FRAXE IN THE NEWFOUNDLAND POPULATION

by

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Abstract

Children, especially boys, can present to their primary care physician, pediatrician, or genetics program with developmental delay and/or intellectual disability. FRAXE is a rare cause of intellectual disability caused by expansion and methylation of the CCG repeats in the 5' untranslated region of the *FMR2* gene. Although the phenotype associated with FRAXE is not well defined, it has been reported to include: mild (IQ 50-69) to borderline (70-79) intellectual disability, learning problems, communication problems and overactivity with no consistent dysmorphology.

In the Canadian province of Newfoundland and Labrador, prior to initiation of the current study, two families segregating *FMR2* expansions had been identified. The purpose of this study was to determine the prevalence of FRAXE among boys referred to the Provincial Medical Genetic Program for intellectual disability and/or developmental delay; to characterize FRAXE positive families; and to determine if there is a common ancestor connecting these families. This was accomplished by reviewing the charts of boys seen in the Provincial Medical Genetics Program from 1994 to 2004 that had unexplained developmental delay. This review resulted in the discovery of a FRAXE positive boy and his full mutation sister. During this time two additional FRAXE families were referred to the Provincial Medical Genetics Program. Using microsatellite markers three of the four FRAXE positive families were found to share a common haplotype of 1.8 Mb.

The minimum prevalence rate in this male pediatric population was found to be 1 in 7737 which is a six fold increase compared with the reported prevalence rate for FRAXE of 1 in 50,000 or 1 in 23,423. Our data supports routine testing of *FMR2* expansions in boys with intellectual disability in the NL population.

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Abbreviations

ABI	Applied Biosystems
ADD	attention deficit disorder
ADHD	attention deficient hyperactivity disorder
ARVD5	arrhythmogenic right ventricular cardiomyopathy/dysplasia type V
ASD	autism spectrum disorder
ATV	all-terrain vehicle
BMP15	bone morphogenetic protein 15
C	Celsius
CD	compact disc
CGH	comparative genomic hybridization
cm	Centimeters
CP	cerebral palsy
CRC	colorectal cancer
CT	computerized tomography
CYP19A1	cytochrome P450 family 19 subfamily A member 1
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dH_20	deionized water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DSM-5	Diagnostic and Statistical Manual for Mental Disorders 5
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid

EEG	electroencephalogram
ESR1	Estrogen Receptor 1
ESR2	Estrogen Receptor 2
EtOH	ethanol
ExoI	exonuclease I
FAP	familial adenomatous polyposis coli syndrome
FASD	fetal alcohol spectrum disorder
FMR1	Fragile Mental Retardation - 1
FMR2	Fragile Mental Retardation - 2
FMRP	Fragile X mental retardation protein
FOXL2	Forkhead Box L2
FOXO3	Forkhead Box O3
FRAXA	Fragile X A mental retardation
FRAXA	folic acid type, rare, fra(X)(q27.3) A
FRAXE	Fragile X E mental retardation
FRAXE	folic acid type, rare, fra(X)(q28) E
FSH	follicle stimulating hormone
FSHR	Follicle Stimulating Hormone Receptor
FSIQ	full scale IQ
FXS	Fragile X syndrome
FXTAS	Fragile X-associated tremor ataxia syndrome
GTP	guanosine triphosphate
ICD	inner canthal distance
ID	intellectual disability
IDS	iduronate 2-sulfatase

INHA	inhibin alpha subunit
IQ	intelligent quotient
IVF	in vitro fertilization
kb	kilobase
kg	kilogram
KO	knock out
LHR	Luteinizing Hormone Receptor
M	molar
Mb	megabase
MCP	medical care plan
MEN1	multiple endocrine neoplasia type 1
$MgCl_2$	magnesium chloride
mL	millilitre
MLPA	Multiplex Ligation Dependent Probe Amplification
mM	millimolar
MOU	Memorandums of Understanding
MR	mental retardation
MRI	magnetic resonance imaging
MS-MLPA	Methylation-specific Multiplex Ligation Dependent Probe Amplification
NaCl	sodium chloride
NF1	neurofibromatosis type 1
NGD	Newfoundland Genealogy Database
NH_4Cl	ammonium chloride
NL	Newfoundland
NS-XLID	nonsyndromic - x-linked intellectual disability

OCD	obsessive compulsive disorder
p	p value
PCR	polymerase chain reaction
PD	Parkinson's disease
PDD	pervasive development delay
pH	potential of hydrogen
PMGP	Provincial Medical Genetics Program
pmol/µl	picomol per microliter
POF	premature ovarian failure
POI	premature ovarian insufficiency
PTRG	Population Therapeutics Research Group
rpm	revolutions per minute
SAP	shrimp alkaline phosphatase
SD	standard deviations
SDS	sodium dodecyl sulfate
SEN	special education needs
SF1	splicing factor 1
SLITRK2	*SLIT* and *NTRK*-like family member 2
SNP	single nucleotide polymorphism
SNS	special needs school
S-XLID	syndromic - x-linked intellectual disability
TE	tris EDTA
UCSC	University of California, Santa Cruz
µg	micrograms
µl	microliters

US	United States
UTR	untranslated region
UV	ultra violet
WAIS-III	Wechsler Adult Intelligence Scale 3
WES	whole exome sequencing
WGS	whole genome sequencing
WIAT-II	Wechsler Individual Achievement Test 2
WIAT-III	Wechsler Individual Achievement Test 3
WISC-III	Wechsler Intelligence Scale for Children 3
yrs	years

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Chapter 1 – Introduction

1.1 Developmental Delay

Developmental delay is defined as a significant lag in physical, cognitive, behavioural, emotional, and/or social development. The delay must be present in at least two of these areas and persistent. These conditions can be attributed to a multitude of diagnoses including intellectual disability (ID), cerebral palsy (CP), Down syndrome and autism spectrum disorder (ASD) while many others cannot be attributed to a specific diagnosis. The 2012 Canadian Survey on Disability reported that 0.6% of Canadian adults (aged 15 years and older) were identified as having a developmental disability[1]. The most prevalent underlying causes were autism spectrum disorder (ASD), cerebral palsy (CP), and Down syndrome.

1.2 Intellectual Disability

Intellectual disability (ID) is defined by the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) as deficits in intellectual functioning and adaptive functioning in the conceptual, social, and practical areas of living. Intellectual functioning is considered an individual's ability to learn through both academics and experience. It captures reasoning, problem solving, abstract thinking and judgement. Adaptive functioning refers to deficits in activities of daily living, including but not limited to

communication, participation, independent living and social interaction at home, school, and work[2]. To be diagnosed with ID, the individual's symptoms must date back to early development and not have occurred later in life due to an injury, which would be referred to as a neurocognitive disorder.

The intelligence quotient (IQ) is a common tool used to measure intellectual function. ID is characterized by an IQ < 70 and levels of ID are referred to as mild (IQ = 50-70), moderate (IQ = 35-50), severe (IQ = 20-35), profound (IQ ≤ 20) or unable to classify[3]. The most recent version of the DSM has removed the reference to IQ scores to indicate the level of ID severity and has proposed using the individual's adaptive functioning level across all aspects of life[2]. However, many practicing clinicians still refer to IQ scores and they are commonly reported in the literature.

ID is the most frequent reason for referral to pediatric genetic services[4]. ID can have a wide variety of causes including exposure of the fetus to toxins during development such as drugs and/or alcohol or maternal infection. Prematurity at birth or problems during the birthing process such as oxygen deprivation can also cause ID. Illness during development, injury to the brain and/or poor social conditions also can alter normal childhood development, particularly in terms of a child's cognitive abilities. Genetic factors are thought to contribute to 25 to 50% of all cases of ID[5]. ID can be isolated or part of a broader syndrome. The worldwide prevalence of ID is estimated to be 1%[4]. Approximately 75 to 90% of those with ID have mild ID.

1.2.1 Common forms of Intellectual Disability

As described above there are several causes for ID but the most common include Fetal Alcohol Spectrum disorder (FASD), Down syndrome and Fragile X syndrome.

1.2.1.1 Fetal Alcohol Spectrum Disorder

FASD is the leading cause of preventable ID[6]. It is caused by the mothers' consumption of alcohol during pregnancy. Health Canada estimates that more than 3,000 babies a year are born in Canada with FASD[7]. The severity of the effects of alcohol consumption depends on numerous factors, including the amount of alcohol consumed, the duration and timing of the consumption. Features of FASD include a small head (microcephaly), facial dysmorphology, prenatally and/or postnatally restricted growth, learning disabilities, ID, hyperactivity, attention deficiencies, problems with hearing and vision, and a wide variety of congenital malformations including renal and cardiac. Dysmorphic facial features characteristic of FASD include short palpebral fissures with widely spaced eyes, a short nose and a bow-shaped mouth with thin upper lip. The facial abnormalities associated with FASD are due to the consumption of alcohol during critical periods of development for the fetus, therefore not all children with FASD will exhibit facial dysmorphology[8].

1.2.1.2 Down Syndrome

Down syndrome, also known as, Trisomy 21, is the most common form of ID worldwide and occurs in approximately 1 per 1,000 babies born each year in the United States[9]. A survey of Down syndrome rates in Canada between 2005 and 2013 reported that 1 in 750 live-born babies in Canada has Down syndrome[10]. Ninety-five percent of individuals with Down syndrome have an extra copy of chromosome 21. The remaining 5% of cases are due to either a Robertsonian translocation, whereby a portion of chromosome 21 is translocated to another chromosome (usually chromosome 14 or 22), or mosaicism, whereby only a proportion of an individual's cells have an additional chromosome present. Although Down syndrome is a genetic condition, it is rarely inherited. It is possible for an unaffected parent harbouring a balanced Robertsonian translocation to pass on this rearrangement to his or her offspring which can become unbalanced resulting in a loss or gain of genetic material[11].

Down syndrome has a distinct phenotype consisting of mild to moderate ID, characteristic facial features and often specific major congenital malformations, usually involving the heart and/or gastrointestinal tract. Specific facial features include up slanting palpebral fissures, epicanthic folds, flat nasal bridge and a protruding tongue. Other typical dysmorphia include single palmar creases, short curved fifth fingers, and a wide space between first and second toes. Additional phenotypic features include low muscle tone and short stature[9].

1.2.1.3 Fragile X syndrome – Historical delineation

Fragile X syndrome (FXS), was originally published as Martin-Bell syndrome. It has been referred to in the literature as fragile X mental retardation syndrome and as marker X syndrome. It was first described in 1943 by James Martin and Julia Bell[12] and affected males were ascertained because of the presence of a cytogenetic fragile site on the X chromosome. This specific fragile site is referred to as folic acid type, rare, fra(X)(q27.3) A or FRAXA. In general, cytogenic fragile sites refer to chromosomal regions that are susceptible to breaks or gaps and were first identified in 1969 by Herbert Lubs. These fragile sites become apparent after exposing dividing white cells to certain culture conditions, such as a folate-deficient medium or by treating with chemical agents such as aphidicolin[13]. Over 100 different fragile sites have been identified and common fragile sites are considered to be present in all humans. Rare fragile sites occur in less than 5% of the population and have been associated with ID and also have implications in cancer genetics[14]. FXS was the first disease described to be associated with a specific fragile site. However, the FRAXA fragile site is not consistently expressed in all males with FXS.

It is the second most common cause of ID, the most prevalent cause of ID in males and the most common cause of inherited ID[15].

1.3 Genetics and Intellectual Disability

As stated in **section 1.2**, 25 to 50% of ID cases are thought to be attributed to genetic factors[5]. Over 700 genes have been linked to X-linked, autosomal dominant and autosomal recessive forms of ID[4].

Briefly, inheritance is the process by which genetic information is passed on from a parent to his or her offspring. Mendelian inheritance is defined by two factors – the chromosome on which the mutated allele resides (autosome or sex chromosome) and whether the phenotype is dominant or recessive. Dominant diseases require only one mutated allele in order to cause disease whereas recessive conditions are expressed when both parents pass on the mutated allele to their offspring. Monogenic diseases are autosomal dominant, autosomal recessive, X-linked dominant, X-linked recessive or Y-linked[12]. X-linked diseases carry important implications for males, who carry only a single X chromosome; X-linked recessive mutations are typically expressed in males, whereas females with one X-linked mutation are usually healthy carriers and do not express disease features.

1.3.1 Syndromic vs non-syndromic X-linked Intellectual Disability

Historically, the X chromosome was the main focus for studying ID due to the unbalanced sex ratio of ID incidence, with males being more commonly affected than females[16]. X-linked intellectual disability (XLID) accounts for 5 to 10% of male cases of ID[17]. At least 102 genes that have been linked to 160 different XLID diseases (**Figure 1.1**). XLID can be further subdivided into syndromic (S-XLID) and non-syndromic ID

(NS-XLID). To be considered a syndromic form of ID, one or more clinical features must be present in addition to the ID, for example dysmorphic facial features and/or birth defects. ID is considered to be non-syndromic when ID is the sole clinical feature present[5]. The majority of the 102 XLID-associated genes identified reported in Lubs et al. (2012) are associated with S-XLID; however, overlap exists between mutations in these genes and S-XLID and NS-XLID forms of the disease **Figure 1.1.A**[17].

FXS, the most common cause of inherited ID, is the most widely recognized and common form of S-XLID[15]. It is caused by mutations in the Fragile X mental retardation 1 (*FMR1)* gene, which maps to Xq27.3 and contains 17 exons spanning 38 kilobases (kb). Ninety-nine percent of individuals with FXS have a *FMR1* loss-of-function mutation due to expansions of the polymorphic CGG repeat (> than 200 repeats) in the 5' untranslated region (5' UTR) of the gene[15]. This hypermethylation in the *FMR1* promotor results in inhibition of the *FMR1* transcript and subsequently complete loss of, or a significant reduction in the level of, the Fragile X mental retardation protein (FMRP). There are four classes of *FMR1* repeats: normal (5 to 44 repeats), intermediate or grey zone (45 to 54 repeats), premutation (55 to 200 repeats) or full mutation (>200 repeats). Clinical manifestations associated with each repeat size in discussed in detail below (**section 1.3.2**).

Prevalence rates of Fragile X full mutations have been reported as 1 in 7000 or 1 in 5000 to 7000 males depending on the source[15,18]. Prevalence rates of full mutations in females have been reported as 1 in 11,000 or 1 in 4000 to 6000 females[15,18].

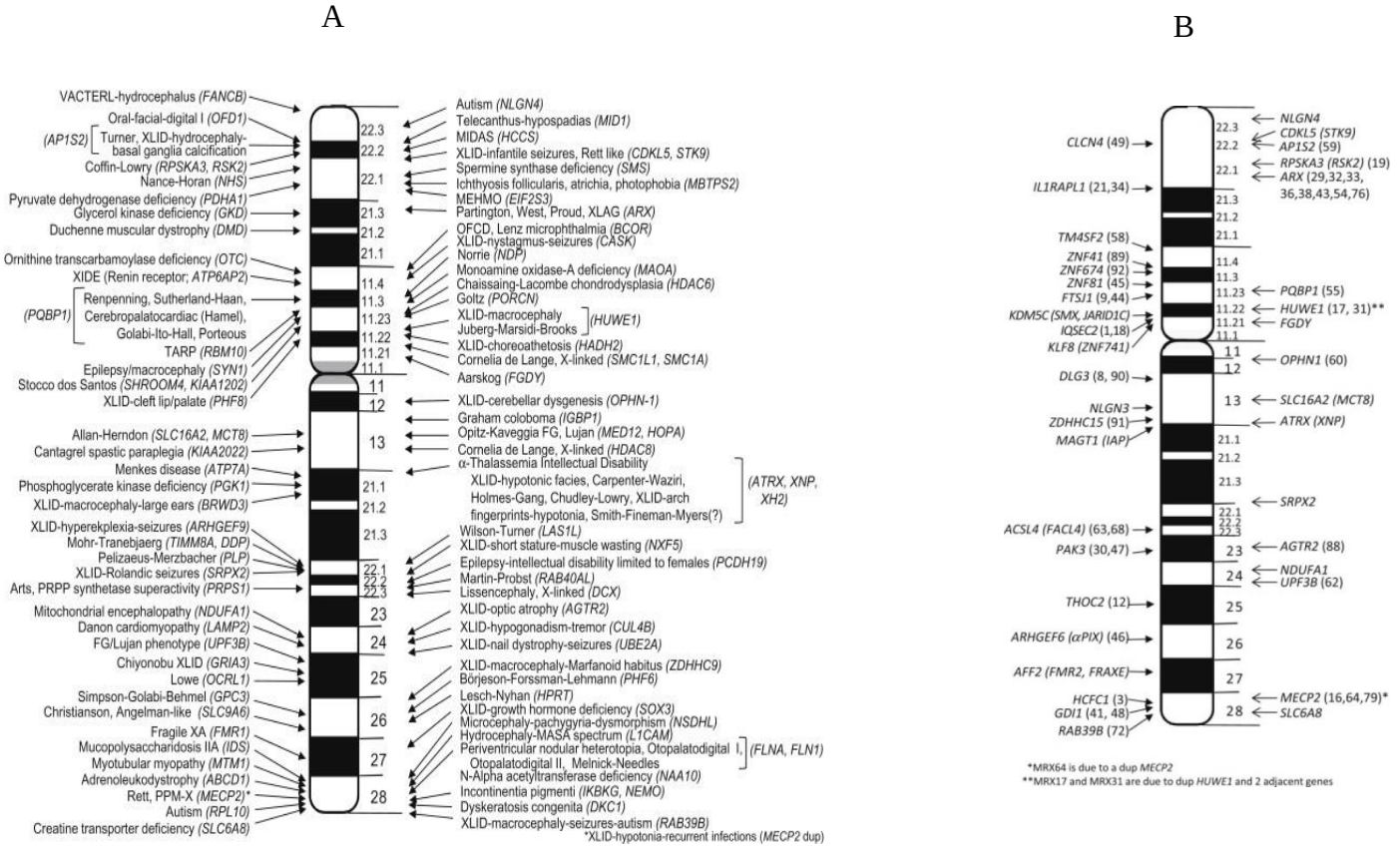


Figure 1.1 – A. Genes with identified mutations that cause S-XLID. B. Genes with identified mutations that cause NS-XLID. The genes listed on the right of the figure are associated with both S-XLID and NS-XLID. Reprinted with permission from Fragile X and X-Linked Intellectual disability: Four Decades of Discovery Lubs, Stevenson, Schwartz (2012)[17].

Approximately two thirds of XLID cases are thought to be non-syndromic[3]. Thirty-nine genes identified to be associated with NS-XLID are shown in **Figure 1.1B**[17]. The genes listed on the right of the figure are associated with both S-XLID and NS-XLID.

Unlike FXS, which is the most common form of syndromic ID, Fragile X E mental retardation (FRAXE), is a rare and non-syndromic form of ID. FRAXE is caused by triplet repeat expansions in the 5' UTR region of the Fragile X mental retardation 2 (*FMR2*) gene, which is approximately 600 kb downstream of the *FMR1* gene, and maps to Xq28. It is composed of 21 coding exons with several possibilities of alternative splicing for exons 2, 3, 5, 7 and 21[19]. The gene encodes a protein that localizes in neurons in the neocortex, Purkinje cells of the cerebellum and granule cell layer of the hippocampus[20]. It is highly expressed in the placenta and the adult and fetal brain[21].

As in FXS, FRAXE has a folate-sensitive fragile site, folic acid type, rare fra(X)(q28) E (FRAXE); however, the FRAXA and FRAXE fragile sites are undistinguishable from each other using cytogenetic methodology[22]. Both fragile sites, FRAXA and FRAXE, are not consistently expressed in all males with full *FMR1* and *FMR2* expansions.

FRAXE was first described in 1992 by Sutherland and Baker and initially was not considered to be associated with a disease phenotype. The identification of the *FMR2* gene in 1996 allowed for the link between FRAXE and ID to be recognized[23-25]. Since that time a total of 40 probands have been reported in the literature. **Section 1.3.8**

provides a complete an in-depth review of the literature with an emphasis on the phenotype of FRAXE positive cases.

Insight from the *Fmr2* knock-out (KO) mouse model indicates that *Fmr2* is expressed in several tissues besides the brain including bone, cartilage, hair follicles, lung, tongue, tendons, salivary glands and major blood vessels[26]. As well, *Fmr2* knock out mice were found to have impaired learning and memory. *FMR2* is part of a family of genes which also includes AFF1/AF4, AFF3/LAF4 and AFF4/AF5q31[27]. Redundancy within this family of genes may explain the fact that many males with *FMR2* full mutations have a mild to borderline phenotype[28].

As with FXS, FRAXE has four classes of *FMR2* repeats: normal (6 to 30 repeats), premutation (61 to 200 repeats) and full mutations (>200 repeats). There is some debate as to what is considered an intermediate allele, with some papers suggesting 31 to 60 repeats[29,30] and others 41 to 60 repeats[31,32].

In 1996, Brown estimated the prevalence rate of FRAXE to be rare at 1 in 50,000 males[33]. This rate was based on prevalence of FXS in the population and an expected ratio of FRAXE to FRAXA at approximately 4%. In 2000, Youings calculated an updated prevalence of 1 in 23,423 based on their five-year study in combination with a large sample set from another study, Crawford et al.[34].

Due to the differences in frequency between FRAXA and FRAXE in the population it is not surprising that the amount of clinical testing for FRAXE is much lower than for FRAXA. Just two years after *FMR2* was identified as the gene involved in FRAXE Brown made a strong case against routine FRAXE screening, stating that all

individuals thus far who had a FRAXE positive result had a positive Xq27 fragile site test but were negative for an *FMR1* expansion[33]. Therefore, FRAXE testing should be offered only when a fragile site was identified and *FMR1* was negative. Knight and colleagues (1996) felt that FRAXE was a relatively rare, but significant, cause of ID in the population and screening should be offered where appropriate.

1.3.2 Clinical Manifestations

1.3.2.1 Fragile X syndrome – Syndromic X-linked Intellectual Disability

Males with a *FMR1* full expansion, accompanied by aberrant methylation, typically have moderate ID. Following puberty, full expansion males often have a characteristic appearance. Distinct features include: a long narrow face with prominent forehead (macrocephaly), a prominent chin, a high arched palate, large, protruding ears and large testes (macroorchidism) after puberty[35]. A recent review of the literature by Ciaccio *et al*. (2017) demonstrated that full mutation males have a multi-system condition which includes musculoskeletal, cardiovascular, central nervous system, neuropsychiatric and eye features. Over 74% of full mutation males had electroencephalogram (EEG) anomalies, 50% had joint hypermobility, and 50% had pectus excavatum. Psychomotor delay and ID was seen in all patients. Ninety percent of individuals had aggressive behaviours, 74 to 84% had attention problems, 50 to 66% were hyperactive and 58 to 86% had anxiety disorder. ASD was noted in 30 to 50% of cases and 30% had sleep problems[15]. Approximately 30% of young children with FXS do not have obvious

dysmorphic features, however, delayed developmental milestones in young children, particularly in regard to psychomotor ability and speech are often apparent[15,35].

Females heterozygous for a full *FMR1* expansion may have the physical and behavioural features seen in full expansion males but at a lower frequency and with milder involvement than males[35]. Full expansion females have a 30% chance of having normal intelligence and a 25% chance of having ID (IQ <70)[15].

1.3.1.2 FRAXE – Non-syndromic X-linked Intellectual Disability

FRAXE is a rare cause of ID caused by expansion and methylation of the CCG repeats in the 5' UTR of the *FMR2* gene. Although the phenotype associated with FRAXE is not well defined, it has been reported to include: mild (IQ 50 to 69) to borderline (70 to 79) ID, learning problems, communication problems, and overactivity, with no consistent dysmorphology[36]. There have been very few reported females affected by FRAXE due to fully methylated expansions.

1.3.3 *FMR1*-related Disorders

A *FMR1* repeat size between 55 and 200 is considered a premutation and presents itself as a very different disease than FXS. *FMR1* premutations have been associated with fragile X-associated tremor/ataxia syndrome (FXTAS) in males and to a lesser extent in females. Females may also exhibit premature ovarian failure (POF). In the normal population 1 in 850 males and 1 in 300 females carry a *FMR1* premutation[18].

1.3.3.1 Fragile X-associated tremor ataxia syndrome (FXTAS)

FXTAS is a neurodegenerative disorder characterized by intension tremor, cerebellar ataxia, progressive neurodegeneration, parkinsonism and cognitive decline[37]. Individuals harbouring a premutation allele (55 to 200 repeats) of the *FMR1* gene have an increased risk of developing FXTAS later in life. Age is a significant risk factor for FXTAS development in males; approximately one third of male premutation carriers older than 50 are affected by FXTAS, and over 50% of affected by age 70 to 90 years of age. In comparison, FXTAS was detected in only 16.5% of female premutation carriers[38].

FXTAS has major and minor diagnostic criteria with both neuroradiologic and clinical signs. A definite diagnosis of FXTAS requires the presence of a premutation in the *FMR1* gene, as well as, white matter lesions in the middle cerebellar peduncles and/or brain stem on magnetic resonance imaging (MRI) (two major neuroradiologic signs), in addition to either intention tremor or gait ataxia (two major clinical signs). Individuals can also be given a probable diagnosis of FXTAS, which requires one major neuroradiologic sign and one minor clinical sign or two major clinical signs. A possible FXTAS diagnosis includes one minor neuroradiologic sign and one major clinical sign. Minor neuroradiologic signs include white matter lesions in the cerebral white matter on MRI and moderate to generalized atrophy. Clinical minor signs include parkinsonism, moderate to severe working memory deficits and executive cognitive function deficits[35].

1.5.3.2 Primary Ovarian Failure (POF)

Primary ovarian failure (POF), also known as, primary ovarian insufficiency (POI) is defined as the cessation of menses for 6 months before the age of 40 associated with follicle-stimulating hormone (FSH) levels observed in the menopausal range[39]. POF incidence is estimated at 1 in 1000 women under the age of 30, 1 in 250 women around 35 years old, and 1 in 100 women at 40 years of age. Overall, the prevalence of POF in the general population is approximately 1 to 2%[40]. A number of genes have been associated with POF including *FMR1* and *FMR2*; the transforming growth factor beta family members bone morphogenetic protein 15 (*BMP15*) and inhibin alpha (*INHA*); the gonadotropin receptors for luteinizing hormone (*LHR),* and FSH (*FSHR*); transcription factors such as forkhead box L2 (*FOXL2*) and forkhead box O3 (*FOXO3)*; the nuclear hormone receptors estrogen receptor alpha (*ESR1*) and beta (*ESR2*) and the orphan splicing factor 1 (*SF1*); and aromatose, or cytochrome P450 family 19 subfamily member 1 (*CYP19A1*)[41].

The prevalence of *FMR1* premutation females in the normal population is approximately 1 in 300[18] with the estimate of POF in *FMR1* premutation carriers ranging from 12.9% to 21%[42,43]. Women with *FMR1* full mutations do not exhibit POF. Exactly how the presence of *FMR1* premutations, but not full mutations, leads to ovarian dysfunction is unknown; however, the FMRP protein is highly expressed in the germ cells of the fetal ovary[44,45]. One theory is that elevated levels of *FMR1* may lead to increased oocyte development, resulting in an overall decrease in the number of oocytes. Another

theory is that the mRNA produced by the premutation alleles may have a toxic effect, leading to elevated levels of follicular atresia[41].

In a study on 507 women, *FMR1* repeat size was compared with ovarian dysfunction[42]. The authors found the highest prevalence of POF in women with 80 to 99 repeats, which they termed a *medium size premutation*. Individuals with higher premutation sizes (>100) and lower premutation sizes (59 to 79), exhibited significantly lower rates of POF, 12.5% and 5.9% respectively, in comparison to individuals with 80 to 99 repeats (18.6%)[42]. Furthermore, they found that carrying an intermediate or low premutation contributed to reducing the age of menopause onset. In addition, younger premutation women were similar to non-carriers with respect to FSH levels, suggesting that significant fertility problems may not be an issue for these women before the age of 30.

Table 1.1 – Summary of *FMR1* repeat size and associated disease phenotype in males and females

***FMR1* Repeat Size**	**Associated Phenotype Male**	**Associated Phenotype Female**
Normal (5 to 44)	Normal	Normal
Intermediate (45 to 54)	Normal	Normal
Premutation (55 to 200)	At risk of developing FXTAS	At risk of developing POF At risk of developing FXTAS
Full mutation (>200)	FXS (100% with ID)	~ 25% with ID ~ 30% with normal intellect

1.3.4 *FMR2* related associations

Unlike *FMR1*, no known diseases have been associated with premutations in *FMR2*. However, several research groups have investigated the effect of the four *FMR2* repeat allele classes on phenotype manifestations.

1.3.4.1 Intermediate Alleles and Learning Disabilities

Murray *et al*. (1998) tested 992 boys with learning disabilities and compared them to controls which were derived from the non-transmitting X chromosome of the mothers. The authors found a significant excess of *FMR2* intermediate alleles in the test population (p=0.036) suggesting a link between intermediate alleles and learning disabilities[39].

In 1999, Crawford and colleagues assessed the frequency of *FMR2* intermediate and premutations alleles in a large cohort of children between the ages of 7 and 10 (n=2,652) who attended special-education needs (SEN) classes in public school. The control group was the non-transmitting X allele from the mother. They found no excess of intermediate or premutation alleles in the SEN population and no significant difference between the cases and the controls[46].

Finally, in a screening of 276 boys aged 2 to 18 years with mental impairment or learning disabilities, three intermediate alleles (31 to 60 repeat sizes) were identified in the at-risk group compared with none in 207 controls; however, this difference did not reach statistical significance[31].

With conflicting evidence, it is difficult to determine if intermediate alleles in *FMR2* play a role in learning disabilities. In order to confirm or dispute the findings from Murray additional studies are needed in individuals with learning disabilities.

1.3.4.2 Intermediate Alleles and Parkinson's disease

A study by Annesi *et al*. (2004) examined 203 males with idiopathic Parkinson's disease (PD) and compared *FMR2* allele size with a healthy male cohort of 370 individuals. The authors did not identify any premutations or full mutations in either population, but did observe an excess of intermediate alleles (31 to 60 repeats) in the PD cohort which was statistically significant. In the Parkinson's cohort, thirteen intermediate alleles (6.4% [13/203]) were observed compared with only one in the controls (0.3% [1/370]). The authors also investigated the test population in terms of clinical phenotype including cognitive performances, but did not find a difference between those who had an intermediate allele and those with an allele in the normal range[29].

A follow up study in 2011 did not support the previous study's findings. Costa and colleagues (2011) tested 206 PD patients and 227 controls and compared allele sizes across both groups, defining intermediate alleles as being in the size range of 31 to 61 repeats. Although they found twice as many intermediate alleles in the test group (2.9% [6/206]) as to the control group (1.3% [3/227]), these frequencies did not reach statistical significance[32].

Overall the information available for *FMR2* intermediate alleles is limited and what is available is conflicting. More information is needed to determine if *FMR2* intermediate alleles have any phenotypic effect on an individual.

1.3.4.3 Microdeletions and Primary Ovarian Failure

It has been suggested that the *FMR2* gene may play a role in POF, particularly with respect to microdeletions involving the gene. In 1998, Murray's research group published their work on 147 women with POF, of whom 17% had familial POF, investigating the role of the *FMR1* and *FMR2* genes. They found 2% of chromosomes in the POF group carried *FMR1* premutations ($p = 4.3x10^{-3}$); 8% of chromosomes harboured *FMR1* premutations if the data was restricted to only include individuals with familial POF ($p = 0.0086$). No *FMR2* premutations or full mutations were identified in this cohort. They also noted that the POF group contained a significant excess of minimal alleles (<11 repeats) in *FMR2* in comparison to the controls ($p = 0.046$)[39]. As one woman showed a smaller *FMR2* amplicon than what would be expected from an allele with no CCG repeats, the authors speculated she must have carried a small deletion. A follow up investigation specifically interrogating *FMR2* microdeletions in a POF population identified six (2.8%) women with minimal *FMR2* alleles (allele size of ≤11); of these six, three had deletions within the gene[47]. In comparison, of 2,434 women in the control group, 41 had minimal alleles yet only one had an *FMR2* microdeletion. In the study group, two out of three deletions were near or included the *FMR2* transcription start site.

There have been no additional findings in the literature to support the theory that microdeletions or premutations in the *FMR2* gene cause POF. In two recent published review articles exploring the causes of POF, *FMR2* was listed as an associated gene[41,48]. However, both reviews only reference the original work by Murray[39].

Similar to the work published on intermediate alleles in individuals with learning disabilities and Parkinson's disease, more research is needed to determine if microdeletions in *FMR2* play a significant role in women with POF. For women who have POF, *FMR2* testing may be warranted, especially in there is a family history of ID or developmental delay.

1.3.5 Mechanism of Disease

Although FXS is classified as a syndromic form of ID and FRAXE as a non-syndromic form of ID, mutations in the *FMR1* and *FMR2* genes are similar in their mechanism of action - both are triplet repeat disorders with expansions of the polymorphic nucleotide repeat leading to gene silencing.

1.3.5.1 Triplet Repeat Disorders

A new class of genetic disease, termed triplet repeat disorders, was recognized in the 1990s[49,50]. These disorders are characterized by an expansion within the affected gene of a segment of DNA that contains a repeat of three nucleotides. In the general population, the repeat range in the associated gene are polymorphic however, lower

repeat numbers are typically observed. As the gene is passed from one generation to the next, the number of repeats is unstable (dynamic mutation), and can increase during replication leading to an alteration of gene expression and function[11]. These triplet repeat disorders can vary in the nucleotides associated with the repeat (e.g. CCG, CGG, AAG), the location of the repeat with respect to the gene (exon, intron, 5' UTR, 3' UTR), the mechanism of disease (gain-of-function, loss-of-function) and the inheritance pattern (autosomal dominant, autosomal recessive, X-linked). Trinucleotide repeat expansions account for 16 neurological disorders ranging from ID syndromes to neurodegenerative disease[51].

In terms of FXS, this is a loss-of-function triplet repeat disorder caused by expansions of the polymorphic CGG repeat in the 5' UTR of *FMR1* with an X-linked inheritance pattern. FRAXE is also a loss-of-function mutation with an X-linked inheritance pattern, however, it is caused by expansions of CCG repeats in the 5' UTR of the *FMR*2 gene. For both diseases, an expansion of repeats greater than 200 triggers methylation of the CpG islands in the gene's promotor. This hypermethylation causes the chromatin to condense preventing the binding of specific transcription factors and therefore silencing the gene[52]. It is the silencing of the gene and therefore loss of or reduction of the protein that causes the phenotype associated with FXS or FRAXE depending on the gene involved.

1.3.5.2 Deletions

Less than 1% of individuals with FXS have a partial or full deletion of *FMR1*[35]. Deletions in *FMR1* were first reported in 1992 through the identification of individuals with a Fragile X phenotype, but normal *FMR1* CGG repeat size[53,54]. Since that time, only a handful of additional cases have been observed with deletions involving *FMR1* ranging from 355 base pairs (bp) to 13 megabases (Mb)[52].

Like *FMR1*, there have been rare cases of mutations in the *FMR2* gene not associated with methylation/silencing of the gene. In 2007, Honda *et al*., reported two patients with chromosomal abnormalities involving the *FMR2* gene. The first patient, a 3 year old girl with mild ID, had a *de novo* balanced translocation, 46,XX,t(X;15) (q28;p11.2) resulting in a disruption between exon 3 and 4 of *FMR2*, with the intact X chromosome being predominantly inactive. This young girl had delayed development, was nonverbal and had febrile convulsions. The second child, a 23-month-old boy, had a deletion starting between exons 9 and 10 of *FMR2* and up to and including five other genes, one of which was iduronate 2-sulfatase (*IDS*), a gene known to be responsible for the lysosomal storage disease, Hunter syndrome. He presented as a floppy infant with coarse facial features, unusually enlarged tongue, low muscle tone and abdominal swelling. Although it was difficult to distinguish which features were associated with the *FMR2* deletion compared to the *IDS* deletion, there were several features that were present in this boy that have not been associated with Hunter syndrome, such as muscular hypotonia, hypothyroidism and gastroesophageal reflux[55].

A deletion resulting in the complete loss of exon 3 in *FMR2* was identified in two brothers with mild ID[56]. Both children had developmental delay in the areas of motor and language by their second year. Autistic features including impaired social interaction and communication as well as hand flapping, restricted interests and repetitive behaviours were noted. In both brothers, a 121 to 145 kilobase (kb) deletion in the *FMR2* gene was identified by array comparative genomic hybridization (CGH) platform (Agilent 244K). Their younger brother was unaffected by developmental delay, but did have mild attention deficit and hyperactivity disorder (ADHD), and he did not harbour the deletion. The mother was a carrier for the deletion and was considered unaffected. A maternal aunt and uncle, who both had mild ID, were also screened for the deletion using Multiplex Ligation-dependent Probe Amplification (MLPA); only the uncle was found to harbour the same deletion as the brothers. The maternal aunt's phenotype which was described as mild ID, was considered significantly different than the brothers and she had no behaviour abnormalities. The phenotype of the deletion-positive uncle was considered very similar to the brothers and he also exhibited autistic behaviours.

In another study, two unrelated males with microdeletions in the *FMR2* gene were identified using whole genome oligonucleotide array[57]. One patient had a loss of approximately 240 kb within exons 2 to 4 of *FMR2*. He presented at age 21.5 months with microcephaly. The parents reported that some early milestones were delayed, particularly speech. At 35 months, he was examined by a geneticist and had some dysmorphic features, including mild flattening of the occiput, prominent nasal tip, and bilateral fifth finger clinodactyly. The second patient had a 499.3 kb deletion that

included exons 1 and 2 of *FMR2*, along with an additional 343 kb segment upstream of *FMR2*. This patient also had a heterozygous 358 kb duplication of the 1q21.1 region. He was referred at 3 years 7 months of age with global developmental delay and behavioural problems and was not dysmorphic. He had substantial deficits in areas of social interaction, expressive and receptive language, and fine motor skills. He was withdrawn, unresponsive to affection, adverse to new situations and exhibited hand flapping. He had a positive family history for developmental delay, as his two older brothers had speech and language delays; they were unavailable for testing[57].

Several cases of deletions that involve both the *FMR1* and *FMR2* genes have been identified[58-62]; however, the phenotype associated with these deletions was attributed by the authors as being mainly due to the deletion of *FMR1*. Then in 2007, a female patient was reported who had ID and a large deletion which included *FMR1*, *FMR2* and *IDS*[63]. Because there was sufficient iduronate 2-sulfatase (*IDS*) enzyme in her blood, the authors concluded that her phenotype was due to the deletion of *FMR1* and *FMR2*.

Recently, a male and his mother have been reported with a deletion that included *FMR1*, *FMR2*, and *SLITRK2* (the *SLIT* and *NTRK*-like family member 2). This deletion was located within a paracentric inversion[64]. This male was described as not having the typical fragile X facial dysmorphology.

In summary, when a deletion spans multiple genes, it is difficult to determine which genes contributed to which phenotypic features of the patient in question, especially when such events are rarely seen. Overall, deletions are an uncommon cause of FXS and FRAXE, but an important consideration when a patient presents with

unexplained ID and phenotypic features of these diseases. As microarray analysis becomes a more routine diagnostic test, more deletions may be identified. Moreover, whole genome sequencing (WGS) can more precisely define the breakpoints of deletions than microarrays and can identify structural rearrangement of the genome that are missed by other technologies. Hence over the next few years, there may be more reported cases that involve deletions and other structural variants (e.g. inversions) that involve *FMR1* and *FMR2*.

1.3.5.3 Point Mutations

Point mutations account for less than 1% of reported FXS cases[35]. The first report of an *FMR1* point mutation associated with disease was in 1993 in a large Dutch family affected with X-linked liver glycogenosis[65]. One individual within this family presented with a large forehead, asymmetric long face, large ears, thick lips and macroorchidism without the presence of a fragile site, expansion of the *FMR1* gene or hypermethylation. Sequencing of *FMR1* identified a point mutation, c.1100T>A, resulting in a missense mutation (p.Ile376Asn). This was determined to be a *de novo* mutation as it was not present in the parents or any of the neurotypical siblings[65]. No functional work was presented in the paper to support this genotype-phenotype relationship.

More recently, a point mutation in *FMR1* (c.797G>A; p.Gly266Glu) was identified in a 16 year old male with FXS[66]. The characteristic Fragile X dysmorphic features were noted in conjunction with disruptive outbursts, ADHD and ASD. Algorithms predicted this highly conserved amino acid change as damaging. Functional

analysis using an *Fmr1* KO mouse model, western blotting, and co-immunoprecipitation showed the mutated protein could not perform normal key functions including mRNA binding and polyribosome association, suggesting that the variant was responsible for the phenotype of this patient[66].

In 2014, Handt *et al.* published a cohort of 508 males who met the criteria of Fragile X-like phenotype, including ID/developmental delay, and who were negative for *FMR1* and *FMR2* full mutations. These males were tested for point mutations in *FMR1* using high resolution melting analysis, and only two missense variants were identified in three clinically affected males. The first boy, who had psychomotor delay, language impairment and attention issues as well as above average height (98^{th} centile) carried a c.1444G>A (p.Gly482Ser) variant. The other two unrelated boys carried a c.1601G>A (p.Arg534His) variant; their phenotype was similar with respect to developmental delay and learning difficulties. One of these two boys also exhibited low attention span and behavioural outbursts and was macrocephalic with height and weight close to the 97^{th} percentile[67]. The authors described all three boys as having a FXS like phenotype. The relevance of these variants requires further functional work to determine potential pathogenicity.

Overall, *FMR1* point mutations appear to be rare leading to less than 1% of the FXS observed in the population. However, testing for point mutations may be warranted for males presenting with a phenotype consistent with FXS who have an *FMR1* repeat size that is within the normal range.

There have been no reported cases of point mutations causing disease in *FMR2*. The literature on FXS is vast in comparison with FRAXE publication, and given the rarity of *FMR1* point mutations, it is possible that *FMR2* point mutations do contribute to the FRAXE phenotype but have not been reported in the literature.

1.3.6 Genetic Phenomenon Associated with Fragile X

1.3.6.1 X-inactivation

The mode of inheritance also contributes to the complicated etiology of FXS and FRAXE. X-linked diseases are caused by mutations in genes that are found on the X chromosome. A karyotypically normal female carries two X chromosomes. In order to compensate for gene dosage, one of the two X chromosomes is inactivated early in embryonic development, through a methylation process called X-inactivation. Under normal circumstances, the selection of which X is inactivated is random. The inactivated X chromosome can be observed cytologically as a heterochromatin mass called the Barr body.

If a female carries a mutation on one of her two X chromosomes and random X-inactivation occurs, one expects to see the normal allele expressed in approximately 50% of her cells and the mutant allele expressed in the other 50%. This is known as random X-inactivation. This explains why the majority of females who carry a mutation in an X-linked gene are "healthy carriers". Females who are carriers of an X-linked mutation occasionally have disease manifestations, and the commonest mechanism through which

this occurs is skewed X-inactivation. In this scenario, the X chromosome with the normal allele is silenced in more than 50% of the cells. X-inactivation studies are typically performed on lymphocytes, and females with manifestations of X-linked disorders typically have a ratio of at least 90% to 10%[12].

As illustrated in **Table 1.1** 25% of females with *FMR1* full expansions have ID, indicating that most females with full expansions do not have FXS. The associated phenotype, or lack thereof, in females is believed to be strongly connected to their X-inactivation pattern[15]. In a recent case report, two daughters affected with FXS were found to carry a full *FMR1* expansion with random X inactivation[68]. Their mother, who also carried a full expansion but who was phenotypically normal, had a non-random X inactivation pattern with the normal allele being predominantly active.

There have been very few reports of females with full expansions of *FMR2* who are affected with FRAXE. In one French family with seven individuals segregating full expansions, one female was identified to be severely affected[69]. This child had idiopathic West syndrome (infantile spasm syndrome), speech delay and mild to moderate ID (IQ 50 to 60). She carried an expansion of 800 repeats, and had 22 to 28% cytogenetic fragile site expression. In comparison, her mother did not have ID or psychological problems yet had a *FMR2* expansion of 750 repeats, with 12-15% fragile site expression. In contrast, in another study of two large families with FRAXE, four phenotypically normal females were identified who carried full *FMR2* expansions that were fully methylated[70].

1.3.6.2 Anticipation

Anticipation, the phenomenon whereby the symptoms of a disease appear earlier or in a more severe form from one generation to the next generation, is another common feature of triplet repeat disorders. The size of the repeat is often an indicator of the severity of the disease, and in some cases, is also associated with its instability. In general, premutations are often not large enough to cause disease, but are large enough that they may become unstable when passed from one generation to the next[12]. The sex of the transmitting parent often also plays a role in the stability or more importantly, the instability of a premutation.

In FXS, full expansions are only transmitted to offspring maternally, either by transmission of a full mutation allele or through expansion of a premutation allele[18]. Males with a premutation only transmit a premutation allele to their daughters. As males with FXS are severely affected, there is little data on male transmission of full expansions to their offspring; however, in a small study of four males with full expansions, only premutations were observed in their sperm[71].

1.3.6.3 Mosaicism

Another feature associated with X-inactivation which can be observed in these diseases is mosaicism, in which two or more populations of cells with different genotypes exist within one individual. Mosaicism has been noted in males who have two or more different cell populations for either *FMR1* CGG or *FMR2* CCG repeats. Males who are size mosaics can have both a full mutation and premutation size repeat detected in blood.

Males who are methylation mosaics have also been reported. These individuals carry full mutations in both a methylated and unmethylated state[72]. Mosaicism for full mutations and premutations has been reported to occur in 12 to 41% of males with FXS[72,73]. This information is not available for *FMR2*.

1.3.7 Testing for *FMR1* and *FMR* 2 Mutations

Historically, Fragile X positive patients were identified by the presence of a fragile site on Xq27.3 following exposure of cultured cells to a folate deficient medium. At that time, the *FMR2* gene had not been identified, its association with disease was not known, and it was not possible to distinguish between the *FMR1* and *FMR2* fragile sites using cytogenetic means. With the identification of the *FMR1* gene in 1991[74-77], came a new approach for testing for FXS – Southern blots.

Southern blotting is a technique for detecting specific DNA sequences by transferring restriction enzyme treated DNA fragments that have been separated by gel electrophoresis onto a filter membrane. This is then hybridized with a radioactive labelled probe such as StB12.3 or Ox1.9[12]. First described by Rousseau *et al*. (1991), double digestion by restriction enzymes such as EcoRI (non-methylation sensitive) and EagI (methylation sensitive) allows for the identification of a *FMR1* and *FMR2* expansion, as well as, associated methylation status. All wild type males have an EcoRI-EagI fragment of 2.8 kb, whereas all wild type females have two fragments – the 2.8 kb fragment and an inactive 5.2 kb fragment. EagI is inhibited by DNA methylation, and therefore, a full mutation male sample treated with EagI will not be digested by EagI and will be greater

than 5.2 kb in size[78]. Although Southern blotting has good sensitivity for detecting expansions, it does not give a precise repeat size. Southern blotting is also a labour intensive and expensive methodology with a long turnaround time that uses radioactivity, requires a large amount of DNA, and is technically difficult.

For these reasons, polymerase chain reaction (PCR) has been the test of choice for FXS and FRAXE for a number of years. PCR can detect allele sizes within the normal, intermediate, and small premutation range, therefore eliminating the need for Southern blotting for a large number of *FMR1* and *FMR2* negative patients. Larger premutations and full mutations cannot be detected by PCR as they fail to amplify; therefore, any male sample that does not produce an amplicon, or female samples who appear homozygous in the normal range, must undergo further investigation using Southern blotting.

Recently, several companies have produced kits that allow for the amplification of the CG-rich repeat sequences. The most widely used is Amplidex™, produced by Asuragen, which can accurately detect full mutations up to 1,300 CGG repeats[22] (Asuragen, Austin, TX USA). This assay was approved for clinical use in Canada in 2013 and is currently used in many of the clinical laboratories across the country. Although Amplidex™ detects repeats in the full mutation range, it can only accurately size repeats in the premutation range (<200 repeats) and cannot detect methylation status, therefore, Southern blot is still a necessary step to determine methylation status. The Amplidex™ kit is only used for interrogating the *FMR1* gene and the company does not produce a similar kit for *FMR2*.

Another technique, Methylation-specific Multiplex Ligation Dependent Probe Amplification (MS-MLPA) can be used to investigate the methylation status of the CpG islands in the 5' UTR of the *FMR1* and *FMR2* genes (MRC-Holland, Amsterdam, Netherlands). The MS-MLPA ME029 kit can only detect methylation of the *FMR1* and *FMR2* gene in males and cannot indicate repeat size. The kit also tests for deletions or duplications for some of the exons of *FMR1* and *FMR2*.

As technology advances, new methodologies for investigating the *FMR1* and *FMR2* genes will emerge. For now, standard protocol for most laboratories is PCR, followed by Southern blot, if warranted.

1.3.8 FRAXE – An Elusive Phenotype

1.3.8.1 FRAXE Studies in the Literature

36 studies screening various cohorts for *FMR2* expansions have been published. The at-risk populations screened have included children (male and female) with ID and/or academic difficulty and individuals with ASD, obsessive compulsive disorder (OCD), Parkinson's disease (PD), and premature ovarian failure (POF) (**Appendix L - Supplementary Table S.1**). Individuals with *FMR2* full mutations were identified in eight of these 36 studies, specifically in seven ID cohorts and one OCD cohort with a frequency of 0.027% to 3.85%. A summary of the positive FRAXE findings are shown below.

Table 1.2 – Summary of the FRAXE male positive cases identified in *FMR2* studies in the literature

FRAXE Screening Studies							
Ref	**# of Individuals**	**Normal**	**Intermediate**	**Premutation**	**Mutation**	**Cohort Criteria**	**Origin**
79	911 M 903 / F 8	908			1^{+} (+3)	Negative for *FMR1* expansions	United Kingdom
80	105 (sex N/A)				1	ID patients	Northeast Italy
81	180 (sex N/A)	179			1	ID (unknown etiology)	Italy
82	222 M 182 / F 40			0	1	4-20 yrs in SNS for ID	Spanish
83	232 (sex N/A)				1	ID patients	Italian
34	3738 All Male	3687	41	2	1	boys 5-18 yrs in SNS	England
84	114 M 73 / F 41				1	9-16 yrs in SNS with mild-mod ID	Croatia
85	26 M 15 / F 11	25			1	males & females with OCD	Utah, US

M = males; F = females; N/A = unavailable; SNS = special needs school; yrs = years; OCD = obsessive compulsive disorder. When available, the number of individuals tested is broken down in male and female. [+] Reference 79: Two related females were found to harbour an *FMR2* expansion; only one positive *FMR2* expansion was identified in the screened cohort, however, the article reports on three additional FRAXE positive cases; Most authors used the term MR (mental retardation) when the paper was published and, as this is no longer acceptable terminology, MR has been changed in the above table to ID.

1.3.8.2 FMR2 Positive Cases

Since the *FMR2* gene was identified in 1992[86], 40 probands have been reported in the literature (**Table 1.3**)[34,69,70,79-85,87-98]. A total of 80 full *FMR2* expansion males and 71 full *FMR2* expansion females have been identified in these 40 families.

Table 1.3 – Summary of FRAXE positive male probands in the literature and full expansion alleles identified in their family members

Ref	# of Probands (male)	Type of Report: Family or Case Study	# of Full Expansion Male Relatives	# of Full Expansion Female Relatives
87	2	Family 1	4	6
		Family 2	2	0
88	1	Family	9	7
89	2	Family 1	2	9
		Family 2	4	7
90 +	4	Family 1	2	1
		Family 2	4	2

Ref	# of Probands (male)	Type of Report: Family or Case Study	# of Full Expansion Male Relatives	# of Full Expansion Female Relatives
		Family 3	1	2
		Family 4	1	2
91	4	Family A	1	2
		Family B	2	0
		Family C	5	1
		Family D	1	3
92 +	3	Family 1	2	5
		Family 2	4	5
		Family 3	2	5
79	4	Case	4	N/A
80 ^	1	Case	1	N/A
81 ^	1	Case	2	0
93	3	Family A	3	1
		Family B	1	1
		Family C	2	1
82	1	Case	1	N/A
70	2	Family 1	3	4
		Family 2	1	1
94	2	Case	1	1 *
		Case	1	N/A

Ref	# of Probands (male)	Type of Report: Family or Case Study	# of Full Expansion Male Relatives	# of Full Expansion Female Relatives
83	1	Family	2	2
95	1	Case	1	N/A
34	1	Case	1	N/A
96	2	Case	2	0
84	1	Case	1	N/A
97	1	Family	1	1
69	1	Family	3	3
85	1	Family	2	0
98	1	Case	1	N/A
Total	**40**		**80**	**71**

[+] Reference [90] - Family 5 and 6 not listed here; description available in reference [92] (equivalent to Family 1 and 2). ^ These articles are from the same research group and it was not possible to determine if they referred to the same or different probands. ***** Not part of a family but a separate case (female) with a *FMR2* expansion. N/A = not available or not applicable.

Each article was reviewed and all information regarding the phenotype for each positive FRAXE male was recorded in a spreadsheet. Two articles provided no phenotypic information about the proband[34,80]. A condensed version of this chart is shown below (**Table 1.4**). Information was recorded based only on the description given by the author.

A blank field indicates that this information was missing from the description provided in the paper. If an individual was recorded as having no dysmorphic features this is indicated as 'no' under the dysmorphic heading; if there was no information regarding this feature, that field is blank. If the author described clinical features of the individual, but considered the person to be non-dysmorphic, this is also indicated. The presence of dysmorphism was commented on in 26 cases and 20/26 (77.9%) were considered to be nondysmorphic. A more detailed description of the dysmorphic features is summarized in **Table 1.5**.

Although IQ and ID can be overlapping terms and IQ values can be used to determine ID, to avoid confusion they are recorded separately. In some articles, mild ID may have been reported, however, formal testing may not have occurred. The ID category is not inferred from the IQ testing value but instead refers to the authors' specific comments on the individual's level of ID. For most of the literature the term used was not ID but mental retardation (MR). As MR is no longer considered socially acceptable and has recently been replaced by the term ID, ID is indicated in the summary table below instead. In many instances, the authors referred to the disability as mild or severe MR.

IQ scores were reported in 28 cases with a range of 45-128 and mean of 69. The presence or absence of ID was recorded in 41 cases: 2/41 (4.9%) had borderline ID, 12/41 (29.3%) had mild ID, 5/41 (12.2%) had moderate ID and 5/41 (12.2%) had severe ID. Moderate to severe ID was recorded for one case (1/41 [2.4%]), and four cases (4/41 [9.8%]) were just reported as having ID but the level of ID was not defined. Normal intelligence was recorded in 11/41 (26.8%) cases. One case (1/41 [2.4%]) was recorded as

having variable ID, but details were not specified. Combining all instances of ID, from borderline to severe, 73% of the cases (30/41) had some level of ID.

The learning category indicates whether the affected individual was reported as having any difficulties in school. The abnormalities ranged from mild learning difficulties, such as alternative curriculum pathways for specific subjects, to attending a special school for individuals with ID. The presence or absence of learning problems was commented on for 29 of the cases, of which 27/29 (93.1%) reported there was some degree of academic difficulty. Of the 27 individuals reported to have learning problems, ten attended a special school (37%) for children with learning problems and/or ID.

The language and speech category includes any problems related to communication such as delayed speech, stutter, repetitive speech and echolalia. If the individual just had delayed speech, that later resolved with age or therapy, this is indicated in the table as “delayed speech”. Language and/or speech abnormalities were recorded in 33/78 (42.3%) of the cases. In two cases, the language issue was delayed speech that resolved as the child got older.

Fifteen individuals (15/78 [19.2%] had features of ADD or ADHD – three individuals were recorded as overactive, ten cases had attention problems and two males were recorded to have both overactivity and attention difficulties.

Behavioural problems were noted in 17 cases and a description of the behavioural issue was noted in the comment section, if available.

Although only two cases were reported as having a formal diagnosis of ASD, several authors reported autistic features in their cases. Combining the cases described as

having socialization deficits, hand mannerisms and/or echolalia with the cases described as having autistic features or ASD, fifteen individuals had features commonly associated with ASD. The role of FXS in ASD is well documented and may be an important point to consider for FRAXE as well[99].

Table 1.4 – Condensed phenotype review of all *FMR2* full mutation males in the literature

Reference	Individual ID from Paper	Dysmorphic	IQ	ID	Learning	Lang/Speech	Overactivity	Attention	Developmental delay	Behavioural	*FMR2* Repeat size	Comment
87	F1 IV-8	N		Mild	Y Mild					Y Sev		obesity
	F1 III-8 (uncle)									Y		
	F1 III-21 (Rel)			Nor	Y SS							
	F1 II-6 (GU)					Y				Y		quick temper abn. socialization
	F2 III-1	N			Y SS	Y						
	F2 I-2 (GR)	N		Nor								
88	P II-5										800 bp M	obesity
	II-4										800 bp M	obesity
	II-8											
	II-10										120, >760 M	
	II-19		65	Sev				Y Sev				obesity

Reference	Individual ID from Paper	Dysmorphic	IQ	ID	Learning	Lang/Speech	Overactivity	Attention	Developmental delay	Behavioural	*FMR2* Repeat size	Comment
	III-1				Y SS						265 M	obesity
	III-3				Y	Y					>1000 bp M	obesity
	III-11											
	III-18		64	Sev				Y Mild				
89	P F1-ID8	N		Mild							200 M	
	F1 – ID10 (Cous of 1)	N		Nor							~ 2.3 kb M	
	P F2-ID3	N		Var	Y	Y Mild				Y Sev	~ 6.5 kb	obesity abn. socialization
	F2-ID10 (GU)				Y SS					Y	1.5 kb	delayed milestones autistic features
	F2-ID11				Y						1.3 kb	
	F2-ID12				N	Del						abn. socialization
90	F1 I-1 (GF)		53	Y		Y					Δ 1.2-1.8 FM	"nervous breakdown" stutter
	F1 III-2 (GS)		104	Nor								
	P F2 IV.1	N		Mild	Y SS						Δ 1.3	

Reference	Individual ID from Paper	Dysmorphic	IQ	ID	Learning	Lang/Speech	Overactivity	Attention	Developmental delay	Behavioural	*FMR2* Repeat size	Comment
	F2 IV.2 (BR)			Nor							Δ 1.1 PM	
	F2 IV.3 (BR)			Nor	Y						Δ 0.9, 2	
	F2 III.5 (uncle)			Nor							Δ 0.8-2.8	
	P F3 II.1			Mild		Y				Y	1 kb FM	autistic features sexually overactive intrusivism aggressive
	P F4 III.1			Y							1.3-3.1	multiple spider angiomata
91	FA II-3			Mild							200-1015	
	P FB II-1			Mod-Sev			Y				590 FM	XXY karyotype
	FB II-3		70	Mild		Y			Y		450 and 600	psychomotor delay
	FC II-1		68	Mild	Y	Y					225 PM	
	FC II-3			Bord							155 PM	
	P FC II-8			Mild		Y					315 FM	
	FC II-9			Mild		Y		Y			415-815 FM	

Reference	Individual ID from Paper	Dysmorphic	IQ	ID	Learning	Lang/Speech	Overactivity	Attention	Developmental delay	Behavioural	*FMR2* Repeat size	Comment
	FC II-10					Y	Y				515 FM	
	FD II-6	Y	70			Y					350-750 PM	psychomotor delay
92	P F1 - II-6			Mod							1-2 kb FM	
	F1 -II-9 (Cous)			Mod							0.8 kb FM	
	P F2 III-1	Y		Sev			Y				6.2 and 8 kb M smear	
	F2 III-4 (BR)		62	Mod	Y		N				5.6 and 7.2 kb M 3.1 kb UM mosaic	
	F2 II-7 (uncle)			Mild	Y SS						5.8, 8 kb M smear	
	F2 II-8 (uncle)			Mild							6.8, 8 kb smear M	
	P F3 II-3			Sev			N				6.8 kb M	
	F3 III-3 (Nep)			Nor							6 to 9.5kb smear M	
79	C1	Y			Y SS	Y			Y		~ 1 kb	
	C2	N			Y SS	Y				Y		clumsy poor coordination hand mannerism

Reference	Individual ID from Paper	Dysmorphic	IQ	ID	Learning	Lang/Speech	Overactivity	Attention	Developmental delay	Behavioural	*FMR2* Repeat size	Comment
	C3	N	66		Y	Y						
	C4				Y	Y			Y	Y	Δ ~ 2.9 kb	aggression autistic features social integration sensory awareness
81	C		68	Y		Y Sev					900-1600 bp M	
	BR	N	92	Nor							600, 1200 bp FM	
93	FA-III-1	N	54	Bord		Y		Y		Y	3 kb	OCD autistic features
	FA-III-2 (BR)	N	50			Y		Y		Y	1.4 kb	aggression autistic features
	FA III-3 (BR)	N	54					Y			2.2 kb	
	FB II-1	N	65			Y	Y	Y		Y	1.8 kb	social withdrawn depression immaturity somatic complaints scizoidness aggression autism
	FC III-1	N	88		Y Mild	Y	Y	Y	Y	Y	1.8 kb	OCD aggressive

Reference	Individual ID from Paper	Dysmorphic	IQ	ID	Learning	Lang/Speech	Overactivity	Attention	Developmental delay	Behavioural	*FMR2* Repeat size	Comment
												delayed echolalia repetitive speech
	FC III-2 (BR)	N			Y	Y					2.2 kb	IgG3 subclass deficiency
82	C	Y	52		Y SS	Y			Y	Y	100 +/- 30 Mos M	aggressive abn. socialization personality disorder
70	P F1 III-7		46	Mod	Y Sev	Y		Y Sev			1700-1900bp smear FM	obesity
	F1 III-9 (Cous)		N/A								1300, 1700bp FM	
	F1 III-3 (Cous)		61	Mild	Y Mild			Y Mild			500, 1300bp Mos	
	F 2 II-1			Mod								obesity
94	C1		69									
	C2		73									
83	P	Y	++	Sev							>400 FM	epilepsy
	Twin	N	128	Nor							>400 FM	
95	C		62		Y	Y					N/A	abnormal EEG
96	C1		45			Y			Y			epilepsy abnormal EEG
	C2		71	Y						Y		schizophrenia

Reference	Individual ID from Paper	Dysmorphic	IQ	ID	Learning	Lang/Speech	Overactivity	Attention	Developmental delay	Behavioural	*FMR2* Repeat size	Comment
												abnormal EEG
84	C		Mild			Y				Y	N/A	epilepsy
97	II-4	N		Nor	Y SS	Y					N/A	
69	II-7	Y	52		Y	Y		Y	Y	Y	630-1150 PM	psychological aggression abn. socialization anxiety
	III-2	N	94 Nor		Y	Del		Y			600-1000 PM	psychological obsessive fears
	II-6				N						420 PM	abn. socialization
85	P1				Y						>300 M	OCD
	Uncle to 1					Y					~ 630-1200 M	
98	P	N	85 low Nor			Y			Y	Y	>200 M	autism

Table legend. [++] Could not be evaluated; Y = Yes; N = no; Nor = normal; Bord = borderline; SS = special school; Var = variable testing result seen; Mod = moderate; Sev = severe; Del = delayed; P = proband; Rel = relative; GU = great uncle; GF = grandfather; BR = brother; Nep = nephew; F=family; C= case; FM = full methylated; PM = partial methylation; Mos = mosaic; OCD = obsessive compulsive disorder; EEG = electroencephalogram. Note: a blank field indicates that this information was missing from the description provided in the publication.

Of the 80 full expansion males reported in the literature, the presence or absence of dysmorphic features was commented on for 39 males (48.9%) from 40 families. Authors included an overall statement about dysmorphology in 15 cases, six of whom were classified as dysmorphic (including one mildly dysmorphic). The published descriptions were reviewed by a geneticist (BF). Based on the presence of multiple minor physical abnormalities described in the manuscript, the geneticist classified certain additional individuals as dysmorphic, which is indicated in the table below as "dysmorphic based on the reported features". This resulted in an additional 11 cases being classified as dysmorphic. Therefore, the total number of males that were dysmorphic were 17/39 (43.6%). Of the seventeen cases that were dysmorphic, 9/17 (52.9%) had at least two dysmorphic features that are common features of FXS (macrocephaly/tall broad forehead, large ears, long facies). The description of the dysmorphic features reported by the authors for the full expansions males is given below (**Table 1.5**).

Table 1.5 – Dysmorphic features observed in 39 FRAXE full expansion males from 80 full expansion males reported in the literature

Ref	Individual ID from publication	Dysmorphology	Dysmorphic (as per the authors) ^	Dysmorphic Based on Reported Features	At least two FXS features
87	Fam1 IV-8 Proband	• narrow and high arched palate	No		
	Fam1 III-8 (uncle)	• broad forehead			
	Fam2 III-1 Proband	• high, broad forehead • epicanthic folds	No		
88	II-5 Proband	• macrocephaly • long narrow face		Yes	Yes

Ref	Individual ID from publication	Dysmorphology	Dysmorphic (as per the authors) ^	Dysmorphic Based on Reported Features	At least two FXS features
		• midfacial hypoplasia • high arched palate			
	II-4 (twin brother)	• macrocephaly • long narrow face		Yes	Yes
	II-8 (cousin)	• macrocephaly • midfacial hypoplasia			
	II-10 (cousin)	• macrocephaly • midfacial hypoplasia			
	II-19 (cousin)	• macrocephaly • long narrow face • midfacial hypoplasia • high arched palate • long neck		Yes	Yes
	III-1 (nephew)	• macrocephaly • long narrow face • midfacial hypoplasia • high arched palate • long, narrow ears • prominent jaw • long neck		Yes	Yes
	III-3 (nephew)	• macrocephaly • long narrow face • midfacial hypoplasia • high arched palate • large ears • long neck • height (>3rd centile) • weight (>3rd centile)		Yes	Yes
	III-18 (cousin's son)	• long narrow face • midfacial hypoplasia • high arched palate • long neck		Yes	No
89	Fam1 ID8 Proband	• encephalopathy	No		
90	Fam1 I-1 (grandfather)	• macrocephaly • narrow lower face • prominent eyebrows • large ears • Dupuytryn contracture		Yes	Yes
	Fam1 III-2 (grandson)	• high arched palate			

Ref	Individual ID from publication	Dysmorphology	Dysmorphic (as per the authors) ^	Dysmorphic Based on Reported Features	At least two FXS features
	Fam3 II.1 Proband	• macrocephaly • hypotelorism • epicanthic folds • small ears • prominent upper incisor • high arched palate • nipples were low placed • small hands/feet		Yes	No
91	FamA II-3 Proband	• cleft lip/palate		No	No
	FamD II-6 Proband	• hypertelorism • broad nasal bridge • anteverted nares • protruding tongue	Yes	Yes	No
92	Fam2 III-1 Proband	• brachycephaly with normal head circumference • broad midface • narrow sloping forehead • prominent eyebrows • mild telecanthus • high arched palate • clinodactyly • narrow chest with pectus deformity (excavatum of lower sternum and carinatum of upper sternum) • blepharophimosis • broad nose and nares • wide nasal septum • mild kyphoscoliosis	Yes	Yes	No
	Fam2 III-4 (Brother)	• brachycephaly • narrow sloping forehead • prominent eyebrows • broad neck • telecanthus, left • epicanthic fold • slight micrognathia • broad nose and nasal bridge with anteverted nostrils		Yes	No

Ref	Individual ID from publication	Dysmorphology	Dysmorphic (as per the authors) ^	Dysmorphic Based on Reported Features	At least two FXS features
		• wide nasal septum • pectus deformity (excavatum of lower sternum and carinatum of upper sternum) • mild kyphoscoliosis			
	Fam3 II-3 Proband	• microcephaly • brachycephaly • long narrow face • narrow forehead • flattened occiput • testis abnormal (vl l-50ml, r-35 ml) • high arched palate • pectus excavatum • carinatus			
79	Proband	• microcephaly • stellate irides • thick lips and long upper lip • misplaced teeth • height (< 3rd centile) • weight (< 3rd centile) • hoarse voice	Yes (Mild)	Yes	No
	Proband	• clinodactyly • café-au-lait	No		
	Proband	• 2 white patches in hair	No		
	Proband	• height (>97th centile) • macrocephaly			
81	Proband	• midface hypoplasia • hypotelorism • epicanthic folds • micrognathia • abnormal dental morphology • clinodactyly			
93	FamC III-1 (Brother of Proband)	• small, simple pinnae • failure to thrive	No		
	FamC III-2		No		
82	Proband	• Marfan like • long narrow face	Yes	Yes	No

Ref	Individual ID from publication	Dysmorphology	Dysmorphic (as per the authors) ^	Dysmorphic Based on Reported Features	At least two FXS features
		• high arched palate • low posterior hairline • thoracic scolosis			
70	Fam1 III-7 Proband	• midfacial hypoplasia • blepharophimosis			
	Fam1 III-9 (cousin)	• midfacial hypoplasia			
	Fam1 III-3 (cousin)	• long narrow face • midfacial hypoplasia • long nose			
	Fam2 II-1 Proband	• macrocephaly • long face • midfacial hypoplasia • blepharmophimosi		Yes	Yes
83	Proband	• long face with high forehead • prominent jaw • mild hypertelorism • strabismus • low set ears • hypoplastic alae nasi • prominent lower lip • high arch palate • crowded teeth • narrow sloping shoulders • longer slender hands/fingers	Yes	Yes	Yes
	Twin Brother		No		
96	Proband	• cleft palate			
84	Proband	• prominent high forehead • large prominent ears		Yes	Yes
69	II-7 Proband	• hypertelorism • unfolded left ear	Yes	Yes	No
	III-2 (cousin's son)	• flat feet • length asymmetry of limbs	No		
	II-6 (Brother)	• cleft lip			

^ Comment from author if the individual was dysmorphic or not. Blank cell = no comment from authors.

1.8.2.3 Repeat range in the Published Data

A review of the literature revealed the most common *FMR2* allele size was 15 with a mode range of 13 to 20 repeats depending on the population examined[29,30,46,89,100-110].

Table 1.6 – Repeat range of the *FMR2* allele observed in the literature

Reference	Population	Repeat Range		Mode
89	At risk	6	25	15
109	At risk	7	35	16
	Control	10	23	13
100	At risk	3	35	15
111	At risk	7	25	16
30	At risk	8	87	15
	Control	8	41	15
102	At risk	5	38	15
101	At risk	4	39	16
	Control	12	34	16
103	At risk	N/A	N/A	16-18
104	At risk	N/A	N/A	16
110	At risk	1	43	16
46	At risk	7	54	17.59
105	Control	5	44	15
106	At risk	8	48	20
107	At risk	3	27	15

Reference	Population	Repeat Range		Mode
	Control	8	27	15
108	Control	N/A	N/A	15
29	At risk	5	54	14
	Control	11	37	14

1.4 Study Rationale and Objectives

In the Canadian province of Newfoundland and Labrador (NL), prior to initiation of the current study, two families segregating *FMR2* expansions had been identified. This was unexpected due to the province's relatively small population of 519,720[112] and the reported rarity of FRAXE. We hypothesized this may be due to NL's unique genetic architecture. The province is composed of a collection of genetic isolates. Its first colonies were established in the 17th century from approximately 20,000 individuals from two main immigrant populations, Roman Catholics from the south of Ireland and Protestants from the south-west of England[113]. These settlers came to the island because of its rich fishing grounds and established coastal homes. Until relatively recently, travel between these communities was difficult and occurred mostly by sea. In addition, to geographical isolation and little emigration, religious segregation also occurred as individuals tended to marry into the same denomination. This, combined with large family sizes, makes NL an ideal place to study genetic disease.

Genetic isolates like NL often have a unique distribution of genetic disorders, reflecting those that were present in the population's founders. Certain diseases may be over represented compared with more heterogeneous populations, while others may not be present at all. NL has the highest Canadian rate of colorectal cancer[114] and the highest incidence of type 1 diabetes mellitus[115]. Founder effects have been identified for certain monogenic diseases including familial adenomatous polyposis coli syndrome (FAP)[116], Lynch syndrome[117], arrhythmogenic right ventricular cardiomyopathy/dysplasia type V (ARVD5)[118] and hereditary diffuse gastric cancer syndrome[119].

On the other hand, FXS due to expansions in the X-linked *FMR1* gene, which is the most common cause of inherited ID in most populations[4], is virtually absent in NL: only one NL family with an *FMR1* expansion has been identified through Eastern Health's Province Medical Genetics Program (PMGP).

1.7.1 Purpose and Objectives

Building on a strong foundation of solving genetic disease, which is in partly due to the unique genetic isolates and large family size, the health care structure here is ideal. The PMGP is a centralized clinical service for investigating genetic disorders for the entire province. This, coupled with the local expertise in developmental delay and ID, as well as, the resource of the Newfoundland Genealogy Database, offers a unique opportunity to study FRAXE in the population. The proportion of identified *FMR2* to

FMR1 mutations in NL was unusual and made it plausible that this population is enriched for *FMR2* mutations.

The purpose of this study was to determine the prevalence of FRAXE among boys referred to the PMGP for ID and/or developmental delay. Specifically, the objectives of this research were:

1) To determine the prevalence of *FMR2* mutations in *FMR1*-negative individuals through a comprehensive chart review; and
2) To characterize *FMR2* positive families and determine if there is a common ancestor connecting these families.

Chapter 2 – Methods

The purpose of this research project was to explore FRAXE in the Newfoundland and Labrador (NL) population. The recruitment phase of this study involved three different cohorts: 1) two known FRAXE NL families; 2) boys referred to the Provincial Medical Genetics Program (PMGP) with developmental delay of unknown etiology and negative *FMR1* testing (referred to as the retrospective cohort); and 3) children with mild intellectual disability (ID) seen in the Child Development Clinic at the Janeway Children's Health and Rehabilitation Center, St. John's, who typically would not be referred for assessment by a clinical geneticist (referred to as the Janeway cohort).

FMR2 allele size was compared between the retrospective test population and controls, as well, the NL population was compared to the reported allele size in the literature. A review of the clinical phenotypes of the previously reported males with FRAXE was compiled.

Recruited participants were tested for *FMR2* allele size through PCR and expansions were confirmed using an outside research facility. As NL has a unique genetic structure it was postulated the FRAXE families may share a common ancestor. To determine if this was the case, families were analyzed using the Newfoundland Genealogy Database. Haplotype analysis was performed on these families to determine if they shared a common haplotype region. A description of how this was accomplished is detailed below. Memorial University's Health Research Ethics Board (HREB) approved this project [05.175].

2.1 Participant Recruitment

2.1.1 Known FRAXE Families

At the time of this study, there were two unrelated, geographically separate NL families known to the PMGP. Both families were contacted by a genetic counsellor at the PMGP and asked if they would be interested in speaking to someone about a new research project investigating FRAXE.

2.1.2 Retrospective Cohort

2.1.2.1 Chart Review and Recruitment

The SHIRE database from the PMGP was used to identify all individuals who were referred to genetic services from 1994 to 2004 for unexplained developmental delay. The charts were reviewed and participants were recruited using the following inclusion criteria: male; referred to genetics with development delay; received *FMR1* testing and remained without a specific genetic diagnosis and was between the ages of 2 and 19 years at the time of testing. A parent was contacted and given a brief introduction to the study. Parents were informed that their participation was completely voluntary and if they agreed to participate, they could withdraw at any time (**Appendix A**). If the parent agreed, the family's address was confirmed and 2 copies of the consent and a stamped return envelope were mailed.

In an Access database the following was recorded: child's name, MCP (medical care plan), date of birth and DNA number; parents' names and address; date of phone call, along with some details regarding the contents of the call; and date on which the consent form was mailed. The child was then given a study number and the chart was pulled for follow-up.

A follow-up call was made two weeks later to confirm that the consent was received and to provide the parent with an opportunity to ask questions. Parents were asked to provide consent for their child. If the child was 12 years or older and could comprehend the details of the study, then he was included in the consent process (**Appendix B**). If the parent agreed to participate and the consent was not received within one month from the follow-up call, an additional call was made to remind the parent that testing for their child would not proceed until the signed consent was received. If after two months the consent had not been received and the parent had expressed interest in participating, a reminder letter along with an additional copy of the consent was sent (**Appendix C**). The reminder letter also indicated that if the consent was not received by the date specified, we would assume that the family was not interested in participating and no further attempts to contact them would be made.

2.1.2.2 Chart Extraction

All signed consents were placed in a locked filing cabinet. Once the consent was received, a chart extraction was completed (**Appendix D**). The chart extraction form

included sections for dysmorphology findings, growth parameters (height, weight and head circumference) and the results of biochemical, metabolic, molecular, cytogenetic, radiology and any other relevant testing. Also recorded was the date of first visit to genetics, reason for referral, referring physician, and positive family history of developmental delay, intellectual disability (ID) and/or learning problems.

2.1.2.3 Sample Acquisition

An aliquot of the DNA was requested from the Laboratory Medicine DNA Bank. For some of the participants there was no DNA remaining. In these cases, the parent was contacted and informed there was not a sample available for *FMR2* testing. The parent was given the opportunity to withdraw from the study or to have the child provide a new blood sample. If the parent agreed to have the child's blood drawn, a blood requisition form was mailed out to take to the family's nearest blood collection service. An additional DNA consent form was sent to the parent to indicate options for the child's sample once the study was complete (**Appendix E**). A letter to the parent accompanied the consent form to ensure parents understood the implications of storing their child's DNA. (**Appendix F**). The letter indicated that if no response was received from the parent selecting an option for the child's DNA once the study was completed, then the sample would be destroyed. Contact was made with the appropriate laboratory to arrange shipment of the blood once the sample was taken. DNA was then extracted according to the procedure detailed in **section 2.2.1**.

2.1.3 Janeway Cohort

2.1.3.1 Janeway Recruitment

The third phase of this study was to recruit participants through the Child Development Clinic at the Janeway Children's Health and Rehabilitation Center, the local children's hospital for the province. A brief presentation was given to introduce the child developmental pediatricians to the FRAXE study. The discussion focused on the purpose of the study, the inclusion criterion, what to do if a child met the inclusion criteria and how to track this information. Five child developmental pediatricians agreed to aid in the recruitment of this study.

2.1.3.2 Tracking Form and Inclusion Criteria

Each of the pediatricians received a recruitment package which included the inclusion criteria, a tracking form, and the parents' package (**Appendix G-H**). The inclusion criteria specified that participants must meet all the requirements to be invited into the study (**Figure 2.1**). Learning disabilities or academic difficulties could be as mild as "pathway 2" in school. Behavioural abnormalities included but were not limited to a diagnosis of attention deficit disorder (ADD) or aggressive behaviours.

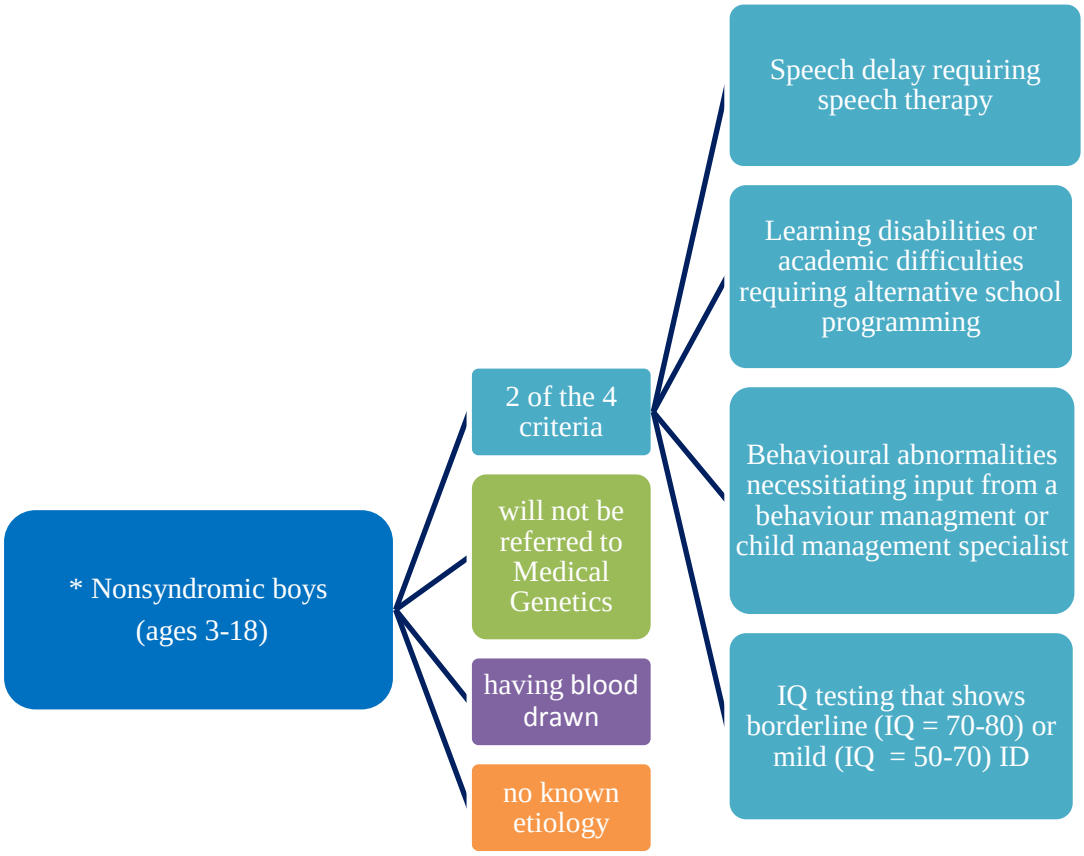


Figure 2.1 – Inclusion criteria for Janeway cohort. *Nonsyndromic refers to a boy who did not appear syndromic as judged by the developmental paediatrician.

The tracking form recorded which criteria were met and allowed the researcher to track the number of patients being seen who did not meet the criteria. Once the pediatrician assessed that the child was eligible, the parent was invited to take an information package. The parent package included a letter explaining the study, the consent form with special DNA consent form attached, a stamped return envelope and a blood requisition form (**Appendix I**).

2.1.3.3 Consent Process

Once a blood sample was received, the parent was then contacted to explain the study in more detail. Although providing a blood sample implied consent, like the retrospective study, a signed consent from the parent was needed before FRAXE testing could be conducted. If a signed consent was not received within several weeks of receiving the blood sample, the parent was re-contacted and reminded if the family was still willing to participate, a signed consent would need to be received before testing could proceed.

2.1.4 Controls

Two hundred and eighty males from Newfoundland who were previously recruited through random-digit-dialing as the controls for a colorectal cancer (CRC) study[120], were used as the control population. When these Newfoundlanders were recruited as CRC study controls, they were not asked specific questions about their intellectual or academical abilities. However, they all completed a detailed enrolment package which implies that they could all read and write. The package included a consent form, a family history questionnaire, a personal history questionnaire and a food frequency questionnaire. All CRC control samples tested were anonymized and hence could not be linked back to the individual person. This process was approved by Memorial University's Research Ethics Board [05.175].

2.1.5 *FMR2* Test Results

Once *FMR2* testing was complete, parents were notified of the result. If the test result was negative, the mutation report along with a letter explaining the result was sent to the parent (**Appendix J-K**). A copy of the test result was also sent to the referring doctor. If the result was positive, the parent was contacted and invited to come into the genetics clinic to speak with a geneticist.

2.1.5.1 Positive FMR2 result

Individuals found to carry a *FMR2* expansion were invited to come into the PMGP to meet with a geneticist and discuss the result. The option was made available for confirmation of the result at the molecular diagnostic lab at the Hospital for Sick Children in Toronto. If the parent(s) agreed, the affected child had a clinical examination to identify possible dysmorphic features which included growth parameters, facial measurements and a clinical photograph. IQ testing was offered by a trained psychologist and the medical chart was reviewed. Additional family members were given the option of having *FMR2* testing (beginning with the male proband's mother) and if a premutation or full mutation was identified, that relative was offered the same evaluation as the proband.

In addition, genetic counselling was offered to the family. Recurrence risks were discussed in relation to the probands' parents and other family members and for the proband if he ever decided to have children.

2.1.6 Literature Review

All papers describing FRAXE positive cases were catalogued by using search terms "FRAXE" and/or "*FMR2*" and/or "*FMR-2*" in Reference Manager. The abstract was reviewed and the paper obtained if a FRAXE mutation had been found or the *FMR2* gene had been investigated. Papers were divided into two categories – *FMR2* case reports and *FMR2* screening studies.

2.1.6.1 Case Reports

For the case reports all individuals who tested positive for a FRAXE mutation were recorded. All phenotypic features were noted in an excel spreadsheet and divided into categories and sub-headings. The phenotypic features extracted from the publications were reviewed by a clinical geneticist (BF) who combined some of these, where appropriate. The number of positive cases of males versus females in each family was recorded.

2.1.6.2 FMR2 Screening Reports

For the studies evaluating *FMR2* status in various cohorts the following was recorded in an Excel spreadsheet: the number of alleles (or in some cases the number of participants), the proportion of individuals in each *FMR2* expansion class (normal, intermediate, premutation and mutation) and the geographical origin of the study subjects.

The minimum, maximum and mode allele sizes were also noted when available. This information was also recorded for the control population.

2.2 Molecular Analysis

2.2.1 DNA Extraction from Blood

Five volumes of warm Red Cell Lysis Buffer (0.14 M NH_4Cl, 0.017 M tris pH 7.65) solution were added to 1 volume blood in a 50 ml centrifuge tube. The tubes were centrifuged at 2500 rpm for 5 minutes. The supernatant was discarded leaving a white cell pellet at the bottom. Ten ml of 0.15 M sodium chloride saline was added to the tube, vortexed and centrifuged at 2500 rpm for 5 minutes. The supernatant was removed and 3 ml of Nuclei Lysis Buffer (10 mM Tris, 400 mM NaCl, 2 mM EDTA) was added to the cell pellet. The mixture was vortexed and transferred into a 15 ml centrifuge tube. 0.25 ml of 10% SDS and 0.6 ml of pronase E solution (3 mg/ml in 1% SDS, 2 mM EDTA) was added and the mixture was incubated in a 37^{o}C water bath overnight. Once removed from the water bath, 1 ml of saturated NaCl was added, shook vigorously for 15 seconds and centrifuged at 2700 rpm for 17 minutes. The supernatant was poured into a 15 ml tube containing 2 volumes of absolute ethanol (EtOH) and inverted several times. The DNA strands were hooked out and washed several times with streams of 70% EtOH. The DNA was air dried, placed in a 1.5 ml tube and dissolved in 200 µl of TE (10 mM Tris, 1 mM EDTA, pH 8). DNA was dissolved overnight and then put on a spinning rotator for 1 hour to ensure complete rehydration. Samples were then quantified by UV spectrometry.

2.2.2 PCR-based *FMR2* Expansion Testing

DNA samples which had been collected previously were retrieved from the Laboratory Medicine DNA Bank. Each DNA sample was mixed with 0.5 µl of 25 pmol/µl of primers 598 (fluorescent labelled) and 603 (**Appendix L – Supplementary Table S.2**), 0.5 µl of Amplitaq (Applied Biosystems) and 11.5 µl of Fragile X Buffer. Fragile X buffer contained 400 µl of 10X buffer II (Applied Biosystems), 240 µl of 25 mM $MgCl_2$, 8 µl of 100 mM deoxyadenosine triphosphate (dATP), deoxycytsosine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP), 160 µl of 5 mM 7-deaza guanosine triphosphate (GTP), 400 µl of DMSO, 800 µl of Betaine and 1058 µl of ddH_2O. Samples were amplified in the thermocycler under the following conditions: 5 minutes at 95°C for initial denaturation, 55°C for 25 seconds, 72°C for 25 seconds, followed by 30 cycles of 95°C for 45 seconds, 65°C for 30 seconds and 72°C for 30 seconds, with a final elongation of 72°C for 10 minutes with a 15°C hold (Protocol from the Hospital for Sick Children, Toronto, personal communication). Amplicons were stored at 4°C until they were visualized on an agarose gel stained with Ethidium bromide using UV light. Samples which did not produce an amplicon were repeated and if a second PCR analysis produced no product, the sample was sent for Southern blotting analysis.

2.2.3 X and Y Chromosome Markers

DXS990, DXS986, DXS1226, DXS1214, DXS8055 and DXS991 (**Appendix L – Supplementary Table S.2**), markers from the Applied Biosystems (ABI) Prism Linkage Mapping Set v2.5 were used. PCR conditions were carried out as follows: 1.0 µl of 100 ng/µl of DNA was mixed with 0.75 µl of 10X PCR Buffer, 0.25 µl of 100 pmol/µl of forward and reverse primer, 0.1875 µl of 10 mM of dNTPs, 0.375 µl of 50 mM $MgCl_2$, 0.075 µl of Platinum Taq (Invitrogen) and 4.6125 µl of dH_2O to make up a final volume of 7.5 µl. Samples were amplified in the thermocycler under the following conditions: 3 minutes at 95°C for initial denaturation, 10 cycles of 94°C for 15 seconds, 55°C for 15 seconds, 72°C for 30 seconds, followed by 20 cycles of 89°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds, with a final elongation of 72°C for 10 minutes with a 4°C hold.

Y chromosome markers DYS570, DYS643 and DYS490[121] (**Appendix L – Supplementary Table S.2**), were run using the same protocol.

2.2.4 Fragment Analysis

PCR products were diluted to 1 in 15 ratio with deionized water (dH_2O) and 1.2 µl of this diluted amplified PCR product was suspended in 8.3 µl of High-Di Formamide (ABI) and 0.5 µl of Liz500(-250) size standard using a 1:1:2 ratio based on which fluorescent label (blue: green: yellow) the marker was tagged with. Samples were denatured in the thermocycler for 2 minutes at 95°C and quickly cooled by immediately

placing on ice. Samples were run on the ABI 3130xl and analysed using ABI's Genemapper software.

2.2.4.1 FMR2 Genotype and Repeat Size

To determine the size of the *FMR2* repeat, samples were first binned using Genemapper software. A subsample of the genotypes were viewed graphically to see where they fell on the X-axis and anything that fell within +/-0.7 was represented by the same value. Once all samples were binned, *FMR2* repeat size was determined by subtracting the PCR amplicon size minus the repeat (290 bp) and dividing by 3 (**Figure 2.2**). Results were verified independently to ensure accuracy.

agctgtccaggctccgccc**cctgtgagtgtgtaagtgtgtgatgctgccg**cggccgccgc
cgccgcctgtgcagccgctgccgccgccgccgccgccgccgccgccgccgccgccgccgc
cgccgctgccgccccggctgccgcgccgcgccgctgcctctgccccggccgcccccgccg
ccgctgccgccgccggcccgcagccagccaggcgggcggcccagcccgcctgagcccgca
gcggctgccgccgcagcgtcgggtcgctgggtgcgcgggctaccgcggaccgagcggacc
cgagtgggcgaccaggcgcttgcccg**cccagtgccactgccgccgcttcctcgc**cggagc

Figure 2.2 - *FMR2* PCR amplicon. Forward primer is colored in green, reverse primer is colored in purple and CCG repeat is highlighted in yellow. To determine repeat size, amplicon size was subtracted from 290 and divided by 3.

2.2.4.2 Comparison of FMR2 Allele Size in Test Population versus Controls

The distribution of *FMR2* allele size was compared between the test population from the retrospective study and the controls by tallying all repeat sizes for each allele size seen, dividing by the total and multiplying by 100 to get the percentage. A Fisher test was applied to determine if there was a significant difference seen between the two populations.

2.4.4.3 Comparison of FMR2 Allele Size in the Newfoundland population versus the literature

Based on the information collected from the literature review the minimum, maximum and mode *FMR2* allele sizes were compared across all studies and compared with the sizes seen in the NL population.

2.2.5 *FMR2* Sequencing

2.2.5.1 FMR2 PCR for Sequencing

A subset of the samples was sequenced to confirm fragment size. PCR for sequencing followed the same protocol as described in **section 2.2.2** except primers 598 and 603 were not fluorescently labelled.

2.2.5.2 PCR Amplicon Purification

Following amplification, excess nucleotides and primers were removed from the PCR amplicon by incubating with EXOSAP: a cocktail of 0.5 µl of exonuclease I (ExoI), 0.5 µl of shrimp alkaline phosphatase (SAP) and 7.5 µl of dH_2O were added to 8.0 µl of amplified PCR product and incubated in the thermocycler for 30 minutes at 37°C, followed by 15 minutes at 80°C.

2.2.5.3 Sequencing Reaction

1.0 µl of EXOSAP product was then added to 0.5 µl of sequencing mix (ABI), 2.0 µl of sequencing buffer, 2.0 µl of 5 pmol/µl of primers 598 and 603 and 14.5 µl of dH_2O. Samples were denatured for 1 minute at 96°C, followed by 25 cycles of 10 seconds at 96°C, 5 seconds at 50°C, 4 minutes at 60°C. The cycle sequencing reaction was stopped with the addition of 5.0 µl of EDTA.

2.2.5.4 Sequencing Amplicon Purification

Immediately following the addition of EDTA, 65µl of 95% EtOH was added and the samples vortexed to allow the DNA to precipitate out of solution. Samples were placed in the dark for 1 hour up to overnight. After precipitation, the sample plate was centrifuged for 30 minutes at 3000 g. Following the spin, the sample plate was inverted on paper towels to decant the ethanol. Samples were then washed with an additional 165µl of 70% ethanol and centrifuged for 15 minutes at 3000g. Following the spin, the

plate was inverted to decant the ethanol and placed in the dark to allow the samples to dry. Once dried, the samples were resuspended in High-Di Formamide, vortexed and briefly spun and placed in the thermocycler to denature at 95°C for 2 minutes. Samples were immediately supercooled by placing on ice and run on a 3130xl ABI Sequencer.

2.2.5.5 Sequencing Analysis

All samples were run using the Sequencing Analysis program and imported into Sequencher to be visually compared to the reference sequence. *FMR2* repeat size was manually determined by counting the number of CCG repeats in each sample and recorded in an excel spreadsheet. Results were read independently by two members of the research team to ensure accuracy.

2.2.6 *FMR2* Expansion and Methylation Testing

8 µg of DNA was sent to Human Genetik, a clinic laboratory in Germany for *FMR2* expansion testing on several members from Family A (IV-1, III-2, III-6, III-8) (see **Figure 3.1** for pedigree) and from the proband of Family C (III-1); these were the two families that had consented at the time of testing. A control sample (C50), a retrospective sample (12273), and a Janeway sample (364), that were inconclusive using PCR-based *FMR2* testing, were also sent for further investigation.

During the course of this work, a research laboratory in Chicago, Rush University Medical Center, started offering both expansion and methylation testing for *FMR2*. As the

results from Human Genetik were inconclusive for some of the samples and this research facility also provided methylation results, the samples were sent there instead. 8µg of DNA was sent to Rush University Medical Center from members of Family A (IV-1, III-2, III-6, III-8), from the proband for Family C (III-1), and the control sample (C50) (see **Figure 3.1** for pedigree).

2.3 Founder Effect Study

2.3.1 KINNECT

2.3.1.1 Data Mining

Pedigrees were created in Progeny and relevant fields such as individual name, middle name, also known as, last name, maiden name, date of birth, place of birth and address were exported as a text file. Field headings between Progeny and KINNECT were matched before importing the data into KINNECT. For example, in Progeny, a female's last name was considered her last name at the time the data was collected, however, in KINNECT a female's last name was her maiden name and if the individual was married, that name was called the surname. Number format for date of birth and date of death were revised to be consistent. Gender was reformatted from male or female to 1 for male and 2 for female. The additional information field in Progeny was reviewed and if it contained place of birth and/or current living location, this information was distributed under appropriate headings. Individuals in the pedigree were given a unique identifier. This

identifier was a combination of the pedigree number and the system ID number assigned by Progeny. All individuals born after 1945 or individuals with only a surname or first name were removed.

2.3.1.2 Generation of KINNECT Results

The families were analyzed using KINNECT[122], and results viewed in an Excel spreadsheet. Each individual in Progeny who was found to have a significant match in the Newfoundland Genealogy Database (NGD) was given both an individual and family match score. Match scores were based on the number of fields matched as well as the family structure of the matched family in the NGD compared to the Progeny family. Results were sorted based on unique identifier, family score and individual score with highest ranking on top. Each record was checked using the unique NGD number, as families in the NGD were recorded by household and community, easily allowing one to determine if this individual match was to the correct family.

Once a positive match was found, all the information for that family was added to the family file. The census data listed all members living in the household and the individual's relationship to the head of the household. This allowed extended family members such as siblings, parents, in-laws, cousins, etc. to be recorded and family members who did not have a positive match but were in the NGD database.

2.3.1.3 Additional Genealogy Resources

All families were researched using Stonepics, a collection of CDs which contain pictures of headstones in most graveyards in the province. Software to accompany Stonepics allows the user to search based on name, community and year of birth. If additional information about the family was found, it was added to the pedigree.

Pedigree expansion was also explored using online resources. Genealogy databases such as ancestory.com and familysearch.org and more Newfoundland based websites such as Newfoundland Grand Banks (http://ngb.chebucto.org/) and the NL GenWeb (http://www.rootsweb.ancestry.com/~cannf/) were explored. Obituaries and birth announcements from local newspapers were searched. The population therapeutics research group (PTRG) had a number of additional online resources catalogued pertaining to Newfoundland genealogy such as books and/or online blogs describing large extended families in certain areas of the province which were created for public interest or for events such as local "Come Home" celebrations.

The PTRG, through Memorandums of Understanding (MOUs) with various church denominations, had digital copies of church registers from across the province. These registers provided records of most births, marriages, and deaths within a community. This resource was used to help clarify parental information, dates of birth, and places of birth through baptismal records. It also helped to identify maiden names of females through marriage records.

2.3.2 Haplotype Analysis

2.3.2.1 X chromosome markers for haplotype analysis

Markers flanking the *FMR2* gene were investigated using the Genome Browser Gateway on the UCSC Genome Bioinformatics website. DXS998, DXS7847, DSX7393, DXS7389, DXS7390, DXS7394, DXS7812, DXS1318, DXS6729, DXS8303, DXS1185, DXS457, DXS1123, DXS1215, DXS548, DXS533 and DXS1193 (**Appendix L – Supplementary Table S.2**), were chosen based on the proximity to the gene and their heterogeneity (**Figure 2.3**). PCR conditions were carried out as previously described in **section 2.2.3** and amplicons were run on the ABI Sequencer as described in **section 2.4**.

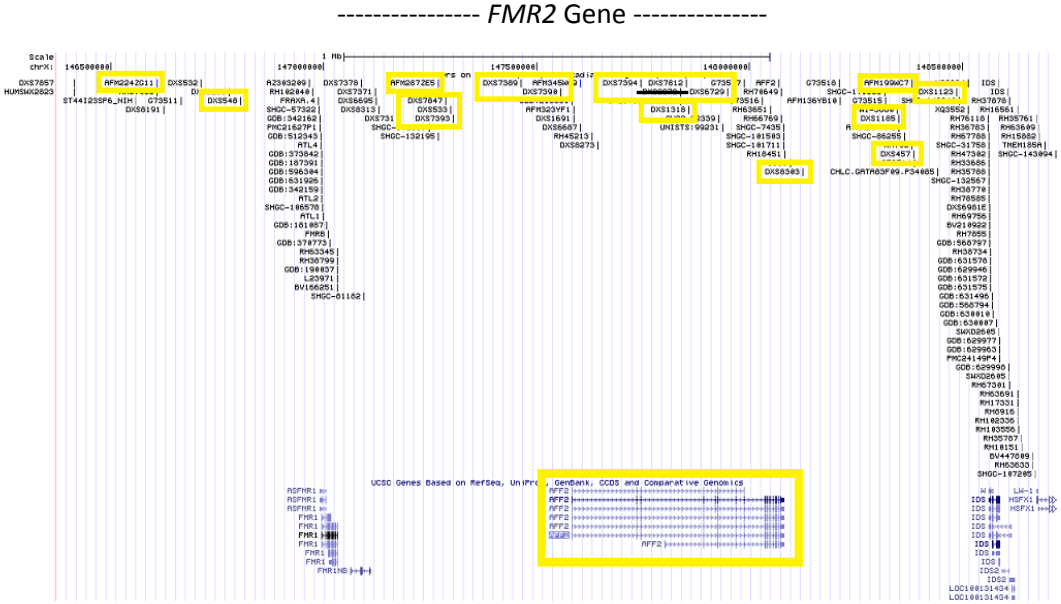


Figure 2.3 – Location of X chromosome markers in relation to the *FMR2* gene for haplotype analysis. X chromosome makers flanking the *FMR2* gene are highlighted in a yellow box.

2.3.2.2 Haplotypes

Genotypes were analyzed and the distances of the markers from *FMR2* were calculated using the UCSC (University of California, Santa Cruz) Genome Browser and plotted on the pedigree to create haplotypes in Progeny. Males were used to create haplotypes for the female parent. Haplotypes between families were compared to determine if they shared a common region. A control sample was tested to compare to the haplotypes seen in the families.

2.3.3 FRAXE Prevalence in the Male Newfoundland Population

For Newfoundland, the minimum prevalence of FRAXE in the pediatric population was calculated using the number of positive FRAXE boys known to be living in the province of NL divided by the total number of boys living in the province of NL between 2 and 18 years of age in 2011.

Chapter 3 - Results

3.1 Participant Recruitment

3.1.1 Families

As stated in **section 2.1.1**, at the time this study was initiated, there were two families known to the Provincial Medical Genetics Program (PMGP) that were segregating *FMR2* expansions and these families were consented to this study.

Two additional families were referred to the PMGP from the genetic outreach clinic in Corner Brook during the course of the study. The genetic counsellor in that area explained the study to those two families in detail and they both agreed to participate in the project.

3.1.1.1 Family A (known to PMGP prior to initiation of the study)

The mother of the proband (III-2) of Family A gave permission to the genetic counsellor to be contacted about the FRAXE study and the study was explained in detail by the research team (**Figure 3.1**). The mother agreed to the study and came in for a follow-up appointment with a geneticist. The proband's parents signed the consent form and additional blood samples were collected for CGH microarray analysis. As well, IQ testing was arranged for the proband and his mother who carried an *FMR2* premutation. The proband's mother spoke to her sisters regarding the study and determined that they

were willing to be contacted by the research team. The mother no longer had contact with her brothers. Individual III-6 (the proband's maternal aunt) was contacted by phone and the study was explained in detail. She signed a consent and gave a blood sample for *FMR2* testing. Individual III-8 (the proband's maternal aunt) was previously seen at PMGP and was known to carry a *FMR2* expansion, as did her son IV-7 (**Figure 3.1**). When contacted by a genetic counsellor, she gave permission for her banked DNA to be used, as well as her and her child's clinical records to be reviewed for research. She was not willing at that time to come into the clinic or provide additional samples.

3.1.1.2 Family B (known to PMGP prior to initiation of the study)

Unfortunately, no contact was made by the genetic counsellor with this family. A telephone message was left asking the family to call the PMGP. After two months of no return call, a letter was sent regarding a follow-up appointment for the child through the clinic, however, the family did not keep this appointment. Therefore, no further workup could be done on this family.

3.1.1.3 Family D (identified by the PMGP following initiation of the study)

The genetic counselor in the area contacted the family and obtained consent on behalf of the research team (**Figure 3.1**). Blood samples were obtained of the proband (III-1) and his premutation mother (II-2) for haplotype analysis. The maternal

grandmother (I-2) gave permission for the research team to use her banked DNA sample for this study.

3.1.1.4 Family E (identified by the PMGP following initiation of the study)

The genetic counsellor made contact with and obtained consented on behalf of the research team (**Figure 3.1**). As in the case with family D, the parents signed a consent form and blood samples were collected from the proband (IV-1) and his premutation mother (III-2). The maternal grandmother (II-2) and great-grandmother (I-2) also signed a consent form and gave permission for their banked DNA sample to be used for the research study.

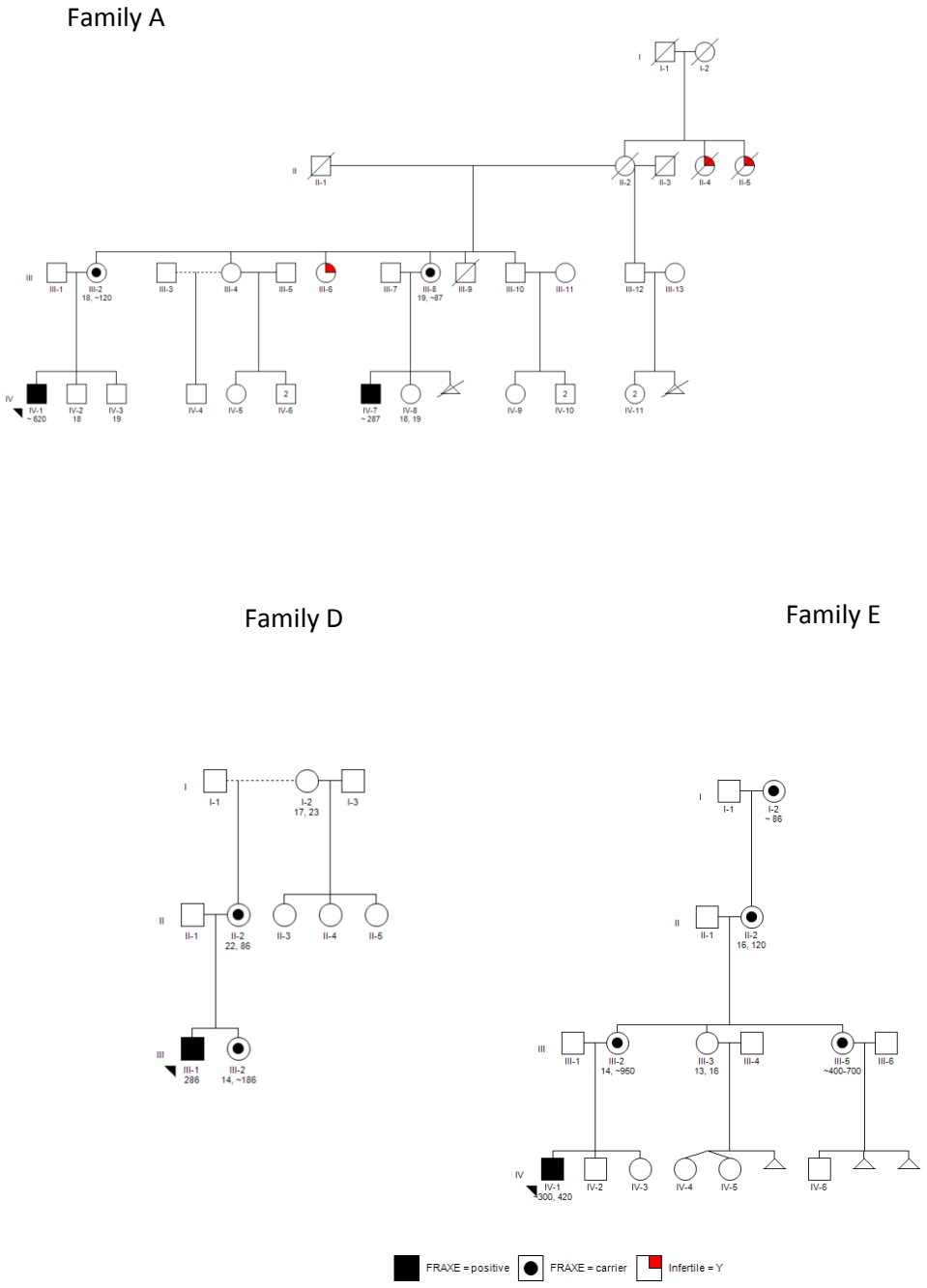


Figure 3.1 – Pedigrees of Family A, D and E. Dark shading indicates an *FMR2* expansion; circle inside the symbol indicates a carrier female; red top right hand shading indicates infertility. The number inside the pedigree indicates the number of offspring, sex unspecified and a small diamond indicates miscarriage. The number under the sample ID indicates the *FMR2* expansion allele size.

3.1.2 Retrospective Cohort

3.1.2.1 Eligible Participants

A total of 357 patient charts were reviewed. Forty five percent (158/357) of individuals were invited into the study based on the inclusion criteria (described in **section 2.1.2.1**), and of these, 92 consented (**Figure 3.2**). 56% (199/357) of the charts reviewed were excluded because they did not meet the inclusion criteria. The majority of those excluded were individuals who were either female (94/199 [47%]) or boys who were already tested for *FMR2* expansions (61/199 [31%]) and found to be negative.

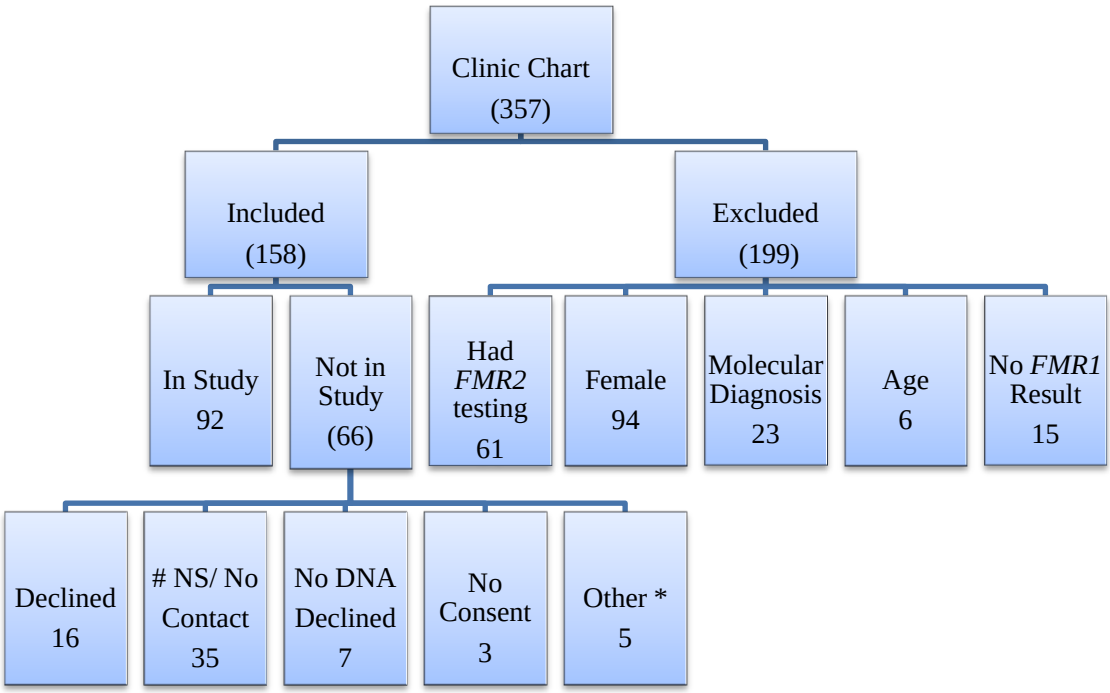


Figure 3.2 – Flow diagram of the charts reviewed for the retrospective study. The number of individuals within each category is shown in parentheses. *Other includes individuals that were excluded from the study due to special circumstances and did not fit any of the categories described.

Twelve percent (44/357) of individuals were excluded based on their age at the time of testing (outside of the included age range of 2 to 19 years), a molecular diagnosis to explain their phenotype or no *FMR1* result present in the chart. Individuals in the latter group had a requisition for *FMR1* expansion testing in their clinical chart, but no test result present. These included individuals whose parent(s) initially agreed to *FMR1* testing as part of the clinical genetic assessment, but did not provide a blood sample, as well as individuals for whom the initial *FMR1* testing by the appropriate laboratory failed and an additional sample was not received by the clinic.

Of the 158 boys eligible as indicated in **Figure 3.2**, 66 individuals were not enrolled in the study because they were lost to follow-up, no sample was available or a signed consent form was not received. An additional five individuals with special circumstances were excluded from the study: two children had died, one individual was living in a long-term care facility, and two individuals were brothers whose parents had indicated they wanted their children's DNA samples destroyed after initial testing. As the inclusion criteria stated that a banked DNA sample must be available for testing, these individuals were not contacted.

The parents/guardians of the remaining 92 males consented to participate in the study. The overall participation rate was 58.2% (92/158).

3.1.2.2 Chart Review

The reason for the child's referral to genetics was indicated in 90 of the 92 male charts in the retrospective study (**Table 3.1**). The reason for referral was broken down

into four categories: 1) *autism spectrum disorder (ASD)*, which included ASD, Asperger's and pervasive development delay (PDD); 2) *developmental delay*, which included developmental delay, speech delay, mental/physical handicap, low IQ, cognitive delay, ADHD, large head/developmental delay, motor delay, seizures, and several syndromes associated with developmental delay and dysmorphism; 3) *fragile X syndrome* or other neuropsychiatric disorders which included psychosis and schizophrenia; and 4) *dysmorphology/visible malformation*, which included cleft lip palate, tremor and hemihypertrophy.

Sixty percent of all individuals were referred because of developmental delay of unknown etiology, compared with only 7.8% where Fragile X syndrome genetic testing was specifically requested. Twenty three percent of individuals were referred because of confirmed or suspected ASD and 9% were referred for dysmorphic features.

Table 3.1 – Reason for referral for 90 boys in the retrospective study

Reason for Referral	**# (%)**
Autism	21/90 (23.3%)
Developmental Delay	54/90 (60.0%)
Possible Fragile X syndrome	7/90 (7.8%)
Dysmorphology/Visible Malformation	8/90 (9.0%)
Not available *	2/92

* For two individuals, the reason for referral was not indicated in the genetics clinic chart.

A review of the genetic charts was completed on all 92 participants enrolled in the study. The mean age of these 92 boys at the time of the clinic genetic assessment through the PMGP was 7.82 years. Accounting for the 18 month to 2 year wait list to be seen by a geneticist during the study period, the majority of boys were at least 5 years old when referred for genetic consultation.

As part of the consult, several assessments were carried out for each child and are summarized in **Table 3.2**. Eighty percent of children were sent for chromosomal analysis and 77% had a biochemical metabolic work up. For those for whom information was available, 45% were dysmorphic and 61% had a positive family history of developmental delay, ID or learning problems**.**

Table 3.2 – Review of genetic charts for 92 consenting boys previously tested for *FMR1* expansions with negative results

	# Assessed (%)	**# with an Abnormal Value (%)**
Dysmorphology present	86/92 (93.5)	39/86 (45.3)
Family History of DD and/or ID and/or LD	77/92 (83.7)	47/77 (61.0)
Head Circumference	61/92 (66.3)	27/61 (44.3)
Height	68/92 (73.9)	15/68 (22.1)
Head CT	53/92 (57.6)	5/53 (9.4)

	# Assessed (%)	# with an Abnormal Value (%)
EEG	51/92 (55.4)	11/51 (21.6)
Biochemical/Metabolic	71/92 (77.2)	4/71 (5.6)
Molecular Tests (excluding FRAXA)	13/92 (14.1)	0/13 (0)
Chromosome Analysis	74/92 (80.4)	3/74 (4.1)
Total	92	

The # assessed indicates the number of individuals that were evaluated for each of the headings divided by the total number of individuals included in the study; percentages in parentheses. Abnormal value indicates the number of individuals that had a test result that was abnormal on the test report or required a follow-up test divided by the total number of test results available. DD = developmental delay; ID = intellectual disability; LD = learning disability; CT = computerized tomography; EEG = electroencephalogram.

3.1.3 Janeway Study

Information was received on 17 boys who had been recruited into the FRAXE study through the Child Development Clinic, Janeway Children's Health and Rehabilitation Center, St. John's. Of these 17 boys, a signed consent form and blood sample was received for 14 individuals. Three individuals did not participate in the study for various reasons: a blood sample was received for one participant but the parent declined the study; another parent verbally agreed to participate but a signed consent form or blood sample were not received; and one parent received an information package from the physician, but contact was never made with the parent and no signed consent form or blood sample were not received.

All individuals in this cohort whose parent(s) consented and provided a blood sample for their child were tested for *FMR2* expansions. A mutation report, along with a letter explaining the result was sent to each parent and the referring pediatrician for the child's medical chart.

3.1.4 Controls

DNA was available from 528 individuals who were recruited as controls through random-digit-dialing for a study on colorectal cancer and who agreed to have their anonymous DNA samples used in other studies[120]. Of the 528 participants, only male participants were included of whom there were 284 (53.8%). Of the 284 male samples available, DNA was received on 277. Seven samples were unavailable due to DNA quantity and/or quality issues.

3.2 Molecular Analysis

3.2.1 PCR based *FMR2* Testing

All DNA samples from the PMGP retrospective (n=92), Janeway (n=14), and control cohort (n=277), as well as the additional family member (n=1) from the recruited FRAXE positive family (Family A) were amplified for *FMR2* gene analysis and calculations were carried out to determine repeat sizes.

Of the 92 individuals meeting the inclusion criteria for the retrospective study, all samples were in the normal range except for 1 sample (03MG438), which failed to produce a visible amplicon. PCR for the *FMR2* repeat for the Janeway cohort produced an amplicon for each of the 14 individuals enrolled in the study. In the control population, one sample (C50) failed to produce an amplicon. The aunt (III-6) of the proband in Family A produced a single allele. There were no intermediate or premutations seen in any of the samples tested. The retrospective sample (03MG438) and the control sample (C50) which failed to produce an amplicon underwent further testing, detailed in **section 3.2.2** (*FMR2* expansion testing).

3.2.1.1 Allele Sizes

Allele size was determined using fragment analysis. Samples were binned and repeat size was determined as described in **section 2.4.4.1**. A subset of the samples, one for each repeat size observed, was sequenced. Fragment analysis size was off by 3 repeats to the actual repeat size observed in the sequencing data (**Figure 3.3**). The allele size seen in each of the cohorts is presented in **Appendix L – Supplementary Tables S.3, S.4 and S.5**. An *FMR2* allele size was not obtained on all samples using PCR. A summary of the results for all three cohorts is presented in **Table 3.3**.

Table 3.3 – Summary of *FMR2* results using fragment analysis for each of the cohorts

Cohort	# of Individuals	*FMR2* result obtained by PCR
Retrospective	92	90
Janeway	14	13
Control	277	267

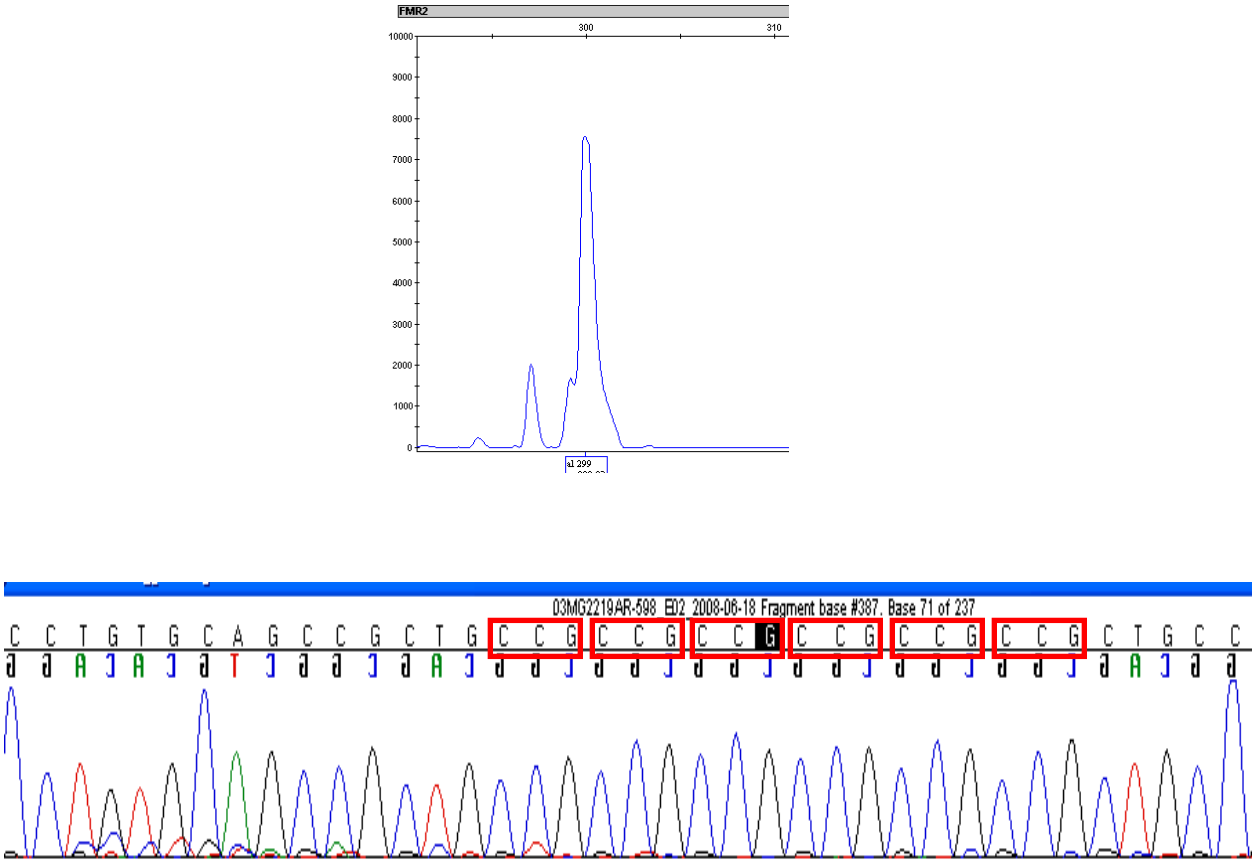


Figure 3.3 – An example of an *FMR2* result obtained for 1 sample using fragment analysis vs Sanger sequencing. Result obtained from fragment analysis was binned to 299 indicating a repeat size of 3 compared with 6 repeats obtained using Sanger sequencing.

For a small number of samples in the retrospective, Janeway, and control cohort an anomaly was seen in the genotyping data - the samples looked like they were heterozygous (**Figure 3.4**). For each of these samples, the two alleles present were within one repeat of each other and the peaks looked to be present in equal amounts. For some of the samples, rerunning the plate on the ABI Sequencer or redoing the PCR with a lower concentration of DNA resulted in the heterozygote phenomenon disappearing. For one sample from the retrospective study, one sample from the Janeway study and eight samples from the control cohort repeating the PCR did not resolve the issue.

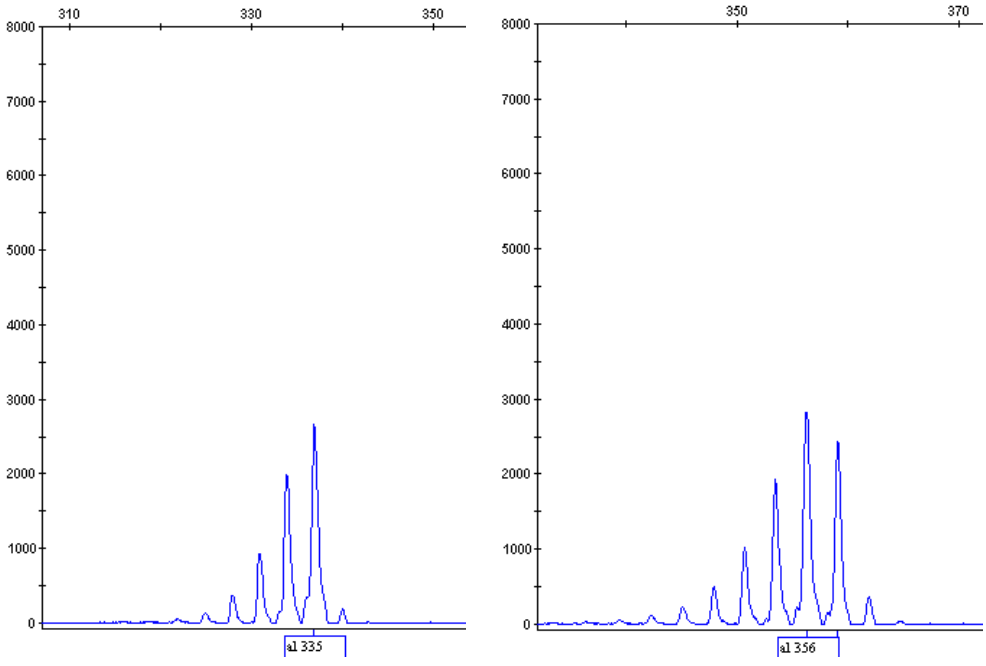


Figure 3.4 – Fragment analysis view of typical *FMR2* allele and heterozygote phenomenon

The two samples from the participant cohorts (retrospective and Janeway) were sent for further evaluation to confirm that only one allele was present. From the control cohort, the eiht samples were removed from the study. For individual III-6 from Family A, the aunt of the proband, a single allele was seen in GeneMapper. As it is not possible to tell from fragment analysis if this female was a homozygote or carried an expansion not detected by PCR, further investigation was required.

3.2.1.2 FMR2 Repeat Range in the Newfoundland Population

The most common allele size in both the retrospective study (n=92) and controls (n=277) in the NL population was 12 (**Figure 3.5**). The distribution of *FMR2* alleles in the test population is very similar to the controls except the test population had two allele sizes (3 and 6) that were not seen in the controls; the smallest repeat in the test population was 3 compared with 8 in the controls. Using a Fisher test the minimal allele size (>10) was not found to be statistically significant in the at-risk population compared with the controls ($p = 0.81$). The highest repeat size seen in the test population was 27 compared with 29 in the controls. The most common allele size seen in the cases and controls in the NL population was 12 compared with a repeat size of 15 in previously published data[29,30,46,89,100-110].

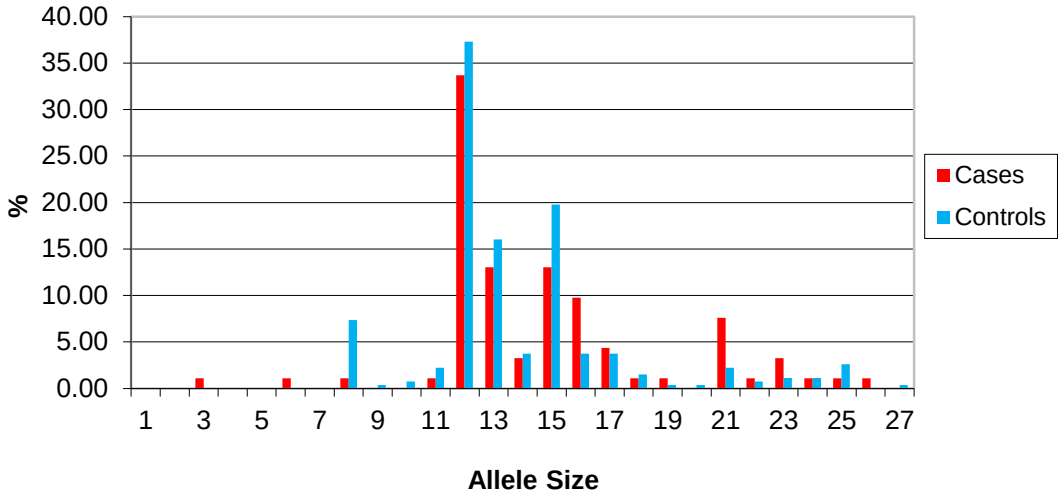


Figure 3.5 – Comparison of *FMR2* repeat range in the retrospective cohort versus the control population in Newfoundland

3.2.1.3 X and Y Chromosome Testing

As discussed above, two samples failed to produce an amplicon – one sample from the PMGP retrospective (03MG438) study and one from the control (C50) cohort. One sample from the retrospective study (12273) and one sample from the Janeway study (364) had two allele sizes which looked like an artifact as the alleles where within one repeat of each other. In addition, one control sample (C49) produced two distinct fragment sizes (15, 22) for *FMR2* (**Figure 3.6**).

For each of the samples, a new aliquot of DNA was obtained and the testing was repeated with the same results seen. To rule out poor quality DNA the samples that failed to produce an amplicon for *FMR2* testing were run using 6 X chromosome markers. As one would not expect to see two alleles for an X chromosome marker in a cohort that

consisted entirely of males, the control sample (C49) was also tested using these markers to determine if this result was something specific to the *FMR2* gene (**Table 3.4**).

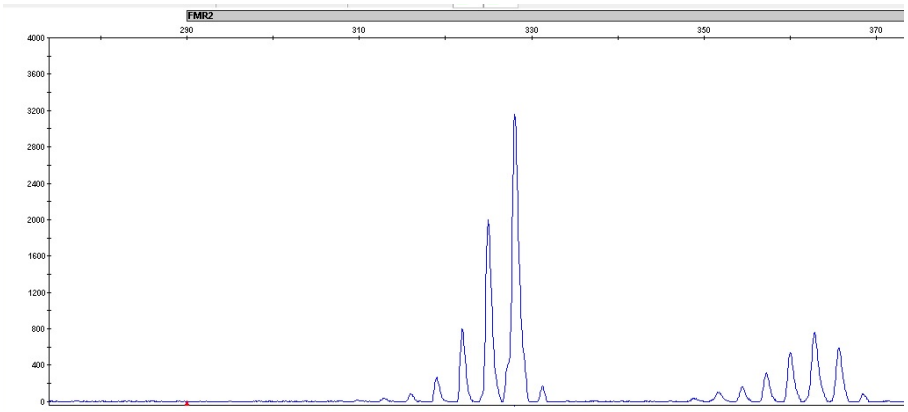


Figure 3.6 – *FMR2* fragment analysis result for control sample C49

Table 3.4 – Fragment analysis testing using X chromosome markers on specific samples from retrospective, Janeway and control cohorts

X Chromosome Markers	03MG438 (Retrospective Cohort)	364 (Janeway Cohort)	C49 (Control)	C50 (Control)
DXS990	124	126	132	130
DXS1226	295	289	301, 307	293
DXS1214	288	296	288, 294	286
DXS986	165	161	165	167
DXS8055	316	314	316	316
DXS991	330	326	330	326

Amplification for all six X chromosome markers were seen in both the retrospective (03MG438) and control sample (C50) that failed to produce an amplicon for *FMR2*. This ruled out poor DNA quality as the possible explanation for lack of amplification for *FMR2*.

Premutations and full mutations cannot be detected using this technique and therefore may be the reason why these samples failed to produce an amplicon. An alternative possibility is that there was a single nucleotide polymorphism (SNP) in either of the primers used for *FMR2* testing causing the primer not to anneal. The samples were then sent to a clinical laboratory to determine their *FMR2* status.

As indicated above, control sample C49 was heterozygous for *FMR2* as well as two out of the six additional X chromosome markers tested. A possible explanation for this is that there was a mix-up in the DNA and the sample was actually female. To exclude this possibility, the sample was genotyped for three Y chromosome markers which all amplified (**Table 3.5**). Therefore, it can be assumed this individual's karyotype is 47, XXY, Klinefelter's syndrome.

Table 3.5 – Fragment analysis testing using Y chromosome markers on sample C49 from the control cohort

X Chromosome Markers	**C49 (Control Cohort)**
DYS490	173
DYS643	133
DYS570	257

3.2.2 *FMR2* Expansion Testing

3.2.2.1 FMR2 Expansion Testing - First Diagnostic Laboratory

As described in **section 2.2.6** samples with possible *FMR2* expansions (03MG438 and C50) were sent to an outside clinic laboratory, Human Genetik, for expansion testing. The retrospective sample (12273) and the Janeway sample (364) which showed a heterozygous result within one repeat size were also sent for further investigation. The aunt of the proband from Family A (III-6) whose *FMR2* result was a single allele was sent to confirm if she was indeed a homozygote or harboured an expansion not detected by PCR method. Additional family members from Family A (IV-1, III-2, III-8) were sent as positive controls (see **section 3.3.1.1; Figure 3.8** for pedigree). The results received from the diagnostic laboratory are shown in **Figure 3.7**.

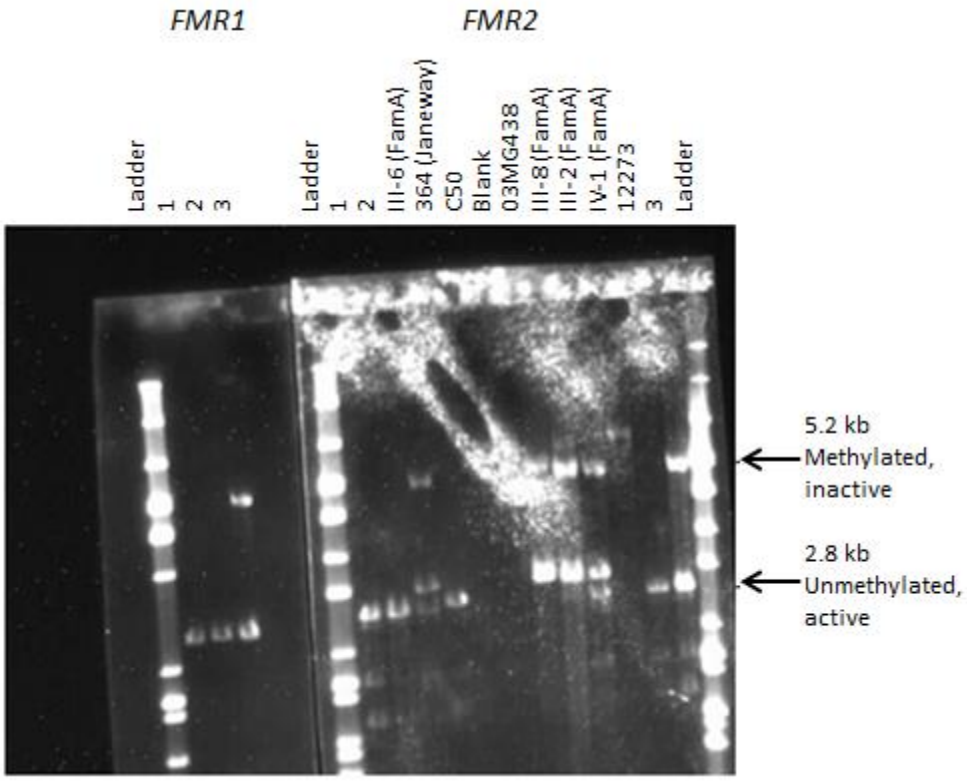


Figure 3.7 – *FMR2* expansion testing using Southern blot on select samples from clinical laboratory. Legend ID – Sample 1 and 2 are normal male and female; sample 3 is a knockout of the gene. The remaining samples are from Family A (III-6 aunt; III-8 aunt; III-2 mother; IV-1 proband), Janeway sample (364), control sample (C50) and the retrospective samples (03MG438, 12273).

The quality of the data received from the outside clinical laboratory was disappointing. A summary of the results can be seen below (**Table 3.6**). For samples 12273 and 364 from the retrospective and Janeway study, respectively, the lab reported no pathogenic mutation. This was as expected as other samples that showed the same allelic pattern when repeated only resulted in one fragment size. These were the only two samples that a mutation report was received from the clinical laboratory.

For two of the samples, the proband from Family A (IV-1) and the control sample (C50), no signal was detected which they suggested was due to low quantity of DNA available. To provide a result a new aliquot for the sample would need to be sent. For the two test samples, 03MG438 and the aunt (III-6) from Family A the lab said star activity was present. This indicated that something present in the sample interfered with the digestion. These samples were extracted using standard salting out method which has been known to interfere with downstream applications. The lab however did not suggest that an additional clean up method would alleviate the problem.

Table 3.6 - A summary of *FMR2* expansion testing on select samples provided by an outside clinical laboratory (Humane Genetik)

Cohort	Sample ID	Clinical Laboratory Testing Result	Previous Testing
Retrospective Cohort	03MG438	Star activity	N/A
	12273	Normal no pathogenic expansion	N/A
Janeway Cohort	364	Normal no pathogenic expansion	N/A
Family A	IV-1 (proband)	No signal	~ 620 repeats
	III-2 (mother)	Star activity	~ 120 repeats
	III-6 (aunt)	Star activity	N/A
	III-8 (aunt)	Star activity	~ 87 repeats
Control	C50	No signal	N/A

Star activity refers to insufficient digestion of assay. N/A = not applicable.

3.2.2.2 FMR2 Expansion Testing – Second Diagnostic Laboratory

During this time, a research laboratory in Chicago, Rush University Medical Center, offered both expansion and methylation testing for the *FMR2* gene requiring the same amount of DNA the clinical laboratory in Germany needed for expansion testing only. As the amount of DNA available was limited and the test required 8 µg, it was determined in the best interest to preserve the valuable sample it would be sent to the Rush University Medical Center. All samples were sent to the research laboratory except for the two samples that were reported not to have a pathogenic expansion. A summary of the results received from Rush University Medical Center is shown below (**Table 3.7**). No Southern blot image was provided. As indicated in the table below sample 03MG438 from the retrospective study was found to harbour an *FMR2* expansion.

Table 3.7 – A summary of *FMR2* expansion and methylation testing on select samples provided by an outside research laboratory (Rush University Medical Center)

Cohort	Sample ID	Mutation Status	Methylation Status
Retrospective	03MG438	small full/premut. (~ 250)	Eco RI site - <10% methylated Eag I site - 10% methylated
Family A	02MG693 (IV-1) Proband	full mutation	Eco RI site - 80% fully methylated; 20% partially unmethylated Eag I site - 20% fully methylated; 80% partially unmethylated
	02MG693 (III-2) Mother of Proband	premutation (~70-80)	unmethylated on active X non-random X- inactivation (Premutation allele active allele 70% cells)

Cohort	Sample ID	Mutation Status	Methylation Status
	377 (III-6) Aunt of Proband	premutation (~ 80-100)	unmethylated on active X
			non-random X- inactivation (Premutation allele active allele 60% cells)
	03MG1104 (III-8) Aunt of Proband	premutation (~60-70)	unmethylated on active X
			random X-inactivation 50/50
Control	C50	Normal (no pathogenic mutation)	

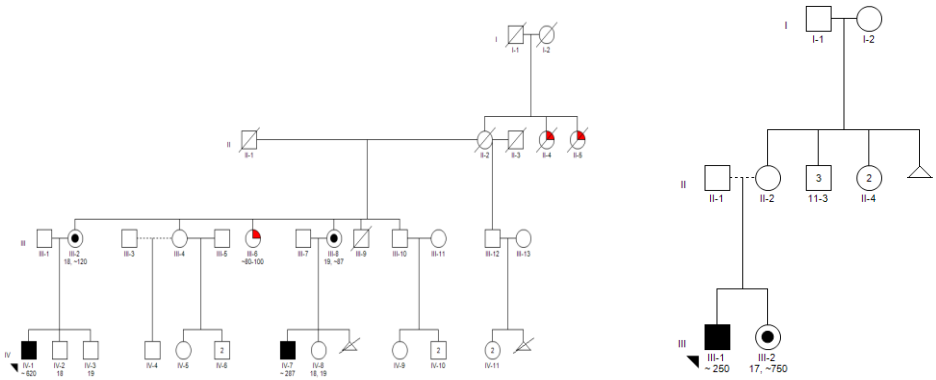
3.3 FRAXE Positive Families and Founder Effect

3.3.1 Clinical Description of FRAXE positive Families

As stated, at the time of this study two FRAXE expansion families were known to the PMGP and one of these families (Family A) consented to be a part of this research project. This retrospective cohort of less than 100 NL boys revealed another FRAXE positive family (Family C). While this study was in progress, two additional FRAXE positive families were referred to the PMGP and were invited into the research project (Family D and E). Below is a description of the four families that consented to research (**Figure 3.8**).

Family A

Family C



Family D

Family E

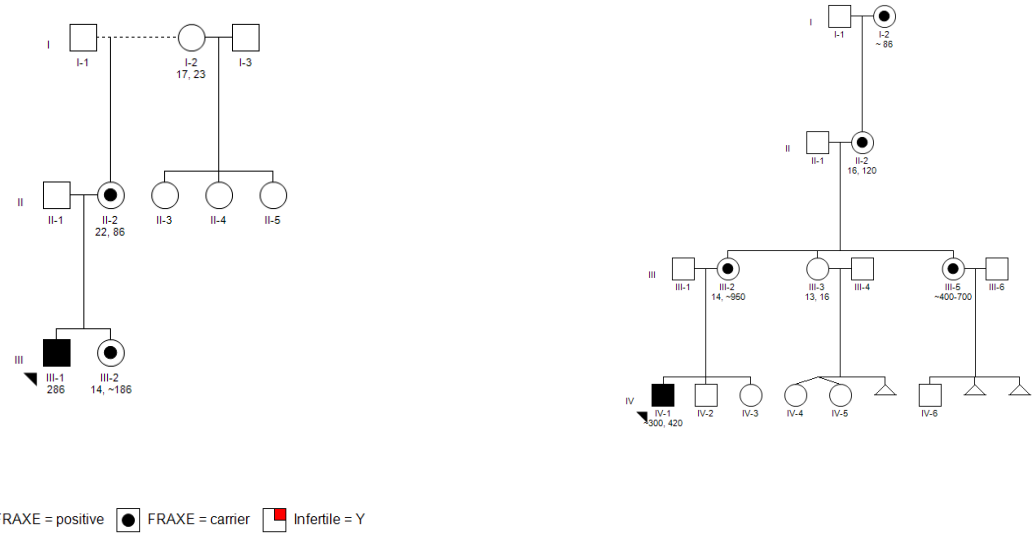


Figure 3.8 – Pedigrees of four FRAXE positive families in Newfoundland

3.3.1.1 Family A

The proband (IV-1, **Figure 3.8**) was investigated at 3 years of age for congenital macrocephaly, hypotonia, developmental delay and unusual facial features. Routine chromosomal analysis revealed a fragile site at Xq27 in 7 of 22 lymphocyte metaphases; the lymphocytes were cultured in a standard way and not in folate deficient media. *FMR1* testing was normal, so *FMR2* testing was arranged and revealed a full *FMR2* expansion (~620 CGG repeats). Methylation testing showed 80% full methylation at the Eco RI site and 20% fully methylated at the Eag I site, with no DNA completely unmethylated. Genome-wide microarray analysis (Agilent's 40k oligonucleotide array) failed to identify any pathogenic genomic deletions or duplications.

The proband remained hypotonic until 4 years. He walked at 4 years and did not speak in sentences until 6 years. His academic curriculum was modified, with a criteria C designation for moderate-severe cognitive delay. At 15 years, the Wechsler Intelligence Scale for Children III (WISC-III) testing showed a standard IQ score of 40, with < 1^{st} percentile ranks in all areas. His visual, motor, academic and cognitive skills were at a kindergarten level. When examined at 16.5 years, IV-1's head circumference was 60.75cm (>> +2SD) and he had several facial features reminiscent of Fragile X syndrome including large ears, a prominent chin and forehead and other dysmorphic findings including a high arched palate and a wide mouth with thick lips.

When examined at 21 years, the proband had macrodolichocephaly with a head circumference of 61.2 cm (>> +2SD); height was 173.5 cm (20^{th} – 50^{th} centile) and weight was 70.5 kg (50^{th} centile). He had male pattern baldness, bitemporal narrowing

and prominent supraorbital ridges. He had brilliant green iridies with long palpebral fissures and ocular hypertelorism; inner canthal distance (ICD) was 6.4cm (well above +2SD). He had a wide mouth with thick lips and a high arched palate. His four upper incisors were wide spaced. Palmar creases, hand length and genitalia were all normal. As an additional measure, a higher resolution microarray was performed in a diagnostic laboratory and the proband was found to carry a heterozygous deletion of the 1p36.33 region. The deletion was also present in his healthy father and is most likely a benign copy number variant.

During this time, an intellectual assessment was done using the Wechsler Adult Intelligence Scale (WAIS-III). The proband's full scale score was IQ 54, which places him in the mild (bordering on moderate) intellectual disability (ID) range. There was no difference between the verbal and performance IQ (58). The subtest scale scores indicated his strengths are mainly in the nonverbal/visual areas and he was very weak in the area of verbal skills. At present, he can be left home alone and can operate an all-terrain vehicle (ATV) which he uses when hunting rabbits.

The proband's mother (III-2) carried a premutation of approximately 120 repeats. Methylation analysis revealed the *FMR2* site was unmethylated and that there was non-random X inactivation. The premutation allele was active in 70% of the cells, whereas the normal allele was active in 30% of the cells. When examined at 49 years, she had borderline large ears and a wide mouth and otherwise non-dysmorphic. She did not complete high school for social reasons, but did not have a history of academic difficulty. Her full-scale IQ score was 84. There was significant discrepancy between her verbal comprehension score (74) and her process speed scores (108) indicating that her visual

processing skills were better developed than her verbal reasoning skills and verbal conceptualization skills. She was employed as a home support worker.

The proband's mother (III-2) had two other sons (IV-2, IV-3) who inherited the normally sized allele from her. IV-2 was 26 years old at the time of the proband's second assessment (at age 21 years). His curriculum was modified at school but he graduated from high school. The proband's other brother (IV-3) was 29 years old at the time of the proband's second assessment. His mother reported that he was behind in school in the early years but did graduate from high school with an A average and completed some post-secondary education.

The proband's mother (III-2) has three sisters, two of whom (III-6 and III-8) carry premutations. III-6 (age 50 years at the time of testing) had a premutation of approximately 80-100 repeats, which was unmethylated. This premutation was identified through this study. The premutation allele was the active allele in 60% of the lymphocytes tested on her blood sample. III-6 reported that she was unable to have children. She worked as a homecare worker with handicapped children.

The proband's mother's other sister (III-8, age 40) also had a premutation of approximately 60-70 repeats which was unmethylated. Random X-inactivation was observed. III-8 has two children, one of whom (IV-7, age 21) had a small full mutation (~287 repeats). He had academic difficulty but finished high school and was employed as a laborer away from home. He was not available for examination or further assessment.

3.3.1.2 Family B

This family did not consent to research and therefore no clinical information is available.

3.3.1.3 Family C

This proband (III-1, **Figure 3.8**) was diagnosed with FRAXE through this study. He was originally referred for genetic consultation at age 7½ years because his biological mother had neurofibromatosis type 1 (NF1). III-1 has no cutaneous features of NF1 and his family was counseled that he was unaffected. At the time, *FMR1* testing and routine chromosome analysis was performed because of academic difficulty. No X chromosome fragile site was identified on standard chromosome analysis. He was not tested for an *FMR2* expansion at the time of the initial clinical assessment.

He was tested for *FMR2* because he fit the criteria for the retrospective part of the study which included male sex; referred to genetics with development delay; received *FMR1* testing and remained without a specific genetic diagnosis. *FMR2* testing revealed a 250 repeat which was mostly unmethylated. Chromosome analysis using a folate-deficient medium showed a fragile site at Xq27.3 in 5/60 metaphases (8%). CGH microarray analysis was normal.

At the time of the initial genetic consult (age 7 ½ years old), the parent reported that he could count to ten, but could not read well. He could print his name at the kindergarten level and was in a modified curriculum at school. He had a few minor physical anomalies (mild brachycephaly, borderline low set ears and mild clinodactyly of the 5^{th} digits) but was not strikingly dysmorphic. He also had congentially fused left

fourth and fifth metatarsals. A detailed family history was not available; however, it was noted that his biological mother did not finish high school and was said to be a poor reader.

During the first 11 years of life, III-1 had poor home circumstances. The medical geneticist reviewed an educational assessment from the school which was done at the end of his grade 6 year (June 2008, age 12 years). The assessment indicated that he was having academic difficulty with all subjects with the exception of Health and Social Studies. He received pathways 2 and 4 support. The pathway 4 support included an alternate course in literacy skills, provided by the special education teacher. Pathway 2 is the provincially prescribed curriculum with student specific strategies and supports, whereas Pathway 4 is a combination of the core curriculum and individually designed curriculum to meet the student's individual needs.

At the age of 12 (grade 7), the Wechsler Individual Achievement Test (WIAT-II) was administered showing a total score of 71 (3^{rd} centile rank), with math reasoning skills at the 10^{th} centile, spelling at the 3^{rd} centile and written expression at the 5^{th} centile. Written spelling was at a grade 3.7 equivalent.

When the proband was examined at 12 years, height was 159 cm (50-75^{th} centile), weight was 70.3 kg (97^{th} centile) and head circumference was 56 cm (+1.5 SD). He had some mild dysmorphic features including mild brachycephaly, borderline ocular hypertelorism with epicanthal folds and hooded eyelids, deep-set nails and slightly low-set ears with thick, fleshy lobules. According to his case worker, he had some aboriginal ancestry and he was not considered to be strikingly dysmorphic by the geneticist.

The proband had his left 5th ray amputated because of foot pain. He also had a tonsillectomy and nasal cauterization for recurrent nose bleeds. He was seen by a neurologist who diagnosed him with simple motor tics that included head flicking.

The proband's case worker described him has having a fairly good attention span and noted improvements in his reading skills since receiving tutoring. At this time of assessment, the boy was living in a group home and still had some academic difficulty.

The proband's sister (III-2) who also had academic difficulty carried an *FMR2* expansion of 750 repeats. Her initial chromosome analysis was normal at the 500 band level. Testing using a folate deficient medium showed an Xq27.2 fragile sit in 7/20 metaphases (35%). III-2 also had a genome-wide microarray (Agilent's Oligonucleotide Array, EmArray Cyto6000 Custom Design) which showed normal DNA copy number.

She was assessed at age 14 ½ years and was also following a modified curriculum at school. Her teachers reported significant improvements in her reading since receiving additional support. She attended speech therapy as a child. On examination at 14½ years, the proband's sister's height was 159 cm (25-50th centile), weight was 53 kg (50th centile), and head circumference was 55.4 cm (+1SD). She was not dysmorphic. A short philtrum was noted (length 1.2 cm, slightly less than the 3rd centile). There were no cutaneous findings of NF1.

An EEG showed diffuse slowing of background waveforms on EEG. Overall, she was very articulate and her academic difficulty had improved significantly since her home environment stabilized.

3.3.1.4 Family D

This 10 year old boy (III-1, **Figure 3.8**) was seen in the genetic clinic with his sister (III-2) who was referred because of a family history of multiple endocrine neoplasia type 1 (MEN1). Genetic testing for *FMR1* and *FMR2* was ordered for this boy because the genetic counsellor noted a history of mild learning difficulties in school. He had a full *FMR2* mutation of 286 repeats. He walked independently at 18 months and had speech therapy due to a speech delay. At age 10 years, all classes were in a special education stream and there were no behavioural difficulties.

When examined at age 10 years, the proband's height was at the 25th centile, weight at the 10th centile and head circumference was at the 10th centile. He had triangular facies. He was mildly dysmorphic with protruding ears, partial syndactyly of the second and third toe. He had right cryptorchidism.

The proband's mother (II-2, age 37) had a premutation of 86 repeats. She reported struggling academically and needing additional support with reading. The proband's sister (III-2, 13) also carried a premutation allele, with a repeat size of 186. The mother reported her daughter (III-2) had some difficulties with reading. The grandmother (I-2) was tested and found to have two normal *FMR2* alleles. One would expect that the mother had inherited the *FMR2* mutated allele from her father; however, there was no contact with this side of the family so further testing was not possible.

3.3.1.5 Family E

This male proband (IV-1, **Figure 3.8**) was referred to genetics at 3.5 months of age because of a petechial rash, decreased platelets and anemia. At birth, he had no spontaneous respirations for two minutes and was intubated shortly after delivery. APGAR scores were 4 at 1 minute, 8 at 5 minutes and 8 at 10 minutes. In the neonatal period, an echocardiogram (ECG) revealed cardiac enlargement and a head CT showed an ill-defined hypodense area in the right occipital region consistent with ischemia. Both tests were normal when they were repeated. He was assessed by a clinical geneticist and no genetic testing was felt to be necessary at the time.

The proband reached his early childhood developmental milestones on time. He used single words at 12 months, crawled at 8 months and walked independently at 15 months. By three years his vocabulary was described as advanced for his age.

At age twelve years, the proband was seen again in the genetics clinic because his maternal aunt (III-5, 39 yrs) had a routine chromosome analysis prior to in vitro fertilization (IVF) that showed a Xq fragile site. The proband had *FMR2* testing which showed mosaicism for a full mutation with sizes of approximately 300 and 420 repeats.

At 11 years, the proband had IQ testing. A Wechsler Intelligence Scale for Children IV (WISC-IV) was administered and full scale IQ was 96 (39^{th} centile – normal range). There was a significant discrepancy seen between his verbal comprehension score (108) and processing speed score (75). During the psychometric testing, he was noted to be highly active, moving in his chair and walking around the room constantly. The examiner concurred with his parents and teachers and suspected higher intelligence given

his strong verbal skills, abstract reasoning and creative ideas. This was supported by the Woodcock Johnson test of Cognitive Abilities III in which he scored in the superior range for verbal ability (121–92th centile) and comprehensive knowledge (121–92th centile). The examiner noted the testing scores would be minimums as he was highly unfocussed during the exam and felt that his true ability was potentially even higher. The examiner also noted some obsessive-like thoughts/behaviours and some unusual vocalizations.

At age 13 years, the proband was diagnosed with attention deficit disorder (ADD), inattentive type. He had long standing attentional difficulties at school and was on a stimulant medication. Printing skills were messy and he was unable to perform cursive writing. He used a voice recognition system to do homework, but followed a regular curriculum with a 70's average. Although he preferred to socialize with adults, there were no behavioural or social concerns.

On physical exam at age 13 years, he was considered to be non-dysmorphic. The proband's height was 164 cm (50th centile), weight 61.3 kg (75th centile) and head circumference was 59 cm (+3SD). He had sandy blonde hair, green irides and triangular facies with a somewhat low frontal hairline. His eyes were normally placed with a normal well developed philtrum. His mouth was small and he had a wide space between his upper central incisors, with minor dental malocclusion. His ears were normal in position and contour. There was no abnormality of the feet and hands. Palmar creases were normal and there was no clinodactyly of the digits. He had hyperextension of the elbows particularly of the left, with cubitus valgus. He had axillary hair, normal genitalia (Tanner stage 5) and there was no macroorchidism.

The proband's mother (III-2) carried a full mutation of approximately 950 repeats. She had two additional children who did not have any learning problems and were not tested for *FMR2* expansions.

The mother (III-2) was hyperactive as a child, but did not have academic difficulty and has several years of post-secondary training. On examination at age 39, height was 158 cm (10-25th centile), weight was 66.9 kg (75th centile) and head circumference was 58 cm (+2SD). She was not dysmorphic.

The proband's maternal aunt (III-5) who had the cytogenetic fragile site, had a mosaic *FMR2* expansion with allele sizes ranging from 400-700 repeats. Her chromosome analysis was ordered as part of work-up for infertility, prior to undergoing in vitro fertilization (IVF). She had no academic difficulty and had post-secondary education.

The proband's grandmother (II-2) had a premutation of 120 repeats and the great-grandmother (I-2) had a premutation of approximately 86 repeats.

In the literature FRAXE is described as being characterized by mild (IQ 50-69) to borderline (IQ 70-79) ID, learning problems, communication problems and overactivity, with no consistent dysmorphic features[36]. A summary of the male probands identified in the Newfoundland population with reference to the FRAXE phenotype description is provided in **Table 3.8**.

Table 3.8 – Summary of male probands from each of the four Newfoundland families with reference to the FRAXE phenotype description

	Family A IV-1	**Family C III-1**	**Family D III-1**	**Family E IV-1**
FMR2 repeat size	620 repeats	250 repeats	286 repeats	300-420 repeats
Cognitive ability	FSIQ - 54	FSIQ -71	*N/A	FSIQ – 96
Learning problems	Yes	Yes	Yes	Yes
Speech Delay	Yes	Yes	Yes	No
Attention problems	No	Yes	Yes (ADD)	Yes (ADD)
Tic disorder	No	No	Yes	No
Presence of Dysmorphology	Yes (like FRAXA)	Minor dysmorphism	Minor dysmorphism	No

*No formal IQ testing. Clinically, he appeared to have mild ID (early milestones delayed and at the age of 10 years, alternate curriculum at school). ADD = formal diagnosis of Attention Deficit Disorder

Consistent with previously published data, the probands in this study had inconsistent dysmorphology. Proband IV-1 from Family A was dysmorphic with physical features highly reminiscent of Fragile X syndrome (*FMR1* testing was normal). He had a long forehead, prominent chin and large ears, high arched palate and macrocephaly which are common features seen in patients with Fragile X syndrome due to *FMR1* expansion mutations. Proband III-1 from Family C had minor dysmorphism including mild brachycephaly, borderline low set ears and mild clinodactyly of the 5th digits. Proband III-1 from Family D also had very mild dysmorphism with protruding ears, mild syndactyly

of the second and third toes and right cryptorchidism. Proband IV-1 from Family E was not dysmorphic.

IQ testing was only available on three out of the four probands. One male (IV-1 from Family E) had an IQ within the normal range (FSIQ-96). A second male had a borderline low IQ (III-1 from Family C, FSIQ-71). The proband from Family A had mild ID (FSIQ-54) and had a more severe phenotype than the other three probands. Learning problems were seen in all four probands even though the degree of disability and extra help needed was variable. Speech delay was seen in each of the probands except IV-1 from Family E. Attention problems were seen in all probands except IV-1 from Family A, and two probands were diagnosed formally with ADD.

Table 3.9 – Summary of full mutation males, full expansion females and premutation females identified in the Newfoundland population

	Full mutation males	**Full expansion females**	**Premutation females**
Family A	IV-1 (~ 620 repeats)		III-2 (~ 120 repeats)
	IV-7 (287 repeats)		III-6 (~ 80-100 repeats)
			III-8 (~87 repeats)
Family B*	Full expansion		
Family C	III-1 (~250 repeats)	III-2 (~750 repeats)	
Family D	III-1 (286 repeats)		III-2 (~186 repeats)
			II-2 (~ 86 repeats)

Family E	IV-1 (~300, 420 repeats)	III-2 (~950 repeats)	II-2 (120 repeats)
		III-5 (~400-700 repeats)	I-2 (~ 86 repeats)
Total	6	3	7

*Family B did not consent to research but proband was known to PMGP to carry a full *FMR2* expansion.

3.4 Founder Effect study

3.4.1 KINNECT

Expansion of the families focused on the side of the family that the disease was passing through; if this information was unknown, both sides of the family were investigated. Connections were made in the Newfoundland Genealogy Database (NGD) and this allowed families A and D to be expanded to 4-6 generations before the proband. A summary of the pedigree expansion for all families is shown in **Table 3.10** below. The expanded pedigrees can be found in the appendix (**Appendix M**). Using the NGD, a common ancestor was not found in any of these families.

Table 3.10 – Number of individuals in each of the four FRAXE pedigrees before and after running through KINNECT

Pedigree	Original	After KINNECT	Additional Family Members
Family A	43	114	71
Family C	61	61	0
Family D	26	117	91
Family E	42	55	13

3.4.2 Haplotype Analysis

The proband and any additional family members whose DNA was available were haplotyped using 15/17 markers flanking *FMR2* to help determine if a common ancestor connected these families. Of those 15 markers, only 7 markers were informative. From these seven markers, there is strong evidence that families A, C and D are related as they share a common haplotype of 1.88 Mb. To further support this theory, three generations of Family D were tested and the grandmother (I-2) who did not give the *FMR2* expansion allele to her daughter (II-2) was shown not to have the disease haplotype.

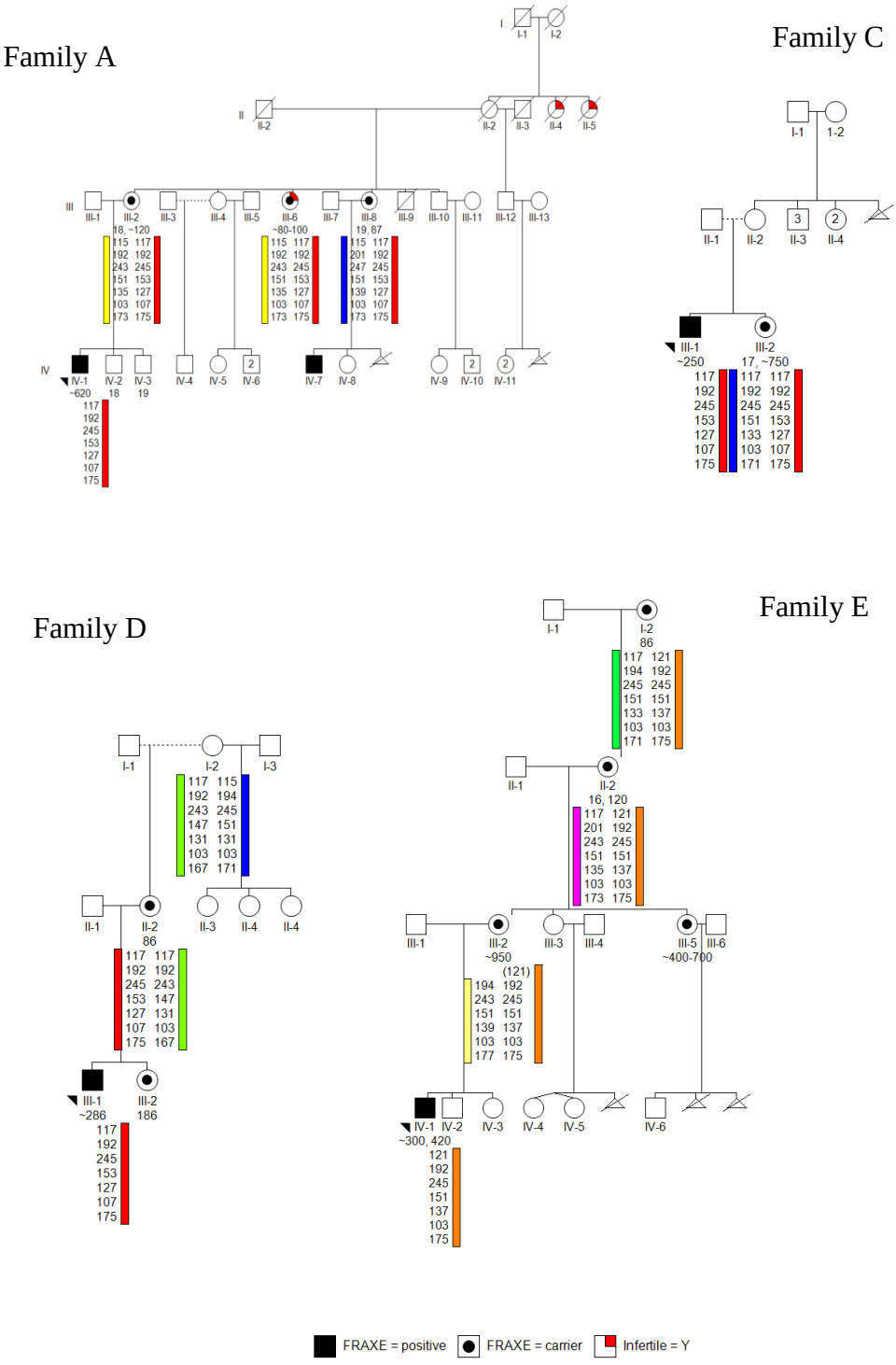


Figure 3.9 – Haplotyped pedigrees of four FRAXE positive families in Newfoundland

3.4.3 Prevalence Rate

For Newfoundland, the minimum prevalence of FRAXE in the male pediatric population (2 to 18 years old) was calculated to be 6 males in 46,420, or 1 in 7,736. The minimum prevalence of FRAXE in the total male population was calculated to be 6 males in 251,901 or 1 in 41,983. The minimum prevalence of expanded *FMR2* alleles in the Newfoundland population was calculated to be 16 in 512,900 or 1 in 32,056.

Chapter 4 - Discussion

Brief summary:

The purpose of this study was to determine the prevalence of FRAXE among boys referred to the Provincial Medical Genetics Program (PMGP) for intellectual disability (ID) and/or developmental delay. FRAXE is exceedingly rare in most populations with a prevalence rate of 1:23,423 males or 0.004%[34]. Because two FRAXE families were known to the PMGP, we hypothesized that FRAXE expansions might be present in additional Newfoundland and Labrador (NL) families with an overall population prevalence that is higher than in the general population. In a cohort of 92 boys referred for developmental delay with negative *FMR1* results, one male (1.2%) was found to carry an *FMR2* expansion. During the length of this study, two additional families became known to the PMGP. In the province of a little over 500,000 people, five Newfoundland families are now known to carry *FMR2* expansions (three families ascertained through PMGP that participated in the research study, one family ascertained through PMGP that did not participate in the research project and one family ascertained through the retrospective cohort). The minimum prevalence rate calculated in the male pediatric population in Newfoundland was 1 in 7,736 or 0.01%.

4.1 Study Design

4.1.1 Inclusion criteria

As the vast majority of Fragile X testing (for *FMR1* expansions) in the PMGP at the time was done on pediatric males, this study included boys between the ages of 2 and 19 years at the time of *FMR1* testing. The inclusion criteria for this study were very similar to what has been reported in the literature. Most research groups investigating *FMR2* targeted individuals with developmental delay and/or ID[31,80-84,102,106-108,123-129]. Several studies looked at males and females with negative *FMR1* testing in combination with developmental delay and/or ID[79,109,124,130], which more closely matched the inclusion criteria for this study. Other reports in the literature were of children with academic difficulties and/or who attended special needs classes[30,34,46,100,104]. Cohorts with other phenotypes have also been screened and these phenotypes included autism spectrum disorder (ASD), Parkinson disease (PD), obsessive compulsive disorder (OCD) and premature ovarian failure (POF)[29,32,39,47,85,110,131]. Although including individuals from these types of broader cohorts was beyond the scope of this project, it is interesting to note that one female from Family E (III-5) was identified when a routine karyotype was ordered as part of an infertility work up. This showed an Xq27 fragile site which turned out to be due to a mosaic *FMR2* full mutation (400-700 repeats).

The retrospective ID cohort from the PMGP consisted of boys with severe enough phenotypes to warrant a clinical genetic assessment. In an effort to mitigate the aforementioned potential limitation and to enrich our chances of findings *FMR2*

expansions, a second cohort was investigated. These were boys assessed through the Child Development clinic at the Janeway Children's Health and Rehabilitation Center for speech delay, learning disabilities or academic difficulties, behavioural abnormalities and/or borderline (IQ = 70-80) or mild (IQ 50-70) ID (see section 2.1.3.2 for inclusion criteria). Many of the children seen at Child Development have milder phenotypes and therefore would not necessary be referred to the PMGP, particularly if the developmental paediatrician considered the child to be non-dysmorphic.

4.1.2 Male preponderance

The chart review for the retrospective study found that of children referred for development delay during the ten-year period (1994-2004), 73.7% (263/357) were male. This finding was consistent with boys being more likely to present to their family physician with developmental delay, ID and/or learning disability. In the literature, it has been reported that 30% more males are diagnosed with ID than females[132]. In 2001, Statistics Canada produced a report that profiled disability in Canadian children and reported that males were more likely to have a speech-related disability than females (46.6% males compared with 37.6% females) and that boys were more likely to be diagnosed with a learning disability than girls (68.9% males compared with 58.0% females)[1]. Common disorders associated with ID such as Fragile X syndrome (FXS) and autism spectrum disorder (ASD) are more common in males than females[18,133]. Girls were excluded from both the test and control cohorts because: 1) testing girls using PCR for *FMR2* mutations presents a problem in the female population as all females carry two X

chromosomes and therefore all alleles that are homozygous for *FMR2* would have required additional testing by Southern blot to rule out a possible expansion; and 2) females with full *FMR2* expansions have milder phenotypes than males with full *FMR2* expansions and were less likely to exist in the PMGP retrospective cohort because the likelihood of being referred for a clinical genetics assessment is relatively low.

4.1.3 Control Cohort

The controls for this study were previously recruited through random-digit-dialing as controls for a study on colorectal cancer. These individuals had agreed to allow their anonymized samples to be used in other research studies. This was an adult control group, the majority of whom were over the age of 40 (704/720 [97.8%])[120]. Ideally the control cohort would have been comprised of neurotypical boys between the ages of 2-19; however, such a cohort was not available. The controls tested were matched for gender and provided a representative sampling of the Newfoundland population. Although it is unknown whether any of the controls had ID, a history of developmental delay or a learning disability, the chance of this was reduced because each participant completed several detailed hand-written questionnaires.

4.1.4 Patient recruitment

Recruitment in the study was lower than anticipated in both the family and retrospective cohorts. The research team did not attempt to enroll Family B as the family was not interested in a follow up appointment with PMGP.

Family A had two boys with *FMR2* expansions, but only one (IV-1 who had a 620 *FMR2* repeat expansion) was seen in clinic. The proband's aunt (III-8, 87 repeat premutation), although willing to give permission for her DNA sample to be used, was not interested in a follow up appointment for herself or her son (IV-7) who carried a 287 repeat expansion. It would have been beneficial to have an assessment on the proband's affected cousin to compare how the disease presented itself within this family. The proband's mother had no contact with her brother (III-10) so it is unknown if he or his daughter (IV-9) carried an *FMR2* expansion. If his daughter (IV-9) carried a premutation or expansion and had a son, he would be at 50% risk of having FRAXE.

The proband for Family C (III-1 who had a 250 *FMR2* repeat expansion) was living in a group home and had little contact with his biological mother and no contact with his biological father. Therefore, no additional family members (apart from his sister, III-2 who had a 750 repeat expansion) could be invited into the study.

In Family E, the 13 year old proband had a mosaic full mutation (~300, 420 repeats). Although multiple generations were available for testing, the proband's mother (III-2, 950 repeat expansion) chose not to have her two other children (IV-2, IV-3) tested

for an *FMR2* allele size. Although the mother reported no issues in these children, they were not assessed clinically and the mother declined genetic testing.

The retrospective cohort had a participation rate of 58.2% (92/158). The participation rate for the Janeway cohort could not be calculated and will be discussed in detail in the limitations section of the discussion. In a review article summarizing participation level in genetic research, the authors reported a range anywhere from 21% to 85% of individuals are willing to participate in genetic research[134]. In general, individuals have mixed views on participating in genetic research studies. A recent study looking at public attitudes about genetic research in NL found that although the majority of participants supported genetic research, there were several areas of concerns[135]. In particular, individuals worried about the storage and protection of genetic information, the potential misuse of genetic information to promote social discrimination and the issue of information to third party groups (in particular, insurance companies)[135].

ID is a very sensitive topic for many families and may have contributed to the overall willingness of parents to participate. Families that have a child with an ID or developmental delay often have additional strains on their time due to numerous appointments (speech therapy, occupation therapy, physiotherapy, etc.) required for the health and well-being of their child and may feel they do not have the time to participate in research, particularly if they perceive that there will be no direct benefit to their family.

4.2 *FMR2* Analysis

4.2.1 PCR-based *FMR2* Testing

Overall, interrogating the *FMR2* locus was problematic. Standard PCR conditions did not work for this locus. It is highly repetitive and has a high GC content. GC rich amplicons are more stable and therefore require a higher than normal melting temperature. In order to alleviate this, multiple experiments were performed under conditions favourable to GC rich amplicons such as higher annealing temperature, higher $MgCl_2$ concentration and polymerase Taq kits specific for GC rich areas[136]. By using 7-deaza-dGTP, in combination with DMSO and betaine, and a slightly longer initial denaturation time, successful amplification was achieved.

All samples tested were in the normal range except for two samples (one from the retrospective cohort and one from the control cohort), which failed to produce a visible amplicon. Possible explanations for this included human error, insufficient DNA quality or lack of amplification of the product either due to a single nucleotide polymorphism (SNP) in the primer preventing binding or due to an expansion in the *FMR2* repeat region. A new aliquot for each sample was obtained, but revealed the same result. Additional X chromosome markers were tested to ensure the DNA was of sufficient quality for amplification and each marker produced a result.

The samples were sent to an outside research laboratory for expansion testing and one sample in the retrospective cohort was found to carry an *FMR2* expansion. It was not surprising that 1.1% (1/92) of boys were found to carry an *FMR2* expansion as it was

postulated that the NL population may be enriched for *FMR2* expansions. Comparing our findings to published reports, 77.8% (28/36) of studies were unsuccessful in finding an *FMR2* expansion in their populations[29-32,39,46,47,100-104,106-111,123-131,137]. A very large study of 3,738 boys only identified one boy with an *FMR2* expansion[34]. The at-risk cohorts that were tested in previous studies included children (both males and females) with ID and/or academic difficulty, individuals with ASD, OCD, Parkinson's disease and POF (**Appendix L – Supplementary Table S.1**). In studies with comparable inclusion criteria to this study, individuals who were negative for *FMR1* expansions and who had developmental delay and/or ID (n = 841), no *FMR2* expansions were observed[102,109,124].

The sample from the control cohort that failed to produce an amplicon was also sent to an outside research laboratory for expansion testing and was found to be in the normal range. As mentioned above a possible explanation for this result may be that there was a SNP in the primer region preventing binding. Since the initiation of this study, the number of known SNPs has grown exponentially. Cross referencing the primer sequence against known SNPs in SNPCheck3 and AluMut Visual®, no SNPs were found. This individual may have a rare SNP in the primer that has not been reported in the literature, perhaps unique to the Newfoundland population. As the primer used by the research laboratory may have been different than the primer used in this study, this may explain why the external laboratory was able to obtain an amplicon.

If a larger Janeway cohort had been assembled it may very well have identified additional boys with *FMR2* expansions as this cohort more closely matched the FRAXE phenotype description. The Janeway cohort was made up of only 14 boys and information

on their eligibility criteria was not forwarded to the research team. The problems with recruitment and lack of tracking information are discussed in greater detail later in the discussion (**section 4.5**). Overall the number of boys enrolled in the Janeway cohort was too small to make any inferences regarding this population of children.

4.2.2 Anomalies within the test results

The genotyping for the *FMR2* repeat looked unusual for a small number of samples in the at-risk populations (the retrospective and Janeway cohorts) as well as the controls. The samples appeared heterozygous; however, in each case the allele size was within one repeat size of the other (i.e., 15, 16). In the case of a true heterozygote allele, which differ by one repeat, the smaller allele will produce a larger peak than the higher allele because it has the combined effect of its allele size and the stutter of the higher allele. However, this was not the case in these samples. By rerunning the plate or repeating the PCR with less DNA for most of these samples, this phenomenon disappeared indicating that it was an artifact of the PCR amplification. The probable explanation for this observation is amplification slippage[138]. If a mistake occurred in the first couple of rounds of PCR amplification, then the true amplicon and the artifact amplicon would be amplified in similar proportions, showing a result that looked like a heterozygote. For the test samples, additional testing was performed by Southern blot to ensure that indeed this was a mistake during PCR replication and not a true heterozygote. The control samples (n = 8) that did not resolve after repeat PCR where excluded from the cohort.

A single control sample (C49) presented with a unique finding – two alleles for *FMR2* were observed. It was speculated that human error when pipetting the sample into the test well was the explanation, as a male sample would only have one allele for an X chromosome marker. To address this possibility, a new sample was requested from the stock tube, which yielded the same result. To determine if this was something specific to the *FMR2* gene, additional X chromosome markers were tested and the sample produced two alleles for markers DXS1226, and DXS1214, indicating the presence of two X chromosomes. It was possible the sample was female and a mix up had been made by the laboratory when the DNA was extracted for the CRC study or alternatively, that the patient had a sex chromosome aneuploidy. To determine if indeed the sample was male, three Y chromosome markers were tested and for each marker an amplicon was seen suggesting the karyotype for this individual was 47,XXY (Klinefelter syndrome).

Klinefelter syndrome is a chromosomal disorder that results when a male has an extra copy of the X chromosome. It often goes undiagnosed until adulthood. An affected male may be identified when he fails to go through normal puberty or when he is unable to have children. The phenotypic features include small testicles, enlarged breasts, sparse facial and body hair with the inability (or reduced inability) to produce sperm. It is the most commonly observed sex chromosome anomaly and occurs in 1 in 500 – 1 in 1000 males[139]. In this particular case, the sample was an anonymized one from the CRC control group and therefore this type of phenotypic information was not available.

4.2.3 Repeat Range in the Newfoundland Population

The most common allele size seen in both the retrospective cohort and the NL controls was 12 CCG repeats and no sample carried an intermediate or premutation allele for *FMR2*. In the literature, the most common allele size identified was 15, although depending on the population mean normal allele sizes of 13, 14, 16 and 17.59 were also seen [29,30,46,89,100-105,107-110]. The retrospective study had two allele sizes (3 and 6) not seen in the controls. To determine if there was a difference in minimal allele size (>10) in the test population versus the controls, a Fisher test was performed showing no significant difference between the two population groups ($p = 0.81$).

It was expected that the Newfoundland population would carry alleles in the higher range as this might be a possible explanation as to why there are more cases reported here than anywhere else in the world. However, this was not the case. In fact, the NL population exhibited a smaller modal size than other reported populations.

As mentioned in the results (**section 3.2.1.1**), the repeat size obtained using fragment analysis differed by three repeat sizes from the result obtained by Sanger sequencing. This is a common anomaly noted in other studies looking at trinucleotide repeats[140]. In order for this study to be compared with the repeat sizes reported in the literature, the repeat size was not corrected for by the sequencing factor. Of all the studies that reported on repeat size only a small number of publications reported they adjusted by sequencing result. This is an important factor to consider when making inferences between different research studies. However, if the repeat size in the NL population was

adjusted for by the sequencing factor, then the most common repeat size is 15 CCG repeats.

Although a common practice in the literature, it is very difficult to compare repeat sizes across different published reports. Repeat sizes can differ between laboratories based on a number of factors including: the primers used, which primer was labelled (forward or reverse), binning criteria, and differences in equipment, chemistry and the fluorophore used. The actual repeat size for an individual is not critical, only which repeat class they fall into – normal, intermediate, premutation or full mutation. For any result that falls near the borderline of two repeat classes, a second method for investigating repeat size should be used.

4.3 Clinical description of FRAXE positive families

Due to the suspected excess of FRAXE cases in Newfoundland, we hoped to refine the FRAXE phenotype that has been reported in the literature. Reviewing the positive cases in this province largely confirmed what is in the literature with regards to the phenotype. All four probands (Families A, C, D and E) had learning problems, three had delayed speech (Families A, C and D), three had attention difficulties (Families C, D and E) and two were formally diagnosed with ADD (Families D and E). These are all known features of FRAXE.

For three of the four probands where formal IQ testing was available, one had mild ID (Family A, FSIQ 54), one had a borderline low IQ (Family C, FSIQ 71) and one had an IQ within the normal range (Family E, FSIQ 96) with a formal diagnosis of ADD. The fourth proband (Family D) had delayed early childhood milestones, and at age 10 years followed an alternative curriculum at school and so very likely had at least a borderline-low IQ, if not mild ID. We suggest that it is important point for clinicians to consider the possibility of an *FMR2* expansion in a boy with an IQ in the normal range who has other neurobehavioral abnormalities (e.g. learning disability, ADD/ADHD), particularly if that boy comes from NL.

Unlike the other children with positive *FMR2* expansions, the Family A proband had a more severe phenotype. He was the only child referred to the PMGP due to developmental delay and a syndromic appearance. He had facial features similar to those observed in FXS and had mild ID by formal psychometric testing.

A comparative genomic hybridization (CGH) microarray was ordered to rule out any additional genetic contributors to his phenotype and the patient carried a small deletion of 1p36.33 (less than 1 Mb). This proband's deletion overlaps a much larger 2.5 Mb that is associated with a contiguous gene deletion syndrome, monosomy 1p36 syndrome. For this syndrome, the size of the deletion influences the phenotypic features[141]. The prevalence of this syndrome is estimated to be 1 in 5,000 to 1 in 10,000 births, with a 2:1 female to male ratio and all affected individuals have developmental delay or ID[142]. 1p36 deletion syndrome has typical dysmorphic features that include microbrachycephaly, midface retrusion, straight eyebrows, deep set eyes, epicanthal

folds, wide and depressed nasal bridge, long philtrum, pointed chin, large anterior fontanel and low-set ears[142].

The Family A proband was very dysmorphic, but his appearance was not consistent with 1p36 deletion syndrome. Instead of having a small head circumference (microcephaly), he had macrocephalic with very different facial features that included a prominent forehead and jaw, a long narrow face, hypertelorism (widely spaced eyes), large ears, high arched palate, thick lips and teeth irregularities. For the Family A proband, it is unlikely that his heterozygous 1p36.22 deletion explains the severity of his phenotype, including his highly dysmorphic appearance, as it is also present in his father who does not have ID and who is not dysmorphic.

The Family A proband had a very syndromic appearance, and the Family C and D probands were mildly dysmorphic, which is in contrast to the fact that FRAXE is classified as a non-syndromic form of ID. Again, this is an important consideration for clinicians, particularly in NL. Since the conclusion of the study, whole exome sequencing (WES) has been introduced into diagnostic laboratories and is recommended as an early investigation for dysmorphic children with either ID or an autism spectrum disorder (ASD)[143]. This technology allows scanning of most of the genome's 22,000 exons for mutations, but fails to pick up triplet repeat disorders. FRAXE syndrome should be included in the differential diagnosis list for a syndromic appearing boy with ID or other neurobehavioral abnormalities, particularly if the child comes from NL, as *FMR2* expansions are often not detected by WES.

The fact that three out of the four FRAXE families were ascertained by the PMGP for indications other than ID in the proband suggested that FRAXE may very well be under diagnosed in the NL population. Only one of the probands (IV-1 of Family A, 620 *FMR2* expansion repeat) was referred to genetics because of ID. This proband had more severe delay than the other five affected boys and was very dysmorphic. Two of the other probands were originally referred to the PMGP due to known genetic conditions in other members of their family. The Family C proband (250 *FMR2* repeat expansion) was referred because his biological mother had neurofibromatosis type 1 (NF1). When he was assessed, there were no features of NF1 on physical examination, but learning difficulties in school were noted. *FMR2* was not standard testing at the time and only *FMR1* testing was arranged. The proband for Family D was seen for a family history of multiple endocrine neoplasia type 1 (MEN-1), of which he was negative. He tested negative for the family mutation, but the genetic counsellor identified that he had academic difficulties, and *FMR1* and *FMR2* testing were ordered. The Family E proband had learning difficulties and a diagnosis of ADD but was not referred for genetic assessment because of either of these. A consultation was requested because his aunt was found to have an Xq27 cytogenetic fragile site as part of a work up for infertility that was due to a large *FMR2* expansion (400-700 repeats).

Another interesting finding was that in two out of the four families, fertility issues were documented. In Family A, an aunt of the proband (III-6) was infertile and she carried an approximately 80-100 unmethylated *FMR2* expansion. In turn, by history two of her aunts (II-4, II-5) were unable to conceive children (their DNA was not tested as

part of this study). In Family E, the *FMR2* expansion was identified through chromosome analysis testing of the aunt (III-5) who was undergoing in vitro fertilization (IVF) treatment as described above.

Primary ovarian failure (POF) is defined as the cessation of menses for 6 months before the age of 40[39] and is well documented in FXS. Women who carry premutations in the *FMR1* gene have a 12.9 to 21.0% chance of having POF[42,43]. Contrary to *FMR1*, the relationship between *FMR2* and POF is not well understood. There have been no published reports that have documented women with premutations in *FMR2* who also had had POF. One study by Murray reported six women with POF that had minimal (<11 repeats) alleles for *FMR2*, three of whom had microdeletions within the gene[47]. This was a follow up study from a previous paper investigating minimal alleles in individuals with POF versus controls in which the authors found a significant difference between the two groups[39].

Our study suggests that the association between POF and *FMR2* allele size should be further investigated. We identified two women with unexplained infertility who carried *FMR2* expansions: In Family A, a maternal aunt of the proband (III-6), who had a premutation allele of approximately 80-100 repeats and in Family E, a maternal aunt of the proband (III-5) who had mosaicism for a full expansion of 400-700 repeats.

4.4 Founder effect

4.4.1 Newfoundland population and founder effect

The province of Newfoundland and Labrador is constructed from a small number of founders and is composed of genetic isolates[113]. As described in more detail in the introduction, this province has a young history with first colonization occurring in the 17th century from only 20,000 settlers. Communities were established along the coast, and travel between communities was restricted due to geography and ruggedness of the area. Religious segregation combined with little emigration resulted in unique genetic populations.

In this study, we report four families that have *FMR2* expansions. One additional family is known to the PMGP to have FRAXE but was lost to follow up. The rarity of the disease, coupled with the genetic structure and history of the province, suggests that these families may have a common founder or ancestor. To test this theory, the Newfoundland Genealogy Database (NGD) was utilized to determine if indeed these individuals shared a common ancestor.

4.4.2 Newfoundland Genealogy Database

It was surprising that a common ancestor could not be identified between these families. The Newfoundland Genealogy Database (NGD) is a unique resource constructed using census records from 1911, 1921, 1935 and 1945. It was complemented by Stonepics, a collection of pictures of headstones throughout the province, historic church records of births and marriages throughout the province from various denominations, as well as numerous online resources containing genealogy information

on families in Newfoundland. Using the NGD, making connections to Family C and E was difficult. The proband in Family C was living in foster care at the time of the study and his biological parents were unavailable to clarify any pedigree questions. The proband's father was born after 1945 and therefore is too young for the NGD. The paternal grandparents are unknown to the proband. The proband's maternal grandmother was placed in foster care at a young age and could not provide any genealogical information. For Family E, DNA was available for four generations of individuals, and therefore, it was possible to determine the side of the family that was segregating the expanded *FMR2* allele. The proband's great-grandmother (I-2) on the maternal side who carried a premutation, could not be found in the NGD and therefore the pedigree could not be extended. However, Family E did not share the same haplotype as the other families, therefore a common ancestor would not be expected.

Although the NGD is a valuable resource there are some limitations to its abilities. Firstly, it is comprised of census data of which there are missing records. The census records at the time were not held in a centralized location and some data was stored in individual's attics and basements. Water damage and fire has destroyed some of the records. The census data was ascertained by individuals hired to go door-to-door to collect the information, therefore, there were undoubtedly mistakes made in transcription. If a household was not occupied at the time of the survey, the family's data would not have been collected. Secondly, none of the information gathered during the census was verified by another source. It was not uncommon at that time for non-paternity issues to

be present in families. Although the NGD could not confirm a common ancestor between Family A, C and D, it did allow for considerable expansion to the pedigrees.

4.4.3 Haplotype Analysis

Although the NGD did not identify a common ancestor, using haplotype analysis seven markers were found to segregate in three (Family A, C, and D) out of the four families proving a common haplotype of at least 1.88 Mb was identified. Eight individuals from three separate families shared this haplotype. In Family A, the haplotype was present in the proband (IV-1), his mother (III-2) and his two aunts (III6, III-8); all three of these women carry premutations. For Family C, no parental DNA was available but the haplotype was present in the proband and his sister (III-2) who carried a full *FMR2* expansion. Three generations of Family D were tested and both the proband (III-1) and his premutation mother (II-2) carried the shared haplotype. The maternal grandmother (I-2) who had two normal *FMR2* alleles had a different haplotype.

Family E did not share the common haplotype seen in Family A, C and D. This family had 4 generations available for testing and the proband (IV-1), his premutation mother (III-2), his premutation grandmother (II-2), and his premutation great-grandmother (I-2) all carried the same haplotype which was different from the haplotype observed in the other 3 families.

A control sample was also haplotyped to compare with the families and this sample did not share either of the disease haplotypes observed in Families A, C and D or Family E.

It was postulated that the four families investigated would have the same haplotype suggesting the possibility of a founder effect; however, this was not the case. It was surprising in a population of approximately 500,000 people that two different haplotypes were observed. This supports the theory that FRAXE is more common here than other populations previously investigated.

4.5 Limitations

This study had several limitations. The purpose of the Janeway cohort was to increase the possibility of identifying additional FRAXE cases in the NL population in a cohort of boys with milder phenotypes than those that would typically lead to a clinical genetics referral. However, recruitment for this part of the study was poor and for the 14 individuals who were recruited, little phenotypic information was received; therefore, they were excluded from the comparison of the *FMR2* repeat size in the at-risk test group versus the NL controls.

The tracking form provided to the pediatricians could have provided information regarding recruitment however, it was never completed. By completing the tracking form the number of boys being seen in the clinic and their eligibility status would have been known. Because these forms were not received, it was not possible to determine if parents were provided information about the study, if parents declined the study and/or if parents were given the study package but did not follow up. Despite continued efforts to improve

recruitment, only 17 individuals were known to the research team, and a signed consent form was obtained from 14 parents.

It is unfortunate that the recruitment issue could not be rectified and it was likely related to the fact that the clinic development clinic is a very busy environment. Currently on average 58 new patients and 441 follow up patients are seen per month in the Janeway's Child Development clinic. Based on the number of patients and the assumption that only half of those would be eligible boys, with a participation rate comparable to the retrospective cohort (58.2%), 200 boys would have been anticipated.

As discussed above, although people accept the idea of genetic research, many individuals do not wish to participate. Parents of children with special needs may be particularly sensitive to participating in such research studies when there is no perceived direct benefit to their child. Although it is difficult to determine why recruitment was so poor the stipulation of having a blood drawn may have contributed. Many children at Child Development are seen by their pediatrician on a regular basis, but would not necessarily have bloodwork done at each appointment. As having a blood draw can be traumatic for many children, especially those with ID, this may have excluded a large number of potential participants. The busy workload of the physicians may also have contributed to the lack of recruitment as they may have been too busy to explain the study. In hindsight, hiring a research nurse would have helped the recruitment phase of this study. The research nurse could have provided information to all parents who were interested in the study and answered any questions, allowing parents to decide if they wished to participate before a blood sample was drawn. The research nurse could also

have completed the tracking form to provide information of eligible criteria and capture the number of individuals who were eligible, but not interested in participating. Unfortunately, the pediatricians each have different clinic days throughout the week and funds were not available for a research nurse for this study.

With regards to the families, ascertainment was also a limitation. As discussed in **section 4.1.4**, many families had key individuals who were unavailable or not willing to participate in the research study. In Family A, updated clinical assessment for the proband's cousin (IV-7) who also carried a full expansion could have provided more information about the FRAXE phenotype in the Newfoundland population. Although the mother reported some academic difficulty in her son, he was working away from home and therefore had a much milder phenotype than the proband, who had mild ID. It would have been helpful to have IQ assessments for the two brothers, IV-2 and IV-3, who did not carry *FMR2* expansions to compare the baseline for intellectual ability within the nuclear family. The proband's mother (II-2) in Family C was unavailable for testing and the proband had no contact with his father (II-1), therefore it could not be determined which side of the family, the maternal or paternal, passed on the expansion to their children. The proband in Family E also had two siblings with unknown *FMR2* status. This proband had IQ testing which was in the normal range. It would have been valuable to compare his IQ testing results with that of his siblings, if negative, to see if the *FMR2* expansion was having an effect on his intellectual ability. If this was the case, one would expect the siblings IQ results to be higher (high normal range) than that of their brother.

As well, there have been reports in the literature of siblings with *FMR2* expansions, who have normal phenotypes[83].

Another limitation of this study was that only males were included. Although the reasoning for this decision was sound, based on the number of premutation females in our families (n = 7) there may be an excess of premutation carriers in the population. In Family E there are two full mutation females and although could not be formally assessed, they had no apparent phenotype. It is possible that additional full mutations females are present in the NL population who either have no manifestations or who are so mildly affected that they do not come to medical attention.

4.6 Summary and Conclusions

We conclude that the minimum prevalence of FRAXE syndrome in the NL population is 6 out of 46,420 or 1 in 7,737 in the male pediatric population which is 6-fold the prevalence seen in the general population.

Our data supports the hypothesis that the NL population is enriched for *FMR2* expansions. Firstly, in our retrospective of 92 boys with negative *FMR1* testing, we identified one boy with a full *FMR2* expansion. Secondly, for three out of the four families ascertained through referral to the provincial genetic service, the *FMR2* expanded allele occurred on a shared 1.88 Mb haplotype, consistent with a founder effect.

For the four FRAXE male probands identified because of referral for a clinical genetic assessment, only one was referred for ID. Two other probands were referred

because of a family history of an unrelated genetic disorder and the fourth was referred because of a cytogenetic fragile site in a maternal aunt. This observation is consistent with our hypothesis that FRAXE expansions are under ascertained in the NL population.

The study confirms that the phenotype in males with full *FMR2* expansions includes ID, but that not all such males have an IQ that is outside the normal range. All four males in whom we identified full *FMR2* mutations had learning problems, three had delayed speech and three had attentional problems; these neurobehavioral abnormalities have all previously been associated with the syndrome.

Although FRAXE syndrome is classified as a non-syndromic form of ID, one NL male proband was strikingly dysmorphic, with features highly reminiscent of the physical features seen in some males with full *FMR1* mutations. Another two males with full *FMR2* expansions were mildly dysmorphic. We therefore suggest that FRAXE syndrome be included by clinicians in the differential diagnosis list for a syndromic appearing boy with ID or other neurobehavioral abnormalities, particularly if the child comes from NL.

Currently when such children are assessed by a clinical geneticist and a syndrome cannot be diagnosed based on clinical features, the first line test is a genome-wide microarray. If the array is normal, Canadian guidelines suggest that the child have whole exome sequencing (WES) and this test will not identify triplet repeat expansion disorders like Fragile X and FRAXE syndromes.

We suggest *FMR2* genetic testing in any NL male (with or without dysmorphism) who has unexplained ID. Our study showed that *FMR2* expansions also exist in NL boys with milder neurobehavioral abnormalities that include learning disabilities and

ADHD/ADD. Consideration should be given to testing this population of NL males, particularly if there is a strong family history of academic difficulties or attentional problems or if that male is seeking preconceptional genetic counselling.

Regarding *FMR2* testing of females in this population, we make the following observations. Firstly, in the four Newfoundland families ascertained through the PMGP, there were three females segregating full mutations: (1) the sister of proband C who had academic difficulty requiring modifications within the school system and (2) the mother and (3) the maternal aunt of proband E both of whom have post-secondary education. Secondly, we identified two women with unexplained infertility who carried *FMR2* expansions: In Family A, a maternal aunt of the proband who had a premutation allele of approximately 80-100 repeats and in Family E, a maternal aunt of the proband who had mosaicism for a full expansion of ~ 400-700 repeats.

There is a known association between POF and *FMR1* premutations, and our study suggests that the association between POF and *FMR2* allele size should be further investigated, particularly in this population.

The indications for *FMR2* genetic testing in NL females are less clear than for males, but we suggest considering the test in girls with a personal and family history of developmental delay and/or ID and in women with POF who have had negative *FMR1* genetic testing.

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Appendix A – Telephone script

Hello, could I speak to _____.

If not available: Thank you. I will call again later. Could you suggest a time that might be better?

Hello _____, My name is _____. I am a research student at Memorial University working with Dr. Fernandez. As you may recall Dr. Fernandez tested your son for Fragile X. We are interested in doing a research study about children who tested negative for Fragile X. I would like to tell you more about this study. Is now a good time to talk?

If no: Could you suggest a better time?

As you know your child tested negative for a FRAXA mutation. We now know about another gene that may cause developmental delay and would like to test your child for it. The gene is called FRAXE. This is a very rare form of Fragile X syndrome. We have recently found several children in Newfoundland with FRAXE. We suspect that FRAXE may be more common in Newfoundland than in other places. If this new mutation is the cause of your child's developmental delay, we ask you to come back to the genetics clinic so that we can fully explain the test result.

Your participation in this study is completely voluntary and you are under no obligation to take part. If you do agree to join the study, you can withdraw at any time.

Are you interested in the study?

If no: Thank you for your time. Goodbye.

I will send you a consent form. It explains the study in more detail and explains your rights and who you can contact for more information. You will have to sign the consent form in order to be in the study. I will send it out to you in the next few days and I will call you in 10 days to see if you received it. At that time we can go through the consent form and answer any questions or concerns you may have about the study. When we have received your signed consent, we will proceed with the study.

Do you have any questions?

Thank you for your time and I will be talking to you soon.

Goodbye.

Appendix B – Consent form

October 2003

Faculty of Medicine, Schools of Nursing and Pharmacy of Memorial University of Newfoundland; Health Care Corporation, St. John's; Newfoundland Cancer Treatment and Research Foundation

Consent to Take Part in Health Research

TITLE: Is FRAXE syndrome over-represented among Newfoundland children with cognitive impairment?

INVESTIGATOR(S): Ms. Amanda Dohey, Dr. Ban Younghusband, Dr. Bridget Fernandez, Ms. Marian Crowley

SPONSOR:

You have been invited to take part in a research study. It is up to you to decide whether to be in the study or not. Before you decide, you need to understand what the study is for, what risks you might take and what benefits you might receive. This consent form explains the study.

The researchers will:

- **discuss the study with you**
- **answer your questions**
- **keep confidential any information which could identify you personally**
- **be available during the study to deal with problems and answer questions**

If you decide not to take part or to leave the study this will not affect your usual health care.

1. Introduction/Background:

In most populations Fragile X (type A) is the most common form of inherited developmental delay. However in Newfoundland there has only been one individual known to have Fragile X (type A). There is new information that suggests another gene may be responsible for mild to moderate developmental delay in our population. In this study we would like to test all children who had negative FRAXA for an alteration in another gene called FRAXE. This will help us not only to determine the number of individuals with FRAXE but also to learn more about individuals who have it.

2. Purpose of study:

- to determine if children with unknown developmental delay have FRAXE mutations
- to describe the characteristics of people with FRAXE

3. Description of the study procedures and tests:

If you decide to take part in this study, your child's banked DNA will be tested for FRAXE. You will be informed of the result of this testing. If the test is positive, you will be asked to

attend a clinic appointment with Dr. Fernandez and Ms. Dohey. Here the result will be explained in more detail. Dr. Fernandez will also re-examine your child at this time. You will receive genetic counselling and the option of genetic testing for other family members will be discussed.

4. Length of time:

For patients who test positive, a visit to the genetics clinic will be requested. The visit will last approximately 1 hour.

5. Possible risks and discomforts:

There are no risks associated with this study. New referrals will have the discomfort of providing a blood sample.

6. Benefits:

It is not known whether this study will benefit you. However it may allow your child to have an additional form of genetic testing which may lead to the identification of the cause of your child's developmental delay.

7. Liability statement:

Signing this form gives us your consent to be in this study. It tells us that you understand the information about the research study. When you sign this form, you do not give up your legal rights. Researchers or agencies involved in this research study still have their legal and professional responsibilities.

8. Confidentiality:

Investigators will strive to maintain your privacy and keep your identity confidential. All computer files containing confidential information will be protected with password only access and all paper files will be kept in a locked filing cabinet.

9. Questions:

If you have any questions about taking part in this study, you can meet with the investigator who is in charge of the study at this institution. That person is:

Amanda Dohey and Phone Number 777-XXXX

Or you can talk to someone who is not involved with the study at all, but can advise you on your rights as a participant in a research study. This person can be reached through:

Office of the Human Investigation Committee (HIC) at 709-777-6974
Email: hic@mun.ca

Signature Page

Study title: Is FRAXE over-represented among Newfoundland children with cognitive impairment?

Name of principal investigator: Amanda Dohey

To be filled out and signed by the participant:

	Please check as appropriate:
I have read the consent [and information sheet].	Yes { } No { }
I have had the opportunity to ask questions/to discuss this study.	Yes { } No { }
I have received satisfactory answers to all of my questions.	Yes { } No { }
I have received enough information about the study.	Yes { } No { }
I have spoken to Ms. Dohey and she has answered my questions	Yes { } No { }
I understand that I am free to withdraw from the study • at any time • without having to give a reason • without affecting my future care [student status, etc.]	Yes { } No { }
I understand that it is my choice to be in the study and that I may not benefit.	Yes { } No { }
I agree that the study doctor or investigator may read the parts of my hospital records which are relevant to the study.	Yes { } No { }
I agree to take part in this study.	Yes { } No { }

_____ _____
Signature of participant Date

_____ _____
Signature of witness Date

To be signed by the investigator:

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study and that he or she has freely chosen to be in the study.

_____ _____
Signature of investigator Date

Telephone number: _____

Assent of minor participant (if appropriate):

_____ _____
Signature of minor participant Date

_____ _____
Relationship to participant named above Age

Signature Page

Study title: Is FRAXE over-represented among Newfoundland children with cognitive impairment?

Name of principal investigator: Amanda Dohey

To be filled out and signed by the participant:

	Please check as appropriate:
I have read the consent [and information sheet].	Yes { } No { }
I have had the opportunity to ask questions/to discuss this study.	Yes { } No { }
I have received satisfactory answers to all of my questions.	Yes { } No { }
I have received enough information about the study.	Yes { } No { }
I have spoken to Ms. Dohey and she has answered my questions	Yes { } No { }
I understand that I am free to withdraw from the study • at any time • without having to give a reason • without affecting my future care [student status, etc.]	Yes { } No { }
I understand that it is my choice to be in the study and that I may not benefit.	Yes { } No { }
I agree that the study doctor or investigator may read the parts of my hospital records which are relevant to the study.	Yes { } No { }
I agree to take part in this study.	Yes { } No { }

_____ _____
Signature of participant Date

_____ _____
Signature of witness Date

To be signed by the investigator:

I have explained this study to the best of my ability. I invited questions and gave answers. I believe that the participant fully understands what is involved in being in the study, any potential risks of the study and that he or she has freely chosen to be in the study.

_____ _____
Signature of investigator Date

Telephone number: _____

Assent of minor participant (if appropriate):

_____ _____
Signature of minor participant Date

_____ _____
Relationship to participant named above Age

Appendix C – Reminder letter

<Insert Date>

Dear Parent(s):

I hope you had a relaxing summer with your family. As you may recall earlier this year we spoke about enrolling your child in the FRAXE study. For your convenience I have enclosed another copy of the consent form in case it has been misplaced over the summer break. It would be much appreciated if you could you please send in your form indicating whether or not you are interested in this study.

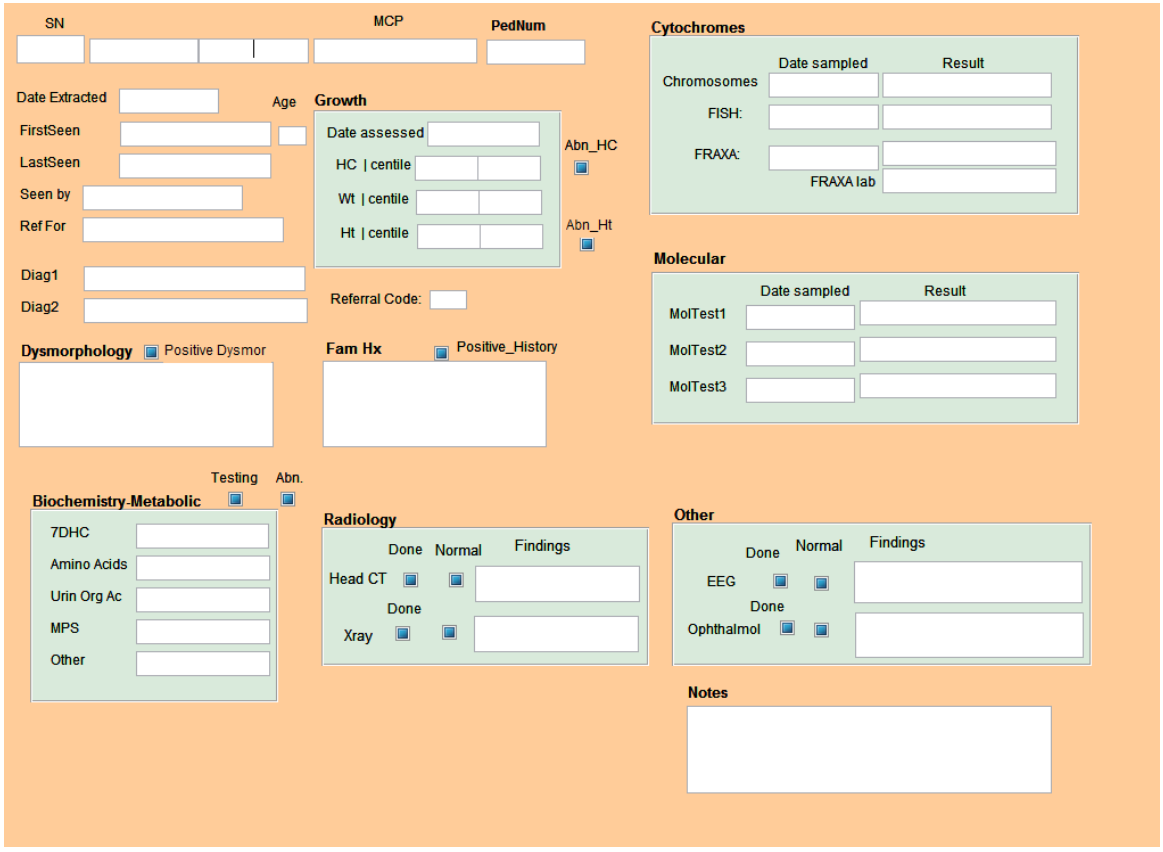
As per our conservation, testing is occurring now and in order for your child to participate, this consent form must be returned by **<insert date>**. If I do not receive the signed consent form by <insert date> I will assume that you are no longer interested in participating in this study and I will not contact you further.

If you have any questions regarding this study or the consent form, please do not hesitate to give me a call at **777-XXXX**.

Sincerely,

Amanda Dohey

Appendix D – Chart extraction form


SN
MCP
PedNum
Cytochromes
Date sampled
Result
Chromosomes
FISH:
FRAXA:
FRAXA lab
Date Extracted
Age
Growth
Date assessed
HC | centile
Wt | centile
Ht | centile
Abn_HC
Abn_Ht
FirstSeen
LastSeen
Seen by
Ref For
Diag1
Diag2
Referral Code:
Molecular
Date sampled
Result
MolTest1
MolTest2
MolTest3
Dysmorphology
Positive Dysmor
Fam Hx
Positive_History
Testing
Abn.
Biochemistry-Metabolic
7DHC
Amino Acids
Urin Org Ac
MPS
Other
Radiology
Done
Normal
Findings
Head CT
Xray
Other
Done
Normal
Findings
EEG
Ophthalmol
Notes

Appendix E – DNA consent form

Child's Name:_____

MCP #: _____

Parents Name: _____

Parents Signature: _____

- **Future use of tissue/DNA samples:**

  In order to preserve a valuable resource, your child's DNA samples may be stored at the end of this research project. It is possible that these samples may be used in a future research project. This project will be related to childhood diseases as determined by a research ethics committee. **Any future research would have to be approved by a Research Ethics Board (REB).**

  Please tick **one** of the following options:

	I agree that my child's DNA sample can be used for approved childhood diseases research project without contacting me again, but **only if my child's name* cannot be linked, in any way, to the sample.**
	Under no circumstances may my child's sample be used for future research. **My child's sample must be destroyed** at **the end of this present project**.
	I agree that I may be contacted in future to be invited to provide consent for the use of my child's DNA in any new approved research project.

  *Includes name, MCP number or any other identifying information.

  The DNA sample from this study will be stored in Dr. Woods lab, in the Discipline of Genetics at the Health Sciences Centre, St. John's, Newfoundland for an indefinite period of time.

You may withdraw your child's sample at any time without affecting your child's future care by contacting Ms. Amanda Dohey at 777-XXXX or Dr. Bridge Fernandez at 777-XXXX at which time we will destroy your child's DNA sample.

Appendix F – Parent letter accompanying DNA consent form

Amanda Dohey
Younghusband Research Lab
Discipline of Genetics, Room 4333
Health Sciences Centre
300 Prince Philip Drive
St. John's, NL A1B 3V6

FRAXE Project
Faculty of Medicine
Health Sciences Centre

Dear Parent:

Enclosed is a special consent form that tells us what to do with your child's DNA sample after the study is over. Please take the time to read this special consent form, choose the option that best suits your family and return in the envelope provided.

DNA is a valuable resource and if you choose to participate in a future genetic study this sample could be used with your permission without having to draw an additional blood sample from your child.

However if I do not receive your response by November 1, 2009 I will assume that you would like your child's DNA destroyed and the sample will be disposed of.

Once again I would like to thank you and your son for your participate. If you have any further questions regarding this form or the study in general please do not hesitate to call me.

Sincerely,

Amanda Dohey
FRAXE Research Co-coordinator

Appendix G – Inclusion criteria information sheet for Janeway cohort

FRAXE STUDY

Is FRAXE over-represented among Newfoundland children with cognitive impairment?

Investigators: Ms. Amanda Dohey
Dr. Bridget Fernandez
Dr. Ban Younghuband
Ms. Marian Crowley

Inclusion Criteria

Nonsyndromic boys between the ages of **3-18** with the following criteria:

1. two of the four criteria

   1) Speech delay requiring speech therapy;
   2) Learning disabilities or academic difficulties requiring alternate school programming at school (could be as mild as "pathway 2" school);
   3) Behavior abnormalities necessitating input from a behavior management or child management specialist (including a diagnosis of attention deficit disorder, aggressive behaviors);
   4) IQ testing that shows borderline (IQ = 70-80) or mild (IQ = 50-70) MR
   and

2. will not be referred to Medical Genetics *and*

3. having blood drawn *and*

4. no known etiology.

Contact Information: Amanda Dohey
Room # - H4333
Email address - acareen@mun.ca
Phone # - 777-XXXX

Appendix H – Tracking form for Janeway cohort

Dr: _____

FRAXE STUDY

Amanda Dohey - Investigator (777-6989)

Patients Name	Two of the four criteria				Not Referred to Genetics	Having Blood Drawn	No Known Etiology	Eligible	Package
	Speech	Learning	Behavior	IQ (<80)					

Appendix I – Parent letter for Janeway cohort

Dear Parent,

My name is Amanda Dohey and I am a masters student at Memorial University. I work with Dr. Bridget Fernandez, Geneticist at the Medical Genetics Department and Dr. Ban Younghusband, Head of the Discipline of Genetics at the Faculty of Medicine.

Thank you so much for your interest in this study. This study looks at a gene called FRAXE which is found on the X chromosome. Defects or mutations in this gene have been found to be associated with various learning problems in children. It is through willingness of people like your family that we may eventually learn more about FRAXE and learning problems in general.

I will be contacting you shortly to talk to you more about this study. Rest assured that your child's sample will not be used until I have spoken with you and I have received the signed consent form. If you have any questions before that, you can call me at **777-XXXX**. Thank you for taking the time to read this letter and I look forward to talking to you soon.

Sincerely,

Amanda Dohey
Masters Student
Memorial University of Newfoundland

Appendix J – Mutation report

Amanda Dohey
Younghusband Research Lab
Discipline of Genetics, Room 4333
Health Sciences Centre
300 Prince Philip Drive

FRAXE Project
Faculty of Medicine
Health Sciences Centre

FRAXE Mutation Screening Report

<Insert Date>

Patient Name:
DOB:
MCP:
DNA #:

Molecular analysis indicates that *(Child's name*) does not have an expansion at the FRAXE locus.			
Patient Name	Date Obtained	*FMR*-2 (CCG)n repeats	Expansion Range

Disease Background: FRAXE is a rare form of the Fragile X syndrome. Individuals with FRAXE tend to be slow learners, be hyperactive and exhibit language delay. The FRAXE gene is located on the X chromosome. The normal gene contains a three base pair sequence (CCG repeat) which varies in the general population. The normal range of CCG repeats is 6-30 repeats, whereas individuals affected with the disease have expansion ranges greater than 200 repeats.

Analysis: (*Child's name)* was tested using PCR analysis for the expansion of the FRAXE gene.

Disclaimer: The testing for FRAXE expansions was performed in a research laboratory, not a licensed medical laboratory. Therefore the accuracy and quality of the results may not be as reliable as those from a licensed service lab.

Amanda Dohey

Research Co-coordinator for the FRAXE Project

Appendix K – Mutation letter

Amanda Dohey
Younghusband Research Lab
Discipline of Genetics, Room 4333
Health Sciences Centre
300 Prince Philip Drive

FRAXE Project
Faculty of Medicine
Health Sciences Centre

Dear _____,

Enclosed is your mutation report from the FRAXE study. As we previously discussed FRAXE is a rare form of the Fragile X syndrome. In this study we looked at defects or mutations in the FRAXE gene. In your child, the FRAXE gene was found to be in the normal range. Therefore your child's medical problems are not the result of a FRAXE mutation.

I would like to thank you for your participation in this study. It is through willingness of people like you that we may eventually learn more about FRAXE and learning problems in general.

A copy of this mutation report has also been forwarded to Dr. Bridget Fernandez, Director of the Provincial Medical Genetics Program. If you have any questions regarding this result please do not hesitate to give me a call at (709)777-XXXX.

Once again thank you for your participate.

Sincerely,

Amanda Dohey
FRAXE Research Co-coordinator

Appendix L – Supplementary Tables

Table S.1 – Summary of 36 studies evaluating *FMR2* status

Ref	Population	# of Individuals	Normal	Intermediate	Premutation	Full Mutation	Cohort Criteria	Origin
109	At risk	300	300	0	0	0	males negative for *FMR1* expansions	Canada
	Control	28	28	0	0	0	healthy normal controls	
131	At risk	57	57	0	0	0	families with ≥ 2 children with autism, PDD or Aspergers	Canada
102	At risk	397	397	0	0	0	males and females living in an institution with developmental delay and negative for *FMR1* expansions	
129	At risk	737	737	0	0	0	males and females with ID or 1st degree relative with ID	Germany
79	At risk	911	908			1+3	negative for *FMR1* expansions	United Kingdom
100	At risk	462	462	0	0	0	7-10 yrs in SNS	Atlanta, US
80	At risk	105				1	ID patients	Italy
81	At risk	180	179			1	ID (unknown etiology)	Italy
30	At risk	992 boys	981	10	1	0	boys in school with learning difficulties	
	Control	725	723	2	0	0	non transmitting X chr from mother	
111	At risk	37 chr	37 chr	0	0	0	Fragile X	Finland
	Control	56 chr	56 chr	0	0	0	normal controls	
101	At risk	206 chr	206 chr	0	0	0	*FMR1* mutations	
	Control	459 chr	459 chr	0	0	0	healthy controls from 3 populations	Euro/American, Chinese, Finish
104	At risk	1014	1014	0	0	0	children 5-18 yrs with academic difficulties	Baltimore City

Ref	Population	# of Individuals	Normal	Intermediate	Premutation	Full Mutation	Cohort Criteria	Origin
82	At risk	222			0	1	4-20yrs in SNS for ID	Spanish
130	At risk	17	17	0	0	0	children identified with fragile site and negative for *FMR1* expansions	United Kingdom
103	Control	610 chr				0	unrelated normal X from 5 populations	Africa, Chinese, Greek/Cyrus, English, Indian
110	At risk	534	525	8	1	0	children 1.5-6 yrs referred for language delay	
39	At risk	147	291 chr	2 chr	0	0[×]	females with POF	London
	Control	1268	1263	5	0	0	non transmitting X of boys with learning difficulties	
128	At risk	257	257				males 3-25 yrs with ID	Greece/Cyrus
127	At risk	150	150	0	0	0	Children 1.6-20 yrs with developmental delay + their mothers + four families with positive fragile site	Chinese
	Control	77	136 chr	0	2	0	healthy adults	
46	At risk	2652	2629	22	1	0	children 7-10 yrs in SNS	Atlanta, US
47	At risk	209	394 chr	0	0	0^	females with POF	London/United Kingdom
	Control	2434	4795 chr	0	0	0~	mom's of boys with learning diff.	
126	At risk	611	611	0	0	0	2-25 yrs with ID (excluded other syndromes)	Cyrus/Greece
125	At risk	62				0	children 1-17 yrs with ID	Mexico
124	At risk	144	144	0	0	0	negative for *FMR1* expansions	Brazil
83	At risk	232				1	ID patients	Italian
123	At risk	206	206	0	0	0	patients with ID in SNS or private care facility	Taiwan
34	At risk	3738	3687	41	2	1	boys 5-18 yrs in SNS	England

Ref	Population	# of Individuals	Normal	Intermediate	Premutation	Full Mutation	Cohort Criteria	Origin
	Control	2968	2917	19	0	0	mothers (other X chr)	
106	At risk	254	246	4	0	0	Boys with developmental delay	
107	At risk	130	157 chr				individuals in SNS or home for ID	New Delhi
	Control	135	225 chr					
108	At risk	146	146			0	males & females living in an institution for ID	
84	At risk	114				1	9-16 yrs in SNS with mild-mod ID	Croatia
31	At risk	276	265	3	0	0	boys 2-18 yrs with ID or learning difficulties	Brazil
	Control	207	204	0	0	0	intellectually/physically normal males	
137	At risk	84	84	0	0	0	males and females 2.6-28.6 yrs referred for preliminary diagnosis of autism	
85	At risk	26	25			1	males & females with OCD	Utah, US
29	At risk	203	190	13	0	0	males with PD	Italy
	Control	370	369	1	0	0	healthy males enrolled for aging study	
32	At risk	198	274 chr	6	0	0	Parkinson's disease	Spain
	Control	220	311 chr	3	0	0		

PDD = pervasive developmental disorder; ID = intellectual disability; yrs = years; SNS = special needs school; chr = chromosome; POF = premature ovarian failure; OCD = obsessive compulsive disorder; PD = Parkinson's disease. [+] Reference 79: Two related females were found to harbour an *FMR2* expansion; only one positive *FMR2* expansion was identified in the screened cohort, however, the article reports on three additional FRAXE positive cases; [×] One female had a small deletion. ^Three women with a small deletion; [~] One women with small deletion in control cohort. Most authors used the term MR (mental retardation) when the paper was published and, as this is no longer acceptable terminology, MR has been changed in the above table to ID.

Table S.2 – Primer Sequences

Primer Name	F Primer (5' to 3')	R Primer (5' to 3')
FRAXE 603F, 598R	CCTGTGAGTGTGTAAGTGTGTGATGCTGCCG	GCGAGGAAGCGGCGGCAGTGGCACTGGG
DXS7847	TTAAACTCTTGGATATGTATGCAC	TTTGTCCTCTCCTCCCTTG
DXS7393	GTCTATTTCAAACAAACACTTACC	AACCTGACCACAGCTATCC
DXS7389	CAACCCATTTCTTTTCCCTTC	AGAAACCAAAAGAGATAACCAAAC
DXS7390	GATTTGAATGCTTCCATTGCC	ATTTCTAAAAATGACATACCCGAC
DXS7394	TTCCCACTAGGTGATTCTG	CGCTTGGAAACTATGATGAAATG
DXS7812	CCTGTTACCTTAGTTTCTTCTG	AAGATGTGCCTTCCTTCTG
DXS1318	CCTCTCTGTGTGTTTCCCC	TCCCCAAGCAAAACCAACATATTC
DXS6729	TAAGACACCTTTATGCCCCA	AAGGATGAGAAAAACAATCTAAGC
DXS8303	TCCACCTCTGCAAAAAACAATTTC	CCTCTGGTGTTTGGGGTTC
DXS1185	TAGGGTCTGGAAATTGCTGA	CACTCCCTCACTCACTCTC
DXS457	CCTGACAATCACATAAGCTA	TCTGGTCACAAGAATTTCAG
DXS1123	TGCCTAAATGTTCGCAAGCCCATTC	ACAAACAGCTGCCTCCTAGAAACCC
DXS998	CAGCAATTTTTCAAAGGC	AGATCATTCATATAACCTCAAAAGA
DXS548	AGAGCTTCACTATGCAATGGAATC	GTACATTAGAGTCACCTGTGGTGC
DXS1215	GGGCAAAACATTAAACCTCTC	GCCCTCTAAGTCATTACGCT
DXS533	AATGTGGCAAGGAAGCCAGCA	CAGTTTTCATTTCTCTGTCTTCAA
DXS1193	AATTCTGACTCTGGGGC	TTATTTTAAGGTGAGTATGGTGTGT
DXS990	AGCTATATGACCAGTACAAACATAC	GACAGAAGGGACATCAACTC
DXS986	CCTAAGTGCTCATCATCCCA	AGCTCAATCCAAGTTGCTGA
DXS1226	CTAAACCCATCTGNCCTC	TTTCCAGCAACTACCTTTCAT
DXS1214	TAGAACCCAAATGACAACCA	AAGATAGCAGGCAACAATAAGA
DXS8055	AGTGGGTCACTCTAGTCATCAT	TCAGGTTTCTGTGTGGACAT
DXS991	ACTTCAACCACAGAAGCCTC	ATCATTTGAGCCAATTCTCC
DYS570	GAACTGTCTACAATGGCTCACG	TCAGCATAGTCAAGAAACCAGACA
DYS643	AAGCCATGCCTGGTTAAACT	TGTAACCAAACACCACCCATT
DYS490	CCTGGCAGGAATTATCCAGA	GCAGAGCTTGCACTGAGCT

Table S.3 – Results of *FMR2* PCR testing for the 92 boys from the retrospective cohort

Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele
00MG1022	14	01MG1617	14	03MG2219	3	11781	14
00MG1052	12	01MG1684	12	03MG411	21	11820	12
00MG1115	15	01MG318	12	03MG438	**	11823	22
00MG265	6	01MG818	13	03MG682	13	11987	13
00MG267	12	01MG948	19	03MG979	13	12108	12
00MG428	12	02MG1008	12	04MG1051	12	12134	17
00MG457	12	02MG1106	21	04MG1495	12	12213	15
00MG505	18	02MG1142	12	04MG1563	12	12273	++
00MG56	21	02MG1229	12	04MG1870	17	12346	12
00MG732	21	02MG139	12	04MG1871	17	12375	23
00MG740	13	02MG1616	12	04MG452	12	12382	12
00MG814	12	02MG1639	21	04MG742	16	12395	23
00MG832	13	02MG1698	12	05MG319	8	12679	16
00MG885	16	02MG1708	13	10570	12	12714	16
00MG949	15	02MG2248	15	10573	12	12881	15
00MG964	15	02MG580	12	11140	13	221	16
01MG100	15	02MG948	13	11158	16	225	15
01MG1028	13	03MG1146	12	11187	12	263	23
01MG1029	16	03MG1298	25	11305	26	284	15
01MG1070	21	03MG138	17	11312	21	306	15
01MG1192	13	03MG1814	12	11324	13	311	15

Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele
01MG1290	12	03MG2095	11	11757	15	317	12
01MG1497	12	03MG2097	12	11769	16	363	16

** = no amplicon; ++ = 2 alleles obtained; further investigation required

Table S.4 – Results of *FMR2* PCR testing for the 14 boys from the Janeway cohort

Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele
330	9	365	16	370	16	375	18
346	29	367	15	371	15	376	19
347	15	368	23	372	15		
364	++	369	12	374	20		

++ = 2 alleles obtained; further investigation required

Table S.5 – Results of *FMR2* PCR testing for 277 Newfoundland controls

Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele
C1	9	C72	12	C141	13	C212	12
C2	12	C73	12	C142	12	C213	17
C3	10	C74	16	C143	12	C214	12
C4	13	C75	25	C144	12	C215	12
C5	25	C76	15	C145	12	C216	13
C6	15	C77	21	C146	14	C217	14
C7	15	C78	27	C148	24	C218	21
C8	10	C79	17	C149	16	C219	15
C9	15	C80	12	C150	15	C220	12
C10	18	C81	12	C151	12	C221	11
C11	13	C82	25	C152	15	C222	13
C12	12	C83	15	C153	14	C223	15
C13	24	C84	12	C154	23	C224	15
C14	12	C85	25	C155	12	C225	13
C15	11	C86	15	C156	15	C226	12
C16	25	C87	15	C157	++	C227	13
C17	13	C88	12	C158	11	C228	15
C18	15	C89	15	C159	++	C229	12
C19	13	C90	12	C160	12	C230	12
C20	12	C91	13	C161	14	C231	12
C21	12	C92	17	C162	21	C232	12

Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele
C22	18	C93	13	C163	15	C233	12
C23	12	C94	15	C164	12	C234	13
C24	15	C95	12	C166	12	C235	12
C25	12	C96	15	C167	16	C236	12
C26	12	C97	12	C168	12	C237	12
C27	16	C98	12	C169	15	C238	12
C28	13	C99	12	C170	15	C239	14
C29	12	C100	12	C171	16	C240	12
C30	13	C101	12	C172	17	C241	++
C31	13	C102	13	C173	12	C242	16
C32	13	C103	12	C174	++	C243	++
C33	17	C104	8	C175	15	C244	12
C34	15	C105	13	C176	15	C245	15
C35	13	C106	16	C177	12	C246	12
C36	13	C107	18	C178	15	C247	21
C37	13	C108	15	C179	12	C248	16
C38	12	C109	13	C180	13	C249	12
C39	15	C110	13	C181	17	C250	8
C40	14	C111	14	C182	12	C251	15
C41	12	C112	12	C183	++	C252	23
C42	23	C113	22	C184	15	C253	15
C43	15	C114	8	C185	12	C254	12

Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele
C44	12	C115	15	C186	13	C255	12
C45	13	C116	22	C187	15	C256	12
C46	15	C117	15	C188	12	C257	15
C47	12	C118	12	C189	15	C258	12
C48	11	C119	12	C190	12	C259	17
C49	12, 25	C120	17	C191	12	C260	15
C50	**	C121	12	C192	12	C261	13
C51	13	C122	12	C193	12	C262	17
C52	15	C123	13	C194	12	C263	12
C53	15	C124	14	C195	13	C264	16
C54	17	C125	21	C196	12	C265	12
C55	13	C126	15	C197	++	C266	12
C56	8	C127	12	C198	13	C267	13
C57	12	C128	11	C199	16	C268	++
C58	24	C129	13	C200	18	C269	12
C59	15	C130	15	C201	15	C270	15
C60	12	C131	15	C202	13	C271	13
C61	13	C132	13	C203	13	C272	14
C62	12	C133	12	C204	14	C273	13
C63	12	C134	13	C205	12	C274	19
C64	20	C135	15	C206	13	C275	12
C65	12	C136	12	C207	8	C276	12

Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele	Sample ID	*FMR2* Allele
C67	13	C137	25	C208	15	C277	12
C68	12	C138	12	C209	++	C278	12
C69	15	C139	12	C210	15	C279	12
C70	15	C140	12	C211	12	C280	11
C71	21						

****** = no amplicon; **++** = 2 alleles obtained, further investigation required

Appendix M – Expanded pedigrees

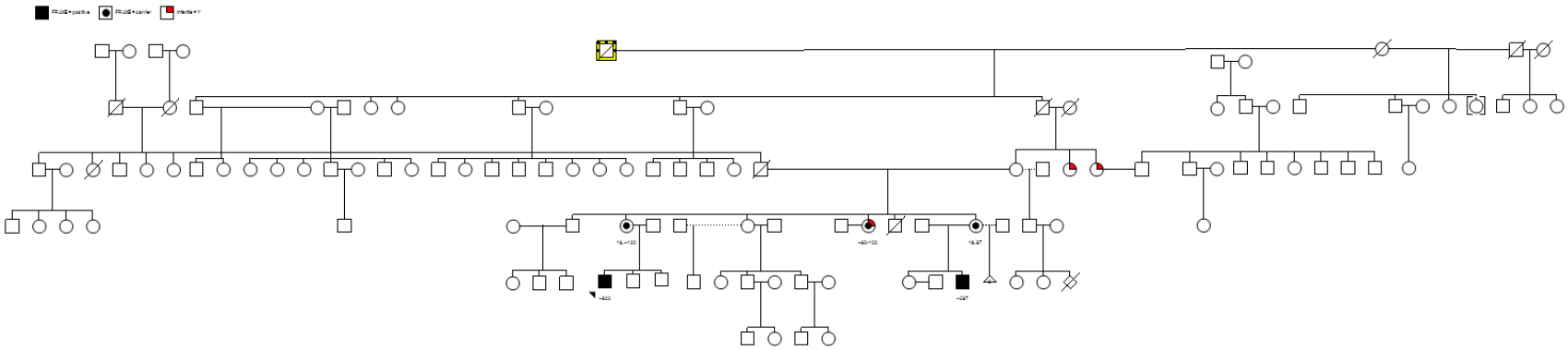


Figure A.1 – Expanded pedigree for Family A

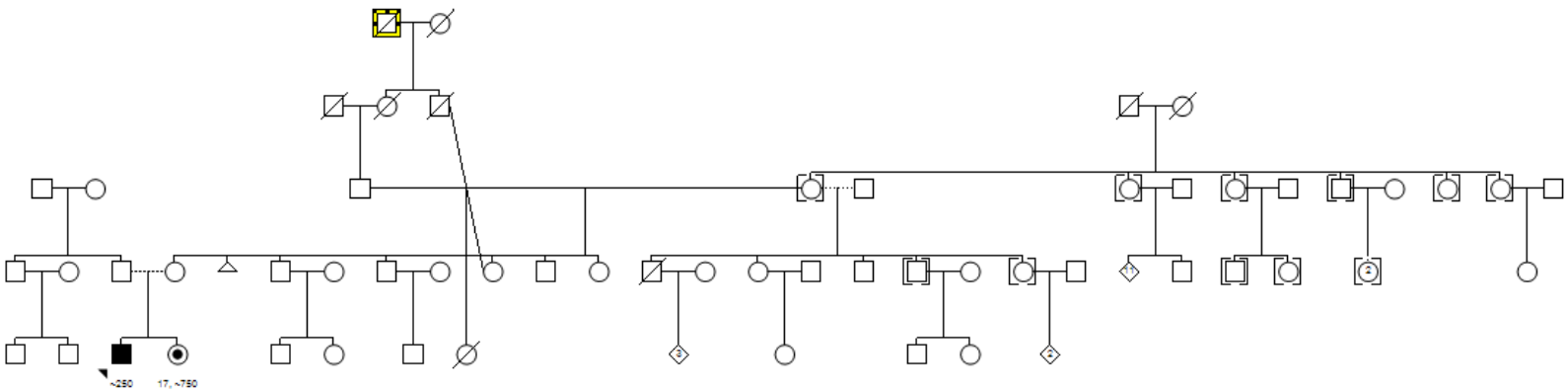


Figure A.2 – Expanded pedigree for Family C

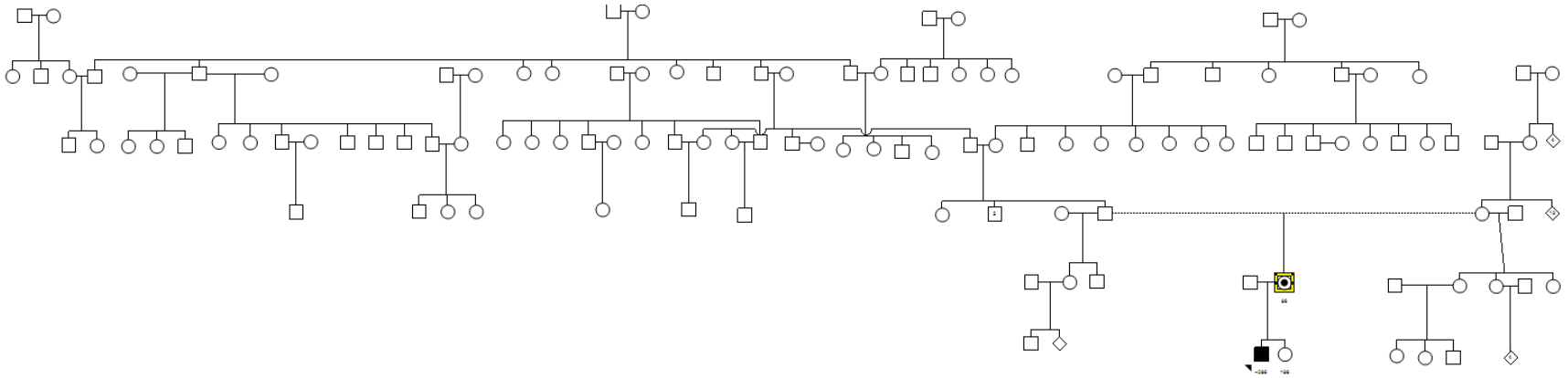


Figure A.3 – Expanded pedigree for Family D