQ H_2O up to 10 ml of total volume.

For amplification of the *FMR1* region, two reagent premixes were prepared.

- Top reagent master mix (500µl) contained:

  12.5µl of Tris sulphate 1.0 pH 9.0 (Sigma, USA),

  25.0 µl of ammonium sulphate 1.0 M (Sigma, USA),

  100.0µl of betaine 5.0 M (Sigma, USA),

  5.6µl of redistilled 2-pyrrolidinone 99+% (Sigma, USA),

  5.6µl of magnesium sulphate 1.0 M (Sigma, USA),

  20.0µl of 20 µM fragile X PCR primer (+) (5`- CGG AGG CGC CGC TGC CAG G - 3`) (Applied Biosystems, USA),

  20.0µl of 20 µM fragile X PCR primer (-) (5`- TET-TGC GGG CGC TCG AGG CCC AG - 3`) (Applied Biosystems, USA),

  100 µM Apo E oligo primer (+) (5`-VIC-CGC CTG GCA GTG TAC CAG GCC GGG G-3`) (Applied Biosystems, USA),

  100 µM Apo E oligo primer (-) (5`-GCC GGC CAG GGA GCC CAC AGT GG-3`) (Applied Biosystems, USA).

- Bottom reagent master mix contained:

  234.0µl of milliQ H_2O,

  10.0 µl of Tris sulphate 1.0 pH 9.0 (Sigma, USA),

  10.0 µl of 100 mM dCTP (Sigma, USA),

  10.0 µl of 100 mM dTTP (Sigma, USA),

  10.0 µl of 100 mM dGTP (Sigma, USA),

  10.0 µl of 100 mM dATP (Sigma, USA),

  193.5µl of betaine 5.0 M (Sigma, USA),

  6.0µl of Vent (exo-) DNA polymerase 2 U/µl (New England Biolabs, UK).

Approximately 100 diploid copies (0.67 ng) per µl of genomic DNA were used to carry out the reaction. This reaction was performed in 10 µl of Bottom reagent master mix, 8 µl of Top reagent master mix and 2 µl of diluted genomic DNA using the "Hot start" technique with Amli Wax (Applied Biosystems, USA). Amplification conditions were as follow:

	Temperature	Time	No. of cycles
Denaturation	98.0°C	30 sec	
Anneling	56°C	4 min	14
Extension	69°C	6 min	
Denaturation	98.5°C	30 sec	
Anneling	56°C	4 min	7
Extension	69°C	6 min	
Denaturation	99.0°C	30 sec	
Anneling	56°C	4 min	10
Extension	69°C	6 min	

PCR products were separated on an ABI Prism® 310 genetic analyzer (Applied Biosystems, USA) under two different electrophoresis conditions. To prepare PCR products for electrophoresis, fragile X sample load master mix was used.

According to the Applied Biosystems protocol:

- Fragile X sample load master mix contains (in total volume of 10 ml):

  0.0501g of tetra-methyl-ammonium hydroxide (Sigma, USA),

  3.18 µl of milliQ H_2O,

  0.0318 g of trans-1,2-diaminocyclohexane tetra-acetic acid (CDTA) (Sigma, USA),

  4.0 mg of recristallized disperse red 1(Sigma, USA),

  168.5 µl of 500 µM Apo E blocking oligonucleotide (5`-CCA CTG TGG GCT CCC TGG CCG GC-3`) (Applied Biosystems, USA),

  56.1 µl of 500 µM fragile X blocking primer (5`-CCT GGC AGC GGC GCC TCC G-3`) (Applied Biosystems, USA),

  1.12 ml of Fragile X size standard, (Applied Biosystems, USA),

  6.0µl of 1-octyl-2-pyrrolidinone (Sigma, USA).

For PCR products separation on the ABI Prism® 310 genetic analyzer, 47cm x 50 µm (36 cm well-to-read) capillary and POP-4™ polymer were used. For separation of

normal, grey and permutation zone alleles, 1 µl of PCR products, 8 µl of fragile X sample load master mix, 5 µl of milliQ water and NGP data electrophoresis parameters were used (filter set C, 15 sec of injection time and 20 min of run time). For separation of large permutation and mutation zone alleles, 6µl of PCR products, 8 µl of fragile X sample load master mix and PE data electrophoresis parameters were used (filter set C, 45 sec of injection time and 55 min of run time).

Genotyping results were analysed by GeneScan™ software (Applied Biosystems, USA). The corresponding peaks length was calculated according to the calibration curve of the fragile X size standard (50 bp-2500 bp). The black peak of the apolipoproteine E (*ApoE*) gene corresponding to 121 bp – 123 bp was used as an internal control for amplification. This CG rich fragment of *Apo E* indicates successful amplification and template quality. Results of genotyping for male with normal CGG allele are shown in Fig.3.2. Results of genotyping for heterozygote female with two CGG alleles within normal range are shown in Fig.3.3. Results of genotyping for heterozygote female with one allele in the normal range and one allele in the premutation range are shown in Fig.3.4.

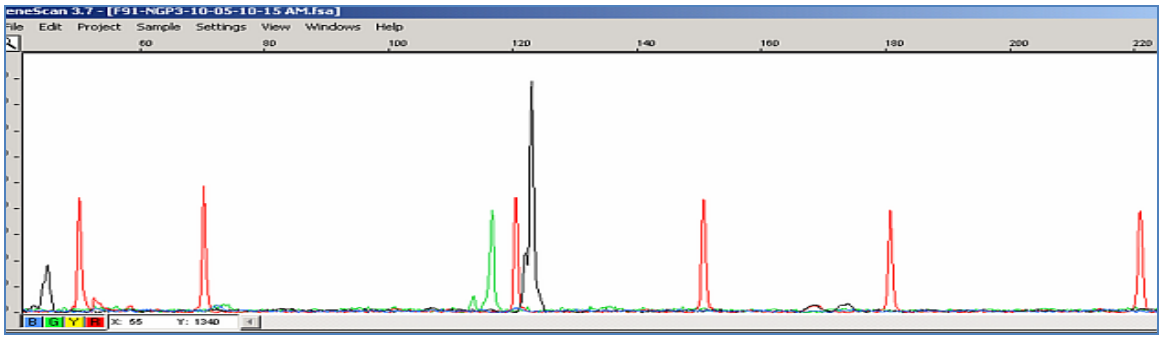

Fig.3.2. Genotyping results for male with 23 CGG repeat allele. Red peaks – fragileX size standard (Applied Biosystems, USA); black peak – internal control fragment of *ApoE* gene; green peak – *FMR1* gene CGG repeat fragment.

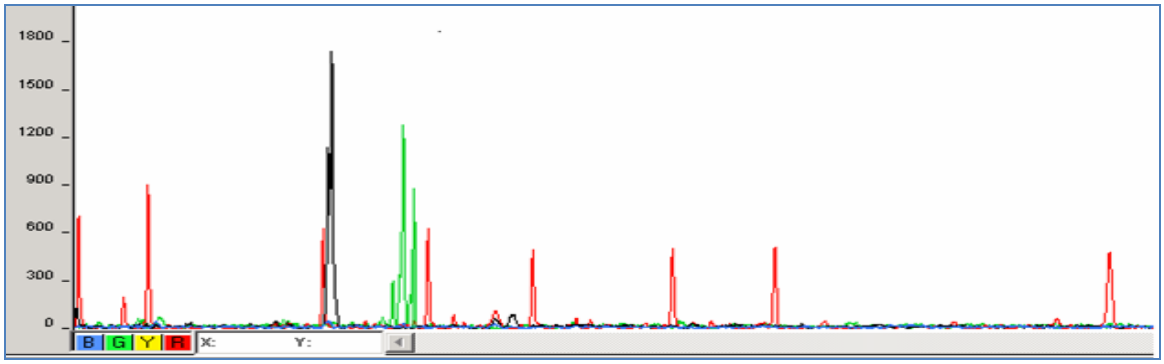

Fig.3.3. Genotyping results for female with 32/33 CGG repeat alleles. Red peaks – fragileX size standard (Applied Biosystems, USA); black peak – internal control fragment of *ApoE* gene; green peaks – *FMR1* gene CGG repeat fragments.

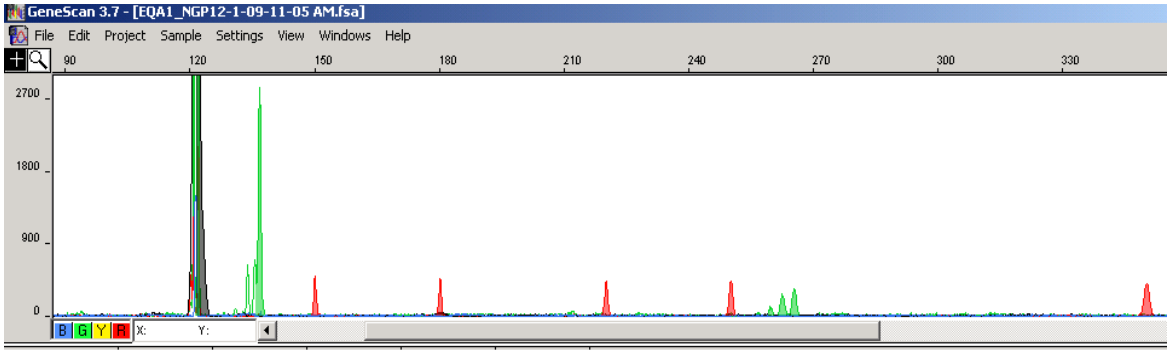

Fig.3.4. Genotyping results for heterozygous female with 30/75....77 CGG repeat alleles. Red peaks – fragileX size standard (Applied Biosystems, USA); black peak – internal control fragment of *ApoE* gene; green peak – *FMR1* gene CGG repeat fragments.

3.2.4. Southern blotting

The FXS diagnosis was confirmed by sizing of repeat array using methylation specific restriction enzyme digestion and genomic Southern blot hybridization according to the described protocol (Dracopoli and Haines, 1994).

For Southern blot analysis, 4 - 6 µg of genomic DNA were used.

To perform sizing of the repeat array, two different digestion reactions were used – EagI/EcoRI (methylation specific) and PstI. Products were separated on 0.8% agarose gel (at ~ 0.35 V/cm, overnight) and transferred to positive charged Nylon membrane by capillary transfer. To detect DNA fragment, labelled [^{32}P] StB12.3 hybridisation probe was used (Fig. 3.5.). Unlabeled StB12.3 probe obtained from Prof. J. L. Mandel, Strasbourg. Labelling of the probe was done according to the described protocol

(Sambrook and Russell, 2001). Analysis performed in the DNA Laboratory, Department of Medical Genetics, Ullevål University Hospital, Oslo, Norway.

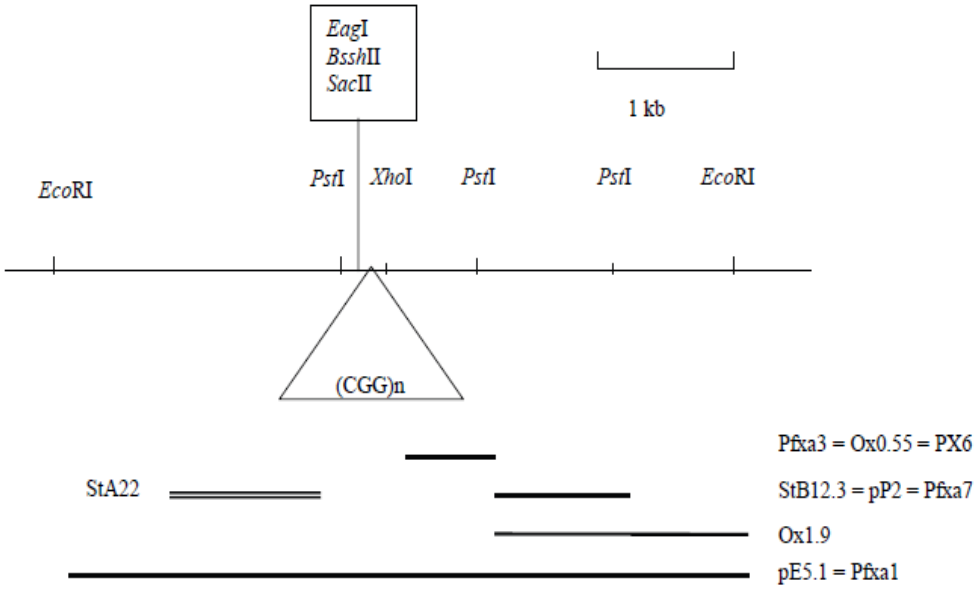

Fig. 3.5. Probes used in the diagnosis of FXS (adapted from EMQN, 2006)

Interpretation of results for EagI/EcoRI digestion was made according to the protocol.

Fig. 3.6. shows schematic representation of the hybridisation patterns detected by probe StB12.3 in EcoRI+EagI double digests. These patterns correspond to those observed in a Southern blot assay for any of following: normal subjects, carriers of a "permutation", carriers of a "full mutation", and "mosaics".

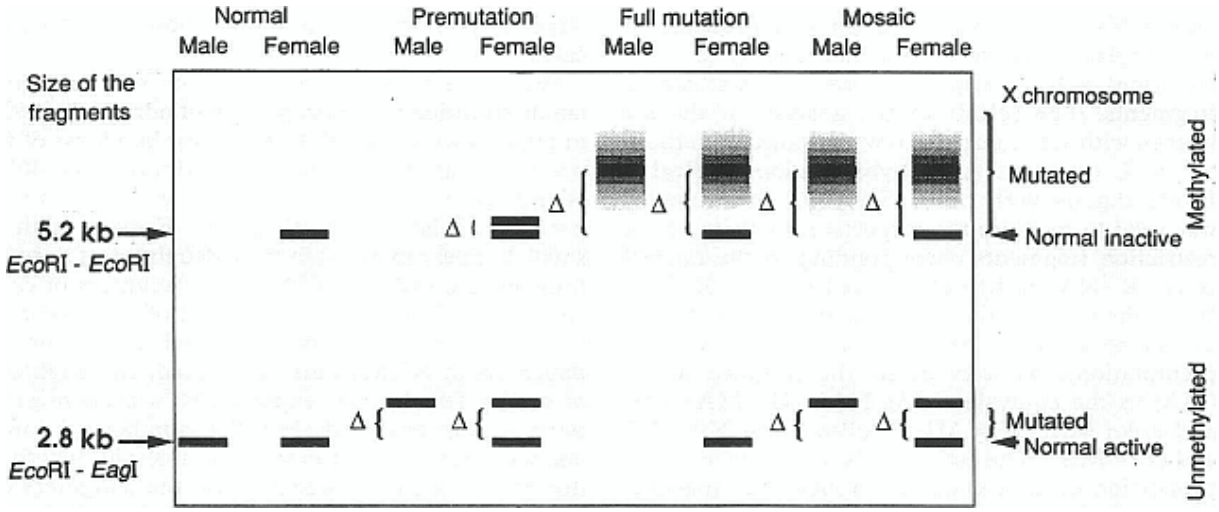

Fig. 3.6. Schematic representation of the hybridisation patterns detected by probe StB12.3 in EcoRI+EagI double digests (adapted from Rousseau et al., 1991).

The normal 2.8 kb and 5.2 kb fragments detected by probe StB12.3 are indicated on the left (plain horizontal bands). Mutated fragments are detected as additional bands with a size increase indicated by "Δ". A heterogeneous pattern of mutated fragments may appear as a smear (gradual range of greys). Interpretation in terms of mutation and methylation is indicated on the right.

Southern blot analysis for patients and their family members using probe pAO365 was performed in the DNA Diagnostic Laboratory, University Medical Center Nijmegen, The Netherlands.

3.2.5. AGG interspersion pattern analysis

Twenty-six alleles of grey zone (35-50 CGG repeat) were analysed for CGG repeat patterns. The AGG interspersion pattern was determined by sequencing of the CGG-repeat array. In brief, the CGG repeat and surrounding DNA sequences were amplified from genomic DNA by *Pfu* polymerase (Fermentas, Lithuania) with the PCR protocol described previously (Chong et al. 1994). The PCR products were run on 2.5% agarose gel at 5.5 V/cm for 60 min to check for amplification of a single allele. The

PCR products were concentrated and purified for sequencing by the Montage PCR centrifugal filter device (Millipore, USA).

The sequencing reaction was performed using concentrated and purified PCR products by the BigDye® Terminator v3.1 kit (Applied Biosystems, USA) according to the manufacturer's protocol. Due to high G/C content of the template, 1 µl of dimethyl sulfoxide (DMSO) and 0.5 µl of glycerol were added to the sequencing reaction. The forward primer used in the sequencing reactions was 5`- GAC GGA GGC GCC GCT GCC AGG -3` (Crawford et al., 2000) (Appendix 1). The cycling conditions consisted of an initial denaturation of 10 min at 98°C, followed by 25 cycles of 30 sec at 96°C, 15 sec at 50°C and 4 min at 60°C. The reaction was carried out on the PCR Mastercycler (Eppendorf, Germany). Subsequent purification of the sequencing products were performed as recommended by the manufacturer.

All sequencing reactions were run on an ABI Prism® 310 genetic analyzer using 61cm x 50µm (50 cm well-to-read) capillary with POP-6™ polymer and analyzed by ABI DNA™ sequencing software.

The sequence pattern of the CGG repeat array was red from the first exon of *FMR1* gene. DNA sequence was established by visual interpretation of the electropherograms. Nucleotides were assigned in the sequence based on the highest fluorescence signal at each position, provided that the nucleotide peak exceeded the background level. To describe the sequence of the *FMR1*, the number of CGG repeats is denoted as a number, while AGG interruption is denoted as "+". Electropherogram of the CGG sequence containing two AGG interspersions is shown in Fig.3.7.

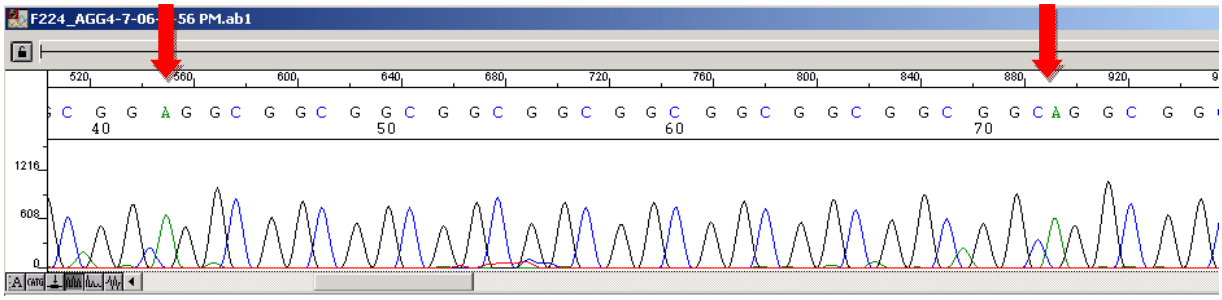

Fig.3.7. Electroferogram of the CGG sequence containing two AGG interspersions (marked with red arrows).

3.2.6. Single nucleotide polymorphism analysis

The ATL1 polymorphism (alleles A/G located 5613bp upstream CGG repeat) was analysed by the allele-specific oligonucleotide PCR protocol described by Dombrowski et al., (2002). The reaction for each polymorphism was performed in a final reaction volume of 25 µl containing 0.5 µM of forward primer, 0.5 µM allele-specific reverse primer, 0.2 mM of each dNTP (Fermentas, Lithuania), 70 ng of genomic template, 1 X HotStart *Taq* DNA polymerase reaction buffer (200 mM Tris-HCl (pH 8.3 at 25°C), 200 mM KCl, 50 mM $(NH_4)_2SO_4$), 1.5 mM $MgCl_2$ and 1.25 U of HotStart *Taq* DNA polymerase (Fermentas, Lithuania).

The primers sequence was as follow:

Forward primer ATL1F: 5`-TCA TCA AGT CCT TGG TAA TAG AA-3`
Allele-specific reverse primer ATL1A:
5`-GAC ACA GAA TCA TAA ATG T-3`
Allele-specific reverse primer ATL1G:
5`- GAC ACA GAA TCA TAA ATG C-3`

The sequence and the location of PCR primers are shown in Appendix 2.

Cycling conditions were an initial 5 min at 96°C followed by 30 cycles of: 30 sec at 96°C, 30 sec at 55°C, 1 min at 72°C. The final extension was performed for 10 min at 72°C. PCR products were visualised on 1% agarose gel using ethidium bromide staining. Presence of PCR product in length of 385 bp was interpreted as a positive result for the specific allele. A lack of PCR product was interpreted as a negative result for a specific allele. The ATL1 SNP was identified by performing two PCR reactions for each chromosome.

3.2.7. Haplotype Analysis

For haplotype analysis among normal and mutant chromosomes, the microsatellite markers DXS548, FRAXAC1 and FRAXAC2 were used. The DXS548 microsatellite is located 189895bp downstream CGG repeat. The FRAXAC1 microsatellite is located 7221bp downstream CGG repeat and the FRAXC2 microsatellite is located 12418bp upstream of the CGG repeat.

Multiplex PCR for DXS548 and FRAXAC2 was performed in a total reaction volume 15 µl containing 1x PCR reaction buffer (75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20) (Fermentas, Lithuania), 1.5 mM $MgCl_2$ (Fermentas, Lithuania), 0.2 mM of each dNTP (Fermentas, Lithuania), 7% glycerol, 2 pmol of each DXS548 primer, 1 pmol of each FRAXAC2 primer and 0.15 U True start™ Hot Start *Taq* DNA polymerase (Fermentas, Lithuania).

The primers sequences of DXS548 locus are as follow (according to Chiurazzi et al. 1999 (Appendix 3)):

DXS548A: 5`-HEX-AGA GCT TCA CTA TGC AAT GGA ATC-3`
DXS548B: 5`- GTA CAT TAG AGT CAC CTG TGG TGC-3`

The primers sequences of FRAXAC2 locus are as follow (according to Chiurazzi et al. 1999 (Appendix 4)):

FRAXAC2A:
5`-6-FAM-GAC TGC TCC GGA AGT TGA ATC CTC A-3`
FRAXAC2B:
5`-CTA GGT GAC AGA GTG AGA TCC TGT C-3`

PCR was carried out for an initial 2 min at 95°C followed by 10 cycles of: 30 sec at 95°C, 1 min at 60°C, 1 min at 72°C. This was followed by a second round of amplification for 25 cycles of: 30 sec at 95°C, 1 min at 55°C, 1 min at 72°C and a final extension for 7 min at 72°C.

The FRAXAC1 microsatellite marker was amplified separately in a total reaction volume of 15 µl containing 1x PCR reaction buffer (75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 0.01% Tween 20) (Fermentas, Lithuania), 1.5 mM $MgCl_2$ (Fermentas, Lithuania), 0.2 mM of each dNTP (Fermentas, Lithuania), 7% glycerol, 4.5 pmol of each FRAXAC1 primer and 0.15 U True start™ Hot Start *Taq* DNA polymerase (Fermentas, Lithuania).

The primers sequences of FRAXAC1 locus are as follow (according to Chiurazzi et al. 1999 (Appendix 5)):

FRAXAC1A:
5`-NED-GAT CTA ATC AAC ATC TAT AGA CTT TAT T-3`
FRAXAC1B:
5`-AGA TTG CCC ACT GCA CTC CAA GCC T-3`

PCR was carried out for an initial 2 min at 95°C followed by 10 cycles of: 30 sec at 95°C, 1 min at 60°C, 1 min at 72°C. This was followed by a second round of amplification for 25 cycles of: 30 sec at 95°C, 1 min at 55°C, 1 min at 72°C and a final extension for 7 min at 72°C.

A volume of 0.5 µl of each reaction product was mixed with 0.5 µl of GeneScan™ ROX 500™ size standard (Applied Biosystems, USA) and 24 µl of deionized formamide. Product fragments length was detected on an ABI Prism® 310 genetic analyzer.

Genotyping results were analysed by GeneScan® Analysis software. The corresponding fragment length was calculated according to the calibration curve of the GeneScan™ ROX 500™ size standard. An electropherogram of the three microsatellite markers fragment length analysis is shown in Appendix 6. Nomenclature for alleles was adjusted to the nomenclature recommended by Macpherson et al. (1994) and Eichler et al. (1996) (Fig. 3.8.).

Validation of genotyping results was made by direct sequencing of random alleles for each microsatellite marker. For alleles of each marker the same PCR conditions, as described above for genotyping, was used. The exception was reverse primers A, which was not labelled with fluorescent dye. The PCR products obtained in reaction were concentrated and purified for sequencing reaction by the Millipore Montage PCR filter device.

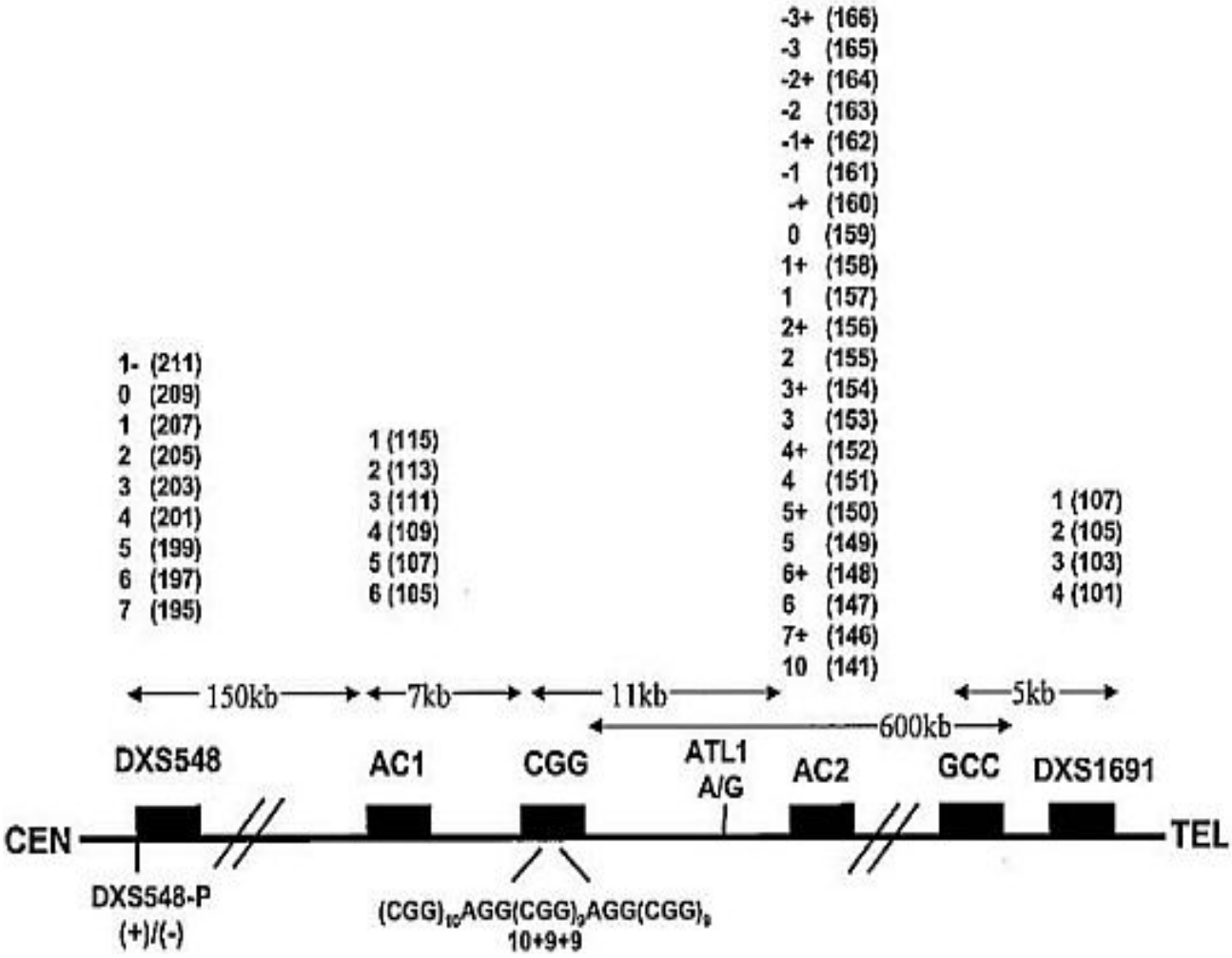

Fig. 3.8 .The location and nomenclature of CGG repeat pattern, STR- and SNP-based haplotypes according to Eichler et al. (1996), adapted from Crawford et al. (2000).

The sequencing reaction was performed using concentrated PCR products and the BigDye® Terminator v3.1 kit (Applied Biosystems, USA). The cycling conditions consisted of an initial denaturation of 10 min at 98°C, followed by 25 cycles of 30 sec at 96°C, 15 sec at 50°C and 4 min at 60°C. The manufacturer suggested PCR conditions were changed due to high contain of C/G nucleotides in the sequence. The reaction was carried out on PCR Mastercycler (Eppendorf, Germany). Subsequent purification of the sequencing products was performed as recommended by the manufacturer.

All sequencing reactions were run on an ABI Prism® 310 genetic analyzer using 61cm x 50µm (50 cm well-to-read) capillary with POP-6™ polymer and analyzed by ABI DNA™ sequencing software.

The DNA sequence was established by visual interpretation of the electropherograms. Nucleotides were assigned in the sequence based on the highest fluorescence signal at each position, provided that the nucleotide peak exceeded the background level. Sequencing data of DXS548 allele 6 and FRAXAC1 allele 2 are shown in Appendix 7. Complex polymorphism sequence of the FRAXAC2 allele 4 is shown in Appendix 8.

Deviation of fragment length described by Macpherson et al. (1994) was detected by sequencing results. Nomenclature was set based to the repeat number in microsatellite markers sequence. Deviation in fragment length was found for FRAXAC1 (Table 3.1.) and DXS548 (Table 3.2.) loci. FRAXAC2 locus alleles corresponded to the previously described fragment length in bp. For the DXS548 locus fragments in length of 193 bp and 192 bp were denoted as allele 6, because both fragments contain 20 CA repeats and the 1bp difference rose from one extra G in forward amplified sequence. Nomenclature of polymorphic FRAXAC2 locus is show in table 3.3.

The haplotype of each chromosome was formed by combining microsatellite markers and single nucleotide polymorphism according to their position at the FRAXA locus. STR and SNP marker haplotypes were combined as follows: DXS548-FRAXAC1-ATL1-FRAXAC2.

Table 3.1.

Nomenclature of FRAXAC1 alleles

Allele	Allele Chiurazzi et al.	Fragment length (bp) Macpherson et al.	Fragment length (bp) Eichler et al.	Fragment length (bp) This study	GT repeats
1	T42	114	115	113	21
2	T40	112	113	111	20
3	T38	110	111	109	19
4	T36	108	109	107	18
5	T34	106	107	105	17
6	T32	104	105	103	16

Table 3.2.

Nomenclature of DXS548 alleles

Allele	Allele Chiurazzi et al.	Fragment length (bp) Macpherson et al.	Fragment length (bp) Eichler et al.	Fragment length (bp) This study	CA repeats
1	T52	206	207	203	26
2	T50	204	205	201	25
3	T48	202	203	199	24
4	T46	200	201	197	23
5	T44	198	199	195	22
6	T42	196	197	193	21
7	T40	194	195	191	20

Table 3.3.

Nomenclature of FRAXAC2 alleles

Allele	Allele Chiurazzi et al.	Fragment length (bp) Macpherson et al.	Fragment length (bp) Eichler et al.	Fragment length (bp) This study	GT-TA-T repeats
3+	T63	154	154	154	18-7-13
3	T62	153	153	153	17-7-14
4+	T61	152	152	152	17-7-13
4	T60	151	151	151	16-7-14
5+	T59	150	150	150	16-7-13
5	T58	149	149	149	15-6-16 15-7-14
6+	T57	148	148	148	15-7-13
6	T56	147	147	147	14-7-14
7+	T55	146	146	146	14-7-13
7	T54	145	145	145	13-7-14

3.2.8. Statistical Data Analysis

Analysis of molecular variance (AMOVA) is a method specifically developed to analyse haplotype frequencies in haploid organisms and by extension is applicable to hemizygous haplotypes from genes on the X and Y chromosomes in human males. This method can be used to estimate population differentiation directly from molecular data and for testing hypotheses about such differentiation. For hemizygous loci each haplotype is treated as a single allelic locus.

Fst measures the effect of population subdivision, which is the reduction in heterozygosity in a subpopulation due to genetic drift. *Fst* is the most inclusive measure of population substructure and is most useful for examining the overall genetic divergence among subpopulations. It is also called coancestry coefficient (q) or 'Fixation index' and is defined as the correlation of gametes within subpopulations relative to gametes drawn at random from the entire population (**s**ubpopulation within the total population). It is calculated by using the subpopulation (average) heterozygosity and total population expected heterozygosity. *Fst* is always positive; it ranges between 0 = panmixis (no subdivision, random mating occurring, no genetic divergence within the population) and 1 = complete isolation (extreme subdivision). *Fst* values up to 0.05 indicate negligible genetic differentiation whereas >0.25 means very great genetic differentiation within the population analyzed. *Fst* is usually calculated for different genes, and then averaged across all loci, and all populations. For human populations, the average value of *Fst* for a large number of DNA polymorphisms is 0.139 (and 0.119 for non-DNA polymorphisms).

The distribution of haplotype diversity was measured using the analysis of molecular variance (AMOVA, Excoffier et al., 1992) as variation within and between population groups.

The significance of the results was tested by 10000 permutations. For AMOVA analysis, level of heterozygosity for all polymorphisms and calculation of pairwise genetic distances (*F*st) the Arlequin 3.5 package (Excoffier and Lischer, 2010) was used. To detect critical value of *F*st, the on-line statistical calculator for critical values of F-statistics by BioKin, Ltd. was used. The “degrees of freedom numerator” was set to 1. The “denomenator” was set to 132.

Analysis of 27 haplotypes, derivates from a total 133 chromosomes, was carried out by population splitting in two subgroups based on normal/mutated FRAXA alleles.

The case-control study data was analysed by Fisher's exact test of 2×2 contingency tables and chi-square using GraphPad QuickCalcs on-line calculator. For statistical significance of results, the *p*-value was set less than 0.05. The Bonferroni correction was applied for multiple testing (Bland and Altman, 1995). According to the Bonferroni correction in four markers, the haplotype analysis *p*-value was set less than 0.0125.

For calculation of results 95% confidence interval QuickCalcs on-line calculator was used. For the proportion calculation CI 95% was applied according to the modified Wald method by Agresti and Coull (1998).

In the retrospect study, estimation of FSX prevalence was based on data from the Central Statistical Bureau of Latvia and data obtained from this study. Prevalence per 100 000 males was calculated:

$$\text{Prevalence} = \left[\frac{\text{number of estimated male patients during the period}}{\text{average number of male population during the period}} \right] \text{X } 100\,000$$

Prevalence expressed as one affected male to the number of male persons in population was calculated:

$$\text{Prevalence} = \frac{\text{average number of male population during the period}}{\text{number of estimated male patients during the period}}$$

4. RESULTS

4.1. Prevalence of the Fragile X Syndrome

The prevalence of the fragile X syndrome was estimated in retrospective survey for the male individuals with mental retardation and developmental disabilities. In this study the estimation of the population prevalence was restricted to the data from male population, because females with a full mutation in the *FMR1* gene show an intellectual development from severely retarded to normal and cannot be picked up just by clinical data.

In the group of unrelated, mentally retarded males (n = 374), 10 (95% CI 4.80 – 18.39) fragile X syndrome patients were newly diagnosed, for a relative prevalence of 0.0267 (2.67%) in ten years time period. According to the data from the Central Statistical Bureau of Latvia, during ten years (from 1998 to 2007), 10503 patients with psychological development disorders or behavioural and emotional disorders were diagnosed (with onset usually occurring in childhood and adolescence, new cases excluded those caused by alcoholism and dependency upon narcotic and psychoactive substances) (Appendix 9). Gender structure for the diagnosed cases was not stated. With theoretical gender structure of the population (1:1) and the developmental disabilities diagnosis of 1.25 male to 1 female (Raymond, 2006), we assumed 6295 male patients (95% CI 5690-7430) with diagnosis of developmental disabilities in Latvia. According to the calculated relative prevalence of disease from our laboratory data, 168 (95% CI 143 – 195) FXS male patients were estimated to be in this patient group.

For Latvia, with an average 1 079 941 male residents (based on data of the Central Statistical Bureau of Latvia during 1998 - 2007; Appendix 10), and assumed 168 male patients with the fragile X syndrome, gives a result in prevalence of 1/6428 males (95% CI 5538-7552) or 15.55/100 000 males (95% CI 13.24 – 18.05).

4.2. Variation of CGG Trinucleotide Repeats

In total, 374 patients were analysed with PCR screening and for all those patients an exact CGG repeat number within *FMR1* gene were detected. Distribution of alleles was as follow: 90.37% of alleles fell in group of normal CGG repeat number, 6.95% were grey-zone alleles and 2.67% of alleles revealed full CGG repeat expansion

(Appendix 11). The highest incidence among all analysed chromosomes were observed for allele 30 (29.95%), allele 31 (13.10%) and allele 29 (12.83%).

From 374 patients, analysed with PCR screening, 364 were detected having CGG repeat alleles within a non-pathogenic range (5 – 50 repeats). Twenty-six different alleles were observed. The smallest repeat size identified within the normal range was 16 CGG repeats. There were absences of alleles with 17; 18; 19; 44; 46; 48 and 49 CGG repeats. The most common allele in the normal range was allele 30 (30.77%). Comparably prevalent were alleles 29 (13.19%), 31 (13.46%), 23 (8.52%) and 24 (6.32%). Distribution of non-pathogenic range CGG repeat alleles is shown in Fig. 4.1. The raw data of normal alleles distribution are shown in Appendix 12.

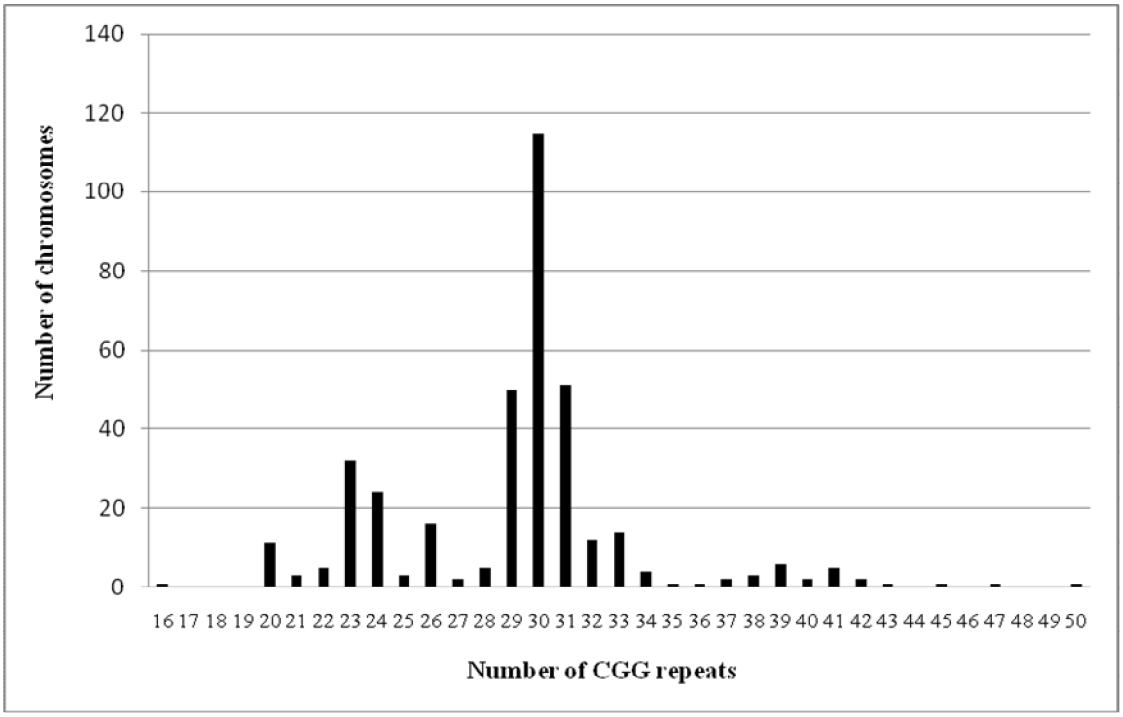

Fig. 4.1. Distribution of CGG alleles within non-pathogenic range.

4.3. ATL1 SNP

The control group CGG repeat alleles (n = 122) were analysed with respect to ATL1 SNP alleles. Sixty-two chromosomes in total had ATL1 polymorphism A which results in a frequency of 56%. Sixty chromosomes were detected with ATL1 polymorphism G (44%). Distribution of ATL1 polymorphism A and G among control group CGG repeat alleles is shown in Figure 4.2. Polymorphism A was observed in 17 different individual CGG repeat alleles and the G polymorphism in 19 alleles respectively. Statistically significant association for individual CGG alleles and ATL1 SNP was found for alleles 29 and G (p = 0.001); 30 CGG repeats and A (p < 0.0001) and allele 31 with A (p = 0.0013). For allele 23 significant associations with the ATL1 polymorphism G was not confirmed.

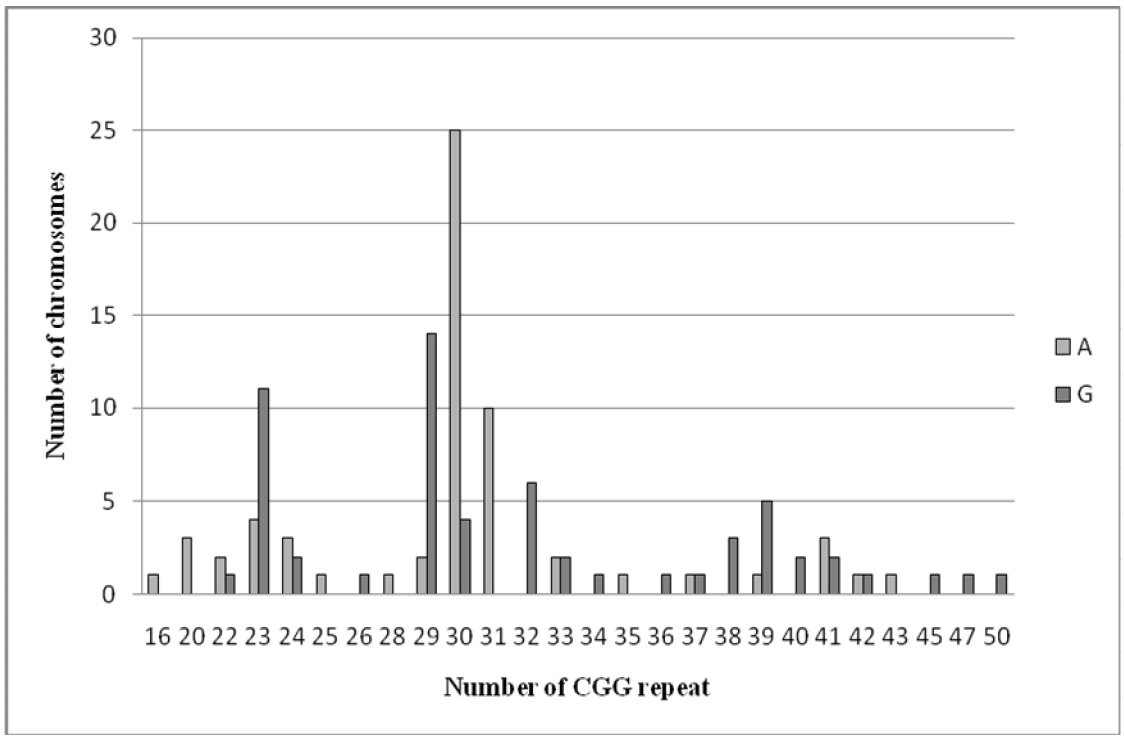

Fig. 4.2. Distribution of ATL1 polymorphism A and G among non-pathogenic CGG repeat alleles.

To discover ATL1 SNP distribution among stable CGG repeats and presumably unstable CGG repeats, the control group was divided into two subgroups according to CGG allele repeat number – normal size alleles and grey-zone alleles (Table 4.1). In grey-zone subgroup polymorphism A and polymorphism G were found with a relative frequency of 0.31 (30.76%) and 0.69 (69.24%) respectively. Distribution of polymorphism G significantly differ between normal size CGG alleles and grey-zone CGG alleles ($p = 0.0271$).

Table 4.1.

Frequencies of ATL1 SNP`s among CGG Alleles

	Normal CGG alleles (16-34 repeats)			**Grey-zone CGG alleles (35-50 repeats)**		
ATL1	**n**	**RF**	**%**	**n**	**RF**	**%**
A	54	0.5625	56.25	8	0.3076	30.76
G	42	0.4375	43.75	18*	0.6924	69.24
Total	96	1.000	100	26	1.000	100

n – number of chromosomes; RF – relative frequency; * - $p < 0.05$

All FXS group chromosomes were exclusively found to be associated with ATL1 polymorphism G and this association was statistically significant ($p = 0.0008$).

4.4. Repeat Structure of Grey-Zone Alleles

26 grey-zone alleles were analysed using direct sequencing, to characterise CGG repeat interruption by AGG trinucleotides. The CGG pattern of each allele and linked haplotypes are shown in Table 4.2.

Table 4.2.

The AGG Interspersion Pattern and Linked DXS548-FRAXAC1-ATL1-FRACXAC2 Haplotype Fraquencies of Grey-Zone CGG Alleles

DXS548	FRAXAC1	ATL1	FRAXAC2	CGG	AGG	n	RF
2	2	G	4	38	9+9+18	8	0.308*
				39	9+29		
				40	9+9+20		
				40	9+9+20		
				41	9+9+21		
				45	9+9+25		
				47	9+9+27		
				50	9+9+30		
7	4	A	5+	39	10+9+9+8	6	0.231
				41	10+9+9+10		
				41	10+9+10+9		
				41	10+9+10+9		
				42	10+9+21		
				43	10+9+22		
6	5	G	7+	37	9+10+6+9	2	0.077
				38	Pure		
7	4	G	6+	39	9+9+9+9	2	0.077
				39	9+9+9+9		
7	4	G	5	39	9+9+9+9	2	0.077
				39	9+9+9+9		
6	4	G	5	41	9+9+21	2	0.077
				42	9+9+22		
6	5	A	7	37	9+10+6+9	1	0.038
6	4	A	5+	35	10+6+8+8	1	0.038
3	2	G	4	38	9+9+18	1	0.038
7	4	G	5+	36	10+9+5+9	1	0.038
Total						26	1.000

RF – relative frequency; AGG – pattern of CGG tract, the digit correspond to CGG repeats number and "+" denote the AGG interspersion position; * - $p < 0.01$

In twelve chromosomes, a CGG interspersion pattern with three AGG`s was detected. Twelve chromosomes with two AGG, one chromosome with one AGG and one pure CGG tract were also detected. In total 10 different AGG interruption patterns were detected (Table 4.3.). For all chromosomes, loss of AGG was detected on 3` end of the sequence.

Table 4.3.

The Structure of Grey-Zone Allele CGG Array`s

Pattern of AGG interruption	Number of chromosomes	Rel. frequency
Pure	1	0.038
9+n	1	0.038
9+9+n	10	0.384
9+9+9+n	4	0.154
9+10+6+n	2	0.077
10+9+n	2	0.077
10+9+9+n	2	0.077
10+9+10+n	2	0.077
10+9+n+n	1	0.038
10+n+n+n	1	0.038

AGG pattern of CGG tract, the digit to the CGG repeat number, “n” corresponds to an uninterrupted CGG repeat number and “+” denote the AGG interspersion position.

The CGG repeat structure was analysed with respect to ATL1 SNP alleles. Significant associations were found, firstly for the polymorphism A and a repeat array with a 10+n structure (p = 0.001) and secondly for the polymorphism G and a repeat array with 9+n structure (p = 0.004). Distribution of the CGG repeat structure for the ATL1 polymorphism A is presented in Figure 4.3. Distribution of the CGG repeat structure for the ATL1 polymorphism G and is shown in Figure 4.4.

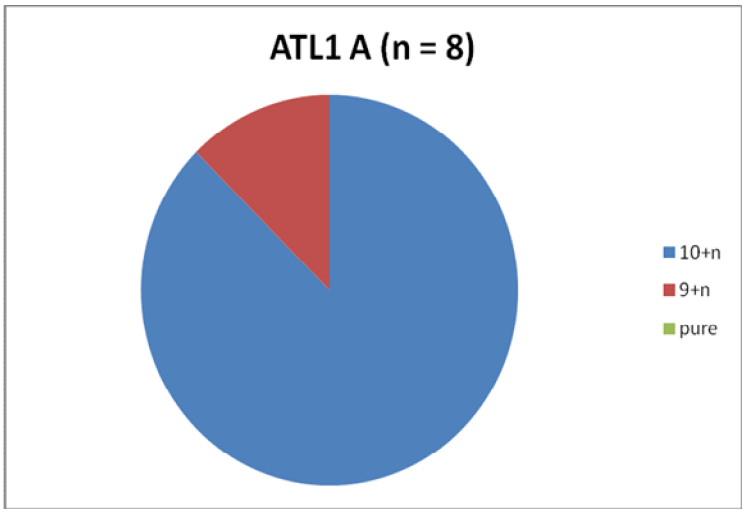

Fig. 4.3. Distribution of CGG repeat arrays associated with the ATL1 allele A.

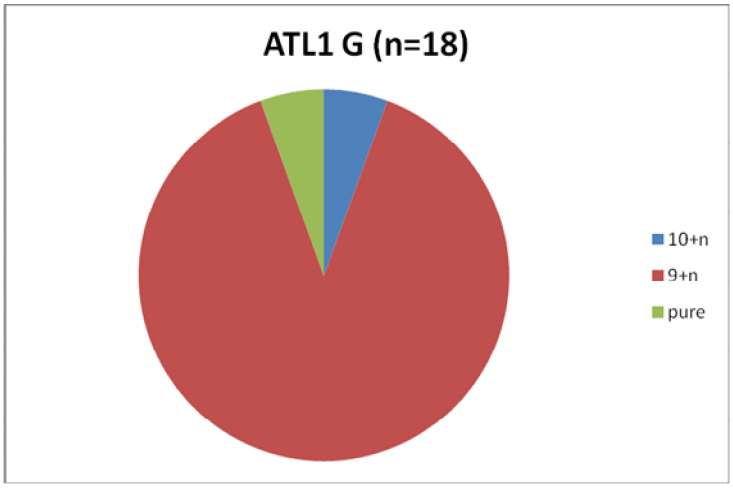

Fig. 4.4. Distribution of CGG repeat arrays associated with the ATL1 allele G.

4.5. DXS548-FRAXAC1-ATL1-FRAXAC2 Haplotypes.

Microsatellite markers and ATL1 SNP were analysed in both a control group and FXS patient group.

Seven different microsatellite alleles of the DXS548 locus were detected (Table 4.4.). Among the control group chromosomes, the prevalent allele of the DXS548 locus was allele 7 (63.9%), for FXS patients allele 2 was the most common allele (90.9%).

Table 4.4.

Frequencies of DXS548 Alleles among Control Group and FXS Group

	Control		**FXS**	
Allele	n	%	n	%
2	9	7.4	10	**90.9**
3	4	3.3	0	-
5	2	1.6	0	-
6	25	20.5	0	-
7	78	**63.9**	1	9.1
8	3	2.5	0	-
9	1	0.8	0	
Total	122		11	

n = number of chromosomes; % = frequency

Four different alleles of the FRAXAC1 locus were identified (Table 4.5.). For the FRAXAC1 locus, allele 4 was the most common allele (66.4%) in our control group. Regarding Latvian FXS chromosomes, we found allele 2 to be the most common allele (81.8%).

Table 4.5.

Frequencies of FRAXAC1 Alleles among Control Group and FXS Group

	Control		**FXS**	
Allele	n	%	n	%
2	12	9.8	9	**81.8**
3	1	0.8	0	-
4	81	**66.4**	2	18.2
5	28	23.0	0	
Total	122		11	

n = number of chromosomes; % = frequency

At the FRAXAC2 locus nine different alleles were found (Table 4.6.). Allele 5+ was the most common allele at this locus within our control group, detected at the frequency of 46.7%. The prevalent allele in our FXS patients was allele 4 (81.8%).

Table 4.6.

Frequencies of FRAXAC2 Alleles among Control Group and FXS Group

	Control		**FXS**	
Allele	n	%	n	%
3	1	0.8	0	-
3+	1	0.8	0	-
4	12	9.8	9	**81.8**
4+	3	2.5	0	-
5	11	9.0	2	18.2
5+	57	**46.7**	0	-
6+	10	8.2	0	-
7	19	15.6	0	-
7+	8	6.6	0	-
Total	122		11	

n = number of chromosomes; % = frequency

The haplotype of each chromosome was formed by combining microsatellite markers and single nucleotide polymorphism according to their position at the FRAXA locus. STR and SNP marker haplotypes were combined as follows: DXS548-FRAXAC1-ATL1-FRAXAC2. In total 27 different haplotypes were detected – 26 in the control group and three in the FXS group. Only one haplotype from the FXS group was unique (Table 4.7.).

Among the FXS patients, haplotype 2-2-G-4 was found at a relative frequency of 0.818 ($p < 0.0001$) (Figure 4.5). The most common haplotype among control group chromosomes was 7-4-A-5+ (RF = 0.327; $p = 0.0336$) (Figure 4.6.). Corrected by Bonferroni this haplotype association is not significant for stable CGG repeat alleles in our population.

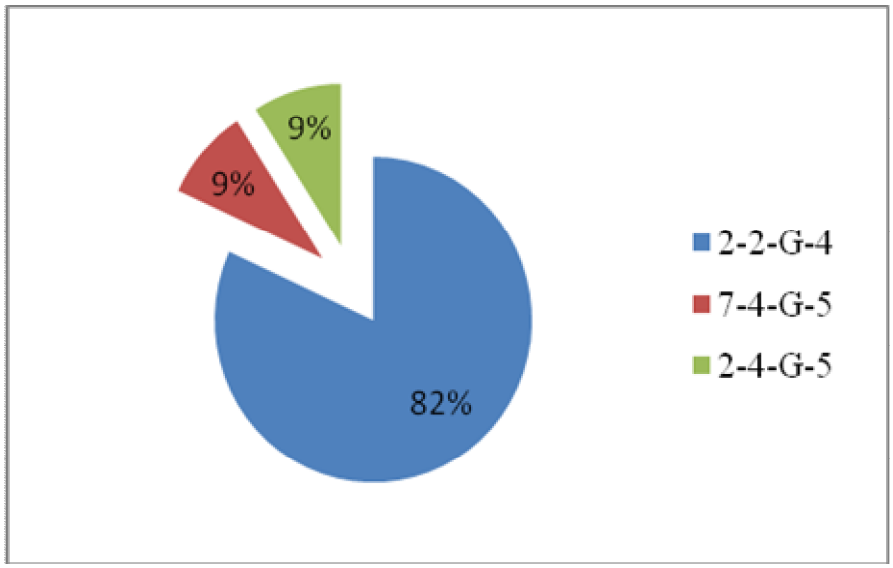

Fig. 4.5. Distribution of *FMR1* Linked Haplotypes in the FXS Group

Table 4.7.

Detected DXS548-FRAXAC1-ATL1-FRAXAC2 Haplotypes in Control group and FXS Group

Haplotype				Control group			FXS group		
DXS548	**FRAXAC1**	**ATL1**	**FRAXAC2**	**n**	**RF**	**S.D.**	**n**	**RF**	**S.D.**
7	4	A	5+	40	0.327	0.043	0	-	-
7	5	G	7	11	0.090	0.026	0	-	-
2	2	G	4	9	0.074	0.024	9	0.818*	0.122
7	4	G	5	8	0.066	0.023	1	0.091	0.091
7	4	G	6+	8	0.066	0.023	0	-	-
6	4	A	5+	7	0.057	0.021	0	-	-
6	5	G	7	6	0.049	0.020	0	-	-
6	5	G	7+	6	0.049	0.020	0	-	-
8	4	A	5+	3	0.025	0.014	0	-	-
3	2	G	4	3	0.025	0.014	0	-	-
5	4	A	5+	2	0.016	0.012	0	-	-
7	5	G	7+	2	0.016	0.012	0	-	-
7	4	G	5+	2	0.016	0.012	0	-	-
7	4	A	4+	2	0.016	0.012	0	-	-
6	4	G	5	2	0.016	0.012	0	-	-
6	5	A	5+	1	0.008	0.008	0	-	-
3	4	G	6+	1	0.008	0.008	0	-	-
7	4	A	5	1	0.008	0.008	0	-	-
7	4	G	7	1	0.008	0.008	0	-	-
7	3	A	5+	1	0.008	0.008	0	-	-
6	4	A	4+	1	0.008	0.008	0	-	-
7	4	A	3	1	0.008	0.008	0	-	-
7	4	A	3+	1	0.008	0.008	0	-	-
9	4	A	5+	1	0.008	0.008	0	-	-
6	5	G	6+	1	0.008	0.008	0	-	-
6	5	A	7	1	0.008	0.008	0	-	-
2	4	G	5	0	-	-	1	0.091	0.091
Total				**122**	**1.000**	**0.374**	**11**	**1.000**	**0.304**

n - number of chromosomes; RF - relative frequency; SD - standard deviation; * - $p < 0.0001$.

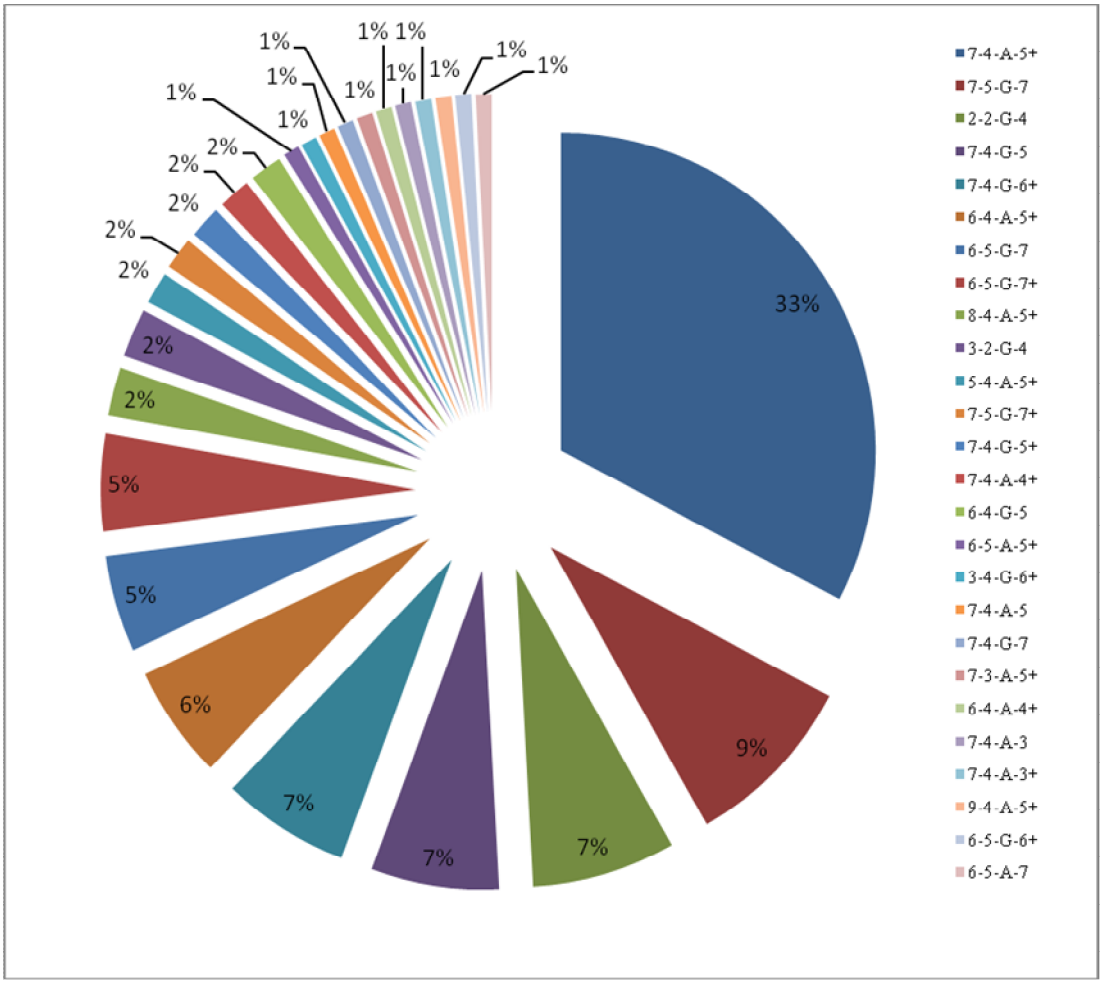

Fig. 4.6. Distribution of *FMR1* Linked Haplotypes in the Control Group

To discover haplotype distribution among stable CGG repeats and presumably unstable CGG repeats, the control group was divided into two subgroups according to CGG allele repeat number – normal size alleles and grey-zone alleles (Table 4.8.).

Table 4.8.

Frequencies of DXS548-FRAXAC1-ATL1-FRAXAC2 Haplotypes in Normal CGG alleles (16 – 34) and Grey-Zone (35 – 50) CGG Alleles

Marker alleles				16-34 CGG		35-50 CGG	
DXS548	**FRAXAC1**	**ATL1**	**FRAXAC2**	**n**	**RF**	**n**	**RF**
7	4	A	5+	34	0.354	6	0.231
7	5	G	7	11	0.115	0	0.000
2	2	G	4	1	0.010	8	0.308
7	4	G	5	6	0.063	2	0.077
7	4	G	6+	6	0.063	2	0.077
6	4	A	5+	6	0.063	1	0.038
6	5	G	7	6	0.063	0	0.000
6	5	G	7+	4	0.042	2	0.077
8	4	A	5+	3	0.031	0	0.000
3	2	G	4	2	0.021	1	0.038
5	4	A	5+	2	0.021	0	0.000
7	5	G	7+	2	0.021	0	0.000
7	4	G	5+	1	0.010	1	0.038
7	4	A	4+	2	0.021	0	0.000
6	4	G	5	0	0.000	2	0.077
6	5	A	5+	1	0.010	0	0.000
3	4	G	6+	1	0.010	0	0.000
7	4	A	5	1	0.010	0	0.000
7	4	G	7	1	0.010	0	0.000
7	3	A	5+	1	0.010	0	0.000
6	4	A	4+	1	0.010	0	0.000
7	4	A	3	1	0.010	0	0.000
7	4	A	3+	1	0.010	0	0.000
9	4	A	5+	1	0.010	0	0.000
6	5	G	6+	1	0.010	0	0.000
6	5	A	7	0	0.000	1	0.038
Total				**96**	**1.000**	**26**	**1.000**

n – number of chromosomes; RF – relative frequency;

Haplotype 7-4-A-5+ with a relative frequency of 0.354 was found to be prevalent among normal CGG repeats (16 - 34) (Figure 4.7). Compared to distribution of haplotypes in grey-zone alleles, this finding was not significant.

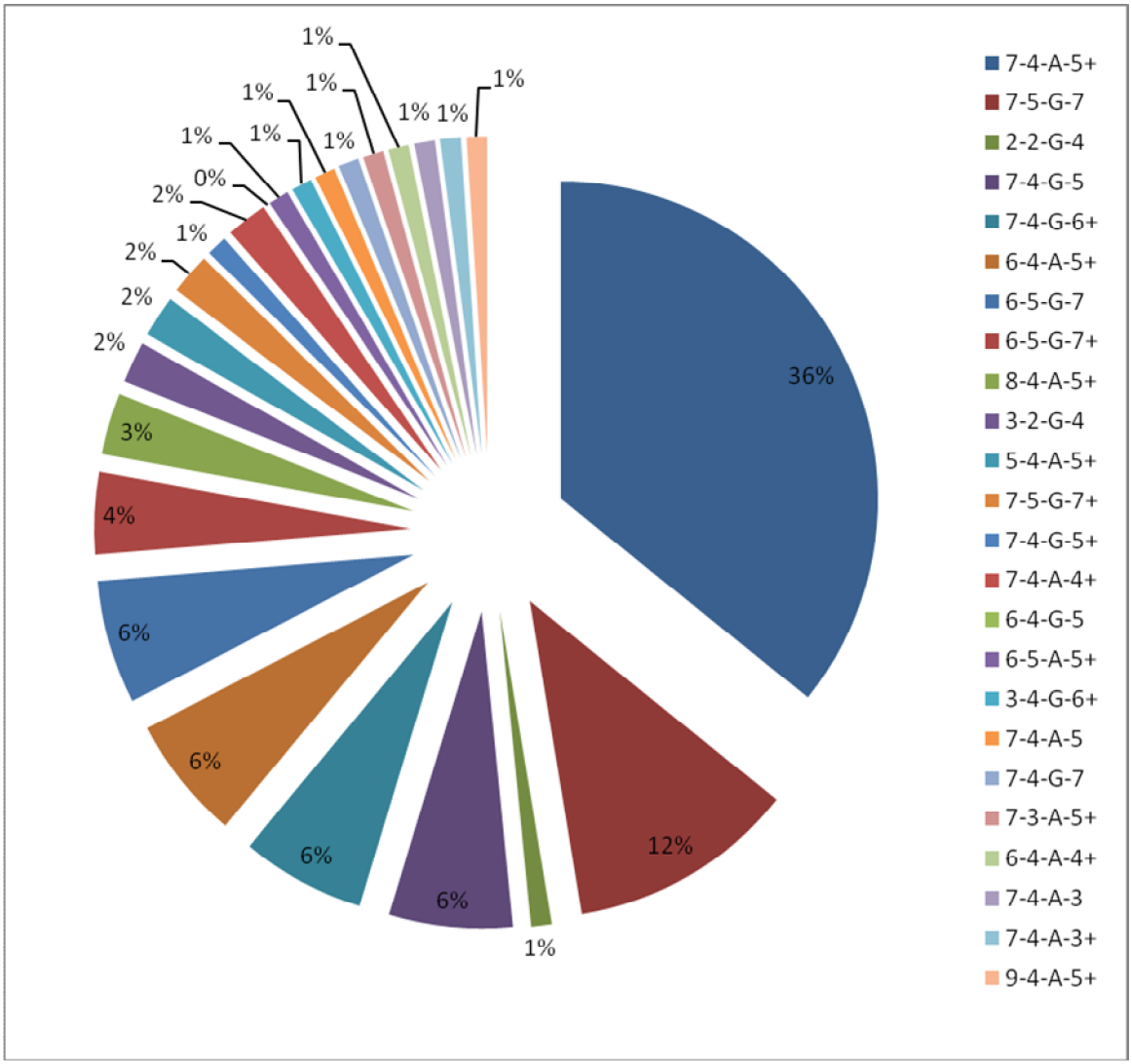

Fig. 4.7. Distribution of *FMR1* Linked Haplotypes among Stable CGG Alleles

Haplotype analysis in grey zone alleles showed the following results (Figure 4.8.). The most common haplotypes in this subgroup were 2-2-G-4 with a relative frequency of 0.308 and 7-4-A-5+ with a relative frequency of 0.231. All alleles with the 2-2-G-4

haplotype had long (> 18 CGG repeat) uninterrupted sequence on 3` end (p = 0.0022). Six alleles out of 18, associated with other haplotypes, had the same, uninterrupted CGG repeat pattern (Table 4.2.).

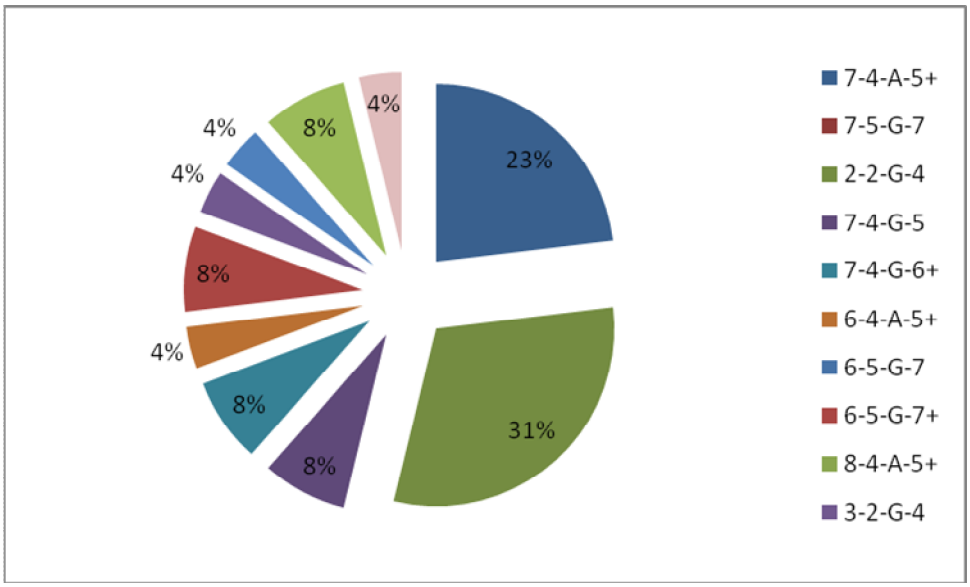

Fig. 4.8. Distribution of *FMR1* Linked Haplotypes in Grey-Zone Alleles

All grey-zone alleles associated with the haplotype 2-2-G-4 showed a CGG tract pattern where the first AGG interspersion occured after nine CGG triplets and this association was considered to be statistically significant (p = 0.0233). Significant association was also found for grey-zone alleles linked with the haplotype 7-4-A-5+ and a CGG tract pattern where first AGG interspersion occured after ten CGG repeats (p = 0.0001).

4.6. Analysis of Molecular Variance

The level of heterozygosity for all polymorphisms was examined under finite island model and compared. Expected heterozygosity was calculated within control and FXS groups based on allele frequency (Table 4.9.).

Table 4.9.

Expected Heterozygosity and Observed Heterozygosity among the Control and FXS Groups

Locus	Control H_E	FXS H_E	Mean	S.D.	H_T	H_O	X^2	p-value
ATL1 SNP	0.504	0.000	0.252	0.356	0.501	0.696	20.332	<0.0001
FRAXAC1	0.501	0.327	0.414	0.123	0.545	0.863	53.654	<0.0001
FRAXAC2	0.734	0.327	0.530	0.288	0.757	1.055	62.591	<0.0001
DXS548	0.546	0.182	0.364	0.258	0.594	1.009	94.310	<0.0001

H_E - expected heterozygosity; H_T - total heterozygosity; H_O - observed heterozygosity; S.D. - standard deviation; X^2 - chi-square.

AMOVA analysis revealed that molecular variation among groups was 27.04%. Molecular variation within groups was 72.96%. The fixation index *Fst* was calculated based on haplotype frequencies between control and FXS groups and found to be 0.27042 ($p < 0.001$). The critical value of *Fst* to confirm the null hypothesis, was calculated to be 0.0683 ($\alpha = 0.01$).

4.7. Genotype-Phenotype Correlation

Clinical data based on case-records of twelve confirmed FXS male patients were analysed. The age of patients at the moment of diagnosis varied between two and sixteen years (average = 7.33 ± 4.46). Molecular diagnostic results for these patients revealed different patterns of CGG repeat expansion. Full repeat size mutation (> 200 CGG repeats) with fully methylated gene promoter region was found in nine patients. Two patients showed premutation/full repeat size mutation mosaic with methylation mosaicism. One patient had full repeat size mutation with methylation mosaicism (up to 80% unmethylated). The frequencies of FXS patient's genotype data are shown in Table 4.10.

Table 4.10.

CGG expansion and methylation pattern of FXS patients (n = 12)

Genotype	n	Rel. Frequency
Repeat size		
Full mutation	10	0.83
Full mutation/premutation mosaic	2	0.17
Methylation status		
Full methylation	9	0.75
Mosaic	3	0.25

Major clinical symptoms of FXS were analysed for twelve patients (Table 4.11.). A detailed clinical picture for each patient is shown in Appendix 13. Eight patients out of twelve were tested for IQ. Results revealed, IQ level of patients ranged from 34 to 74 with an average IQ level of 52.75 (± 12.75). In the group of psychomotor symptoms mental retardation, learning difficulties, speech delay and attention-deficit/hyperactivity were observed all patients. From other clinical symptom groups only hypotonia was found in all examined patients.

Table 4.11.

Clinical symptoms presented by FXS patients (n = 12)

Clinical symptoms	n	Rel. Frequency
Psychomotor symptoms		
Mental retardation	12	1.00
Learning difficulties	12	1.00
Motor development delay	11	0.92
Speech delay/difficulties	12	1.00
Autistic features	7	0.58
Attention-deficit/hyperactivity	12	1.00
Dysmorphic features		
Long face	11	0.92
Large ears	7	0.58
High, wide forehead	10	0.83
Prognatia	0	0.00
Connective tissues		
Hyper elasticity of joints	10	0.83
Flatfoot	7*	0.70
Hypotonia	11**	1.00
Recurrent ARI/otitis	9**	0.82
Neurological symptoms		
Seizure	5	0.42
Balance disturbance	1	0.08
PW-like phenotype	3	0.25

* = 10 patients were examined

** = 11 patients were examined

In order to assess the genotype – phenotype correlation among full mutation alleles and CGG repeat size and/or methylation mosaicism alleles, clinical symptoms were compared between patients with full mutation in lymphocytes and patients with repeat size and/or methylation mosaic (Table 4.12.).

Table 4.12.

Genotype – phenotype comparison among patients with full mutation and patients with mosaic

Clinical symptoms	Full mutation n = 8	Rel. Frequency	Mosaic n = 4	Rel. Frequency
Psychomotor and neurological symptoms				
Mental retardation	8	1.00	4	1.00
Learning difficulties	8	1.00	4	1.00
Motor development delay	7	0.88	4	1.00
Speech delay/difficulties	8	1.00	4	1.00
Autistic features	6	0.75	1	0.25
Attention-deficit/hyperactivity	8	1.00	4	1.00
Seizure	2	0.25	2	0.50
Balance disturbance	1	0.13	0	0.00
PWS-like phenotype	3	0.38	0	0.00
Dysmorphic features and connective tissues				
Long face	7	0.88	4	1.00
Large ears	4	0.50	3	0.75
High, wide forehead	6	0.75	4	1.00
Prognatia	0	0.00	0	0.00
Hyper elasticity of joints	6	0.75	4	1.00
Flatfoot	4*	0.66	3	0.75
Hypotonia	8	1.00	4	1.00
Recurrent ARI/otitis	5	0.63	2	0.50

* = 6 patient examined

Genotype-phenotype comparison did not reveal significant differences among patients with full mutation of *FMR1* CGG repeats and patients with CGG repeats and/or methylation status mosaic.

For recognition and screening of fragile X syndrome among mentally retarded patients, a clinical questionnaire check-list was adapted from literature and translated into Latvian, for use by family doctors, paediatricians, child neurologists and child psychiatrists. The checklist is shown in Appendix 14.

5. DISCUSSION

5.1. Prevalence of the Fragile X Syndrome

Ten years of experience with molecular diagnostic of the fragile X syndrome in Latvia and a comparable low number of diagnosed patients with this disease led to the question, how prevalent is fragile X syndrome in Latvian population? Lack of studies in our geographical region was one more factor that inspired us for this study.

As with other published studies, the target population of our study were patients with mental retardation and/or developmental disabilities which are the main symptoms of FXS. It is of prime importance to screen patients demonstrating symptoms of fragile X syndrome and whilst at the same time increase the detection rate for this disease.

This study of mentally retarded males results in 2.67% of prevalence in the target population. These results are in line with findings from other research group studies of populations with a similar clinical symptom range. Our result proves the importance of clinical symptoms recognition related to FXS syndrome in clinical practices and necessity to suggest a check-list of symptoms for clinical specialists to allow easier detection of patients with suspected FXS.

In this study, to assess prevalence of FXS in entire male population, we attributed our detected prevalence of FXS in target population to the total number of patients with psychological development disorders or behavioural and emotional disorders (with onset usually occurring in childhood and adolescence, new cases excluded those caused by alcoholism and dependency upon narcotic and psychoactive substances) diagnosed in Latvia in same time period. These data were obtained from published data source of the Central Statistical Bureau of Latvia. The gender structure and a detailed overview of included diagnosis were not available, but the overall patient description was the most appropriate for the comparison with a target population of our study. This can be a source of inaccuracy of calculated prevalence in entire male population. As it was mentioned before, the calculated prevalence in different studies correlate with the spectrum of clinical symptoms chosen for target population.

Most reports are concerned with the prevalence of FXS in a target population, but there are also publications that provide prevalence of full mutation in the general population. The prevalence of FXS full mutation in the European descent population is approximately 1/4000 males to 1/6000 males (Crawford, 2001). Orphanet data (2010)

on the prevalence of rare diseases in Europe, suggest a prevalence of FXS 14.25/100 000.

Our results are consistent with these findings. Crawford and colleagues in their fragile X syndrome epidemiology review indicated the necessity of a large population screening for the complete ascertainment of disease prevalence. Just very few publications are based on population screening. We completely agree with such a necessity of large population screening to discover the true prevalence of fragile X syndrome and, even more important, to find out the prevalence of the premutation carrier women in a population of different ethnical backgrounds.

5.2. Variation of CGG Trinucleotide Repeats

In our study we analysed the distribution of normal CGG repeat alleles among unrelated mentally retarded male patients. The prevalent allele detected in this study agrees with reports from populations across Europe and Western European descents from America, and it is allele 30. If we compare results from our study with results from a study by Estonian colleagues, there are no significant differences. Allele 30 was found in 29.30% of all chromosomes in Estonian patients (Puusepp et al., 2008), and 29.95% of all chromosomes in Latvian patients.

The distribution of normal CGG repeat alleles are described in different populations. In Western European descent populations allele 30 is the prevalent allele (Arrieta et al., 2003; Chiurrazi et al., 1999; Kunst et al., 1996). In Asian descent populations, allele 29 has been reported as common (Faradz et al., 2001). Diverse results are reported by two groups of researchers from Japan. Arinami and colleagues (1993) reported prevalence of alleles 28; 29 and 35 in contradiction to Otsuka et al. (2010) who reported a prevalence of alleles 27; 26 and 28. It is possible that this discrepancy of data in one single repeat unit rose from different methods used for CGG number detection and genotyping errors.

Distribution of CGG repeats in Latvian X chromosomes did not reveal any significant differences among our data and data from European populations. The total heterogeneity of CGG allele distribution in our population was assumed to be in line with data from European populations.

5.3. ATL1 SNP

Previous studies have suggested linkage of CGG tract instability with three factors: the G allele of ATL1 SNP; specific microsatellite marker haplotypes; and a CGG tract AGG interspersion pattern exhibiting a long uninterrupted CGG repeat at the 3' end (Arrieta et al., 2003; Crawford et al., 2000; Curlis et al., 2005; Dombrowski et al., 2002; Eichler et al., 1996; Gunter et al., 1998; Zhou et al., 2006).

Gunter and colleagues published an interesting study based on the hypothesis of ATL1 SNP allele polymorphism origin. They analysed normal repeat length chromosomes in several isolated African populations, in the African-American origin open population and in male chimpanzee's chromosomes. All chromosomes had prevalent allele ATL1 G. This finding led to the hypothesis that allele A originated as a mutation in the 30 CGG repeat array linked with the haplotype 7-3-4+. Through either selection or genetic drift, the polymorphism A become the prevalent allele associated with normal CGG repeats in Western European descent populations (Gunter et al., 1998). If we compare this finding with results from our study, the haplotype 7-4-5+ is the most prevalent among the 30 CGG alleles (51.72%) and all alleles with this haplotype were associated with ATL1 polymorphism A.

Our results revealed a statistically significant prevalence of the ATL1 polymorphism G among grey zone alleles and full mutation alleles, which is an indicator of instability.

5.4. Repeat Structure of Grey-Zone Alleles

One of the principal tasks in this study was to detect the structure of CGG repeats among X chromosomes with normal CGG repeat alleles. Since normal range CGG repeats (5 – 34) is considered to be stable in transmission, we decided to detect the CGG array just in grey-zone alleles (35 – 50). Grey-zone alleles are normally expressed and do not leads to FXS phenotype but, these alleles may show instability in transmission. For this reason these alleles are good a target for the study of instability factors.

The absence of AGG interruptions in long tracts of CGG repeats have been described as the main factors related to this instability (Rife et al., 2004). It is hypothesised that CGG expansion occurs only at the 3` end of the triplet array. There are various patterns of AGG interruptions of the array that are believed to be

responsible for "stabilizing" the alleles (Dombrowski et al., 2002). Normal alleles carry an interspersed AGG on every ninth or tenth CGG. Interspersed AGG has been proposed to be the anchor which prevents DNA slippage during DNA replication. The loss of interspersed AGG results in a long pure CGG repeat sequence at the 3′ end which contributes to DNA instability. In general, the longer the 3′ pure CGG repeats, the more susceptible is the CGG to further expansion in later generations (Dombrowski et al., 2002; Poon et al., 2006). The possible role of AGG interspersions was studied by Weisman-Shomer, Cochen and Fry (2000) with *in vitro* experiments of biomolecular tetrahelical structures formation of CGG oligomers. Results suggested that diminished formation and stability of tetraplex structures of AGG interspersed CGG tracts might restrict their expansion in normal alleles. Mulvihill and colleagues (2005) proposed association of trinucleotide repeat nucleosome assembly with genetical instability.

In our study ten different CGG arrays for alleles with 35 to 50 CGG repeats were found. One allele had a pure CGG tract. The most common pattern of grey-zone alleles was 9+9+n. The association of CGG tract pattern results and specific haplotypes is discussed in section 5.5.

5.5. DXS548-FRAXAC1-ATL1-FRAXAC2 Haplotypes

In the present study, we characterised the microsatellite markers DXS548, FRAXAC1 and FRAXAC2, the ATL1 SNP and the corresponding haplotypes in a mentally retarded male population from Latvia with normal and expanded *FMR1* gene CGG repeats. To achieve this task a case-control study was made. The data obtained was analysed using analysis of molecular variance (AMOVA). The AMOVA data based on calculated F_{st} suggested that the differences between detected haplotypes within the control and FXS groups were significant and both our population subgroups show different genetical backgrounds.

Several studies have identified specific haplotypes associated with FXS patients chromosomes and normal CGG repeat alleles across European populations (Arrieta et al., 2003; Dokic et al., 2008; Malmgren et al., 1994; Peixoto et al., 1998; Pekarik et al., 1999; Rajkiewicz, 2008). In Caucasians, haplotypes 6-4-4; 6-4-5 and 2-1-3 were reported as haplotypes positively associated with full CGG repeat expansion (Eichler et al., 1996). However, only a limited number of these studies focused on populations from Eastern and North-eastern Europe.

Different microsatellite markers were used for these haplotype analyses in European Caucasian populations. Thus, comparison of our results with these analyses would prove difficult. Therefore, we compared our results presented here with single locus data in the literature.

Allele 7 at the DXS548 locus was the most common allele (RF = 0.639) in our control group. Similar findings, albeit with slightly different frequencies, have been reported from Sweden (Malmgren et al., 1994), Czech Republic (Pekarik et al., 1999), Finland (Haataja et al., 1994), France (Oudet et al., 1993), Croatia (Dokic et al., 2008), Russia (Drozd et al., 2003), Norway (Larsen et al., 2001) and Poland (Rajkiewicz, 2008). In contrast, our finding with respect to the most common allele in FXS patients (allele 2, RF = 0.909) was in agreement with only one of the analyses (Polish population, allele 2; Swedish population, alleles 7 and 6; Czech population, alleles 2, 6 and 7; French population, alleles 2, 6 and 7; Finnish population, almost exclusively allele 6; Croatian population, alleles 7, 6 and 3; Norwegian population, alleles 6 and 2).

For the FRAXAC1 locus, allele 4 was the most common allele (RF = 0.664) in our control group. Analysis of this locus in control chromosomes of a Czech population revealed allele 3 to be the most common allele (Pekarik et al., 1999). This was also the case in populations from Russia (Drozd et al., 2003), Norway (Larsen et al., 2001) and Croatia (Dokic et al., 2008), while alleles 3 and 4 were prevalent in a Polish population (Rajkiewicz, 2008). Regarding Latvian FXS chromosomes, we found allele 2 to be the most common allele (RF = 0.818). However, this was not in line with the findings from other populations (Norwegian, allele 4; Polish, allele 4; Croatian, allele 3; Czech, alleles 3 and 4).

Compared to the microsatellite marker FRAXAC1, the FRAXAC2 locus has been more widely used in studies. Allele 5+ was the most common allele at this locus in our control group, detected at a RF of 0.467. This finding was not replicated in other populations: Finnish, allele 3 (Haataja et al., 1994); Swedish, Czech and French, allele 4+ (Malmgren et al., 1994; Oudet et al., 1993; Pekarik et al., 1999); Polish, alleles 7 and 7+ (Rajkiewicz, 2008). The prevalent allele in our FXS patients was allele 4. Allele 3 was identified in half of the investigated chromosomes in a Finnish FXS patient group, while alleles 4+ and 5 were prevalent in a Swedish population, alleles 4+ and 4 in a Czech population, alleles 3 and 4 in a French population, and allele 7 in a Polish population.

In summary, our finding regarding allele 7 being the most common allele at the DXS548 locus in Latvian control patients is in line with several other European population control groups. Furthermore, our FXS patient group finding (i.e. allele 2 being the prevalent allele at this locus) is consistent with data from a Polish FXS population. The FRAXAC1 and FRAXAC2 loci results for our control and FXS group differ to varying degrees from the data reported for other European populations. Alleles found in our study are not unique, they are found in studies from other European populations too, but show more similarity with populations from our geographical region.

As the analysed microsatellite loci and nomenclature assigned to alleles in the literature are different, confusion arises, which may lead to bias in the interpretation of literature data comparing haplotypic results from different populations.

In Latvian population, 7-4-A-5+ was determined as the prevalent haplotype for normal CGG alleles. However, after the Bonferroni correction, this finding was not considered to be statistically significant. From literature data the prevalent haplotypes in English population and Western European descended populations of the USA were 7-3-4 and 7-3-4+. These haplotypes were not detected in our control group. Though, taking in to account that most published data from European populations analysed two microsatellite markers, we can therefore compare our results with two marker haplotypes. The haplotype 7-4 constructed from two microsatellite markers DXS548 and FRAXAC1 respectively, was the prevalent one in studies from Basque valleys, Czech, Croatia, Poland and Portugal (Arrieta et al., 2003; Dokic et al., 2008; Pekarik et al., 1999; Peixoto et al. 1998; Rajkiewicz, 2008). In our study, DXS548-FRAXAC1 haplotype 7-4 was found in 72 out of 122 control group chromosomes. This finding shows prevalence of the haplotype 7-4 in our control group.

Furthermore, haplotype 2-2-G-4 was found to be in positive association with full mutation CGG alleles in Latvian FXS chromosomes. In contrast, haplotypes 6-4-4; 6-4-5 and 2-1-3 were reported as positively associated with FXS in Western European descents. These haplotypes were not detected in our FXS group but, nevertheless haplotype 6-4-5 was detected in two grey-zone alleles (41 and 42 CGG repeats respectively). Both alleles were associated with ATL1 polymorphism G, and both had CGG tract pattern 9+9+n. All these findings for 6-4-5 haplotype linked alleles may provide evidence of possible instability for these alleles in later generations.

Grey zone alleles featuring a long (≥18 repeats) uninterrupted CGG tract at the 3' end were found to be in positive association with the haplotype 2-2-G-4. In grey-zone alleles haplotype 2-2-G-4 was detected in 8 alleles. All these alleles had a CGG tract pattern where the first AGG interspersion occurred after nine CGG repeats (9+ structure). This finding is in line with Gunter`s and colleagues suggested "positively" associated haplotypes with the fragile X mutation. Haplotype 7-4-A-5+ was found in six grey-zone alleles and all alleles had CGG tract pattern where the first AGG interspersion occurred after ten CGG repeats (10+ structure). This haplotype might be a "protective" haplotype for CGG tract stability.

These findings imply that, in our population, haplotype 2-2-G-4 is a marker of CGG tract instability. Grey zone alleles with a long uninterrupted CGG tract at the 3' end associated with this haplotype have a higher likelihood of increasing the number of CGG repeats, leading to either premutation or mutation over generations.

To the best of our knowledge, specific *FMR1*-linked haplotypes in the Baltic State region and North-eastern Europe have not been previously described. The present study is the first to report Latvian population *FMR1* haplotype data. Comparison of the data with those obtained from geographically close European populations highlights differences, particularly with the FXS patient group. Indeed, haplotype 2-2-G-4 appears to be exclusively found in Latvian FXS chromosomes. We conclude that a founder effect could not be an explanation of our findings, on the basis of heterogeneity exhibited in the Latvian population and on the basis of a lack of studies across this geographical region. The small number of FXS chromosomes analysed in this study was restricted by a low pick up rate of the fragile X syndrome in our population. It could however, be a source of incomplete data for the FXS chromosomes linked haplotypes. A larger study of *FMR1*-linked haplotypes in Eastern and North-eastern European regions may provide more accurate data. Nevertheless we consider that our data provide evidence of a specific mutational pathway for unstable CGG alleles in our geographical region.

5.6. Genotype-Phenotype Correlation

Clinical symptoms are crucial for patients with fragile X syndrome detection among the mentally retarded population. Recognition of these symptoms is a first step towards a successful diagnosis of the fragile X syndrome and the subsequent cascade

testing among family members. Assessment of a genotype – phenotype correlation among diagnosed patients can help predict a prognosis for FXS patients and allow exploration for a diversity of symptoms.

There are a number of publications reviewing cases of fragile X syndrome with a various phenotype compared to the genotype data. Publication from the de Vries group (1996) presented three related male patients with full mutation in the *FMR1* gene but with different proportion of methylated alleles based on a study in patient`s leucocytes. One patient had 90% of unmethylated alleles, others 35% and 15%. For the patient, who showed a 90% lack of methylation, normal mental status was observed, however some minor FXS facial features were seen. Two other patients showed a typical fragile X syndrome phenotype, including typical behaviour, face dysmorphism and signs of connective tissue weakness.

One of the patients described in our study had similar genotype – DNA study in leucocytes revealed a full mutation in the *FMR1* gene and around 80% of alleles were found to be unmethylated. Unfortunately this patient at the age of 4 years already showed signs of FXS phenotype typical for his age group. In our study we did not have a chance to measure the level of FMRP. Based on clinical data and the genotype of our patient we can conclude that expression of FMRP is absent or at a very low level. Discrepancy of phenotype data and genotype in leucocytes for our patient can be explained by mitotic instability of expanded CGG tract and also mosaic.

In our study we detected three patients with repeat size/methylation mosaic. The observed unmethylated premutation repeat size varied from 78 to 150 CGG repeats. The phenotype of patients with repeat size/methylation mosaic did not revealed significant differences from full mutation FXS phenotype. The only remarkable observation was a lack of autistic features for these patients. At the same time we should admit that for one patient with full mutation autistic features also were also not observed. Neither of our patients diagnosed with repeat size and /or methylation mosaicism showed any signs of milder phenotype which was apparent for patients diagnosed with full mutation and methylation. This can probably be explained by mitotic instability of the expanded CGG tract and possible mosaic in different tissue. Based on these observations, we do not suggest making prognostic predictions on patient clinical phenotype solely based on genotype data obtained from leucocytes DNA study.

There is a report of eight FXS patients with “Prader-Willi like” phenotype (de Vries et al., 1993). The patients had features resembling the Prader-Willi syndrome

(PWS), such as truncal obesity, hypogenitalism, and small hands and feet. Consequently, these fragile X patients might be erroneously diagnosed as having Prader-Willi syndrome. However, some major differences are observed between the classical Prader-Willi syndrome and the PW-like sub-phenotype in these fragile X patients. Unlike PWS patients, PW-like FXS patients have a normal birth weight and show no hypotonia with feeding problems during infancy. Furthermore, seven patients developed a sudden gain of weight at the age of 5 to 10 years without any change in diet. This is not observed in PWS patients who become obese because of a change in eating pattern which often occurs at a younger age. Another diagnostic difference is the typical fragile X behaviour, including poor eye contact, hyperactivity, short attention span, and preservative speech, which is expressed by the fragile X patients with the PW-like sub-phenotype, but not in the case of PWS.

In our study three patient with “PW- like” phenotype were diagnosed having fragile X syndrome. Three patients out of twelve diagnosed with “PW- like” phenotype is remarkable number. In our opinion all patients with PWS phenotype showing mental retardation and/or autistic features should be tested for fragile X syndrome.

Several case reports described in literature lead to the proposition, that a "Sotos-like" phenotype of the fragile X syndrome might exist (de Vries et al., 1995). Among patients examined in our study a “Sotos-like” phenotype was not observed. Nevertheless clinicians should bear in mind that tallness can be a sign for fragile X syndrome along with mental retardation and other signs of FXS.

The low detection rate for patients with fragile X syndrome demonstrated in our study led to the conclusion that fragile X syndrome is generally clinically unrecognised. To help recognise patients with fragile X syndrome among the mentally retarded male population we adapted clinical check list based on a review of relevant literature and translated it into Latvian (Appendix 14). This check list is designed for use by family doctors, paediatricians, children neurologists and children psychiatrists. The check list covers the major symptom groups. For correct evaluation of symptoms, the age of examined patients should be kept in mind due to symptoms onset. The phenotype is subtle in young children and evolves with age. In childhood notice should be taken of delayed developmental milestones, delayed speech, signs of mental retardation, an unusual behavioural pattern, hyperactivity and autistic spectrum features. It should be noted that dysmorphic face features appear more prominent in teenage years or even

adulthood. Macroorchidism is an important feature in post-pubertal age. However, it is not present in all FXS males, but it is specific to FXS.

In our opinion, the low number of diagnosed patients was not only due to the failure to clinically recognise fragile X syndrome, but also due to the attitude of society toward mentally handicapped people and their families. In our experience, families with diagnosed FXS patients refuse to inform relatives at risk thereby preventing family genetic consultation. For clinical specialists, a lack of specific treatment for fragile X syndrome put this diagnose in the line with other psychiatric diagnosis with symptomatic treatment.

6. CONCLUSIONS

1. The prevalence of fragile X syndrome in the Latvian male population was estimated to be 1/6428 (95% CI 5538-7552) or 15.55/100 000 males (95% CI 13.24 – 18.05). The prevalence of the fragile X syndrome among mentally retarded male patients was estimated to be 2.67%.
2. The highest incidence among all analysed normal CGG repeat chromosomes was observed for allele 30 (29.95%), allele 31 (13.10%) and allele 29 (12.83%).
3. For individual CGG alleles with a normal CGG repeat number, a statistically significant association with ATL1 SNP was found for allele 29 and G (p = 0.001); allele 30 and A ($p < 0.0001$) and allele 31 with A (p = 0.0013). Polymorphism G was found to be associated with grey-zone CGG alleles (p = 0.0271) and exclusively associated with all FXS alleles.
4. In the case-control study, haplotype 7-4-A-5+ was determined as the prevalent haplotype for normal CGG alleles in the Latvian population. However, after Bonferroni correction, this finding was not considered to be statistically significant. Results of this study imply that in the Latvian population, haplotype 2-2-G-4 is a marker of CGG tract instability. AMOVA results revealed distinct genetic background for FXS chromosomes.
5. Analysing the structure of grey-zone alleles, revealed ten different CGG arrays. The most common pattern of grey-zone alleles was 9+9+n. The CGG array with a 9+n structure associated with the haplotype 2-2-G-4 was recognised as the pattern positively associated with CGG repeat instability. The CGG array with 10+n structure associated with the haplotype 7-4-A-5+ was recognised as a “protective” pattern.
6. The results of genotype-phenotype analysis did not revealed significant correlation among clinical symptoms, observed in FXS patients, and distinct patterns of CGG repeat expansion, obtained from leucocytes DNA analysis.

7. PUBLICATIONS

Publications

Zanda Daneberga, Zita Krūmiņa, Baiba Lāce, Daiga Bauze, Rita Lugovska. Fragilās X hromosomas sindroms pacientiem ar neskaidras etioloģijas garīgo atpalicību Latvijā. *RSU Zinātniskie raksti 2007*. RSU, 2008: 224-227

Zanda Daneberga, Zita Krūmiņa, Baiba Lāce, Daiga Bauze, Natālija Proņina, Rita Lugovska. Fragile X syndrome in mentally retarded patients from Latvia
Proceedings of the Latvian Academy of Science, Section B, 2009 (63):70-72

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Abstracts

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Bauze D., Ķevere L., Kronberga Z., Riževs A., Jeļisejevs S., Daneberga Z., Dzalbs A., Ločmele Dz., Krūmiņa Z., Andrēziņa R., Lāce B. Autisma un autiskā spektra traucējumu fenotipisko pazīmju, antropometrisko mērījumu un bioķīmisko rādītāju salīdzinājums un analīze. RSU scientific conference, Abstracts, 2011, pp.239.

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Daneberga Z., Krumina Z., Lace B., Pronina N., Lugovska R. Molecular diagnosis of fragile X syndrome in mentally retarded patients in Latvia. *Laboratorine Medicina* 1(29), 2006, pp.20

Daneberga Z., Krumina Z., Lace B., Pronina N., Lugovska R. Molecular diagnosis of fragile X syndrome in mentally retarded patients in Latvia. *European Journal of Human Genetics* Vol 14 Supp. 1, 2006, pp.251

Approbation

Pre-defence of this thesis was held in joint meeting of Department of Biology and Microbiology, Riga Stradins University, Latvian Society of Medical Genetics and Latvian Association of Human Genetics. July 1st, 2011. Department of Biology and Microbiology, Riga Stradins University, Riga, Latvia.

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Appendix 1

Sequence and the location of PCR primers for routine PCR screening (A and 571R), AGG interspersion pattern analysis (forward) at the Homo sapiens fragile X mental retardation syndrome protein (*FMR1*) gene. Location:13500-14500

5` CAAGCCCGCGCACGCCCGGCCCGCGCGTCTGTCTTTCGACCCGGCACCCCGGCCGGTTCC
CAGCAGCGCGCATGCGCGCGCTCCCAGGCCACTTGAAGAGAGAGGGCGGGGCCGAGGGGC
TGAGCCCGCGGGGGGAG Primer A GGAACAGCGTTGATCACGTGACGTGGTTTC AGTGTTTACACCC
GCAGCGGGCCGGGGGTTCGGCCTCAGTCAGGCGCTCAGCTCCGTTTCGGTTCACTTCCG
GTGGAGGGCCGCCTCTGAGCGGGCGGCGGGCCGACGGCGACGCGGGCGGCGGCGGT Primer forward GAC
GGAGGCGCCGCTGCCAGGGGGCGTGCGGCAGCG **(CGGCGGCGGCGGCGGCGGCGGCGGCGG**
CGGAGGCGGCGGCGGCGGCGGCGGCGGCGG) CTGGGCCTCGAGCGCCCGCAGCCCACC
TCTCGGGGGCGGGCTCCCGGCGCTAGCAGGGCTGAAGAGAAGATGGAGGAGCTGGTGGTG
GAAGTGCGGG GCTCCAATGGCGCTTTCT Primer 571R ACAAGGTACTTGGCTCTAGGGCAGGCCCC ATC
TTCGCCCTTCCTTCCCTCCCTTTTCTTCTTGGTGTCGGCGGGAGGCAGGCCCGGGGCCCT
CTTCCCGAGCACCGCGCCTGGGTGCCAGGGCACGCTCGGCGGGATGTTGTTGGGAGGGAA
GGACTGGACTTGGGGCCTGTTGGAAGCCCCTCTCCGACTCCGAGAGGCCCTAGCGCCTAT
CGAAATGAGAGACCAGCGAGGAGAGGGTTCTCTTTCGGCGCCGAGCCCCGCCGGGGTGAG
CTGGGGATGGGCGAGGGCCGGCGGCAGGTACTAGAGCCGGGCGGGAAGGGCCGAAATCGG
CGCTAAGTGACGGCGATGGCTTATTCCCCCTTTCCTAAACATCATCTCCCAGCGGGATCC
GGGCCTGTCGTGTGGGTAGTTGTGGAGGAGCGGGGGGCGCTTCAGCCGGGCCGCCTCCTG
CAGCGCCAAGAGGGCTTCAGGTCTCCTTTGGCTTCTCTTTT 3`

Adapted from EMBL-Bank: L29074.1 (http://www.ebi.ac.uk/ena/data/view/L29074)

Appendix 2

Sequence and the location of PCR primers (ATL1F and ATL1A/G) for ATL1 SNP detection at the Homo sapiens fragile X mental retardation syndrome protein (*FMR1*) gene. Location:18999-19999

5` CTGTTGGTAAGATACTTTACTAAGGGAAGGAATGTGAGGTGTCGCTGG
GGAGAGTTTACCCAAATAAGGATGGACTTTCTGTCTTTGTT Primer ATL1F
TCATCAGTCCTGGTAATAGAATGTTTGAATAGATAGCTCTAGGCATTACATACTTT
CATAAATATGATTATTGTAATTACCTCTTTGGCCCAGTTGCTAGTAAATTAGGGACCCCTTAA
TGATTTATTTCCTGTTTATTCACCCTGATGAAGAACTTGTATCTCTTTTAAACTGTACTTTATC
GCCTTTCTCAAATTCCAAGATTCTCATCACATTTTTTTTCTTCCCAAACTCTAAATAACCTTTT
AATATTAAGTATCTTTGTGGAAACATTGTTTTCTTTTTCTATCCCAATTTTTAAAGCTTTTTTA
AAAAAAAGAGTGCTTTTGTTGGGATGTACATTTTCCAAATGCAAAA Primer
ATL1A/ATL1G**A/G**CATTTATGATTCTGTGTCTCTTATAAAATATGACACTCTCTACTTTTCTCTCATT
TATTTAGTGCCACCTATGTGTGTAATTTCATTACCCACAGCAGTCTTAGGAGGCTGGTGAGTT
CCTTATTTGCAGATGAGGAATCTGAGGTCCAGAGATCACTTCTTGGTGAGAGTCTCACAGCT
ATTAAGTATTAGAGCCAAGATTTTGAACGTAGGTCTGATTCACAGCAAAACCGTTAACCACT
AAGTACACTGACTCCAGTAAGAGCCCTAGTCCTCACCCAATACACTTTAATTCCCCTGTGCA
TTCATTCAAATTCATTGAATTTGCTGCTTTGGAAACCTCTCAGGAACCTCCTCAACCTCTCTT
CTCTACAGACATCAGCTTTGCCTATAGGTAGGGATCATAGCAAAACACAGTTTTCCAAGGTG
GTGATAGGTGGAGTGATAGTGCTCTGGAGATGGCCAAAGAAGGAAGGTATGAGTGTATCTG
TGGGTGGGTGAGTGGTGGATAAGGGGAAGGACAGAGCCAAAAGCGACGGCTATTGGAAAA
A 3`

Adapted from EMBL-Bank: L29074.1 (http://www.ebi.ac.uk/ena/data/view/L29074)

Appendix 3

Sequence and the location of PCR primers (DXS548A and DXS548B) for DXS548 microsatellite marker detection at the Homo sapiens fragile X mental retardation syndrome protein (*FMR1*) gene.

5`....Primer DXS548A GTACATTAGA GTCACCTGTG GTGCTTTTAA AACCCAGGAT TCCTAGAA**(CC CC)**G**(CCCCCCC CCC)**AGTTTAG ACCAATTAAA TCAGAATAGT CTCTGGGGTG GATCTCGTGC GTGCTTGC**(GT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGT)**GG CTTCACAAGT Primer DXS548B GATTCCATTG CATAGTGAAG CTCT.....3`

Adapted from Chiurazzi et al. 1999

Appendix 4

Sequence and the location of PCR primers (FRAXAC2A and FRAXAC2B) for FRAXAC2 microsatellite marker detection at the Homo sapiens fragile X mental retardation syndrome protein (*FMR1*) gene. Location:26000-27000

5` AGGCCCTAATCAGATTTCCACAAATTCTGACTTAATATTTGCCCGCTTATATAACAGCTC
TTCTTTAACAAAAACAAGTACTTTTCTCAATAGAATTTTACTAAGAAAGCTCTTTAGTAA
AACATCGACATTATACATACAACATATCTCAGTATCTGCTGATGAAGAACACCAAAAAGA
ACCCAGATGT[PrimerFRAXAC2A]GACTGCTCCGGAAGTTGAATCCTCAGTATTTTTGCAAAGTTTGTC
TTTCAGTATTTTATTT**(GTGTGTGTGTGTGTGTGTGTGTGTGTGTGT)**C**(TATATATATA**
TATA)(TTTTTTTTTTTTT)AA[PrimerFRAXAC2B]AGACAGGATCTCACTCTGTCACCTAGGCTGGAGT
GCAGTGGCATGATCATGGCTCACTGTAACCTTGAACTCCTGAGCTTGAGCTATCCTCCCACCT
CAGCCTCCCGAGTAGCTGGGACTATAGGCACATACCACTGCACCTAATTTTTTTTTTTTTTA
ATAATTTGTTGTAAAGATCAGGTCTTACCTTGTTGCCCAGGCTGCTCTTGAAGTCCTGGCCTG
AAGCAGTGCTCCCACCTCAGCCTCCCAAAGCTCTGGGATTATAGGCTTGAGCCACCGCATCC
TAATATTTTATATTTTTATGGATATAAAAAATAATTTGGTATCTTTCAGAGTTGTTTAATATC
ATTTTAAATTTAAAAACATAGGCAACTTAAACTCCTATAGGCTGTCTCCATCGGGTTTCTGTG
GTTTAGGAGACCCCACCATCCCAGTGCATGCTGATAACGTCATACTGATCAGCATCCAGCTA
CCCACAGCAAGAATTGACCACCTCGTGGGATCTAAAATTTAAAGGGGGAAAAGTGAGTTGT
GAATTGCTAATGTGCTGATAGCCCCATTTTGCTTGGGAATTAGAGGGCAGTTTTTGTGGTCCT
TGGAATGTGGTTAAAATTCTTCTGCAAGTGGAAGCATATTTATATTACTAACAATTACTGGT
ACTAATATTCAAATATTGAAGGAAATTTC 3`

Adapted from EMBL-Bank: L29074.1 (http://www.ebi.ac.uk/ena/data/view/L29074)

Appendix 5

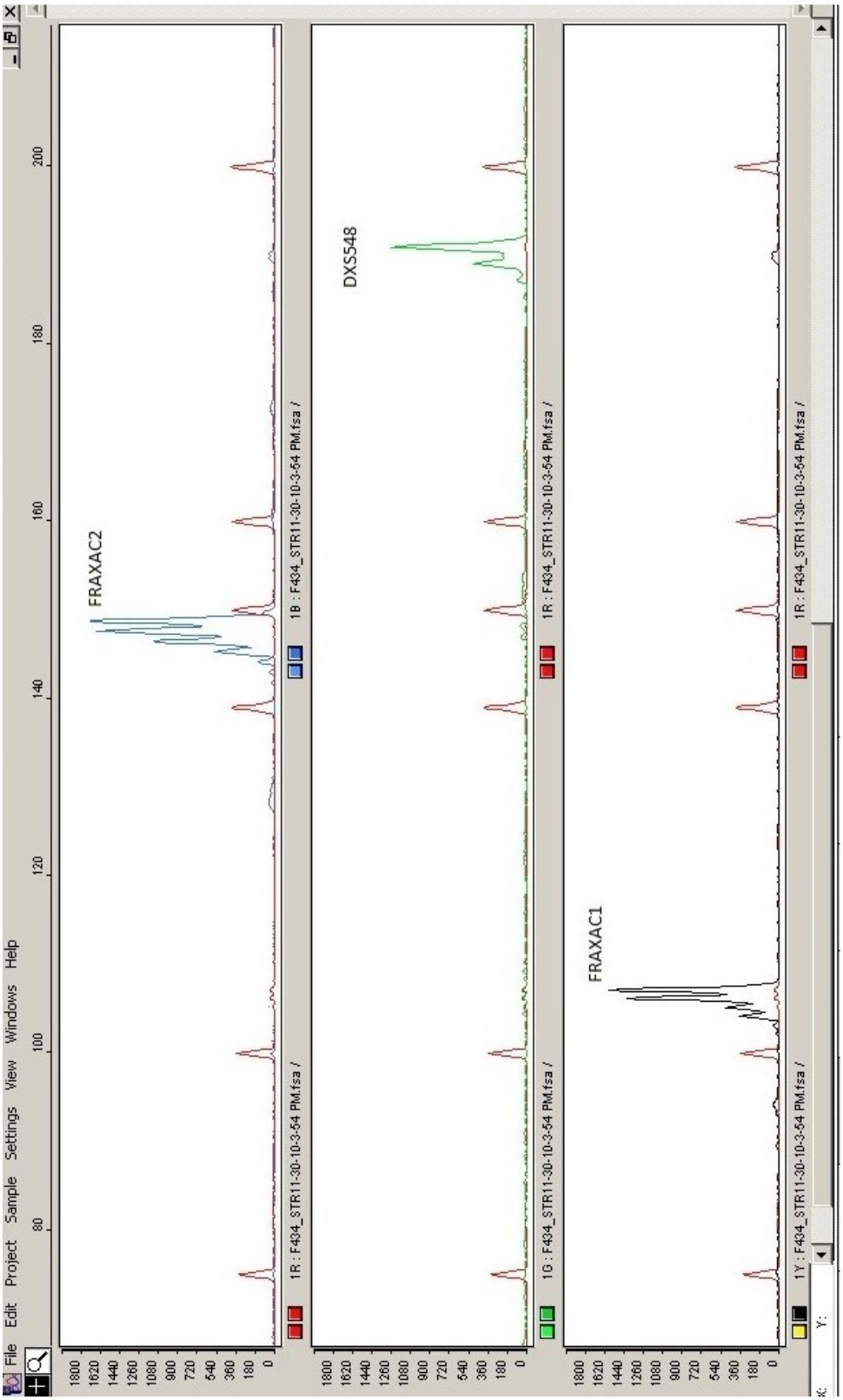
Sequence and the location of PCR primers for FRAXAC1 microsatellite marker (FRAXAC1A and FRAXAC1B) at the Homo sapiens fragile X mental retardation syndrome protein (*FMR1*) gene. Location:6500-7500

5`ACACCTCTCTGTCAGTAATTTACAGATATAGCCAAAAACATCAGTAAGGATATAGTTGAT
GCTGAACATCCTTATCGATCAACTT Primer FRAXAC1A GATCTAATCAACATCTATAGACTTTAT
(GTGTGTGTGTGTGTGTGTGTGTGTATGTGTGTGT)CAGTCTCACTCTGTCACTC Primer
FRAXAC1BAGGCTTGGAGGCAGTGGGCAATCTCTGCTCACTGCAACCTCTGCCTCCCAGCTTCAA
GTGACTCTCATCATGCCTCAGCCTCCTGAGTAGCTGGGATTACAGGCATGCACCACCACACC
CAGCTAATTTTTTGCATTTTTAGTAGAGTCGGCATTTCACTATGTTGGCCAGGCTGGTCTCGA
ACTTCTGGCCTCAAGTGATCCTCCCACCTTAGCCTTGCAAAGTACTGGGATTACAGGCATCA
GCCACTGTGCCTGGCCTGATATTTATAGACTATTTGATCCAACAGAGACAGAATACACATTT
CTTTCATGTTCACATGGAACATTAATCAAGATAGACCACATTCTGTTTCATAAAATTCACCTT
AAAAATTAAAAAAACAGAAATCAAACAAGATATTTTCACAGATTACAATAAAATTAAACTA
GATATTTTTAGAAACCTAGAAATATGCTAGGCAATGCCCTCAAATATTTGGAGATTAAACAA
CACACTTCTAAATAATATATGGATCAAAGAAGATGTTTCAAGAGATATTAAAAATATTTTGA
ACTAAATGAAAAAATAAACTTTTTAAAATTTATGGGATGCAGCAAAAGCAGTGATGAGAGG
GAAATTTATATATCAGCAATGAACAATTGGAATTTGAAATTAAAAACATACCATTCAAACCA
GCACTGAAAAACAAAATATTTAGGTATAAATCTAATAAAATATGTACAGAATCTAGTTCAAC
ACCTTATGAAAGAAATGAAAAATCTAAATAAATTGAGAAATATCCCATGTTCATAAATAGC
AAGACTAATGTTGTTAAACTGTCACTTCTTCC 3`

Adapted from EMBL-Bank: L29074.1 (http://www.ebi.ac.uk/ena/data/view/L29074)

Appendix 6

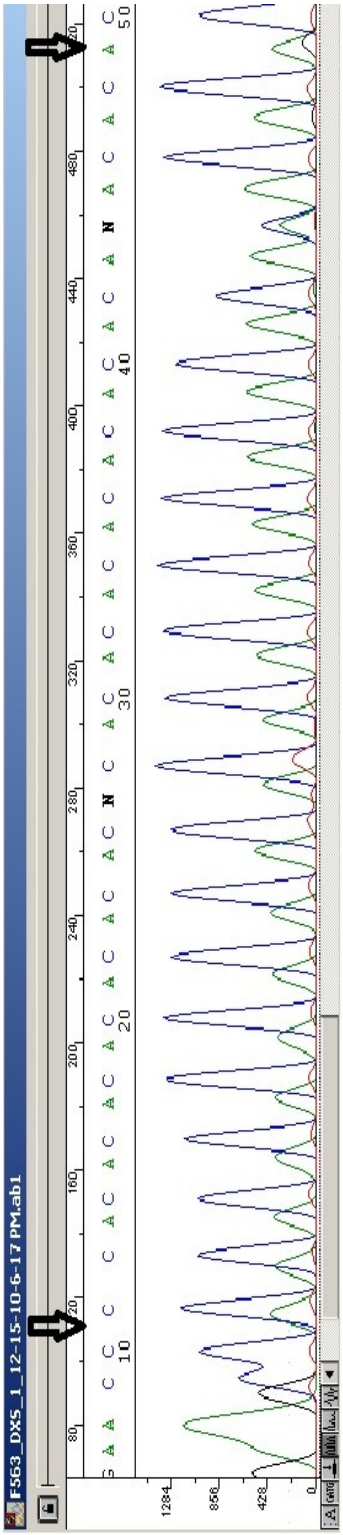
Electropherogram of the three microsatellite markers fragment length analysis on ABI Prism® 310 genetic analyzer



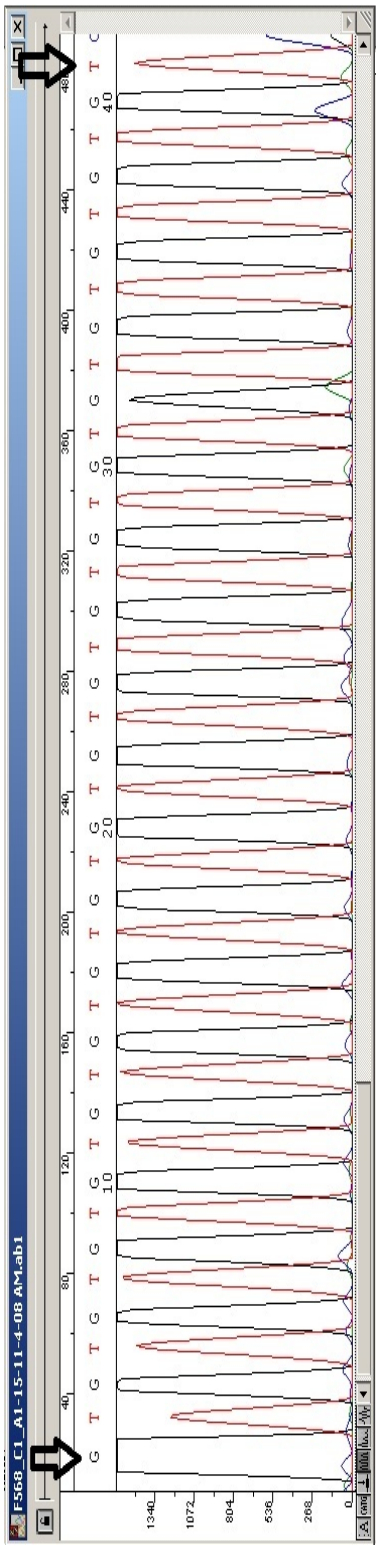
Appendix 7

Electropherograms of validation data from DXS548 and FRAXAC1 loci

DXS548



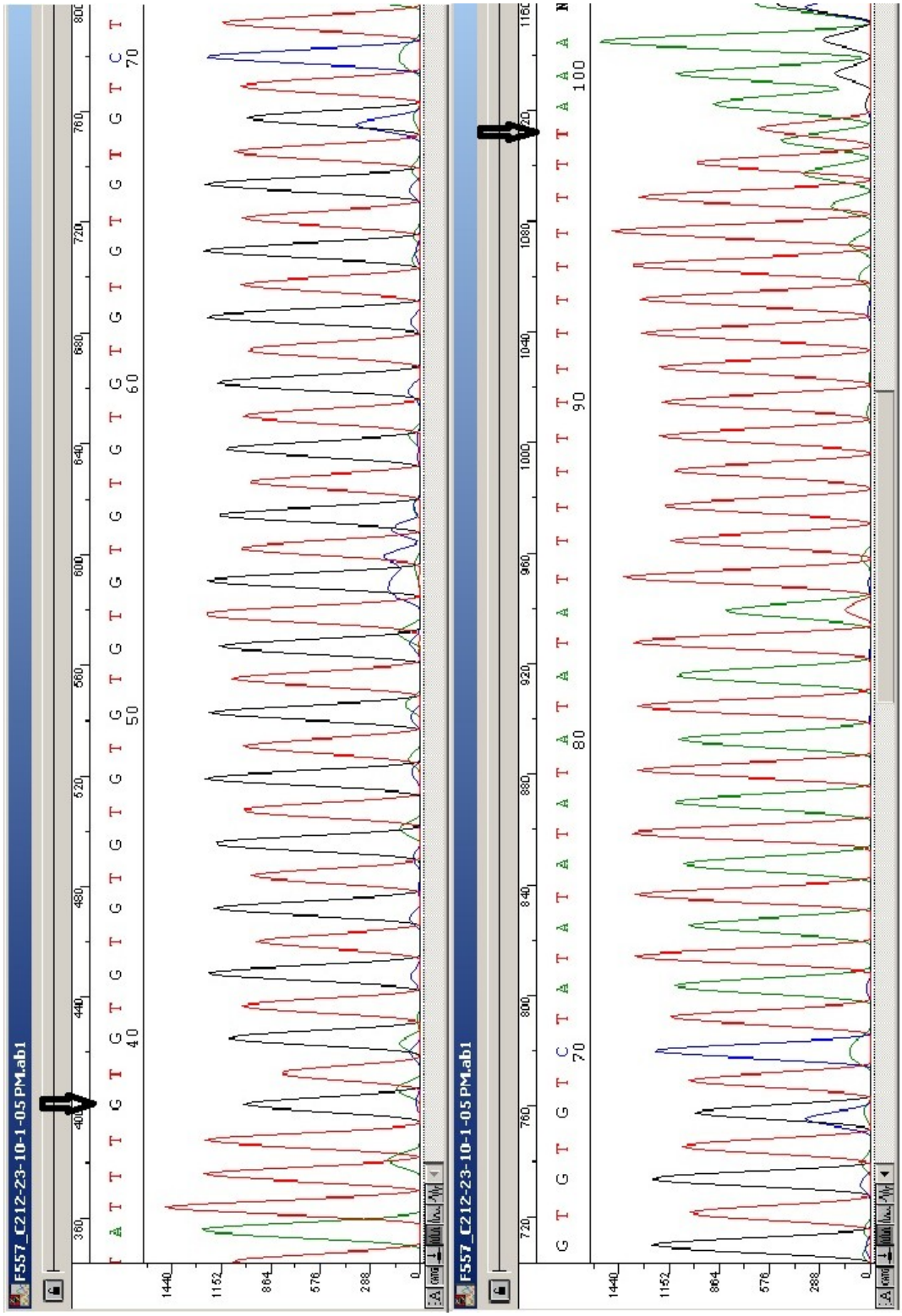
FRAXAC1



Start and end of dinucleotide repeats marked by arrows. 193 bp long fragment of DXS548 locus consist form $[CA]_{21}$ and correspond to allele 6 by Macpherson et al., 1994. 111 bp long fragment of FRAXAC1 locus consist from $[GT]_{20}$ and correspond to allele 2 by Macpherson et al., 1994.

Appendix 8

Electropherogram of validation data from FRAXAC2 locus



Start and end of dinucleotide repeats marked by arrows. 151 bp long fragment of FRAXAC2 locus consist form complex polymorphism $[GT]_{16}$ +$[TA]_7$+$[T]_{14}$ and correspond to allele 4 by Macpherson et al., 1

Appendix 9

Data of Central Statistical Bureau of Latvia

VA06. REPORTED CASES OF MENTAL DISEASES

	TOTAL									
	1998	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**
Disorders of psychological development; behavioural and emotional disorders	1,345	1,312	1,099	982	903	873	997	876	873	812

new cases; excluding alcoholism and dependency upon narcotic and psychoactive substances.

Indicator
Disorders of psychological development; behavioural and emotional disorders
With onset usually occurring in childhood and adolescence.

Number
Slimnieki ar pirmoreiz uzstâdîtu diagnozi; bez alkoholisma un narkomânijas.

Source:

Central Statistical Bureau of Latvia

Copyright:Yes

Contact:

Culture, education, science and health statistics section

Unit:

number

Matrix:

VA0060a

Appendix 10

Data of Central Statistical Bureau of Latvia – Male Population

IS02. RESIDENT POPULATION BY SEX AND PLACE OF RESIDENCE AT THE BEGINNING OF THE YEAR

	males
1998	1,115,483
1999	1,105,438
2000	1,096,888
2000., 31.III	1,094,964
2001	1,088,853
2002	1,080,116
2003	1,073,057
2004	1,068,336
2005	1,062,918
2006	1,057,284
2007	1,051,034

The year 1935; 1959; 1970; 1979; 1989; 2000: Population Census data.
Source:
Central Statistical Bureau of Latvia
Copyright:Yes

Contact:
Population census section
Unit:
number
Matrix:
IS0020a

Appendix 11

Distribution of CGG repeat alleles in mentally retarded patients

	CGG	n	Freq. %	Freq. %
Normal alleles	16	1	0,27	
	17	0	0,00	
	18	0	0,00	
	19	0	0,00	
	20	11	2,94	
	21	3	0,80	
	22	5	1,34	
	23	31	8,29	
	24	23	6,15	
	25	3	0,80	90,37
	26	15	4,01	
	27	2	0,53	
	28	5	1,34	
	29	48	12,83	
	30	112	29,95	
	31	49	13,10	
	32	12	3,21	
	33	14	3,74	
	34	4	1,07	
Grey-zone alleles	35	1	0,27	
	36	1	0,27	
	37	2	0,53	
	38	3	0,80	
	39	6	1,60	
	40	2	0,53	
	41	5	1,34	
	42	2	0,53	
	43	1	0,27	6,95
	44	0	0,00	
	45	1	0,27	
	46	0	0,00	
	47	1	0,27	
	48	0	0,00	
	49	0	0,00	
	50	1	0,27	
Full mutation	>200	10	2,67	2,67
	Total	374	100,00	2,67

Appendix 12

Distribution of normal range CGG repeat alleles

CGG	n	Freq. %
16	1	0,27
17	0	0,00
18	0	0,00
19	0	0,00
20	11	3,02
21	3	0,82
22	5	1,37
23	31	8,52
24	23	6,32
25	3	0,82
26	15	4,12
27	2	0,55
28	5	1,37
29	48	13,19
30	112	30,77
31	49	13,46
32	12	3,30
33	14	3,85
34	4	1,10
35	1	0,27
36	1	0,27
37	2	0,55
38	3	0,82
39	6	1,65
40	2	0,55
41	5	1,37
42	2	0,55
43	1	0,27
44	0	0,00
45	1	0,27
46	0	0,00
47	1	0,27
48	0	0,00
49	0	0,00
50	1	0,27
Total	364	100,00

Appendix 13

Clinical symptoms of fragile X syndrome patients

Code		FXS1/1	FXS2/1	FXS3/1	FXS4/1	FXS6/1	FXS7/1	FXS8/1	FXS9/1	FXS10/1	FXS10/3	FXS12/1	FXS13/1
Gender		♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂	♂
Genotype		F	F	F	150/F	78/F	F/M	F	F	F	140/F	F	F
Age (at the moment of diagnosis)		7	7	7	8	7	4	2	16	2	14	12	2
Psychiatric symptoms	Mental retardation	+	+	+	+	+	+	+	+	+	+	+	+
	Learning difficulties	+	+	+	+	+	+	+	+	+	+	+	+
	Motor development delay	+	+	+	+	+	+	+	+	+	+	-	+
	Speech delay/difficulties	+	+	+	+	+	+	+	+	+	+	+	+
	Autistic features	-	+	+	-	-	+	+	+	+	-	+	+
	Attention-deficit/hyperactivity	+	+	+	+	+	+	+	+	+	+	+	+
	IQ	34	49	74	49	69	nt	49	49	nt	49	nt	nt
Dysmorphic features	Long face	+	+	+	+	+	+	+	+	+	+	-	+
	Large ears	+	+	-	+	+	+	+	-	+	-	-	+
	High, wide forehead	+	+	+	+	+	+	+	+	+	+	-	+
	Prognatia	-	-	-	-	-	-	-	-	-	-	-	-
Connective tissues	Hyperelasticity of joints	+	+	+	+	+	+	+	-	+	+	+	+
	Flatfoot	nt	+	+	+	-	+	+	-	+	+	-	+
	Hypotonia	+	+	+	+	+	+	+	+	+	+	+	+
	Recurrent ARI/otitis	+	+	+	+	+	+	+	-	+	+	-	-
Other symptoms	Seizure	+	-	-	+	-	+	-	+	-	-	+	-
	Balance disturbance	-	-	-	-	-	-	-	-	-	-	-	+
	PWS phenotype	-	-	+	-	-	-	-	+	-	-	+	-

F - full mutation; 150/F – 150 CGG repeats/full mutation mosaic; F/M – full mutation/methylation mosaic; nt – not tested; "+" – observed; "-" – not observed.

Appendix 14

Clinical check-list

Trauslās X hromosomas sindroma klīniskā karte pacientiem ar neskaidras etioloģijas garīgo atpalicību.

Lielās simptomu grupas – pelēkais krāsojums (vismaz viens no mazajiem simptomiem pietiekams lielā simptoma apstiprināšanai) . „+ / - ” – simptoms konstatēts/simptoms nav konstatēts.
Pacientu vēlams izmeklēt uz trauslās X hromosomas sindromu, ja klīniskie simptomi novērojami vismaz četrās lielo simptomu grupās.

SIMPTOMI	+ / -
Kognitīvi traucējumi	
garīgās attīstības aizture un apalicība	
motorās attīstības aizture	
mācību vielas apguves traucējumi	
Runas attīstība	
valodas attīstības traucējumi agrīnā vecumā	
neskaidra runa	
eholalijas	
Autiska spektra traucējumu pazīmes	
izvairīšanās no acu kontakta	
specifiskas roku kustības	
sociālās komunikācijas traucējumi	
Hiperaktivitāte un/vai uzmanības deficīts	
Dismorfisms	
garena seja	
lielas, atstatus stāvošas ausis	
plata, augsta piere	
izvirzīts žoklis	
strabisms	
Prādera-Villi sindromam līdzīgs fenotips	
Sotosa sindromam līdzīgs fenotips	
Saistaudu vājuma pazīmes	
hiperelasticitāte plaukstās	
plakanā pēda	
hipotonija	
mitrālā vārstuļa prolapss	
makroorhidisms	
biežas ausu infekcijas	
Neiroloģiskie simptomi	
epileptiski krampji	
līdzsvara traucējumi	
Garīgas veselības traucējumi	
depresija	
šizofrēnija	
bipolāri afektīvi traucējumi	
Uzvedības problēmas	
impulsivitāte	
dusmu uzliesmojumi	
lietoti medikamenti uzvedības traucējumiem	
Ģimenes anamnēze	
māsas un/vai brāļi ar līdzīgiem simptomiem	
ģimenes locekļi (mātes radi) ar līdzīgiem simptomiem	

Adaptēts pēc literatūras avotiem (de Vries et al., 1999; McConkie-Rosell et al., 2005; Saul and Tarleton, 1998).