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MOLECULAR ASPECTS OF X-LINKED MENTAL RETARDATION LOCI

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A dissertation submitted to the Faculty of Health Science, University of the Witwatersrand, Johannesburg, in fulfillment of the requirements for the degree of Masters of Science in Medicine

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ABSTRACT

Mental retardation (MR) is estimated to affect ~2-3% of the general population and may result from genetic causes and/or environmental causes. X-linked mental retardation (XLMR) is a heterogeneous group of disorders with a broad range of phenotypes and can be classified into syndromic XLMR (S-XLMR) and nonsyndromic XLMR (NS-XLMR) types. A number of X-linked genes have been identified that are associated with the different forms of XLMR.

In an attempt to refine the diagnostic service to patients with XLMR, the Division of Human Genetics, Molecular Laboratory at the National Health Laboratory Service (NHLS) has investigated a number of associated X-linked genes. The main objective of this project was to investigate three genes, *FMR2*, *XNP* and *ARX*, associated with NS-XLMR and their contribution to XLMR in the South African (SA) population of MR males. Patients from different ethnic groups, referred to the Division of Human Genetics for fragile X MR syndrome that tested negative for the *FMR1* expansion mutation were investigated for mutations in these genes. In addition, a cohort of Black institutionalized males was also investigated.

The *FMR2* expansion mutation responsible for fragile X E syndrome was not identified in 1194 *FMR1* expansion negative MR male patients. *FMR2* allele distribution analysis showed that a GCC repeat size of 15 was common in the MR cohort, accounting for 42% of alleles identified. From a total of 210 *FMR1* expansion negative MR male subjects screened for mutations in a hotspot region (exons 7, 8 and 9) of the *XNP* gene, none was found to have a mutation in this region. Two patients from a cohort of 868 *FMR1* expansion negative MR males were found to have a mutation in the *ARX* gene – one patient tested positive for the common 24 bp duplication mutation and a second patient appeared to have a deletion in the region amplified. These results indicate that the *FMR2*, *XNP* and *ARX* genes do not contribute significantly to MR in the SA population. As a result of this study, routine DNA testing for the *FMR2* expansion, mutation screening in the hotspot region of the *XNP* gene and screening for the common 24 bp duplication mutation in the *ARX* gene in *FMR1* expansion negative MR male subjects will not be implemented.

A retrospective analysis was also done on 1862 probands referred to the Molecular Genetics Diagnostic service from 1992 to 2009 for fragile X MR syndrome testing. The *FMR1* full expansion mutation was detected in 6.2% of probands, higher than reported worldwide figures. *FMR1* allele distribution analysis in a cohort of 1184 *FMR1* expansion negative MR males showed that 29 CGG repeats was the most frequent repeat size observed, accounting for

32.6% of all alleles in the cohort. The analysis of *FMR1* alleles in MR males shows a similar distribution between different ethnic groups and compares well with other reported studies. This study reinforces the presence of fragile X MR syndrome in the SA Black population.

Molecular investigations were also undertaken on 3 patients clinically suspected to have X-linked α-thalassaemia mental retardation syndrome (ATR-X) and extended family members. Mutations were identified in each of the patients – two patients were found to have a novel mutation in the *XNP* gene and the third patient had a common *XNP* mutation. As a result, carrier testing and prenatal diagnosis was made possible in these families.

A large family positive for the *FMR1* expansion causing fragile X A MR syndrome was investigated. As an incidental finding, 2 females were found to be compound heterozygotes for 2 *FMR1* alleles. Extended family members were tested and their *FMR1* status was determined. Haplotype analysis was used to track the high-risk X chromosome in the family. As a result of this investigation, females at risk for premature ovarian failure and fragile X tremor ataxia syndrome have been identified.

The approach to testing genes implicated in NS-XLMR has to be refined to allow for a cheaper and more efficient alternative. The use of newer techniques such as CGH microarray and MLPA has allowed for better detection of mutations. Delineating the causes of MR and their molecular and cellular consequences will assist families but also provide insight into the mechanisms that are required for the normal development of cognitive functions in humans.

DECLARATION

I, Fahmida Bibi Essop declare that this dissertation is my own unaided work. It is being submitted for the Degree of Master of Science in Human Genetics at the University of the Witwatersrand, Johannesburg. It has not been submitted for any other degree at this or any other university.

FAHMIDA ESSOP **DATE**

DEDICATION

This work is dedicated to my parents, Moosa and Zuleikha. I am forever grateful for the encouragement, love and opportunities that you have given me.

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First and foremost I would like to thank The Almighty Allah for always looking over me and guiding me and my family and the spiritual support received in the most incredible ways known and unknown.

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ABBREVIATIONS

5’	Five prime
3’	Three prime
%	Percentage
A	Adenine
ABI	Applied Biosystems
ACD	Acid citrate dextrose
ADD	Helicase and zinc finger domains
α	Alpha
ARMR	Autosomal recessive mental retardation
ARX	Aristaless-related homeobox gene
ATH	Alpha thalassaemia
ATR-X	X-linked α-thalassaemia/MR syndrome
bp	Base pairs
C	Cytosine
CCD	Charge-coupled device
cen	Centromere
CGH	Comparative genomic hybridization
cM	CentiMorgan
cm	Centimetre
CNS	Central nervous system
CNVs	Copy number variations
CpG	Cytosine phosphorylated guanine
CVS	Chorionic villus sampling
^{7}CdGTP	Deaza deoxyguanosine-5’-triphosphate (deaza-dGTP)
°C	Degrees Celsius
dATP	Deoxyadenosine-5’-triphosphate
dCTP	Deoxycytidine-5’-triphosphate
dGTP	Deoxyguanosine-5’-triphosphate
dTTP	Deoxythymidine-5’-triphosphate
dNTP	Deoxyribonucleotide triphosphate
ddATP	Dideoxyriboadenosine triphosphate
ddCTP	Dideoxyribocytosine triphosphate
ddGTP	Dideoxyriboguanosine triphosphate
ddH_2O	Deionised distilled water
ddTTP	Dideoxyribothymidine triphosphate
ddNTPs	Dideoxyribonucleotide triphosphate
DNA	Deoxyribonucleic acid
ds	Double stranded
E	*FMR1* premutation
EDTA	Ethylenediaminetetra-acetic acid
EE	*FMR1* full expansion
EtBr	Ethidium bromide
F	Forward primer
FAS	Fetal alcohol syndrome
FM	Full mutation
FMR1	Fragile X mental retardation gene 1
FMR2	Fragile X mental retardation gene 2
FMRP	Fragile X mental retardation protein
FRA	Fragile site
FRAXA	Fragile X A syndrome
FRAXE	Fragile X E mild mental retardation syndrome
FXTAS	Fragile X tremor ataxia syndrome

G	Guanine
g	Grams
H_20	Water
hr	Hour
HCl	Hydrogen chloride
IQ	Intelligence Quotient
kDa	Kilo Dalton
kb	Kilobases
L	Litre
lod	Logarithm of the odds
M	Molar
mA	Milliamps
MBq	Mega bequarels
MECP2	Methyl-CpG-binding protein 2
mg	Milligrams
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Millilitre
MLPA	Multiplex Ligation-dependent Probe Amplification
mm	Millimetre
mmol	Millimolar
MR	Mental Retardation
mRNA	Messenger RNA
MW	Molecular Weight
$NaCl_2$	Sodium Chloride
ng	Nanograms
NHLS	National Heath Laboratory Service
NS-XLMR	Non-syndromic XLMR
NTM	Normal Transmitting Male
OMIM	Online Mendelian Inheritance in Man
p	'Petit' (short arm of the chromosome)
PAG	Polyacrylamide gel
PCR	Polymerase Chain Reaction
pH	Percentage hydrogen
pm	Picomol
PM	Premutation
POF	Premature ovarian failure
q	Long arm of the chromosome
R	Reverse primer
RE	Restriction enzyme
rpm	Revolutions per minute
SA	South Africa
SAIMR	South African Institute for Medical Research
SDS	Sodium dodecyl sulphate
sec	Second
ss	Single stranded
SSC	Sodium citrate solution
S-XLMR	Syndromic XLMR
T	Thymine
Taq	Thermus aquaticus
TBE	Tris-Boric acid-EDTA
TE	Tris-EDTA
tel	Telomere
TRDs	Trinucleotide repeat disorders
Tris	Tris (hydroxymethyl) methylamine

TTP	Thymine triphosphate
U	Units of enzyme
μg	Microgram
μl	Microlitre
UTR	Untranslated region
UV	Ultraviolet radiation
V	Volts
WBC	White Blood Cells
WHO	World Heath Organization
X	X chromosome
XLMR	X linked mental retardation
XNP	X-linked nucleoprotein
Viz	namely
Y	Y chromosome

PUBLICATIONS/PRESENTATIONS ARISING FROM THIS THESIS

1. **ESSOP F**, GREENBERG J, BASEL D, AND KRAUSE A. Molecular analysis of a fragile X family with two females homozygous for a premutation. (Poster) 51st Annual Meeting of the American Society of Human Genetics, San Diego, California, 12-16 October 2001

2. **ESSOP F**, AND KRAUSE A. Contribution of two X-linked loci, FRAXE and *XNP* to mental retardation in South Africa (Poster). 52nd Annual Meeting of the American Society of Human Genetics, Baltimore, 16 October 2002

3. **ESSOP F**, AND KRAUSE A. Contribution of two X-linked loci, FRAXE and *XNP* to mental retardation in South Africa (Poster). 10th Biennial Congress of the Southern African Society of Human Genetics (SASHG), Durban, South Africa, 11-14 May 2003

4. KUHN K, **ESSOP F**, GIBBONS R, KRAUSE A. Molecular diagnosis of alpha-thalassaemia/mental retardation syndrome (ATR-X) in two South African patients (Poster). Combined Congress of the Southern African Society of Human Genetics and the African society of Human Genetics, 13-17 March 2005, Glenburn Lodge Country Estate, Muldersdrift, Gauteng, 2005

CHAPTER ONE

1 INTRODUCTION

Mental retardation (MR) or developmental delay is said to be one of the most common human disorders and is a serious social and medical problem. It has an estimated prevalence of 2-3% in developed countries (Penrose, 1938; Toniolo, 2000) and is reported to be the most common reason for referral to genetic clinics (Leonard and Wen, 2002).

There is large interest in identifying the causes of MR. It can impose a costly and lifelong burden on the public health sector. Establishing the cause in an individual with MR improves clinical management and facilitates genetic counselling for the family.

MR is reported to have a genetic cause in 25-30% of cases and is observed more frequently in males than females, with an affected ratio of 1.3:1 (Leonard and Wen, 2002). This indicated that defects on the X-chromosome are an important factor in the etiology of MR. X-linked mental retardation (XLMR) represents ~10-15% of all MR in males (Ropers, 2008) and from epidemiological studies a prevalence figure of 1 in every 600 males is estimated (Herbst and Miller, 1980).

XLMR is historically classified into syndromic XLMR (S-XLMR) and nonsyndromic (also referred to as non-specific) XLMR (NS-XLMR) forms (Kerr, Turner, Mulley et al., 1991), although this distinction has become less clear since mutations in a single gene have been associated with both forms. The *FMR1* gene responsible for fragile X MR syndrome is the most common inherited form of MR, accounting for at least 25% of XLMR (Fishburn, Turner, Daniel et al., 1983; Ropers 2006). As the *FMR1* gene is implicated in other disorders, for the purpose of this dissertation, fragile X syndrome resulting in MR from an expansion in the *FMR1* gene is referred to as fragile X MR syndrome. Fragile X E syndrome due to an expansion in the *FMR2* gene is referred to as FRAXE syndrome.

Initial research focussed on delineating the molecular causes of fragile X MR syndrome, while the search for other XLMR genes was relatively slow. The field of XLMR has gained interest and developed rapidly, facilitated by the cloning of the human genome. Significant breakthroughs in identifying genes and the molecular and cellular mechanisms underlying XLMR have been made in the past 10-15 years through the establishment of large collaborations between clinical and molecular research teams such as the European XLMR consortium (EuroMRX website,

2009), the Greenwood Genetics Centre in the U.S.A (GGC website, 2009) and The Genetics of Learning Disability study in the UK (IGOLD website, 2009). To date, >90 XLMR genes have been identified (Gecz, Shoubridge and Corbett, 2009). Delineating the causes of XLMR and their molecular and cellular consequences will provide insight into the mechanisms that are required for the normal development of cognitive functions in humans.

The study of XLMR genes has been of particular interest to the Division of Human Genetics, Molecular Genetics Laboratory at the National Health Laboratory Service (NHLS). This was brought about by the escalating number of genes being involved in XLMR and the increasing number of patients with unexplained MR. The focus has been concentrated mainly on developing and/or refining screening methods with a view to offering them as diagnostic tests in the Division. Previous studies on fragile X MR syndrome had been undertaken in the Division by Goldman (1997). In 2000 a study undertaken by myself, in collaboration with scientists from the Greenwood Genetic Centre showed that a dodecamer duplication in the *HOPA* gene was not associated with MR in South African populations (Friez, Essop, Krause et al., 2000).

This study investigated additional genes associated with XLMR. It was undertaken as part of a worldwide effort to elucidate genes implicated in XLMR and focussed particularly on the South African population and the contribution of 3 NS-XLMR genes, *FMR2*, *XNP* and *ARX*. When this study was initiated, the Division of Human Genetics, Molecular Genetics Laboratory at the National Health Laboratory Service (NHLS) had considered and proposed investigating additional X-linked loci/genes such as *MECP2* and/or other XLMR genes if indicated. As the study progressed many of the XLMR genes discovered were found not to contribute significantly to XLMR. As a result additional XLMR genes were not investigated as part of this study. Further, the current methods are generally too expensive or too unreliable to justify mutation screening of all known XLMR genes in diagnostic testing. It must be noted that this study was started in 2001 and has spanned many years of changing knowledge and technology. The general feeling at the time was that mutations in a few genes would contribute to a large amount of NS-XLMR cases. The approach taken in this study was to screen mutation hotspots as a first target for screening for causes of MR in patients. However through the subsequent years, many genes have been identified with few common mutations, none of which has been reported to contribute substantially to NS-XLMR. Presently such a study would not be performed using the approach taken. Newer technologies have allowed for rapid screening methods at affordable costs.

This study also undertook a retrospective analysis of fragile X MR syndrome referrals to the Molecular Genetics Diagnostic Laboratory, NHLS over a 17 year period (1992 to 2009). It extends the PhD study of Goldman (1997) in which the frequency of fragile X MR syndrome was

assessed and allele distributions were determined in the South African population by investigating a larger cohort of families.

Family studies for X-linked α-thalassaemia mental retardation syndrome (ATR-X) and fragile X MR syndrome were also undertaken. Molecular investigations were undertaken on three patients with ATR-X and their extended family members. The causative mutation was identified and carrier and prenatal testing was performed. As an incidental finding during a routine investigation for the *FMR1* expansion, an interesting result was found. Two siblings were found to have two expanded *FMR1* alleles in the intermediate and premutation range respectively. The segregation of this expansion in the family was examined using haplotype analysis. As this is the first South African report of females with 2 expanded *FMR1* alleles and the fifth family reported thus far in the world, it has been presented in this dissertation.

As there are many facets to this study, each of the genes investigated has been discussed in a separate chapter. Chapter 1 provides an introduction and review of the current literature on MR and a review on each of the genes investigated in this project, viz., *FMR1*, *FMR2*, *XNP* and *ARX*. Chapter 2 outlines the subjects tested and provides an in-depth overview on the methods and materials used. The results obtained for each of the NS-XLMR genes investigated and a discussion of these results is presented in Chapters 3 and 4. Chapters 5 and 6 describe the results on the family studies undertaken for ATR-X syndrome and fragile X MR syndrome. It was decided to present results and discussion for each component of the study in order to try to ensure continuity. Chapter 7 presents a final combined discussion and conclusion on all the genes investigated in this study.

1.1 MENTAL RETARDATION

1.1.1 Features of MR

The American Association on Mental Retardation defines mental retardation (MR) as "…a disability characterized by significant limitations both in intellectual functioning (criterion 1) and in adaptive behavior (criterion 2) as expressed in conceptual, social, and practical adaptive skills. This disability originates before the age of 18 (criterion 3)." (AAMR website, 2007).

Intellectual functioning varies in severity and is classified on the basis of Intelligence Quotient (IQ) scores. The different classes of MR vary in IQ and the criteria set by the International Statistical Classification of Diseases (ICD-10), coordinated by the World Heath Organization (WHO, 1980) are summarized in Table 1.1 .

Table 1.1 IQ scores relative to the degree of severity of MR according to ICD-10 criteria.

DEGREE OF MENTAL RETARDATION	INTELLIGENCE QUOTIENT (IQ)
Borderline	70-85
Mild	50-70
Moderate	35-50
Severe	20-35
Profound	<20

The above classes are usually simplified into 2 groups: severe MR (IQ<50) and mild MR (IQ 50-70). Mild forms are thought to be more frequent with an estimated prevalence of 2-3% and severe MR with an incidence of 0,5-1% (Raymond, 2006; Ropers, 2006).

1.1.2 Aetiology of MR

The causes of MR are diverse and include teratogenic and environmental factors, perinatal asphyxia, genetic causes or a combination of genetic and acquired/environmental factors. Although environmental and teratogenic factors play a role in the occurrence and severity of the disease and are reported to occur in 5-13% of cases, genetic factors have a significant impact, with 25-30% of cases thought to be genetic. Less than 50% of MR cases have a known primary cause (Curry, Stevenson, Aughton et al., 1997).

1.1.2.1 Genetic causes

Chromosome abnormalities (microscopic/submicroscopic, interstitial/subtelomeric) are the most common known causes of MR, occurring in 20% of unexplained MR cases (de Vries, Pfundt, Leisink et al., 2005). The use of newer and powerful technologies such as multiple-ligand-probe amplification (MLPA) and genome wide array based Comparative Genomic Hybridisation (CGH) using tiling path DNA microarrays that detect copy number alterations/variations (CNVs) have a more extensive coverage of the genome and have allowed the detection rates to increase. In patients with dysmorphic features and congenital anomalies, 5-7% are reported to have subtelomeric microdeletions or microduplications (Frints, Froyen, Marynen et al., 2002). In syndromic patients, CGH enables detection of genomic copy number changes in 16% of patients (Stankiewicz and Beaudet, 2007). Recently the diagnostic yield of clinically significant CNVs was reported to be 9.1% (Koolen, Pfundt, De Leeuw et al., 2009).

It is estimated that ~50% of severe cases of MR have a genetic cause (Prieto Garcia, 1998). **X-linked mental retardation** (discussed in Section 1.2) represents 10-15% of all MR (Ropers, 2008) and from epidemiological studies a prevalence figure of 1 in every 600 males is estimated (Herbst and Miller, 1980; Chiurazzi, Schwartz, Gecz et al., 2008).

MR resulting from genes on autosomes has also been described. While there has been significant progress made in identifying X-linked genes, genetic knowledge on autosomal inheritance remains largely unknown due to its extreme genetic heterogeneity. To date five genes have been implicated in causing **autosomal recessive** mental retardation (ARMR). These are *PRSS12* (neurotrypsin; MIM606709, 4q26), *CRBN* (cereblon, MIM 609262, 3p26) and *CC2D1A* (MIM 610055, 19p13), *GRIK2* (glytamate receptor 6, MIM 138244, 6q16) and *TUSC3* (8p22) (Molinari, Rio, Meskenaite et al., 2002; Higgins, Pucilowska, Lombardi et al., 2004; Basel-Vanagaite, Attia, Yahav et al., 2006; Motazacker, Rost, Hucho et al., 2007; Basel-Vanagaite, 2008; Garshasbi, Hadavi, Habibi et al., 2008). The five genes are reported to contribute a small percentage to the total ARMR cases. In addition, 10 new loci have recently been mapped, indicating extreme genetic heterogeneity (Basel-Vanagaite, 2007).

The difficulty in ascertaining large families has made the identification of **autosomal dominant** genes difficult. A possible reason could be that MR results in lower reproductive fitness which makes it difficult to identify families for linkage analysis. Recently, Hamdan, Gauthier, Spiegelman et al. (2009) reported on the *SYNGAP1* gene, which encodes a ras GTPase activating protein critical for cognition and synapse function, as a cause of autosomal dominant nonsyndromic mental retardation. They reported on *de-novo* mutations being present in

approximately 3% of their patient cohort with nonsyndromic mental retardation. Figure 1.1 illustrates the main genetic etiologies of MR.

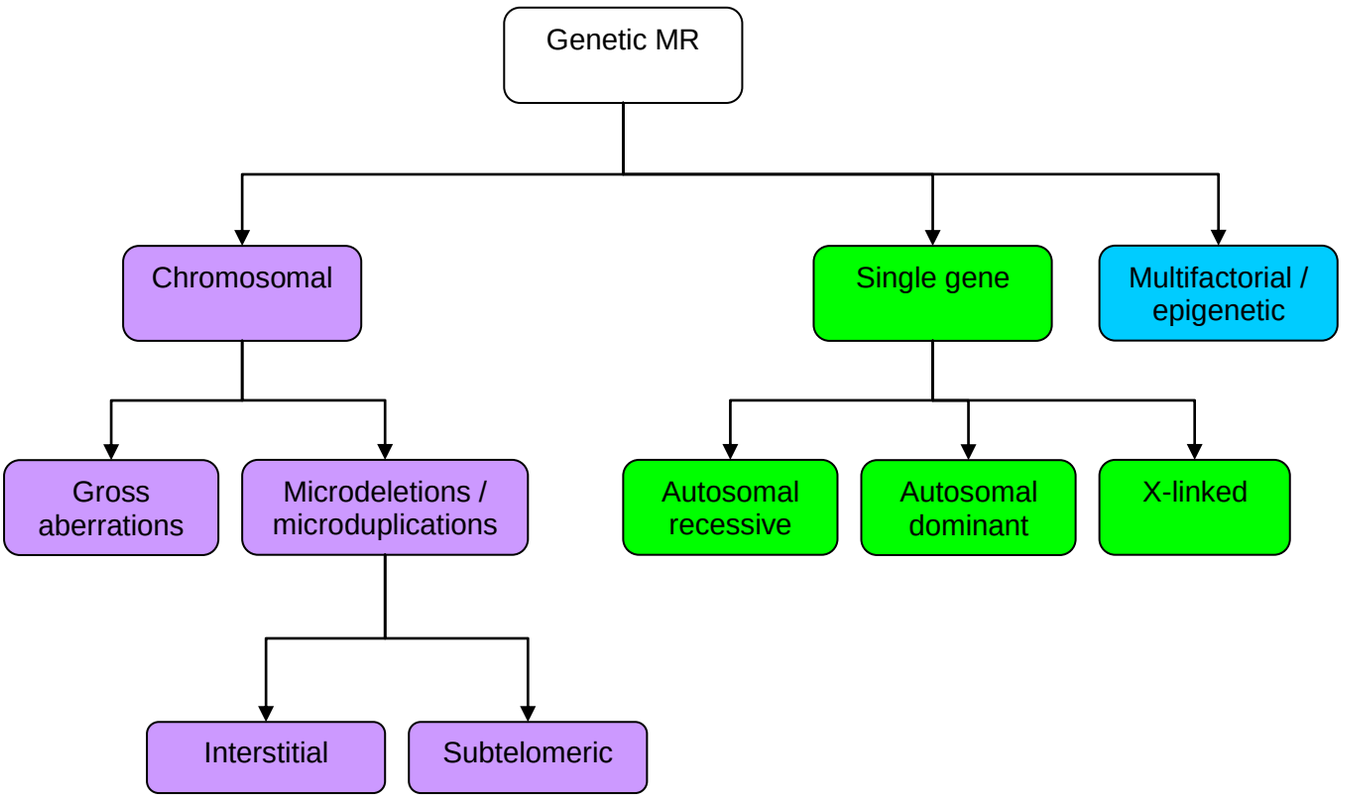


Figure 1.1 Main genetic etiologies of MR
(Basel-Vanagaite, 2008)

1.1.2.2 Environmental causes

The causes of MR differ for each country, with the socioeconomic status of that country having a major influence. In developing countries, poverty and environmental conditions are the major factors responsible for MR. The causes can be divided into biomedical (biologic processes, e.g. genetic disorders, infection or nutrition), social (social and family interaction such as child stimulation and adult responsiveness), behavioural (harmful behaviours, such as maternal substance abuse), and educational risk factors (availability of family and educational support that promote mental development and increases in adaptive skills) that interact during the life of an individual and/or across generations from parent to child (Curry et al., 1997).

1.1.3 MR in South Africa

The prevalence of MR and its epidemiology in developing countries is not well established. Limited information is available for developing countries since most of the research has focused on developed countries. It is estimated that the developing nations have ~70 to 110 million children with MR (Shah, 1991; Roeleveld, Zielhuis and Gabreels, 1997).

The most common cause of MR in South Africa (SA) is fetal alcohol syndrome (FAS). In fact, SA has the highest rate of FAS in the world. In comparison with the USA rate of 0.05 to 2.0 per 1000 births (May and Gossage, 2001), SA has a rate of 46 to 89 cases per 1000 births reported in the Western Cape Province, a rate of epidemic proportions (May, Brooke, Gossage et al., 2000; Viljoen, Gossage, Brooke et al., 2005; May, Gossage, Marais et al., 2007).

Other studies have been conducted to investigate the prevalence of MR in rural and urban populations in South Africa. These are presented below.

The prevalence of severe MR among Coloured children under 18 years in the Cape Town suburb of Athlone (urban residential area) was studied (Power, 1977). The author found the administrative (number of severely retarded children known to social, medical or educational agencies serving the surveyed population) prevalence was 2.62-3.36/1000, a figure lower than that for Europe and North America but comparable to other reported figures (Friedlander and Power, 1982). The authors found that in the Heideveld area of Cape Town (including Heideveld, Vanguard, Surrey and Welcome Estates), the prevalence of severe MR among children less than 18 years of age was 2.5/1000. The prevalence of profound MR was calculated to be 0.4% and 4% for mild to moderate MR, under 19 years of age on the Witwatersrand (Cartwright, Van Rijlaarsdam, Wagner et al., 1988).

A study was also conducted on Black South African children with developmental disabilities at a neurodevelopmental clinic at Baragwanath Hospital, Soweto in Johannesburg (Rubin and Davis, 1986). Their study found that 4% of the etiological diagnoses were prenatal genetic causes, defined as including “children with chromosomal (e.g., Down syndrome), nonchromosomal syndromes of multiple congenital anomalies, and proven cases of congenital infections”.

The aetiology of moderate and severe learning disability in preschool children in Cape Town was determined (Molteno and Lachman, 1996). In 45% the disability was prenatal in origin, in 17% perinatal, in 9% postnatal and in ~29% it was idiopathic.

In 1981 the first South African cases of XLMR were described in which 11 males in 4 families were investigated by cytogenetic studies and were found to have a marker X chromosome (fra (X) (q27)) (Venter, Gericke, Dawson et al., 1981).

The prevalence and types of intellectual disabilities in a rural Black population in South African children aged 2-9 years was investigated in 2002 by Christianson, Zwane, Manga et al. (2002). The ratio of males to females with intellectual disorders was 3:2. The prevalence of severe intellectual disability was found to be 0.64/1000 and mild disability 29.1/1000. Aetiology for MR was determined and 20.6% were found to be due to congenital causes, 6.3% acquired and undetermined in 73.1%. The observed prevalence of intellectual disabilities (35.6/1000) compared well with that found by Molteno, Roux, Nelson et al. (1990) in Cape Town and with the prevalence figure of 30/1000 presented by WHO for developing countries (WHO, 1985b).

1.2 X-LINKED MENTAL RETARDATION (XLMR)

Since there is an excess of males affected with MR compared to females, with approximately 30% more males affected than females, the search for disease-associated genes has predominantly been focussed on the X chromosome (Turner, Turner and Collins, 1970; Lehrke, 1974; Raymond and Tarpey, 2006). In XLMR families, the most common form of XLMR, fragile X MR syndrome (discussed in Section 1.3) accounts for 25% of the mutations (Fishburn et al., 1983, reviewed in Ropers, 2006).

XLMR has been classified into syndromic XLMR (S-XLMR) and non-syndromic or nonspecific XLMR (NS-XLMR) (Kerr et al., 1991). NS-XLMR refers to a heterogeneous group of disorders in which cognitive impairment is usually the only consistent clinical feature seen while S-XLMR refers to a form where MR is associated with other recognizable clinical signs (Gecz and Mulley, 2000). The distinction between these two types appears to be blurred as in some cases the same genes have been identified in both S-XLMR and NS-XLMR (Table 1.3) and also the same gene in different S-XLMR conditions.

The identification of XLMR genes has been an international collaborative effort, involving several groups working in the fields of XLMR, e.g. European XLMR consortium (EuroMRX website, 2009), founded in 1995, comprising Paris, France; Leuven, Belgium; Nijmegen, The Netherlands; Tours, France; Berlin; the Australian associated collaborative group and the Italian XLMR groups of Pavia and Siena (Frints et al., 2002). The Greenwood Genetics Centre in the U.S.A (GGC website, 2009) and the GOLD study group (IGOLD website, 2009) in Cambridge have also contributed to the identification of XLMR genes.

Significant progress has been made, especially in the last 13 years in finding and characterizing genes for XLMR. More than 215 different XLMR disorders have been described, including 149 S-XLMR forms and 66 NS-XLMR forms. To date, >90 XLMR genes have been identified (S-XLMR and NS-XLMR) with detected mutations (Ropers, 2006; Chiurazzi et al., 2008). Table 1.2 gives an updated count of XLMR conditions.

Table 1.2 Count of XLMR conditions by clinical presentation: syndromes, neuromuscular conditions and NS-XLMR conditions.

	TOTAL	MAPPED	CLONED
S-XLMR	**149**	**47**	**66**
X-linked syndromes	98	31	38
Neuromuscular disorders	51	16	28
NS-XLMR conditions	**66**	**50**	**16**
TOTAL XLMR CONDITIONS @ 8 SEPT 2009	**215**	**97**	**82**

A detailed list of all the conditions/syndromes can be found on the XLMR website, 2009
X-linked syndromes refer to X-linked syndromes with MR and multiple congenital anomalies.
Neuromuscular disorders refer to disorders characterized by neurological symptoms such as epilepsy, spastic paraplegia, ataxia, etc.

Given the extensive heterogeneity of NS-XLMR, different strategies have been used for identifying new genes. These include linkage analysis, associations with fragile sites, e.g. FRAXA and FRAXE, breakpoint mapping and cloning in MR patients with balanced X chromosomal rearrangements, characterization of X-chromosome microdeletions at a molecular level and candidate gene strategies by screening for mutations in regions of the X-chromosome involved in neuronal development and function (Ropers and Hamel, 2005; Chiurazzi et al., 2008).

1.2.1 Non-syndromic or non-specific X-linked mental retardation (NS-XLMR)

NS-XLMR is characterized by the lack of distinctive phenotypic features and clinical variability in the degree of MR within families (Chelly, 1999). NS-XLMR appears to be twice as common as S-XLMR (reviewed in Ropers, 2006). The incidence of NS-XLMR has been estimated to be 1.8/1000 or 1 in 600 males (Herbst, 1980; Kerr et al., 1991).

Each NS-XLMR family was initially represented by a single pedigree and the gene segregating in each family was given a distinctive label, MRX by the Human Genome Gene Nomenclature Committee (HUGO website, 2009). If a minimum lod score of +2 was shown between the MR locus and one or more X chromosome markers in that family, the family was assigned a unique number, MRX1, MRX2, MRX3,… (Mulley, Kerr, Stevenson et al., 1992). The first MRX family (MRX1) was characterized in 1988 (Suthers, Turner and Mulley, 1988) and the associated gene

was localized. The first NS-XLMR disorder cloned was Fragile X E mild mental retardation syndrome (FRAXE) (discussed in Section 1.4) (Sutherland and Baker, 1992; Flynn, Hirst, Knight et al., 1993).

Target regions have been identified on the X chromosome where mutations are clustered. Most of these are on proximal Xp and the Xp11 region (Ropers, Hoeltzenbein, Kalscheuer et al., 2003). To date, 38 of the >90 XLMR genes have been implicated in NS-XLMR. Each of these genes (except fragile X MR syndrome, *MECP2* and *ARX*) accounts for 0.2-0.5% of XLMR cases (Gecz et al. 2009; XLMR website, 2009). Table 1.3 lists the 38 genes that have been implicated in NS-XLMR and the frequency of genes commonly associated with NS-XLMR. Table 1.4 lists the genes involved in both NS-XLMR and S-XLMR. Figure 1.2 shows an ideogram of the NS-XLMR genes on the X chromosome with their chromosomal locations.

Table 1.3 NS-XLMR genes identified to date (n=38)

GENES THAT CAUSE NS-XLMR					
OMIM	**PROTEIN NAME**	**LOCUS**	**GENE SYMBOL**	**REFERENCE**	**MUTATION FREQ IN NS-XLMR**
309548	AFF2 protein	Xq28	*FMR2*	Gecz et al. 1996	
300142	p21 Act. Kinase 3	Xq23	PAK3	Allen et al. 1998	1-2%
300104	RABGDIA	Xq28	GDI1	D'adamo et al. 1998	<1%
*300206	IL1 receptor accessory protein-like 1	Xp21.2	IL1RAPL1	Carrie et al. 1999	1-2%
*300267	α-PIX	Xq26.3	ARHGEF6	Kutsche et al. 2000	Rare/very rare
*300096	Tetraspanin	Xp11.4	TM4SF2	Zemni et al. 2000	
*300157	fatty acid-CoA ligase 4	Xq23	FACL4 (ACSL4)	Meloni et al. 2002	<1%
300034	Angiotensin rec. 2	Xq23	AGTR2	Vervoort et al. 2002	Rare/very rare
314995	zinc-finger protein 41	Xp11.3	ZNF41	Shoichet et al. 2003	
*300336	neuroligin 3	Xq13	NLGN3	Jamain et al. 2003	
*300427	neuroligin 4	Xp22.3	NLGN4	Laumonnier et al. 2004	
*314998	zinc-finger protein 81	Xp11.23	ZNF81	Kleefstra et al. 2004	
300189	Discs, large homolog 3	Xq13.1	DLG3	Tarpey et al. 2004	1-2%
*300499	FtsJ homolog 1	Xp11.23	FTSJ1	Freude et al. 2004	1-2%
*300579	Shroom family member 4	Xp11.2	SHROOM4	Hagens et al. 2006	
300573	zinc-finger family member 674	Xp11.3	ZNF674	Lugtenberg et al. 2006	
*312173	Ribosomal protein L10	Xq28	RPL10	Klauck et al. 2006	
*300304	Cullin 4B	Xq23	CUL4B	Tarpey et al. 2007a Zou et al. 2007	
*300576	zinc finger, DHHC-type containing 9	Xq13.3	ZDHHC9	Raymond et al. 2007	
*300553	Bromodomain and WD repeat domain-containing 3	Xq21.1	BRWD3	Field, M. et al. 2007	
*300298	UPF3 regulator of nonsense transcripts homolog B	Xq24	UPF3B	Tarpey et al. 2007b	
*305915	Glutamate receptor, ionotrophic, AMPA3	Xq25	GRIA3	Wu et al. 2007	
*300231	Solute carrier family 9 (sodium-hydrogen exchanger)	Xq26.3	SLC9A6	Gilfillan et al. 2008	
*300697	HECT, UBA- and WWE-domain-containing1	Xp11.22	HUWE1	Froyen et al. 2008	
*300715	Magnesium transporter 1	Xq13.1-q13.2	MAGT1	Molinari et al. 2008	
*314990	Zink finger protein 711	Xq21.1-q21.3	ZNF711	Chiurazzi et al. 2008	0.3%
*300172	Calcium/calmodulin-dependent serine protein kinase	Xp11.4	CASK	Chiurazzi et al. 2008	
*313475	Synatophysin	Xp11.23-p11.22	SYP	Tarpey et al. 2009	0.3%

Table 1.4 Genes that cause NS-XLMR and S-XLMR

GENES THAT CAUSE NS-XLMR AND S-XLMR (OR NEUROMUSCULAR FORMS)					
*300075	Coffin-Lowry	Xp22.1	RSK2 (RPS6KA3)	Merienne et al. 1999	<1%
*300005	Rett	Xq28	MECP2	Couvert et al. 2001	Rare/very rare
*300036	Creatine transporter deficiency	Xq28	SLC6A8	Salomons et al. (2001)	>2%
305400	Aarskog-Scott	Xp11.22	FGD1	Lebel et al. 2002	
*300382	West, infantile spasms	Xp22.1	ARX	Stromme et al. 2002; Bienvenu et al. 2002	>5%
*300463	Sutherland-Haan/MRXS3	Xp11.23	PQBP1	Kalscheuer et al. 2003	Rare/very rare
*300127	Cerebellar ataxia	Xq12	OPHN1	Billuart et al. 1998	
*300032	ATR-X	Xq13.3	ATRX (*XNP*)	Gibbons et al. 1995a	
*314690	JARID1C-related XLMR	Xp11.22	JARID1C / SMCX	Jensen et al. 2005	>2%
#300630	Adaptor-related protein complex 1	Xp22.2-p21.2	AP1S2	Tarpey et al. 2006	

* Gene with known sequence

\# Phenotype description, molecular basis known

No symbol before an entry number - generally indicates a description of a phenotype for which the mendelian basis, although suspected, has not been clearly established or that the separateness of this phenotype from that in another entry is unclear

(Modified from Ropers, 2006; Gecz et al, 2009; XLMR website, 2009)

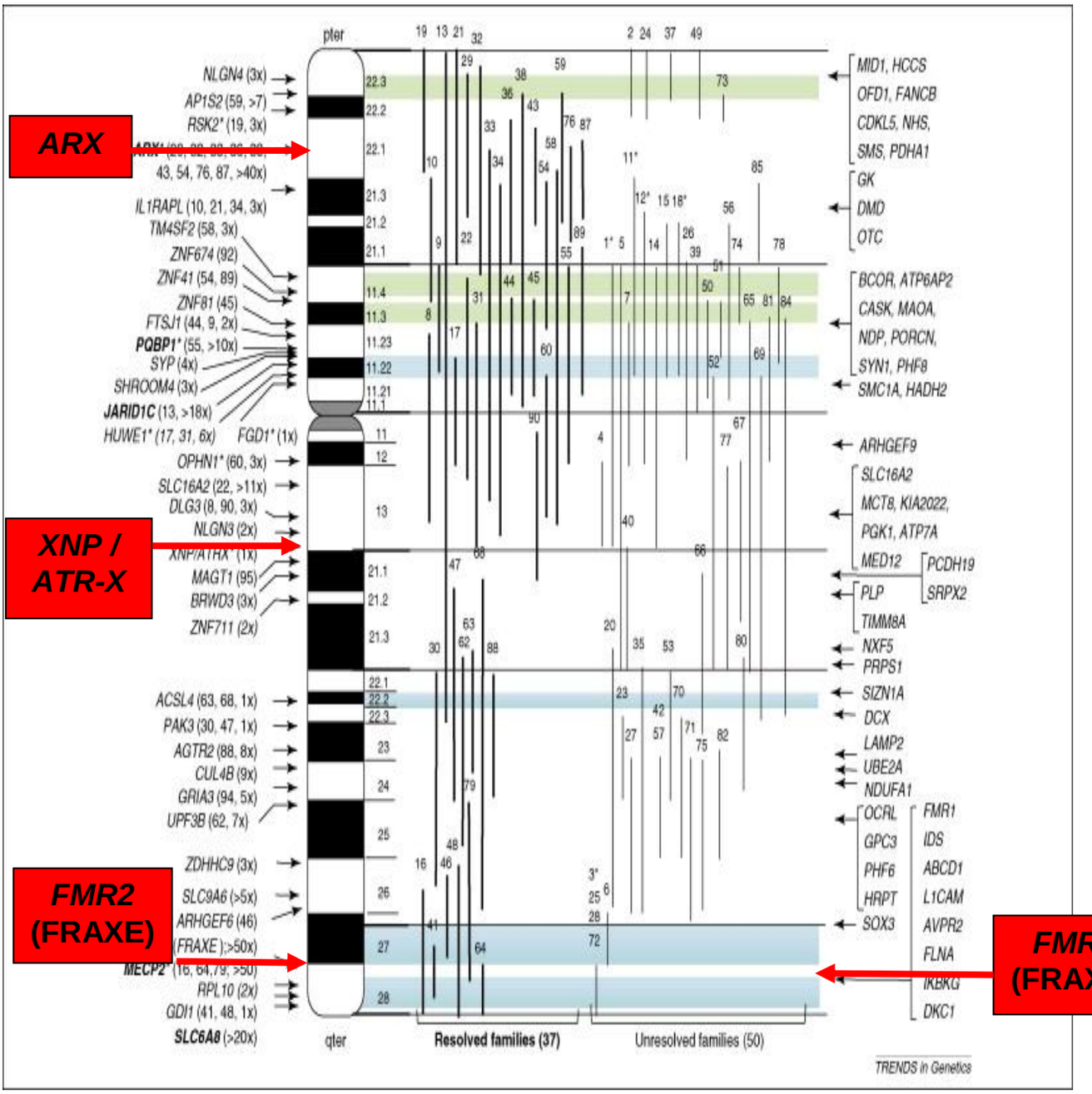


Figure 1.2 Ideogram of the X-chromosome showing the genes implicated in NS-XLMR and S-XLMR.

The 38 genes implicated in NS-XLMR are shown on the left hand side, with arrows indicating position of genes. Genes with high contribution to NS-XLMR are highlighted in bold. The numbers in brackets indicate either the MRX family number or the number of unrelated families with mutations in this gene. The 52 genes on the right hand side are implicated in S-XLMR. The vertical lines shown on the right represent linkage intervals on the X chromosome in MRX families (numbers indicate the MRX family) with lod score >2. Genes involved in S-XLMR and NS-XLMR are shown by an asterisk near the gene. The horizontal boxes indicate regions with frequent deletions (green) and duplications (blue) (Gecz et al, 2009). The genes investigated in this study are indicated by red boxes.

It is noteworthy that mutations in the *ARX* gene have been reported in almost 10% of the 81 large MRX (NS-XLMR) families; far more frequent than mutations in other NS-XLMR genes. Ropers (2006) mentions that the frequency of mutations in the *ARX* gene was much lower (0% to 5%) in smaller families. Mutations in 5 other genes, *JARID1C*, *SLC6A8*, *MECP2*, *IL1RAPL1* and *PQBP1* have each been found to account for ~1% of NS-XLMR genes (reviewed in Ropers, 2006).

Mutations in the *MECP2* (methyl-CpG-binding protein 2) gene are known to cause Rett syndrome, the most common cause of MR in females. It was proposed that mutations in *MECP2* gene might account for ~2% of males with MR of unknown cause (Couvert, Bienvenu, Aquaviva et al., 2001). The authors suggested that the frequency of *MECP2* mutations was comparable to that of fragile X MR syndrome. Subsequent studies (Yntema, Kleefstra, Oudakker et al., 2002a; Bourdon, Philippe, Martin et al., 2003; Ylisaukko-Oja, Rehnstrom, Vanhala et al., 2005) have shown that *MECP2* mutations do not represent a major cause of NS-XLMR and are far rarer than initially thought and the contribution of *MECP2* has probably been overestimated. Recently, Villard (2007) in his review reports that MR caused by *MECP2* mutations is not rare in male patients. An estimated frequency of 1.3-1.7% in males with moderate or severe MR is reported, while the incidence of fragile X MR syndrome is reported to be 2.8% in the same population. Caution should be taken with analysis and interpretation of *MECP2* sequence variants as the pathogenicity of these is questionable. Many non-pathogenic variants were identified in *MECP2* and the gene has a high rate of *de novo* mutations. Also, testing males who are negative for the *FMR1* expansion is not a useful criterion to select a population for *MECP2* mutation screening (Villard, 2007).

It is hoped that elucidating the function of the XLMR proteins will provide insight into brain function. The genes responsible for NS-XLMR are a heterogeneous group and their precise role and function is unknown. XLMR genes that were identified initially were involved in the RhoGTpase pathway as signalling molecules (*GD1, PAK3, ARHGEF6*) or associated with chromatin remodelling (RPS6KA3, ATRX). Genes identified more recently have been involved in synaptic vesicle formation (*SYN1, SLC6A8, NLGN4* and *DLG3*) or are novel transcription factors (*ZNF41, 81* and *674*) (Raymond and Tarpey, 2006).

1.2.2 Syndromic X-linked mental retardation (S-XLMR)

In S-XLMR MR is usually a secondary feature and forms part of a complex syndrome with additional neurological, behavioural, metabolic or somatic abnormalities accompanying MR that define a syndrome (Gedeon, Donnelly, Mulley et al., 1996). Furthermore, S-XLMR can be subdivided into four categories, namely: malformation syndromes, neuromuscular disorders, metabolic disorders and dominant conditions (where males die before birth and females are affected). There have been an increasing number of S-XLMR genes cloned (Figure 1.2) due to the fact that most genes on the X chromosome are haploid in males and mapping genes on this chromosome is relatively simpler (Chelly, 1999). This task has been made easier by the availability of the annotated human genome sequence.

1.3 FRAGILE X A MR SYNDROME (FRAXA)

Fragile X MR syndrome (OMIM#300624, OMIM website, 2009), also known as the Martin-Bell syndrome, was the first XLMR described. It is the most common inherited cause of MR (Hagerman, 1996) accounting for 2-2.5% of MR in males and ~25% of all XLMR (reviewed in Ropers, 2006). A relationship between rare fragile sites and MR was proposed many years ago. In 1969, Lubs described a fragile site on the X chromosome in MR males (discussed in Section 1.3.5). The FRAXA fragile site at Xq27.3 is associated with fragile X MR syndrome (Sutherland, Mulley and Richards, 1993).

1.3.1 Structure and function of *FMR1* gene and protein FMRP

The gene consists of 17 exons and is 38 kb long (Eichler, Richards, Gibbs et al., 1993). It encodes an mRNA of ~3.9 kb composed of a ~0.2 kb 5' untranslated region, a 1.9 kb coding region, and a 1.8 kb 5' untranslated region (Bardoni, Schenck and Mandel, 2001). The cause of MR in fragile X MR syndrome is due to the absence of the *FMR1* gene product, fragile X mental retardation protein (FMRP) (Pieretti, Zhang, Fu et al., 1991).

FMRP was shown to have properties of an RNA-binding protein as it contains two K-homology (KH) domains and an RGG box, found in various RNA-binding proteins (Siomi, Siomi, Nussbaum et al., 1993; Siomi, Choi, Siomi, et al., 1994) and to associate with translating polyribosomes, in particular the 60S subunit (Khandjian, Corbin, Woerly et al., 1996).

FMRP was shown to have a cytoplasmic localization and has been detected in almost every tissue, with highest levels observed in neurons (Devys, Lutz, Rouyer, et al., 1993), human fetal brain (Abitbol, Menini, Delezoide et al., 1993), testes (Bachner, Steinbach, Wohrle et al., 1993), oesophagus, lung and kidney (Khandjian, 1999). FMRP was shown to function as a nucleocytoplasmic shuttling protein that binds to specific mRNas that are involved in dendrite development or synapse function (Weiler and Greenough, 1999). There is evidence that FMRP mediates translational silencing of mRNAs through interaction with microRNAs and the RNA-induced silencing complex (RISC) (Jin, Zarnescu, Ceman et al., 2004; Plante and Provost, 2006). Research in this field has been extensive since it helps in understanding the fundamental aspects of neuronal function (Ropers and Hamel, 2005). The *FMR1* gene is thought to have a function during germ cell proliferation in both males and females (reviewed in Brouwer, Willemsen and Oostra, 2009b).

1.3.2 Molecular basis of fragile X MR syndrome

Fragile X MR syndrome is an X-linked condition. The majority (>99%) of fragile X MR syndrome results from the expansion of a CGG (or CCG) triplet repeat in the 5'-untranslated region (UTR) of exon 1 of the **F**ragile X **M**ental **R**etardation-**1** gene (*FMR1*) (OMIM#309550, OMIM website, 2009) at Xq27.3 resulting in gene methylation and silencing (Kremer, Pritchard, Lynch et al., 1991; Verkerk, Pieretti, Sutcliffe et al., 1991). Fragile X MR syndrome was the first known trinucleotide repeat disorder (TRD) described. TRDs have been implicated in the pathogenesis of more than 20 neurological disorders (reviewed in Brouwer, Willemsen and Oostra, 2009a).

TRDs are repeat regions which are heritable unstable elements that behave differently from static mutations. Their mutation mechanism is dynamic as the repeats become unstable once it exceeds a certain threshold length and undergo an increase in copy number in successive generations (Oberle, Rousseau, Heitz et al., 1991). The resulting phenotype is usually based on the length of the repeat. To date, nine polyglutamine diseases resulting from the expansion of a CTG repeat are known: Huntington disease, spinal and bulbar muscular atrophy, dentatorubral-pallidoluysian atrophy and six forms of spinocerebellar ataxia (Katsuno, Banno, Suzuki et al., 2008). The repeat sequences, location within the genes, ranges of normal and disease-causing repeat lengths and the clinical outcomes differ in the different TRDs (Gatchel and Zoghbi, 2005).

The pathogenic mechanisms underlying TRDs result in gain-of-function and loss-of-function. The timing of the expansion is still under debate, although some attempts have been made to gain insight into this and different models have been proposed (reviewed in Brouwer et al.,

2009a). There has been much speculation as to the mechanism of repeat instability at the molecular level. The mechanism is complex arising through multiple processes (Pearson, Nichol Edamura, and Cleary, et al., 2005). One proposed mechanism suggested is slippage of the replication fork during replication (Kunkel, 1993; Richards and Sutherland, 1994). Proteins involved in lagging strand synthesis, coordination between synthesis of the leading and the lagging strand and restarting of the replication fork play a role in the stability of the microsatellite repeats (reviewed in Mirkin, 2007; reviewed in Brouwer et al., 2009a).

Fragile X MR syndrome shows anticipation, i.e. increased severity and earlier onset in successive generations (Fu, Kuhl, Pizzuti et al., 1991; Oberle et al., 1991). The length of the repeat correlates with the severity of the syndrome (Oberle et al.,1991; Sutherland, Haan, Kremer, et al., 1991b; Yu, Pritchard, Kremer et al., 1991). Figure 1.3 illustrates the range of CGG repeats in the *FMR1* gene.

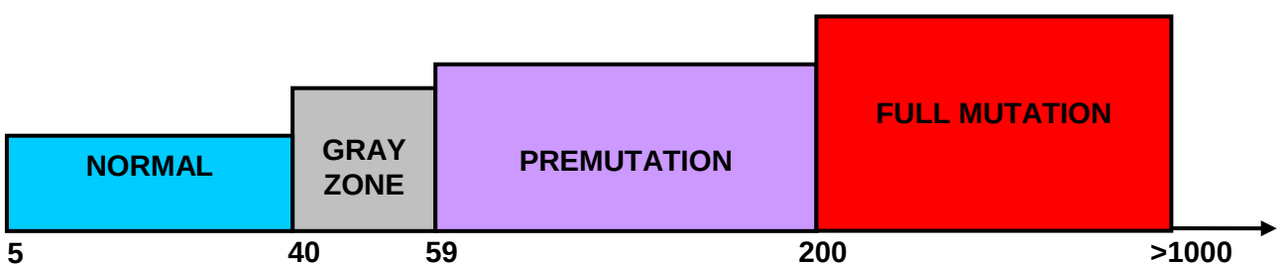


Figure 1.3 Range of *FMR1* CGG repeats

1.3.2.1 *FMR1* normal (N) alleles

The normal number of repeats is polymorphic and varies from 5 to 40 CGG repeats in the *FMR1* gene (GeneReviews website, 2009). These alleles are stably transmitted without any increase or decrease in repeat number. The most common alleles described in different populations range between 28 and 30 repeats and over 98% of all normal alleles are below 46 repeats (Reiss, Kazazian, Krebs et al., 1994). The CGG repeat is interrupted by AGG repeats, which are thought to provide stability to the CGG repeat through the influence of RNA structures (Eichler, Holden, Popovich et al., 1994; reviewed in Brouwer et al., 2009b)

1.3.2.2 *FMR1* intermediate (I) / gray zone alleles

Intermediate alleles are reported to range from 41-58 CGG repeats (GeneReviews website, 2009). The clinical significance of carrying an allele in this intermediate size range is unclear. There is an increased risk of expansion compared to normal alleles, but expansion into the full mutation range is very rare (Morris, Morton, Collins et al., 1995). Alleles above 45 CGG repeats are reported to be more unstable upon transmission than alleles below 45 CGG repeats (reviewed in Brouwer et al., 2009b).

1.3.2.3 *FMR1* premutation (PM) alleles

Premutation carriers have small expansions of 59 to 200 repeats which are not associated with MR (GeneReviews website, 2009). These repeats are unstable (Oberle et al., 1991; Yu et al., 1991) and prone to expand as they pass from one generation to the next. Carrier males have normal, non-carrier sons, but they transmit the CGG repeat unchanged to their daughters, who are all normal, but then obligate carriers of fragile X MR syndrome. The risk of expansion into a full mutation depends on the PM size. The smallest repeat known to expand to a full mutation in a single generation was 59 repeats (Nolin, Brown, Glicksman et al., 2003). This makes the diagnosis of female carriers essential since they are at risk of having offspring affected with fragile X MR syndrome. This illustrates the exclusive maternal inheritance pattern, i.e. the PM expands to a full mutation only when it is transmitted by a female (Fu et al., 1991; Sutherland et al., 1991).

Most PM carriers do not present with a clinical phenotype. However, there have been reports of some PM carriers with mild learning disabilities, developmental delay, obsessive thinking, ADHD, autism and some cases reported to present with MR, social phobia or anxiety disorder (reviewed in Brouwer et al., 2009b).

It is now well established that males with a PM in the *FMR1* gene have an increased risk for developing Fragile X tremor ataxia syndrome (FXTAS) (OMIM#300623, OMIM website, 2009), a progressive neurodegenerative disorder which is clinically distinct from fragile X MR syndrome (OMIM#300624, OMIM website, 2009). Patients with FXTAS generally present with cerebellar gait ataxia and intention tremor but may develop other neurological symptoms (Hagerman and Hagerman, 2004; Jacquemont, Farzin, Hall, et al., 2004). The length of the CGG repeat is thought to influence the severity of the disorder. Female PM carriers are less likely to develop FXTAS and present with a less severe phenotype compared to males with a PM most probably due to the presence of a normal *FMR1* allele. X-inactivation patterns appear to influence the severity of the clinical outcome of FXTAS in females (reviewed in Brouwer et al., 2009b). PM

carriers are reported to have increased *FMR1* mRNA levels in leucocytes with FMRP levels decreasing with increasing CGG repeat length (Tassone, Hagerman, Taylor et al., 2000). A toxic RNA gain-of-function mechanism has thus been proposed for the pathogenesis of FXTAS, similar to that proposed for another TRD, myotonic dystrophy (DM) (reviewed in Brouwer et al., 2009b).

Females with a PM have a 20% risk of developing premature ovarian failure (POF: cessation of menstruation at or before 40 years of age), or more accurately termed, primary ovarian insufficiency (POI), as the range of clinical presentation has broadened since it was first described (OMIM#311360, OMIM website, 2009), compared to 1% in the general population (Sherman, 2000; reviewed in Brouwer et al., 2009b). There is some speculation that RNA-mediated toxicity may also be a cause of POF (reviewed in Brouwer et al., 2009b).

1.3.2.4 *FMR1* disease-associated / full mutation (FM) alleles

Affected individuals have over 200 copies of the CGG repeat (full mutation); up to as many as 2000 CGG repeats (Fu et al., 1991; Kremer et al. 1991; Verkerk et al., 1991). A FM is associated with hypermethylation of an upstream gene promoter region (CpG island), and silencing of *FMR1* gene transcription and thus FMRP is not produced (Oberle et al., 1991; Pieretti et al., 1991). Approximately 1% of mutations include missense mutations, full and partial deletions (Mila, Castellvi-Bel, Sanchez et al., 1996b; reviewed in Garber, Visootsak and Warren, 2008). The absence of the FMRP in neurons is the cause of MR seen in fragile X MR patients (reviewed in Brouwer et al., 2009b).

1.3.3 Clinical features of fragile X MR syndrome

The physical features can be very subtle and therefore difficult to recognize. Clinically, fragile X MR syndrome in males and females can be recognized by intellectual, physical and behavioural features, which include developmental delay, long narrow face, large prominent forehead and chin, protruding ears, macro-orchidism in males (large testes), hyperactivity, hand flapping, attention deficit and autism (Hagerman, Amiri and Cronister, 1991). The phenotypic expression of the syndrome is very variable and MR is the most consistent feature. It is considered a “soft” S-XLMR syndrome. A phenotypic screening checklist for fragile X MR syndrome was proposed by Maes, Fryns, Ghesquiere et al. (2000) to be used in men of any age who have developmental delay of unknown cause (reviewed in Garber et al., 2008).

1.3.4 Frequency of fragile X MR syndrome

It is reported that 1 in 4000 males and 1 in 8000 females are affected with fragile X MR syndrome (Fishburn et al., 1983; Sherman, 1996; Turner, Webb, Wake et al., 1996; Crawford, Meadows, Newman et al., 1999; Biancalana, Beldjord, Taillandier et al., 2004; Ropers, 2006). The condition is present in almost all ethnic groups. The first White South African case was described by Venter and Gericke (1980). A study undertaken in this laboratory (Division of Human Genetics, NHLS, Johannesburg) by Goldman, Krause and Jenkins et al. (1997) showed that the condition occurs in the Black population at a frequency comparable to that in the White population and that it was under-diagnosed in the past.

1.3.5 Cytogenetic analysis of FRAXA and other fragile sites

Fragile X MR syndrome is associated with a fragile site, FRAXA at Xq27.3 (Sutherland, Mulley and Richards, 1993). A fragile site appears cytogenetically as a non-staining gap or break on a chromosome, a chromatid gap, or, less frequently, as a break in metaphase chromosome spreads as shown in Figure 1.4 (Sutherland, 1979; Knight, Flannery, Hirst et al., 1993). These sites are usually induced through culture in a folate deficient medium. Rare fragile sites are characterized by CGG repeat expansion and silencing of any associated gene due to methylation. Four fragile sites have been described at Xq27-28, viz. FRAXA, FRAXE, FRAXD and FRAXF. Seven folate sensitive fragile sites (FRA10A, FRA11B, FRA12A, FRA16A, FRAXA, FRAXE and FRAXF) have been molecularly characterized and involve the expansion of CCG/CGG trinucleotide repeats (Lukusa and Fryns, 2008).

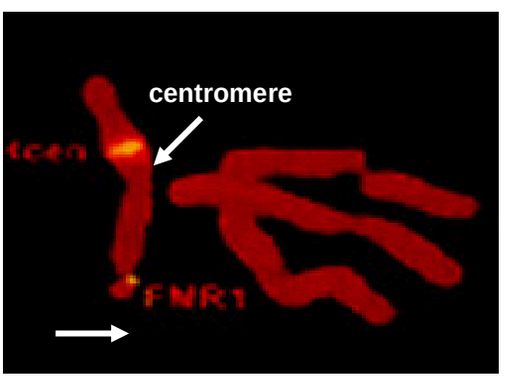


Figure 1.4 An X chromosome showing the FRAXA fragile site responsible for fragile X MR syndrome.

The FRAXE site is situated 600 kb distal to the FRAXA site at Xq28 (Sutherland and Baker, 1992; Flynn et al., 1993). FRAXD (proximal to FRAXA at Xq27) (Hecht and Bixenman, 1990) and FRAXF (distal to FRAXE at Xq28) (Hirst, Barnicoat, Flynn et al., 1993; Parrish, Oostra, Verkerk et al., 1994; Ritchie, Knight, Hirst et al., 1994) are other rare folate-sensitive sites.

Cytogenetic analysis will detect males and females affected with fragile X MR syndrome, but it fails to identify carriers (Jacobs, 1991; Rousseau, Heitz, Biancalana et al., 1991). Also, since other fragile sites like FRAXD, FRAXF with no pathogenic association and the more common FRAXA site cannot be distinguished from each other, cytogenetic analysis can be misleading. Routine karyotyping is still recommended in males referred for fragile X MR syndrome testing to exclude any other chromosomal abnormalities.

1.3.6 Fragile X MR syndrome founder haplotypes

Studies have indicated that fragile X chromosomes are in linkage disequilibrium with a small subset of flanking DXS548-FRAXAC1-FRAXAC2 microsatellite marker haplotypes (Zhou, Tang, Law et al., 2006). Two of the polymorphic AC repeats, DXS548 (150 kb from the CGG repeat on the centromeric side (Riggins, Sherman, Oostra et al., 1992) and FRAXAC2 (10 kb from the CGG repeat on the telomeric side) (Richards, Holman, Kozman et al., 1991) have been described as useful markers, together with the CGG repeat (Goldman, 1997). Figure 1.5 shows the location of these markers relative to the *FMR1* gene.

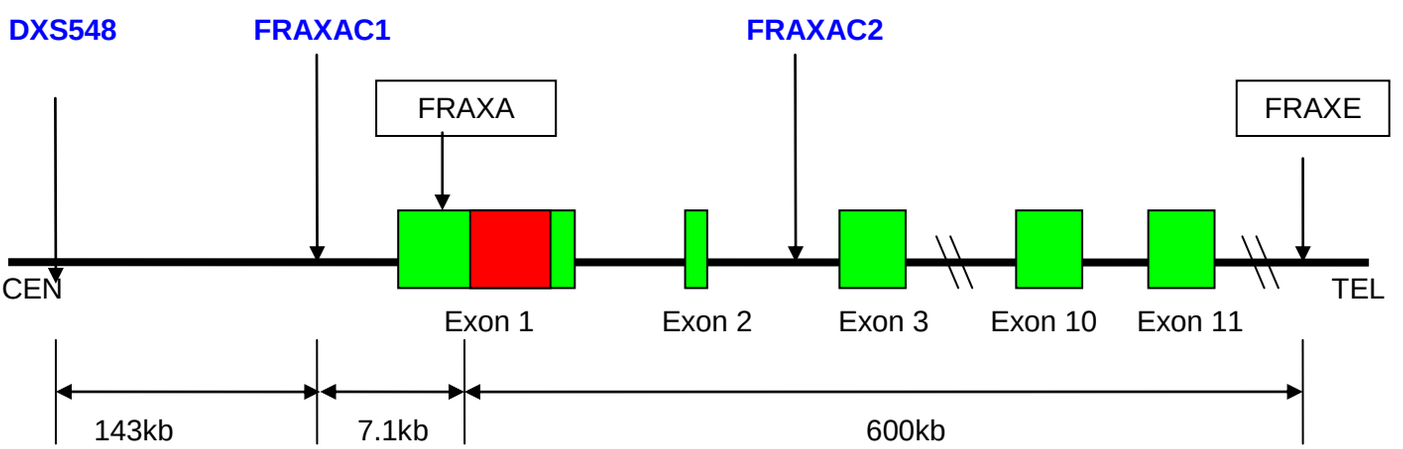


Figure 1.5 Microsatellite markers in and around the *FMR1* gene locus on Xq27.3.
Green boxes represent FMR1 exons. The red box indicates the FMR1 CGG repeat (not to scale) (modified from Zhou et al., 2006).

1.4 FRAGILE X E SYNDROME (FRAXE)

This study investigated the contribution of the *FMR2* gene to NS-XLMR in the South African population. This gene is discussed in detail below as well as its role in NS-XLMR.

1.4.1 Structure and function of *FMR2* gene and protein

The *FMR2* gene at the FRAXE locus at Xq28 was cloned by Knight, Voelckel, Hirst et al. (1994). *FMR2* is a large gene, 500 kb in size and composed of 22 exons, which show a complex pattern of alternative splicing (Gecz, Bielby, Sutherland et al., 1997a; Gecz, Oostra, Hockey et al., 1997b; Gecz and Mulley, 1999). The *FMR2* gene encodes a protein of 1311 amino acids with a major 8.75 kb transcript in placenta, fibroblasts adult and brain and a longer 13.7 kb isoform in fetal brain (Gecz and Mulley, 1999; Gecz, 2000). Gecz, Gedeon, Sutherland et al. (1996) showed that loss of FMR2 expression was correlated with *FMR2* expansion.

The precise function of *FMR2* is unknown, especially its function in processes underlying its association with mild MR (Gecz, 2000). Studies performed in yeast and mammalian cells show that FMR2 is a nuclear protein, with strong transcriptional activation and that it might serve as a downstream effector of Ras/mitogen activated protein (MAP) kinase signalling pathways (Hillman and Gecz, 2001). Recent findings show that *FMR2* is an RNA binding protein implicated in regulating splicing as it co-localizes with the splicing factor SC35 in nuclear speckles, a region where splicing factors are concentrated, assembled and modified. This is accomplished through its interaction with G-quartet RNA, an exonic splicing enhancer (Bensaid, Melko, Bechara et al., 2009).

The mouse fmr-2 shows 88% amino acid identity to the human FMR2 protein (Chakrabarti, Bristulf, Foss et al., 1998), which could help in understanding the function of the protein. Studies on fmr-2 knockout mice indicate no phenotypic or pathologic abnormalities; however, behavioural studies display impairment in conditioned fear and enhanced long term potentiation (LTP), suggesting a role for FMR2 in regulating synaptic plasticity and memory formation (Nelson, Gu, Yamagat et al., 1999; Gu and Nelson, 2003).

1.4.2 Molecular basis of FRAXE syndrome

The presence of a fragile site localised at Xq28 is associated with FRAXE syndrome (FRAXE) (OMIM+309548, OMIM website, 2009), a mild form of NS-XLMR (Sutherland and Baker, 1992; Flynn et al., 1993; Mulley, Yu, Loesch et al., 1995). Similar to FRAXA, a CpG island is found near the FRAXE site. A gene, *FMR2* (**f**ragile X **m**ental **r**etardation gene **2**), was found associated with the FRAXE CpG island and shown to be an unstable expansion of a GCC (or GGC) triplet repeat, which is hypermethylated in individuals with the expanded allele (Gecz et al., 1996; Gu, Shen, Gibbs et al., 1996). This in turn suppresses *FMR2* transcription and leads to absence of the FMR2 protein (Knight et al., 1993). A phenotype of MR is not always expected from an expansion in the *FMR2* gene since it is incompletely penetrant (Gecz and Mulley, 2000). Sometimes, a microdeletion of the *FMR2* gene leads to a truncated FMR2 protein (Gecz et al., 1996; Gu et al., 1996; Gecz et al., 1997b). There have been cases reported of males in large families who were cytogenetically positive, fully methylated, in some cases not expressing FMR2, but mentally normal. It was suggested that the normal phenotype in these individuals might be the result of clinical variability of the disease and of the effect of other genes on the phenotype or of mosaicism and different methylation states in neuronal tissues (Toniolo and D Adamo, 2000).

Unlike fragile X MR syndrome, the GCC repeat of *FMR2* can either expand or contract and is similarly unstable when transmitted through the female germ line (Knight et al., 1993). The *FMR2* repeat is a pure repeat with no unique interspersed sequences, like the AGG triplets in *FMR1*, which are thought to provide stability to the CGG repeat (Eichler et al., 1994; Zhong, Ju, Curley et al., 1996). The range of repeats in the *FMR2* gene is shown in Figure 1.6.

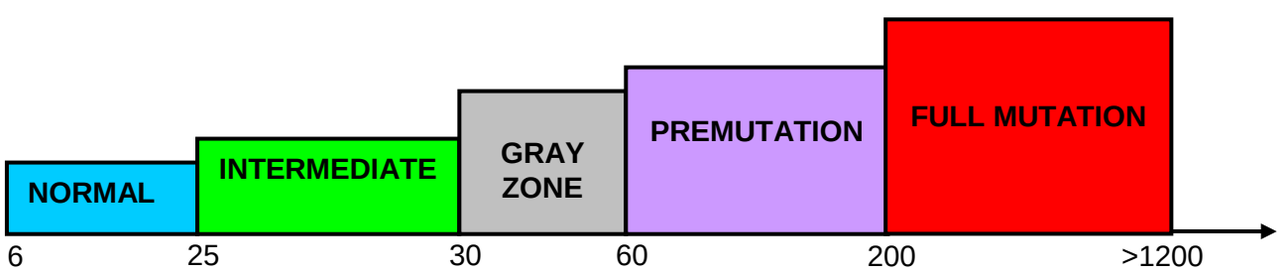


Figure 1.6 Range of *FMR2* GCC repeats in the *FMR2* gene.

1.4.2.1 *FMR2* normal (N) alleles

The GCC (or GGC) repeat is polymorphic in the normal population. Normal individuals have 6 to 25 GCC repeats (Knight et al., 1993).

1.4.2.2 *FMR2* intermediate (I) / gray zone alleles

Intermediate/gray alleles usually range from 26 to 59 GCC repeats. Intermediate-sized expansions are similar to *FMR1* premutations in that they are unmethylated on the active X chromosome and may be unstable upon transmission to offspring (Knight et al., 1994).

1.4.2.3 *FMR2* premutation (PM) alleles

Premutation alleles range from 60 to 200 GCC repeats. The border between normal alleles and premutation alleles is still poorly defined, because the *FMR2* expansion mutation is so rare.

1.4.2.4 *FMR2* disease-associated / full mutation (FM) alleles

Affected individuals have more than 200 GCC copies and the *FMR2* gene is not transcribed due to methylation of the CpG island (Knight et al., 1993). Gecz (2000) cautions not to overlook other causative mutations such as deletions, insertions and point mutations other than the GCC repeat expansion mutation, which can be expected in the *FMR2* gene, as is the case for *FMR1* (Gedeon, Baker, Robinson et al., 1992; De Boulle, Verkerk, Reyniers et al. 1993). However, screening for any other mutations, especially on a large scale is inefficient and costly.

1.4.3 Clinical spectrum of FRAXE syndrome

There has been a spectrum of clinical phenotypes associated with FRAXE syndrome. The disorder is unique among XLMR phenotypes due to its very mild to borderline nature (50<IQ<85). The phenotype associated with the *FMR2* expansion is usually considered as mild or moderate MR, with incomplete penetrance. The characteristic feature of the phenotype is learning difficulties, often a consequence of communication problems (speech delay, poor writing skills) and overactivity (reviewed in Gecz, 2000). However, genotype/phenotype relations have been less characterized.

In addition to the FRAXE/*FMR2* contribution to the aetiology of MR, other phenotypes, some unrelated to MR, were speculated to be linked to the *FMR2* gene. These include mild attention deficit hyperactivity disorder (ADHD) (Abrams, Doheny, Mazzocco et al., 1997), premature

ovarian failure (Murray, Webb, Grimley et al., 1998) and autism (Holden, Wing, Chalifoux et al., 1996b; Longshore, Tarlton, Schwartz et al., 1997). *Fmr-2* knockout mice were shown to display a condition similar to human MR, with impaired learning and memory performance and a hippocampal increased long-term potentiation (Gu, Mcilwain, Weeber et al., 2002).

Wang, Gu, Ferguson, et al. (2003) reported on the *FMR2* expansion in one male patient with obsessive-compulsive disorder, a chronic psychiatric disease. A family member with a phenotype of speech impairment also had the *FMR2* expansion (630-1200 copies). The co-segregation of the full mutation with the apparent neurologic disorders in the same family provides support that FRAXE syndrome is possibly a genetic neurologic condition.

No evidence for an increased risk of POF, FXTAS and additional diseases in *FMR2* mutation carriers was shown in a recent study (Spath, Petersen, Kremer et al., 2007). However, a study undertaken by Sofola, Jin, Botas et al. (2007) showed that that the GCC premutation length repeat leads to an RNA-mediated neurodegenerative phenotype in a *Drosophila* model.

1.4.4 Frequency of FRAXE syndrome

FRAXE syndrome appears to be much rarer than fragile X MR syndrome, but this may be due in part to ascertainment bias for more severe MR than that associated with *FMR2* mutations (Biancalana, Taine, Bouix et al., 1996). FRAXE syndrome has an estimated incidence of at least 1/50 000-1/100 000 males, and more than 50 families are known worldwide (reviewed in Gecz, 2000). As it is not screened for routinely, the number of affected families is expected to be an underestimate.

1.4.5 FRAXE founder haplotypes

A study by Limprasert, Zhong, Currie et al. (1999) demonstrated possible founder effects for *FMR2* alleles as there are for *FMR1* alleles (Richards, Holman, Friend et al., 1992), when they found haplotype associations between *FMR2* triplet repeats and two (DXS8091 and DXS1691) of the five microsatellite markers examined.

1.5 *XNP* AND ITS ASSOCIATION WITH XLMR

The third XLMR gene that was investigated in this study is the *XNP* gene. Interest in this gene was prompted by a study undertaken by Villard, Bonino, Abidi et al. (1999a) in which screening for *XNP* mutations in MR males with a typical facial appearance was suggested. Mutations in this gene were shown not only to be involved in S-XLMR, such as X-linked α-thalassaemia mental retardation syndrome (ATR-X), but also in NS-XLMR (Gibbons, Picketts, Villard et al., 1995a; Villard, Lossi, Cardoso et al., 1997). This gene is discussed in detail below as well as its role in NS-XLMR, ATR-X syndrome and other syndromes.

1.5.1 *XNP* gene

The *XNP* (X-linked nucleoprotein) gene, also called XH2 (X-linked helicase-2) or ATR-X gene (OMIM*300032, OMIM website, 2009) (hereafter referred to as *XNP*), is located at Xq13.3 (Gibbons et al., 1995a). The gene was isolated in 1994 by Gecz, Pollard, Consalez et al. (1994). Villard et al. (1997) identified and sequenced the intron/exon boundaries of the *XNP* gene. The gene contains 36 exons and spans about 300 kb of genomic DNA although there is some uncertainty whether exon 7 is functional, and contains a 4 kb open reading frame (ORF) (Picketts, Higgs, Bachoo et al., 1996).

1.5.2 XNP protein

The *XNP* gene encodes a predicted protein of 2,492 amino acids (Villard et al., 1997). Expression analysis found that *XNP* encodes at least 2 alternatively spliced mRNA transcripts of ~10.5 kb (NM_000489.3 and NM_138270.2) that give rise to slightly different proteins of 265 and 280 kDA, respectively (NP_000480.2 and NP_612114.1). One of these transcripts is expressed predominantly in embryonic tissues (Gibbons, Bachoo, Picketts et al., 1997). A further transcript of ~7 kb represents an isoform that truncates just after intron 11, giving rise to ATRXt, a truncated protein isoform, which is conserved between mouse and man (Garrick, Samara, Mcdowell et al., 2004).

The N-terminal segment has a high degree of conservation between human and mouse (97 of 98 amino acids) and the central and C-terminal regions show 94% conservation (Picketts, Tastan, Higgs et al., 1998). The closest known non-mammalian relative of the *XNP* protein is xnp-1, a *Caenorhabditis elegans* (nematode) gene (Villard, Fontes and Ewbank, 1999b).

At the N-terminal of the protein (see Figure 1.7) there is a zinc finger domain (also called the ADD domain or ATRX DNMT3-DNMT3L domain) which is related to sequences seen in the

DNA methyltransferase 3 (DNMT3) family of *de novo* DNA methyltransferases (Gibbons, 2006). It is thought to be involved in regulating transcription (Aasland, Gibson and Stewart et al., 1995). The central portion of the protein contains motifs that identify *XNP* as a novel member of the sucrose-nonfermenting 2 (SNF2) subgroup of ATPase/DNA helicases. This is a superfamily of proteins that are involved in various regulatory functions, ranging from DNA recombination and repair to control of transcription and mitotic chromosome segregation (Gibbons, Mcdowell, Raman et al., 2000). It is believed that their function is to facilitate these processes by remodelling chromatin. The function of the protein is unknown but the fact that α-globin expression is perturbed in patients with *XNP* mutations suggests that it may play a role in epigenetic regulation of globin gene expression (Gibbons, 2006).

Cardoso, Lutz, Mignon, et al. (2000) showed altered or absent *XNP* expression in cells of affected ATR-X patients with mutations of the zinc finger domain. Although many different types of mutations have been identified, no specific genotype-phenotype correlation appears to exist, suggesting that irrespective of mutation type, gene alteration leads to a loss of function. Recently *XNP* was shown to interact with *MECP2,* a gene responsible for Rett syndrome and XLMR (Nan, Hou, Maclean et al., 2007). The authors suggested that the disruption of the MECP2-ATRX interaction causes a pathologic change that contributes to MR.

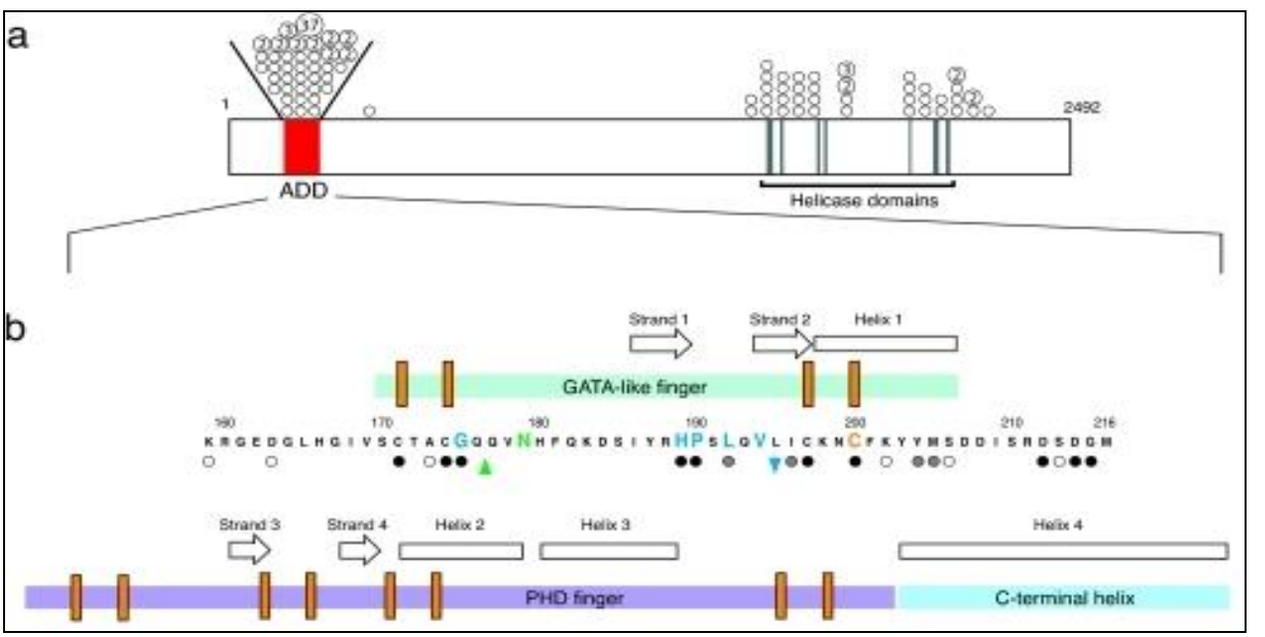


Figure 1.7 (a) ATR-X protein structure illustrating position of the N-terminal cysteine-rich domain and the C-terminal helicase-like domain.
Disease-associated missense mutations are shown by circles. (b) A magnification of the ADD or zinc finger domain, illustrating the locations of mutations and secondary structural elements (N-terminal GATA-like zinc finger, PHD (plant homeodomain) finger and a long C-terminal extension). The orange vertical bars represent the conserved cysteine residues (Argentaro, Yang, Chapman et al., 2007).

1.5.3 *XNP* mutations in NS-XLMR

Guerrini, Shanahan, Carrozzo et al. (2000) proposed that mutations in the *XNP* gene should be considered as a cause of mild MR in male patients that lack specific diagnostic features, e.g. facial dysmorphism. This shows the expanding phenotype of *XNP* mutations-mild to moderate MR patients WITHOUT facial dysmorphism should be screened for *XNP* mutations.

As mutations in the *XNP* gene are reported to lead to a severe MR phenotype, screening for *XNP* mutations in MR males who have a negative family history was suggested (Villard et al., 1999a) and this led the authors to propose large screening of additional patients. The authors reported mutation analysis of the *XNP* gene using direct sequencing of the 300 bp zinc finger coding region, spanning exons 7, 8, and 9. They concluded that this method was suitable for screening individuals in this population.

Mutations are found predominantly in the two protein motifs, the helicase and zinc finger (ADD) domains. The majority of *XNP* mutations identified (~50-60%) for ATR-X cases and related syndromes are clustered in a 'hot spot' region, which is found in the zinc finger (ADD) domain of the gene (Villard et al., 1997; reviewed in Gibbons, 2006). This is a 300 bp region, encoded by exons 7, 8, and the beginning of exon 9 in the *XNP* gene, suggesting that this is a functionally important region. The remaining mutations have been found in the helicase domain, which extends over 3 kb at the C-terminus (COOH) of the protein (Villard et al., 1999a).

1.5.4 *XNP* mutations in S-XLMR

Mutations in *XNP* are associated with several XLMR syndromes, the best known being ATR-X syndrome (Gibbons et al., 1995a). The types of mutations found include, deletions, insertions, missense, nonsense, and splice mutations. Missense mutations are more frequent than frameshift and nonsense mutations (HGMD website, 2009). Twenty percent have the common c.736C>T (p.R246C) mutation. Mutations lead to decreased *XNP* activity (Gibbons and Higgs, 2000).

Mutations in *XNP* have been reported in families affected with Juberg-Marsidi syndrome (MIM 309590) (Villard, Gecz, Mattei et al., 1996a), Carpenter-Waziri syndrome (Abidi, Schwartz, Carpenter et al., 1999), XLMR with spastic paraplegia (R1742K mutation) (Lossi, Millan, Villard et al., 1999), Holmes-Gang syndrome (Stevenson, Abidi, Schwartz et al., 2000), Smith-Fineman-Myers syndrome (Villard, Fontes, Ades et al., 2000) (MIM309580), Chudley-Lowry syndrome (Abidi, Cardoso, Lossi et al., 2005) and MR phenotypes without ATH (Villard, Lacombe and Fontes, 1996b; Villard, Toutain, Lossi et al., 1996c). Mutations in *XNP* have also

been described in other, less well-characterized XLMR syndromes. Despite the clinical variation seen with *XNP* mutations, all male patients have a severe MR associated with microcephaly and facial dysmorphism. It suggests that this gene may be involved in the development of facial morphogenesis (Cardoso et al., 2000). It is unclear if these patients have distinct phenotypes or are part of the spectrum of ATR-X syndrome (Stevenson et al., 2000). Mutations in different regions of the gene have an influence on the phenotype. Mutations in the ADD region appear to be involved with increased psychomotor impairment while mutations in the C-terminus are frequently associated with genital abnormalities (Gibbons, Wada, Fisher, et al., 2008).

XNP is also speculated to interact with genes that are involved in the process of sex determination (Neri and Opitz, 1999). Ion, Telvi, Chaussain et al. (1996) reported on an *XNP* mutation (frameshift mutation, generating a premature stop codon) in a French family in which the affected males presented with features suggestive of ATR-X syndrome (severe MR, dysmorphic features but without ATH), partial ocular albinism and with male-to-female sex reversal (46,XY female).

1.5.5 ATR-X syndrome

Alpha-thalassaemia/mental retardation syndrome (OMIM#301040, OMIM website, 2009) was first reported by Wilkie, Buckle, Harris et al. (1990a) and Wilkie, Zeitlin, Lindenbaum (1990b). Two distinct syndromes, ATR-16 and ATR-X were defined. ATR-16 is a contiguous gene syndrome, in which there are large (1-2Mb) chromosomal rearrangements at 16p13.3 that delete many genes, including the α-globin genes. In ATR-X syndrome, mutations in the *XNP* gene down regulate alpha (α) gene expression rather than delete α genes and possibly also disrupt the expression of other genes (Gibbons et al., 1995a). Clinical features are more variable in ATR-16, whereas ATR-X cases show a very typical phenotype.

Picketts et al. (1996) established that ATR-X downregulates α-globin but not β-globin, possibly due to the fact that α- and β-globin are on different chromosomes and interact differently with regulatory factors and chromatin.

1.5.5.1 Clinical features of ATR-X syndrome

Classical cases of ATR-X present clinically with severe to profound MR (95% of cases), characteristic facial dysmorphism, most easily recognized in early childhood (>90% of cases) - epicanthic folds, flat nasal bridge, small and triangular upturned nose, anteverted nostrils, a triangular mouth and protruding tongue. Other clinical features include muscle hypotonia, most

distinct in neonates and microcephaly. Urogenital malformations are present in 80% of cases - genitalia are usually abnormally small, undescended or dysgenetic testes to external female genitalia, with the affected individuals being defined as male pseudohermaphrodites (Mcpherson, Clemens, Gibbons et al., 1995), shawl-like or hypoplastic scrotum and small penis with hypospadias. Many affected individuals (90%) will have haematological signs of α-thalassaemia (not from deletions of the α genes) (Gibbons, Brueton, Buckle, et al., 1995b; Gibbons and Higgs, 2000; Gibbons 2006). Other features include: skeletal abnormalities (90%), microcephaly (75%), short stature (65%), seizures (30%), cardiac defects (20%), renal/urinary abnormalities (15%) (Gibbons, 2006). Figure 1.8 shows the typical facial characteristics of 2 unrelated boys with ATR-X syndrome.



Figure 1.8 Two unrelated boys with ATR-X syndrome.
Note the flat nasal bridge, small and triangular upturned nose, anteverted nostrils, a triangular mouth and protruding tongue. (Gibbons Laboratory website, 2008)

1.5.5.2 Worldwide prevalence of ATR-X syndrome

The prevalence of ATR-X in the general population is unknown. As of 2006, 168 patients have been reported. An estimate for the prevalence is <1-9/1,000,000 (Gibbons, 2006). In 2000, Wada, Kubota, Fukushima et al. reported on 9 patients (severe MR and typical facial appearance) from 8 independent Japanese families with ATR-X. Stevenson (GeneReviews website, 2009) indicates that there have been no reports on ATR-X occurring at high frequencies in any specific racial or ethnic group. Three patients with ATR-X syndrome were identified in our laboratory and confirmed on molecular investigations. The results obtained will be discussed in Chapter 7.

1.5.5.3 Detection of α-thalassaemia

Confirmation of a clinical diagnosis of ATR-X usually requires a routine haematological screen. The atypical form of α-thalassaemia (ATH) is revealed by the presence of HbH inclusions (β-globin tetramers) in a fresh blood sample (Figure 1.9). This is a technically difficult test to perform.

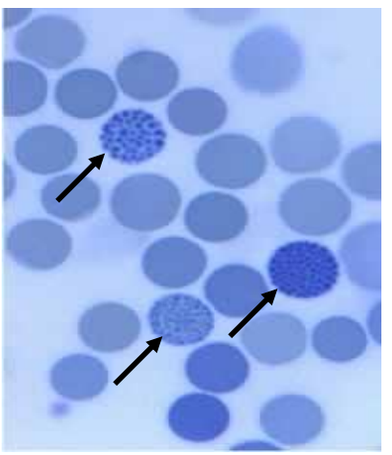


Figure 1.9 HbH inclusion bodies (excess β-chains) or 'golf balls' (shown by arrow) in red blood cells in peripheral blood.
The test uses light microscopy to detect red cells containing HbH inclusions after incubation, at room temperature, of venous blood with 1% brilliant cresyl blue for 4-24 hrs (Gibbons Laboratory website, 2008)

It was thought that 90% of patients with ATR-X mutations have evidence of ATH (Gibbons et al., 1995a). However, this is not always the case, as there have been a number of ATR-X families where the affected members have no signs of ATH (Villard et al., 1996b, 1996c). Patients with the same mutation may have very different degrees of ATH. The effect of ATR-X protein on α-globin gene expression may be modified by other genetic factors (Gibbons et al., 2000). The relationship between ATR-X mutations and ATH remains unclear (Gibbons, 2006).

Haematologically, ATR-X patients are not as severely affected as the classic ATH and there is much variation in the haematological manifestations (Gibbons and Higgs, 2000). ATR-X female carriers are phenotypically asymptomatic and HbH inclusions are only found in ~25% of carriers (Gibbons et al., 1995a), possibly due to a totally skewed X-inactivation pattern of the mutant X chromosome seen in a variety of tissues. A possible direct correlation is postulated between X-inactivation selection and mutation in the gene (Lossi et al., 1999).

1.5.5.4 Mutation testing for ATR-X syndrome

Molecular genetic testing for ATR-X syndrome by sequence analysis of the *XNP* gene can detect disease-causing mutations in ~90% of affected individuals. Approximately 40-50% of known ATR-X mutations are in the region covering exons 7, 8 and the beginning of exon 9 (Villard et al., 1999a) which prompted this study of mutation analysis in this region. The next most commonly affected region is the helicase domain in which 30% of mutations are clustered (Gibbons et al., 2008).

1.6 *ARX* AND ITS ASSOCIATION WITH XLMR

The fourth gene which was investigated in this study is the *ARX* gene. Mutations in this gene are reported to be the second most cause of XLMR after fragile X MR syndrome (reviewed in Gecz, Cloosterman and Partington, 2006). This gene is discussed in detail below as well as its role in NS-XLMR and other syndromes.

1.6.1 *ARX* gene

The *ARX* gene (Aristaless-related homeobox gene) (OMIM*300382, OMIM website, 2009) is located at Xp22.13. It contains 5 exons, spanning 12.5 kb and encodes a 562-amino acid protein. The *ARX* gene is transcribed into an mRNA of about 2.8 kb, with an open reading frame of 1 686 bp (Stromme, Mangelsdorf, Shaw et al., 2002; Gronskov, Hjalgrim, Nielsen et al., 2004).

1.6.2 *ARX* protein

The ARX protein belongs to the largest class of homeoproteins, namely the Prd (paired) class (Bienvenu, Poirier, Friocourt et al., 2002). The ARX protein has two important domains: the homeobox domain that directly binds DNA, and the C-terminal OARb or aristaless domain of unknown function. A conserved octapeptide domain is known to be involved with transcriptional repression and four polyalanine tracts, three of which are encoded in exon 2 and one in exon 4 are thought to be involved with suppressing transcription (Gronskov et al., 2004). Figure 1.10 shows a schematic diagram of the *ARX* gene (Chiurazzi, Tabolacci and Neri, 2004)

The ARX protein is only expressed in the fetal brain and testes during early development, while expression is detected in a wide variety of tissues in adults, including the brain (specifically the forebrain and the floor plate of the developing CNS), heart, liver and skeletal muscles (Suri, 2005). The ARX protein was found to interact via its homeodomain with IPO13, a mediator of nuclear import (Shoubridge, Cloosterman, Parkinson-Lawerence et al., 2007).

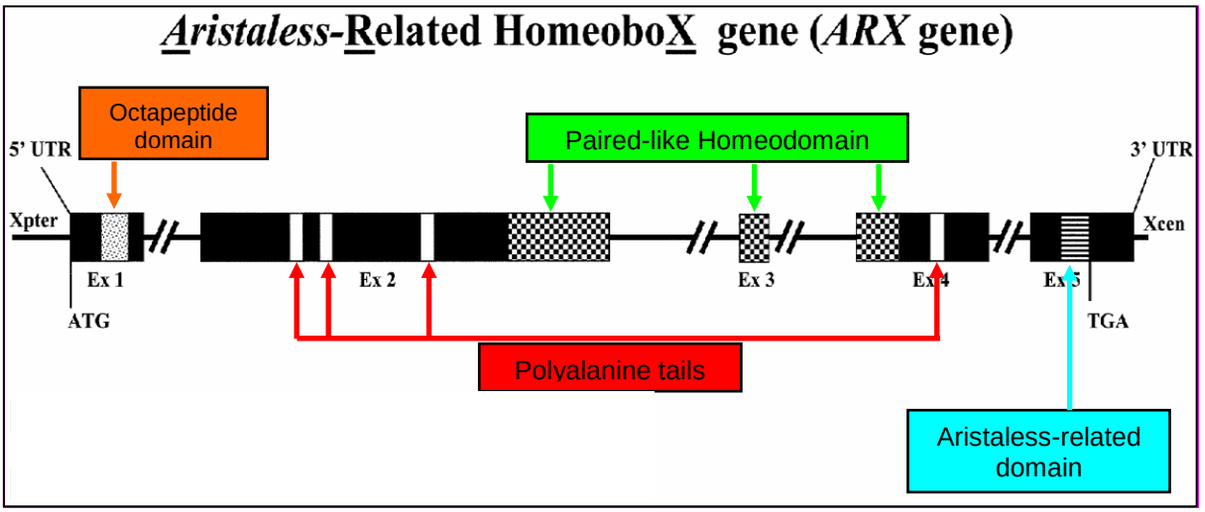


Figure 1.10 Schematic representation of the genomic organization of the *ARX* gene and the corresponding protein domains
(Chiurazzi, et al., 2004)

1.6.3 *ARX* mutations in S-XLMR and NS-XLMR

Mutations in the *ARX* gene have been identified in NS-XLMR and S-XLMR. Mutations in the *ARX* gene are associated with more than 10 different S-XLMR disorders including: West syndrome (also called X-linked infantile spasms, ISSX, characterised by infantile spasms, MR and hypsarrythmia) (Stromme et al., 2002), Partington's syndrome (characterised by MR and dystonic hand movements) (Stromme et al., 2002), MR associated with myoclonic epilepsy and spasticity (Scheffer, Wallace, Phillips et al., 2002; Stromme et al., 2002), X-linked lissencephaly and ambiguous genitalia (XLAG) syndrome (Kitamura, Yanazawa, Sugiyama et al., 2002) and Proud syndrome (which consist of XLMR, agenesis of the corpus callosum and abnormal genitalia) (Kato, Das, Petras et al., 2004).

Mutations in NS-XLMR were described by Bienvenu et al., 2002. It is reported that almost 10% of the 81 large MRX (NS-XLMR) families have mutations in the *ARX* gene; far more frequent than mutations in other NS-XLMR genes. The frequency of mutations in the *ARX* gene is reported to be much lower (0% to 5%) in smaller families (2.2% in affected brother pairs and 0.13% in sporadic males with MR) (reviewed in Ropers, 2006).

A number of mutations have been identified in the *ARX* gene, including polyalanine tract expansions, insertions and deletions, missense and nonsense mutations leading to frameshifts and premature truncation (in the case of XLAG) (Gronskov et al., 2004; Poirier, Lacombe, Gilbert-Dussardier et al., 2006). Almost 90% of *ARX* mutations are situated in exon 2 within the

polyalanine domains and homeodomain. Other mutations have been described in exon 1 within the octapeptide domain (Poirie et al., 2006).

Two recurrent mutations, both leading to expansions of the polyalanine tracts, have been reported, c.431-454 dup24 and c.333-334ins(GCG)7 (Bienvenu et al., 2002; Stromme et al., 2002). A 24 bp duplication, c. 431-454 dup24, also referred to as c. 428-451 dup24 in exon 2, resulting in expansion of a polyalanine (PolyA) tract from 12 to 20 alanines was first identified in individuals with NS-XLMR (Claes, Gu, Legius et al., 1996). This recurrent in-frame duplication is reported to account for about 6.6% of XLMR families and is the most common mutation in *ARX* seen in the majority of the syndromic forms (Mandel and Chelly, 2004). It has been recommended that males with NS-XLMR and S-XLMR who have tested negative for the *FMR1* expansion be tested for the 24 bp duplication mutation in the *ARX* gene, especially in the presence of dystonic hand movements, spasticity and epilepsy (Suri, 2005; De Souza Gestinari-Duarte, Santos-Reboucas and Pimentel, 2006; Nawara, Szczaluba, Poirier et al., 2006).

Disorders associated with brain malformations (XLAG, severe hydrocephalus, Proud syndrome) are reported to result from protein truncation mutations and missense mutations in the homeobox, whereas the non malformation phenotypes (ISSX, Partington syndrome, NS-XLMR) are associated with missense mutations outside the homeobox and expansion of the PolyA tracts (Kitamura et al., 2002; Suri 2005).

Phenotypic heterogeneity within families has been seen with the 24 bp duplication mutation. (Bienvenu et al., 2002; Stromme et al., 2002). Epilepsy is in fact one of the most common symptoms experienced in families with mutations within *ARX* (Friocourt, Poirier, Rakic et al., 2006).

1.7 AIMS OF THE STUDY

The aim of this study was to investigate the contribution of different XLMR genes, particularly the *FMR1*, *FMR2*, *XNP* and *ARX* genes associated with NS-XLMR to XLMR in the South African population. Further, the study was extended to investigate some families with XLMR including a large family positive for the XLMR condition, fragile X MR syndrome and 3 families with *XNP* mutations in subjects with a clinical phenotype of ATR-X syndrome.

1.8 OBJECTIVES OF THE STUDY

1.8.1 FRAGILE X MR SYNDROME

To undertake a retrospective analysis of fragile X MR syndrome referrals to the Division of Human Genetics Molecular Laboratory, NHLS to:

1) Determine the frequency of fragile X MR syndrome due to *FMR1* expansions in South African males with MR of unknown cause
2) To investigate the distribution of all *FMR1* CGG allele sizes in a random cohort of MR males

1.8.2 FRAXE SYNDROME

1) To optimize the PCR assay for FRAXE syndrome on the ABI Genetic Analyzer
2) To validate the PCR assay by investigating Mendelian inheritance and transmission in normal controls
3) To determine the frequency of FRAXE in *FMR1* expansion negative South African males with MR of unknown cause
4) To implement *FMR2* mutation analysis as a routine diagnostic test in MR males if indicated
5) To investigate the distribution of *FMR2* GCC allele sizes in a random cohort of MR males

1.8.3 *XNP*

1) To determine the frequency of *XNP* mutations in *FMR1* expansion negative South African males with MR of unknown cause
2) To implement *XNP* mutation analysis as a routine DNA diagnostic test if indicated

1.8.4 *ARX*

1) To determine the frequency of the common 24 bp duplication mutation in *FMR1* negative South African males with MR of unknown cause
2) To implement *ARX* mutation analysis as a routine DNA diagnostic test if indicated

1.8.5 *FMR1* POSITIVE FAMILY STUDY

1) To determine the *FMR1* (CGG)n repeat length in the available members of a fragile X MR syndrome positive family
2) To perform haplotype analysis in this family using flanking markers
3) To determine the haplotypes associated with the expansion in this family

1.8.6 ATR-X SYNDROME FAMILY STUDIES

1) To undertake diagnostic, carrier and prenatal testing for ATR-X syndrome in three South African White families

CHAPTER TWO

2 SUBJECTS, MATERIALS AND METHODS

This chapter describes the subjects used in this study and the techniques and methods of detection used for screening MR subjects at the 4 NS-XLMR gene loci, *FMR1*, *FMR2*, *XNP* and *ARX*. The patients and their families investigated for fragile X MR syndrome and ATR-X syndrome are also described. The methods including haplotype analysis is presented. All samples used in the study were coded to maintain confidentiality.

SUBJECTS

2.1 SUBJECTS FOR FRAGILE X A MR SYNDROME RETROSPECTIVE ANALYSIS

A retrospective analysis was performed on 1736 probands referred to the Division of Human Genetics, NHLS over a period of 17 years (1992 to 2009) for routine fragile X MR syndrome testing to determine the frequency of *FMR1* expansions. A cohort of 126 institutionalized patients was also included in this analysis. The distribution of *FMR1* alleles was determined by testing a selected cohort of 1184 male subjects. These are described below and summarised in Table 2.1.

2.1.1 Referrals for fragile X MR syndrome testing (1992-2009)

A total of 1803 unrelated probands of all ethnic groups in South Africa were referred for fragile X MR syndrome testing between 1992 and June 2009. Analysis was done only on 1736 probands as no result was obtained on the remaining 67 probands due to poor DNA quality, test cancellation, incorrect blood tubes received, etc. The 1736 probands included 783 White, 617 Black, 194 Coloured and 142 Indian probands. The probands had a suspected diagnosis of fragile X MR syndrome and were referred for diagnostic testing. In some cases, extended family members (mothers of affected individuals, siblings etc.) were referred to determine their *FMR1* status. In total, 2144 subjects were tested for the *FMR1* expansion which consisted of 1736 probands (1471 males and 265 females), 394 extended family members and 14 prenatal samples (chorionic villus sampling (CVS) or amniocentesis).

Each patient/family was given a “FRAX” code, e.g. FRAX200. Most referrals were from the Assessment and Learning Clinics at the Johannesburg Hospital (TMI), Coronation Hospital and

Alexandra Clinic as well as from general practitioners, paediatricians and the medical geneticists at the Genetic Counselling Clinics (NHLS, previously SAIMR, Chris Hani Baragwanath Hospital, Donald Gordon Medical Centre, Coronation Hospital) and genetic nurses. A few cases were referred from doctors in private practice. These patients presented with developmental delay, MR of varying degrees and of unknown cause, or clinical features suggestive of fragile X MR syndrome.

2.1.2 Institutionalized patients

These subjects included a total of 158 institutionalized Black male subjects collected prior to 1997 from 2 institutions caring for MR individuals - The Millsite institution which cared for severely retarded individuals and the Takalani institution which sheltered moderately to mildly developmentally delayed individuals, who were able to care for themselves, as well as individuals who came to the institution daily and worked in a sheltered employment structure. The samples consisted of blood taken from a total of 158 Black, unrelated male patients, 86 from Millsite and 72 from Takalani. From this cohort, *FMR1* results were only obtained on 126 individuals (62 Millsite and 64 Takalani) as the remaining samples were inadequate or of poor DNA quality. Permission to test these individuals was given by the legal guardian of each individual.

2.1.3 *FMR1* expansion negative males for allele distribution analysis

A total of 1184 unrelated MR male subjects were analysed to determine the distribution of non expanded *FMR1* alleles in the different ethnic populations in South Africa. These subjects were selected from the cohort as they had successful PCR results at the *FMR1* locus. These subjects included 1067 unrelated MR male patients of all ethnic groups selected from the cohort of referred probands (described in Section 2.1.1) and 117 institutionalized Black male individuals (described in Section 2.1.2) with non expansions for *FMR1* or *FMR2*.

Table 2.1 Summary of subjects used for *FMR1* retrospective analysis.

SUBJECTS	WHITE	BLACK	COLOURED	INDIAN	TOTAL TESTED
***FMR1* EXPANSION FREQUENCY**					
Probands[1] tested for fragile X MR syndrome					
Male probands	636	536	173	126	1471
Female probands	147	81	21	16	265
Sub total of probands tested	**783**	**617**	**194**	**142**	**1736**
% of total probands tested (N=1736)	45.1%	35.5%	11.2%	8.2%	
Institutionalized patients (males)	0	126	0	0	126
GRAND TOTAL OF PROBANDS TESTED	**783**	**743**	**194**	**142**	**1862**
% OF TOTAL PROBANDS TESTED (N=1862)	42.1% (783/1862)	39.9% (743/1862)	10.4% (194/1862)	7.6% (142/1862)	
***FMR1* ALLELE DISRIBUTION IN NON EXPANSION SUBJECTS**					
Males tested for *FMR1* alleles	506	367	122	72	1067
Institutionalized patients	0	117	0	0	117
TOTAL MALE SUBJECTS TESTED	**506**	**484**	**122**	**72**	**1184**
% OF TOTAL MALE SUBJECTS TESTED (N=1184)	**42.7% (506/1184)**	**40.9% (484/1184)**	**10.3% (122/1184)**	**6.1% (72/1184)**	

[1] Proband refers to the individual per family that was tested

2.2 SUBJECTS FOR FRAGILE X E SYNDROME SCREENING

A total of 1194 MR male subjects were tested for the *FMR2* expansion. These subjects were selected from the cohort as they had successful PCR results at the *FMR2* locus. These included 1069 *FMR1* negative MR male patients and 125 institutionalized males. These are described below and summarised in Table 2.2 which also characterizes the ethnicities tested.

2.2.1 *FMR1* expansion negative MR male subjects

A total of 1069 *FMR1* expansion negative MR male subjects of all ethnic groups (507 White, 368 Black, 122 Coloured, 72 Indian) were tested for the *FMR2* expansion. These were random DNA samples obtained from consecutive patient referrals to the Division of Human Genetics, Molecular Laboratory, NHLS from 1997 to 2009 for routine fragile X MR syndrome diagnostic testing (described in Section 2.1.1) with MR and who tested negative for the *FMR1* expansion mutation.

2.2.2 *FMR1* expansion negative institutionalized patients

These samples consisted of blood taken from a total of 158 Black, unrelated male subjects (described in Section 2.1.2). From this cohort, *FMR2* results were only obtained on 125 individuals (58 Millsite and 67 Takalani) as the remaining samples were inadequate or of poor DNA quality.

Table 2.2 Summary of subjects used for *FMR2* screening

SUBJECTS	WHITE	BLACK	COLOURED	INDIAN	TOTAL TESTED
FMR1 expansion negative MR males	507	368	122	72	1069
FMR1 expansion negative institutionalized males	0	125	0	0	125
TOTAL SUBJECTS	**507**	**493**	**122**	**72**	**1194**
% OF TOTAL SUBJECTS	**42.5%** (507/1194)	**41.3%** (493/1194)	**10.2%** (122/1194)	**6.0%** (72/1194)	

2.2.3 *FMR2* expansion positive control used

No known case of FRAXE syndrome has been reported in South Africa and thus positive controls with known repeat sizes were not available locally for the study. A DNA sample from a male with an *FMR2* expansion was provided by Dr Steve Abbs (DNA Diagnostic Laboratory, Genetics Centre, Guy's Hospital, London). After validating the *FMR1*/*FMR2* multiplex PCR assay, samples that gave consistent fragment sizes on repeated PCR amplification screens at both the *FMR1* and *FMR2* loci were used as controls.

2.2.4 Subjects used for validation of *FMR2* in the multiplex PCR

The PCR was validated using 3 different groups of subjects which are described below.

2.2.4.1 Patients with an *FMR1* expansion

A total of ten fragile X MR syndrome patients with *FMR1* full mutations (8 males and 2 females) were used to validate the FRAXE PCR assay. These subjects are expected to have a normal *FMR2* allele. The *FMR1* status of these subjects was confirmed previously on Southern blot analysis.

2.2.4.2 *FMR1* expansion negative mother-child pairs

A total of ten *FMR1* expansion negative mother-child pairs were tested to track the inheritance and sizing consistency of *FMR2* alleles over two generations. The mothers are expected to have two normal *FMR2* alleles and their children are expected to have either one normal *FMR2* allele if male or 2 normal *FMR2* alleles if female. The *FMR1* status of these subjects was confirmed previously on Southern blot analysis.

2.2.4.3 *FMR1* full mutation positive family (FRAXA family 2)

The *FMR1*/*FMR2* multiplex PCR assay was also validated by tracking *FMR2* alleles in an *FMR1* expansion positive family, consisting of 19 members, 3 of whom were affected with fragile X MR syndrome. The family pedigree is illustrated in the FRAXE/*FMR2* results Chapter 4, Figure 4.1.

2.3 SUBJECTS FOR *XNP* SCREENING

DNA from a total of 210 MR male subjects was tested for *XNP* mutations. These consisted of 100 *FMR1* expansion negative MR White subjects randomly selected from the cohort of consecutive patient referrals to the Division of Human Genetics described in Section 2.1.1 and 110 of the 158 Black institutionalized male patients (48 from Millsite and 62 from Takalani), described above in Section 2.1.2 on whom results were obtained as the remaining samples were inadequate or of poor DNA quality.

2.4 SUBJECTS FOR *ARX* SCREENING

DNA from a total of 868 MR male subjects was tested for *ARX* mutations. These consisted of 737 *FMR1* expansion negative MR subjects (299 White, 282 Black, 98 Coloured, 58 Indian) randomly selected from the cohort of consecutive patient referrals to the Division of Human Genetics described in Section 2.1.1 and 131 of the 158 Black institutionalized male patients (64 from Millsite and 67 from Takalani), described above in Section 2.1.2 on whom results were obtained as the remaining samples were inadequate or of poor DNA quality.

2.5 FRAGILE X MR SYNDROME FAMILY STUDY (FRAXA FAMILY 1)

A large White South African family (Laboratory family code: FRAX 551) that tested positive for fragile X MR syndrome was investigated and their *FMR1* repeat lengths were determined to track the inheritance of *FMR1* alleles in the family. Probands who tested positive for the *FMR1* expansion syndrome and extended family members whose mutation status was known from Southern blot analysis were studied. The family consisted of 24 members, including the probands. The family pedigree is shown in Figure 6.2. In an attempt to elucidate the *FMR1* status of the family members, an interesting finding was observed in two female siblings at the *FMR1* locus. Haplotype analysis using intragenic markers, DXS548 and FRAXAC2 in the *FMR1* gene was used to track the disease chromosomes in the family. The markers used and the detection method are described in Section 2.12.7.

2.6 ATR-X SYNDROME FAMILY STUDIES

A DNA investigation was undertaken on 3 individuals suspected of having ATR-X syndrome. These patients are described below. Consent (documented in the individual patient counselling files) was received from these families to use the photographs in this thesis.

2.6.1 ATR-X Patient 1 (Laboratory Code: FRAX12)

This White family presented to our Genetic Counselling Clinic with a family history suggestive of XLMR. The proband, patient 1, shown in the pedigree Figure 7.1 (II-5) presented with MR. He tested negative for *FMR1* and *FMR2* full expansion mutations. The parents of the proband, I-1 and I-2 are healthy and have 2 healthy daughters. They have a healthy, non-identical male twin to patient 1. They had another son, II-2, who was severely mentally retarded and died of pneumonia at the age of 14 yrs. Figure 7.2.shows the clinical features of patient 1.

2.6.2 ATR-X Patient 2 (Laboratory Code: FRAX549)

This White family presented at the Genetic Counselling Clinic in Johannesburg with a severely delayed child. There was no family history of similarly affected children (see family pedigree in Figure 7.7). He tested negative for *FMR1* and *FMR2* full expansion mutations. Figure 7.8 shows the clinical features of Patient 2.

2.6.3 ATR-X Patient 3 (Laboratory Code: ATRX-1 and FRAX1003)

This is a White patient who presented at the Genetic Counselling Clinic in East London with global developmental delay and family history suggestive of XLMR. He tested negative for *FMR1* and *FMR2* full expansion mutations. The family pedigree and the clinical features are shown in Figure 7.12 and Figure 7.13

2.7 ETHICS APPROVAL

Ethics approval for the use of diagnostic patient DNA samples (anonymised) for this study was obtained (Appendix A). The collection of blood samples from the institutionalized patients was initiated through Goldman's study (1997). An informed consent stating that samples could be used for research purposes was obtained for each of the institutionalized patients. The fragile X MR syndrome and ATR-X families were referred for diagnostic testing. Individual consent forms were obtained to include and present the family results in research and these are filed in each patient's file. The samples were all used in accordance with current regulation on the use of biological material.

MATERIALS AND METHODS

The common methods used in the assays for *FMR1*, *FMR2*, *XNP* (ATRX) and *ARX* are presented in Sections 2.8, 2.9, 2.10 and 2.11 respectively and in Appendices C and D, followed by the detailed methodology used for each of the genes tested. All solutions used in this study can be found in Appendix B.

2.8 DNA EXTRACTION

There are a number of procedures available for extraction of nucleic acids, which are based on a variety of methods and principles. The majority of samples used in this study were extracted using a salting out procedure (Miller, Dykes and Polesky et al., 1988). The samples were extracted as part of the routine diagnostic procedure.

A brief outline of the protocol is described. For a detailed protocol see Appendix C1. A commercial DNA extraction kit from Roche Diagnostics was used to extract DNA from samples where the initial volume of blood received was less than 1ml (see Appendix C2 for protocol). Whole blood (5-10ml in purple-top EDTA or yellow-top ACD vacutainer tubes) was used for the salting out method. The procedure involved washing and lysing the red blood cells with a detergent (sucrose Triton-X buffer). Through a series of washes and centrifugation steps, the red cell debris was removed and the white bloods cells (WBC) pelleted. Degradation of the proteins and the lipid bilayer of the WBC was achieved by an overnight incubation at 37°C with proteinase K, SDS and EDTA. The DNA was recovered with salt and absolute ethanol. The DNA was resuspended in an appropriate volume of Tris-EDTA (TE) buffer (usually 200μl to 1000μl) and stored at 4°C until ready to use.

2.9 DNA QUANTIFICATION

The NanoDrop® ND-1000 spectrophotometer V3.3 is used to quantify DNA samples. The NanoDrop® ND-1000 is a 220-750 nm spectrophotometer based on fibre optic technology. One microlitre of DNA is dispensed onto the end of a receiving fibre optic cable. A second source fiber optic cable is brought into contact with the DNA, allowing the liquid to bridge the gap between the two fibre optic cables. This gap is controlled to both 1 mm and 0.2 mm paths. A pulsed xenon flash lamp provides the light source. A spectrometer with a CCD array analyzes the light passing through the sample and the data is displayed by specialised software. The absorbance at both 260nm (the wavelength at which DNA best absorbs light) and 280nm (the wavelength at which proteins best absorb light) are measured using the 1 mm and 0.2 mm path

lengths. The 260/280 ratio of sample absorbance represents the purity of DNA and RNA. A ratio of ~1.8 generally represents pure DNA. Any ratio lower than 1.8, indicates contaminants such as proteins and phenol, which absorb strongly at 280 nm.

2.10 THE POLYMERASE CHAIN REACTION (PCR)

The Polymerase Chain Reaction (PCR) (Mullis and Faloona, 1987) is a technique that involves the use of specific oligonucleotide primers (18 to 30 mer in length) that flank a DNA sequence of interest to amplify it. PCR was used to amplify *FMR1* and *FMR2* alleles, the hotspot region of the *XNP* gene and the *ARX* region containing the 24 bp duplication. A brief outline of this technique is presented in Appendix C3.

2.11 AGAROSE GEL ELECTROPHORESIS (AGE)

This technique is used to separate DNA molecules by base composition, size and conformation. AGE was used to verify the PCR product after amplifying the *XNP* hotspot region and the *ARX* gene. A brief outline of this technique is provided in Appendix C4.

2.12 FRAGILE X TESTING PROCEDURE

PCR analysis with primers was used to detect the size of the CGG/GCC repeat for *FMR1* and *FMR2*. Southern blot analysis was used to determine the methylation status and to detect full expansions which were too large to be amplified by PCR analysis. The combination of *FMR1* and *FMR2* in a multiplex PCR reaction and Southern blotting has a sensitivity of 99% for *FMR1* and *FMR2* expansion detection. An overview of the strategy as used to test for *FMR1* and *FMR2* is shown in Figure 2.1, followed by a description of the methods used.

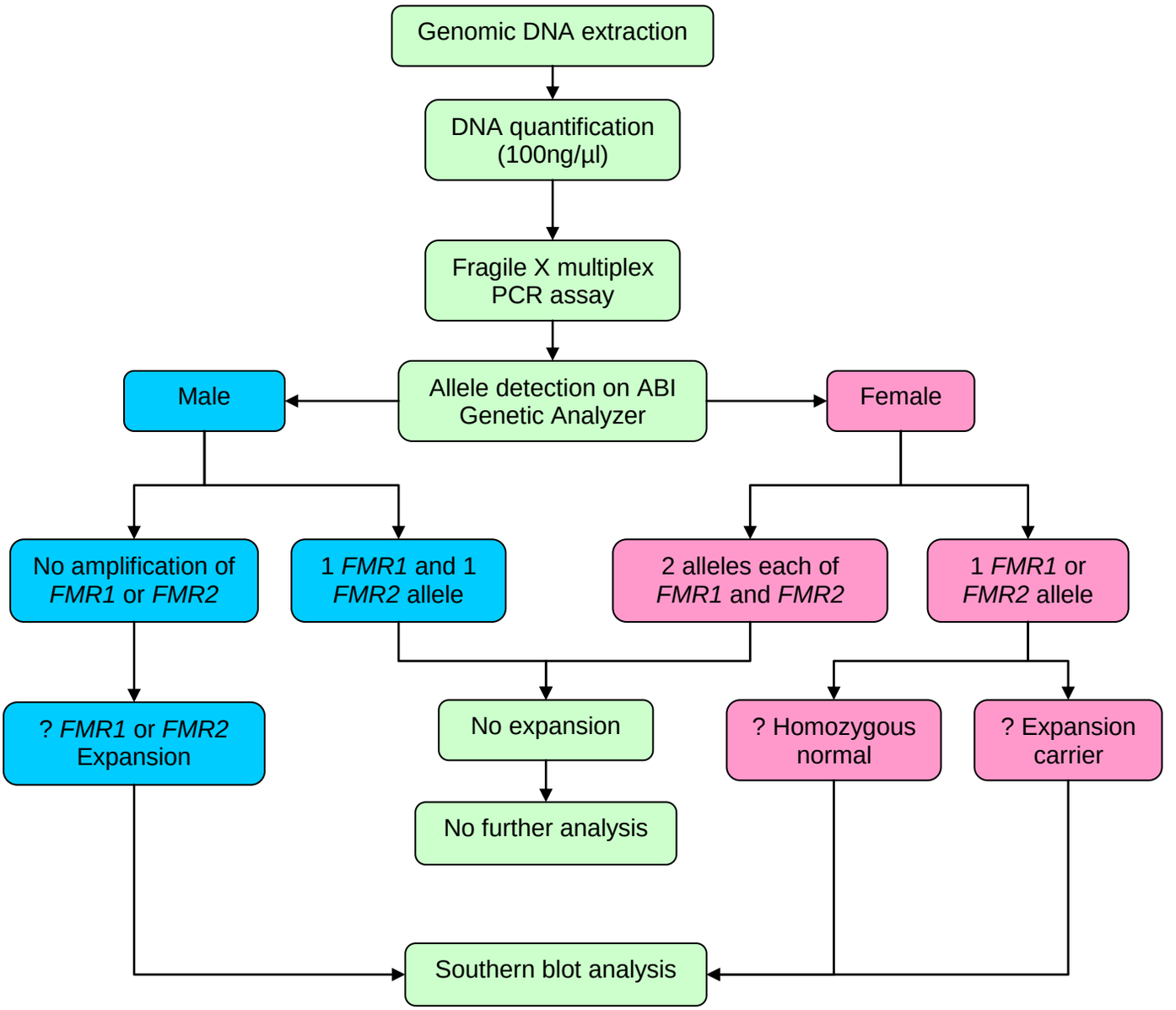


Figure 2.1 An overview of the procedure used for fragile X syndrome testing.
The PCR is set up as a multiplex PCR reaction to detect *FMR1* and *FMR2* repeat and the flow diagram shows the steps followed for each locus.

2.12.1 Methodology for *FMR1*/*FMR2* multiplex PCR screen

The PCR assay is a multiplex reaction, comprising of two primer sets (each labelled with a different fluorescent marker to distinguish the alleles at each locus), which simultaneously detects *FMR1* and *FMR2* alleles in a multiplex reaction.

Genomic DNA was extracted (described in Section 2.8). Since the *FMR1*/*FMR2* multiplex PCR assay is sensitive to DNA concentration, the extracted DNA was quantified to 100ng/μl, using spectrophotometric absorbance readings at a wavelength of 260nm (See Section 2.9). PCR across the CGG/GCC repeats was used as a first-line screen to determine the size of the normal and lower PM *FMR1*/*FMR2* alleles. Alleles were detected on the ABI PRISM™ 377 or ABI 3130xl Genetic Analyzer.

PCR does not amplify the *FMR1* and *FMR2* full expansions. Suspected expansions based on non-amplification (Figure 2.1) have to be confirmed on Southern blot analysis, which is able to detect normal, PMS and FMs reliably. PCR is problematic due to the high GC rich content which promotes formation of alternative and secondary DNA structures and the creation of polymerase pause sites which introduce errors during replication (Kang, Ohshima, Shimizu et al, 1995; cited in Khaniani, Kalitsis, Burgess et al., 2008). The *FMR1* PCR optimised previously makes use of DMSO which is a co-solvent inhibiting secondary structure (Pomp and Medrano, 1991).

For each locus, male patients with one normal-sized allele and female patients with two normal-sized alleles were not analysed further as a diagnosis of fragile X syndrome or FRAXE syndrome due to an *FMR1* or *FMR2* expansion was excluded (see Figure 2.1). If non-amplification was observed at any one locus in a male and if only one allele was observed in a female, Southern blot analysis was performed to identify a possible expansion of the repeat in the PM or FM range.

PCR is capable of determining the exact CGG/GCC repeat number in the normal and lower PM range. This is an effective approach since a diagnosis is made in considerably less time than with Southern blotting. Southern-blot based detection does not provide a precise measure of the number of CGG/GCC repeats in either the *FMR1* or *FMR2* gene. The multiplex reaction controls for PCR failure at the *FMR1* and *FMR2* loci as each locus is used as an internal control for the other. The largest *FMR1* allele detected by far with the PCR assay in this laboratory was 92 CGG repeats, which was tested through the external quality assurance program from the College of American Pathologists. The largest *FMR2* allele amplified was 33 GCC repeats.

2.12.2 Method for validating *FMR2* in the multiplex PCR screen

The *FMR1* PCR validation was done previously as part of my Honours project (Essop, 1999). The *FMR1*/*FMR2* multiplex PCR assay was validated at the *FMR2* locus by performing 'blind' screens on 3 different groups of subjects, viz. patients with an *FMR1* FM, mother-child pairs and a large *FMR1* expansion positive family (FRAXA family 2), as described in Section 2.2.4. Consistent allele sizes observed on at least 2 PCR runs and the stable transmission of normal alleles across generations was used as the criteria for validation.

Primer sequences for the *FMR1*/*FMR2* multiplex PCR assay were provided by Dr Chris Mathew (Guy's Hospital, UK). The primers were synthesized locally by Roche Diagnostics. Each forward primer was labelled with a different ABI Prism fluorescent dye. Table 2.3 lists the primer sequences and the fluorescent dye used to label the primers.

Table 2.3 Primers used in the *FMR1*/*FMR2* multiplex PCR assay.

PRIMER	SEQUENCE
FRAXE 598 – F	5'(FAM) –GCG AGG AAG CGG CGG CAG TGG CAC TGG G -3'
FRAXE 603 –R	5' –CCT GTG AGT GTG TAA GTG TGT GAT GCT GCC G -3'
FRAXA fxc – F	5'(HEX) –GCT CAG CTC CGT TTC GGT TTC ACT TCC GGT –3'
FRAXA fxf – R	5' – AGC CCC GCA CTT CCA CCA CCA GCT CCT CCA -3'

(Wang, Green, Bobrow et al., 1995)

2.12.3 *FMR1*/*FMR2* multiplex PCR optimization

The PCR optimisation for *FMR2* proved to be rather challenging as the region is a G-C rich sequence due to the GCC triplet repeat. The presence of a high G+C content in the target DNA template presents difficulties for PCR amplification across GC rich triplet repeats. To obtain an accurate sizing of the GCC repeat, the conditions must be optimised sufficiently well. An accurate sizing is important and significant as it may give some indication of the stability of the repeat and hence the risk of expansion. This is subsequently helpful for appropriate genetic counselling.

The incorporation of 7-deaza-guanosine triphosphate (deaza-dGTP), a nucleotide analogue, allows successful amplification of GC rich segments by destabilizing the secondary DNA structure (Innis, 1990; Wallace, 1996). A 3:1 ratio of deaza-dGTP:dGTP was used in the multiplex PCR assay. As deaza-dGTP interferes with EtBr intercalation, PCR products with high deaza-dGTP content do not stain efficiently with EtBr (Innis, 1990; Latimer and Lee, 1991) and were therefore not detected on an agarose gel prior to detection on the ABI Genetic Analyzer.

After a series of PCR optimization steps of different reaction conditions and PCR additives, the assay was optimized. A detailed worksheet for the PCR reaction is available in Appendix D1. The GCC repeats are determined using a formula based on primer length and distance. Allele sizes (bp) were rounded off to the nearest GCC repeat number (Appendix D1).

2.12.4 Detection of *FMR1*/*FMR2* alleles on ABI Genetic Analyzer

Products were analyzed on the gel based ABI PRISM^{TM} 377 Genetic Analyzer and more recently on the capillary based ABI 3130xl Genetic Analyzer (Applied Biosystems). PCR products were not detectable by EtBr staining. The labelled products emit fluorescence when excited by a laser. Each dye emits a fluorescent signal within a specific wavelength. The Genetic Analyzer uses the virtual filter sets to detect the dye that is within its wavelength range and displays a peak in the colour that it detects.

On the ABI377 Genetic Analyzer, a polyacrylamide gel is cast, scanned, and prerun, and the GeneScan collection and analysis files are set up. The PCR products are mixed with a size standard, denatured, and loaded onto the prerun gel. After electrophoresis the collected data is transferred to a second Macintosh computer for analysis. A results file is generated and the PCR products are scored and verified. An overview of the procedure for automated genotyping of *FMR1*/*FMR2* on the ABI377 Genetic Analyzer is shown in Figure 2.2. The protocol for microsatellite detection on the ABI3130xl Genetic Analyzer is described in Appendix C5. As the ABI377 Genetic Analyzer model has been discontinued, the PCR products are now detected on the ABI 3130xl Genetic Analyzer which does not require a gel to be cast. The 16-capillary 3130xl Genetic Analyzer (Applied Biosystems/Hitachi) allows simultaneous loading of 16 samples by electrokinetic injection (Huang, Quesada and Mathies, 1992). The capillaries are 0.1 mm in diameter and are filled with an electrolyte-type gel (performance optimized polymer - POP) that allows the separation of analytes based on their size to charge ratio. The DNA migrates through a process called multi-sheath flow. When the DNA migrates through the detection cell, light from a single laser source is split creating a dual beam that illuminates capillaries from both sides of the array. The fluorescent light emitted from each DNA fragment is collected, separated by wavelength and focused onto a charge-coupled device (CCD). The data generated is transferred to a computer linked to the 3130xl Genetic Analyzer where the data is transformed into electropherograms by a program called Foundation Data Collection v3.0. A program called GeneMapper v4.0 is used to visualise and analyse microsatellite data. The protocol for microsatellite detection on the ABI3130xl Genetic Analyzer is described in Appendix C6.

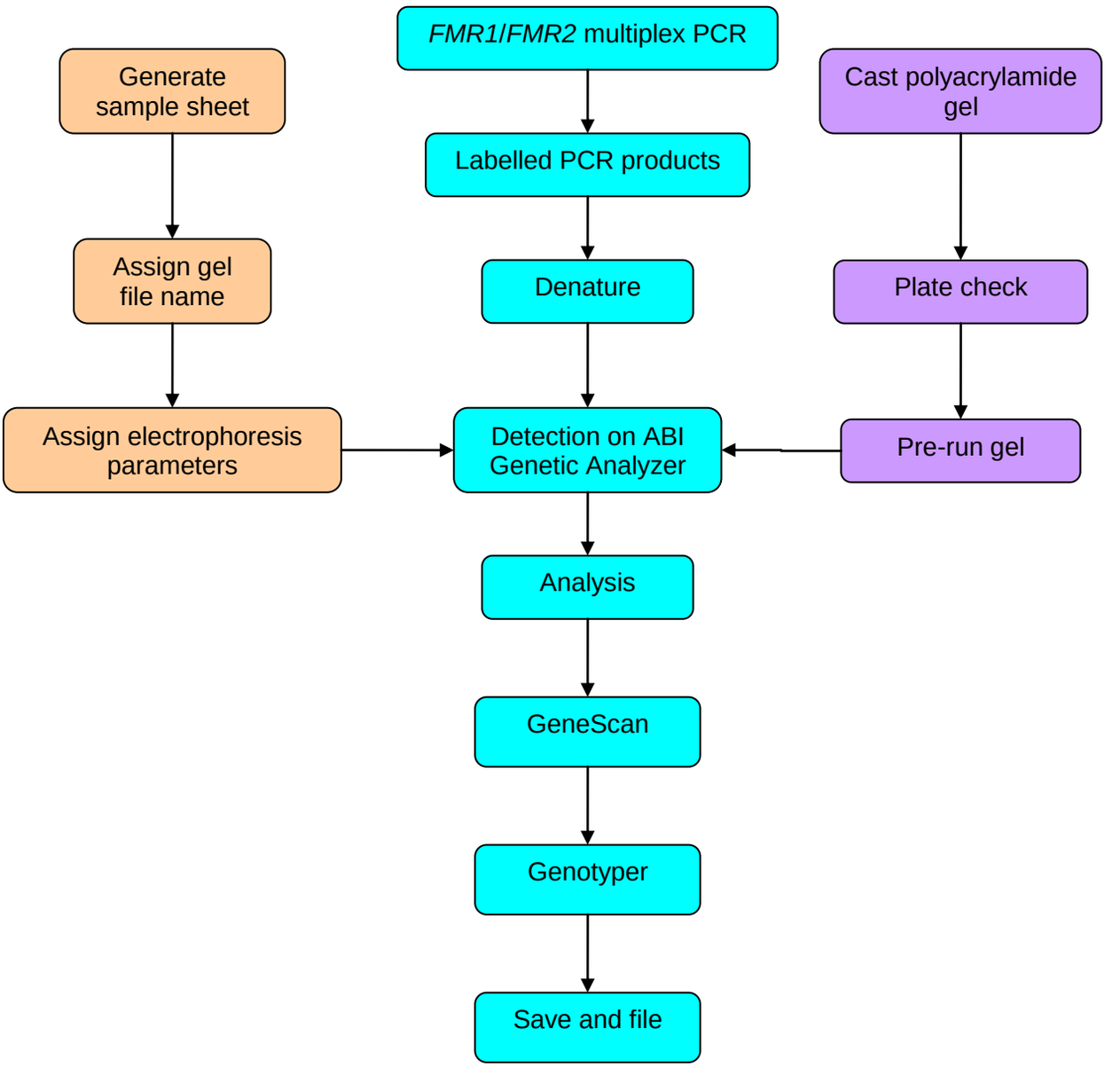


Figure 2.2 Schematic diagram illustrating the procedure for automated genotyping of *FMR1*/*FMR2* alleles on the ABI377 Genetic Analyzer.

2.12.5 Expected *FMR2* allele in males and females

The number of alleles expected to amplify in the multiplex PCR assay in males and females are outlined below. A summary of the number of alleles expected from the *FMR1*/*FMR2* multiplex PCR assay in individuals with different *FMR2* genotypes is listed in Table 2.4.

Table 2.4 Summary of the number of *FMR2* and *FMR1* alleles observed using the multiplex *FMR1*/*FMR2* multiplex PCR assay.

***FMR2* GENOTYPE**	***FMR2* ALLELES OBSERVED ON PCR**	***FMR1* ALLELES OBSERVED ON PCR**
MALE		
Normal (N)	1	1
Carrier (E)	0	1
Affected (EE)	0	1
FEMALE		
Normal heterozygote (N/N)	2	1 or 2
Normal homozygote (N/N)	1	1 or 2
Carrier (N/E)	1	1 or 2
Affected (N/EE)	1	1 or 2
E/E	0	1 or 2
EE/EE	0	1 or 2

A normal male (N) is expected to generate one peak each at the *FMR1* and *FMR2* loci, representing one normal sized *FMR2* GCC allele and one normal sized *FMR1* CCG allele, as shown in Figure 2.3..

A normal heterozygous female (N/N) is expected to generate two peaks each at the *FMR1* and *FMR2* loci, representing two normal sized *FMR2* alleles and two normal sized *FMR1* alleles, as shown in Figure 2.4. If only one allele is present at either locus, it could be interpreted either as a female homozygous for that allele, or the presence of second allele that is expanded, which could not be detected with the PCR assay. In normal heterozygous females there appears to be marked preferential amplification of the smaller allele relative to the larger allele. The greater the difference in size (in bp) observed between the two alleles, the greater the preferential amplification of the smaller sized allele.

Non-amplification at either the *FMR2* or *FMR1* locus in a male implies a premutation (E) or a full mutation (EE), as the PCR technique cannot amplify such large fragment sizes as illustrated in Figure 2.5. These samples were further analysed by Southern blotting (described in section 2.12.6).

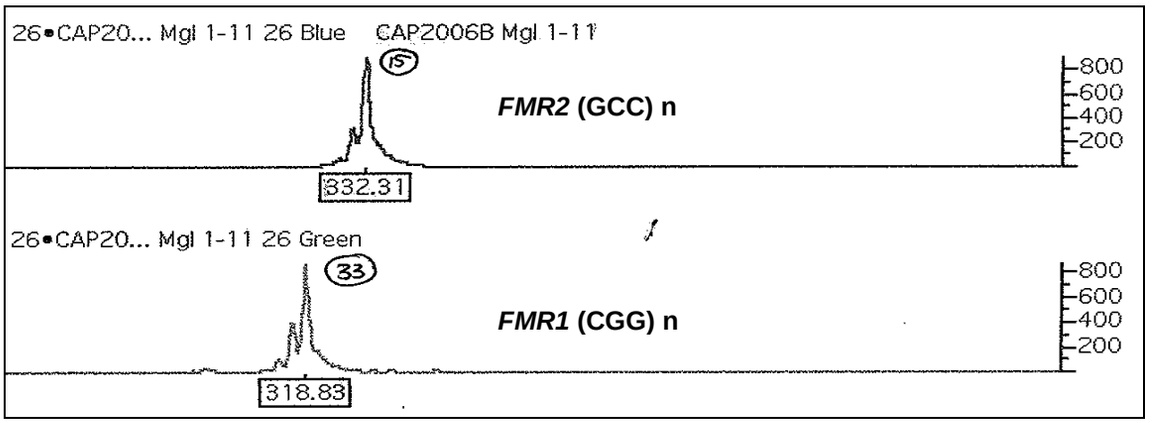


Figure 2.3 Electropherogram of a male with an *FMR2* allele in the normal range.
The diagram shows one *FMR2* allele of 332 bp, corresponding to 15 GCC repeats and one *FMR1* allele of 318 bp corresponding to a 33 CGG repeats, both within the normal range.

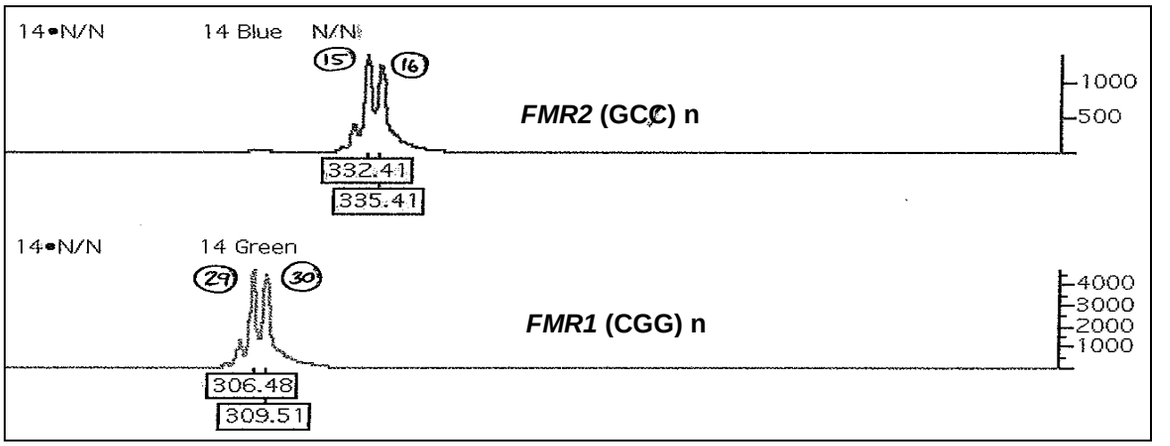


Figure 2.4 Electropherogram of a female with two *FMR2* alleles in the normal range.
The diagram shows two *FMR2* alleles of 332 bp (= 15 GCC repeats) and 335 bp (= 16 GCC repeats) in the normal range and two normal *FMR1* alleles of 306 bp (= 29 CGG repeats) and 310 bp (= 30 CGG repeats) in the normal range.

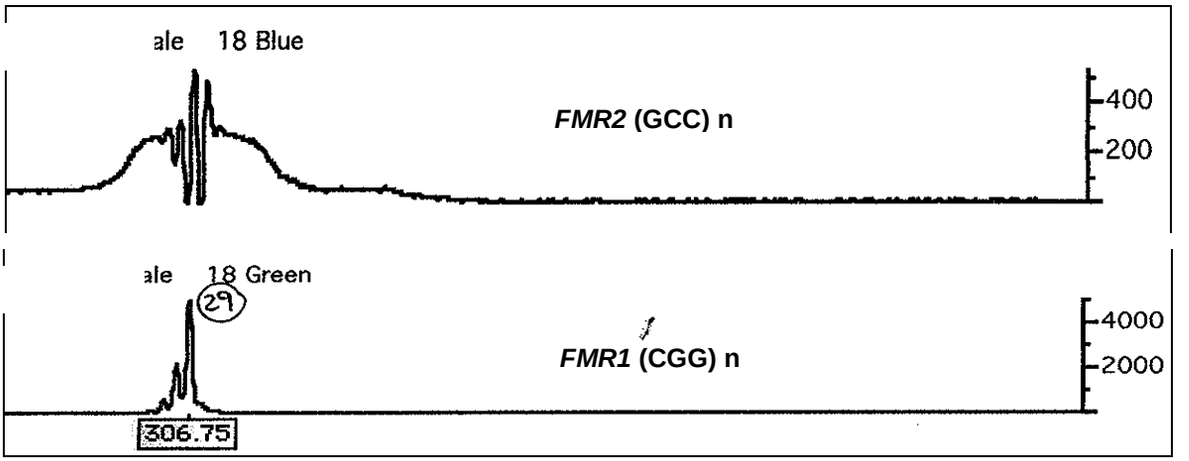


Figure 2.5 Electropherogram of a MALE displaying non amplification of the *FMR2* allele, suggestive of an *FMR2* expansion.
The FAM-labelled (green) *FMR1* allele is 306 bp, corresponding to 29 CGG repeats. The pattern shown at the *FMR1* locus does not represent an allele but is a result of interference from the *FMR2* repeat due to a matrix overlap.

2.12.6 Southern blotting

Southern blotting is a technique that was developed by Southern in 1975. It is a commonly used method for the identification of specific DNA fragments that are complementary to a known DNA sequence. Southern blot analysis was performed on male samples that failed to amplify on PCR analysis and on female samples with only one *FMR2* or *FMR1* allele detected.

2.12.6.1 Restriction enzyme digestion of genomic DNA

Genomic DNA (5-15ug) was digested with one or more restriction enzymes. A single digest using *Hind*III was performed for the detection of the *FMR2* allele. A double digest was performed at 37°C for detection of the *FMR1* allele, using a methylation sensitive enzyme, *Ecl*XI and *Eco*RI (Roche). The digest reaction was verified on a trial 0.8% gel to establish if complete digestion had occurred. The digested samples were loaded onto a 0.8% preparative Southern agarose gel and electrophoresed for ~24 hrs at 1.6 V/cm to resolve fragments sizes adequately. A photograph was taken of the Southern agarose gel. The digest reactions and worksheets for the FRAXE/*FMR2* and FRAXA/*FMR1* are shown in Appendix D2 and D3 respectively.

2.12.6.2 Southern transfer

The gel was denatured for ~30 min in an alkali solution and neutralised for a further 30 min in a neutralising solution (see Solutions in Appendix B). This was followed by soaking the gel in a solution of 20 x SSC for ~10 min. The blot was set up by placing the gel onto a wick draped in 2xSSC. A Hybond nylon membrane (AEC-Amersham) was placed on top of the gel and filter papers and paper towels were stacked on top of this to allow for capillary action to move the DNA out of the gel and onto the membrane. This was allowed to blot for 2 nights after which the membrane was removed and baked for ~1 hr in an 80ºC oven to allow the DNA to fix to the membrane.

2.12.6.3 Pre- and Post-hybridisation

The membrane was pre-treated for ~1 hr with a pre-hybridisation solution (Church-Gilbert pre-hybe buffer, Appendix B) which contained ready to use salmon sperm DNA (Roche). Twenty five nanograms of the probe was labelled with the radionuclide isotope ^{32}PdCTP using the Megaprime™ DNA Labelling Systems kit (from AEC-Amersham) according to manufacturer's instructions (refer to Appendix D4 for detailed labelling protocol). Hybridization was allowed to occur over 2 nights in a Hybaid rotisserie oven. Post hybridisation washes were performed with

2xSSC, 0.1%SDS for 10 min at room temperature (twice), 1xSSC, 0.1%SDS at 65°C for 20 min and three rinses at room temperature with 0.1xSSC. The blot was exposed to Hypersensitive X-ray film (from AEC-Amersham) for at least 2 nights, after which the X-ray film was developed using an Axim developer.

2.12.6.4 Probe preparation

The insert (probe) is contained within a recombinant plasmid. The plasmid containing the probe had been transformed into dH5α competent cells using the method described by Chung, Niemala and Miller (1989). The culture media required for growth is LB broth containing the antibiotic ampicillin at standard concentrations (100μg/ml). The plasmid was extracted using the QIAGEN Plasmid Maxi Kit from QIAGEN (supplied by Southern Cross Biotechnology (Pty) Ltd - South Africa) as per manufacturer's instructions. The insert/probe was excised from the plasmid using a restriction endonuclease which flanks the insert. The probe was purified from a low-melting-temperature agarose gel using QIAquick Gel Extraction Kit from QIAGEN as per manufacturer's instructions.

a) *FMR1* StB12.3 Probe preparation

The membrane was probed with an α-^{32}PdCTP labelled StB12.3 probe. The insert StB12.3 is contained within the recombinant pBluescript II KS plasmid. The plasmid containing the probe had been transformed into dH5α competent cells. The culture media required for growth is LB broth containing the antibiotic ampicillin at standard concentrations (100 μg/ml). The plasmid was extracted using the QIAGEN Plasmid Maxi Kit from QIAGEN. The insert StB12.3 is 1.2 kb in size and was excised from the 2.9 kb plasmid using the restriction endonuclease *Pst*I. StB12.3 detects a ~5.2 kb *Eco*RI fragment in Xq28 of the normal human X chromosome. Figure 2.6 illustrates the restriction map of the *FMR1* region.

b) *FMR2* OxE20 Probe preparation

The membrane was hybridised with an α-^{32}PdCTP labelled OxE20 probe using standard procedures (Rousseau et al., 1991). The insert OxE20 probe was provided by Knight, SJL, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, England. It is contained within the recombinant pBluescript plasmid. The plasmid containing the OxE20 probe had been transformed into dH5α competent cells. The culture media required for growth is LB

broth containing the antibiotic ampicillin at standard concentrations (100 μg/ml). The insert OxE20 is ~800 bp in size and was excised from the plasmid using the restriction endonucleases *EcoR*I and *EaG*I (isoschizomer is *Ecl*XI).

OxE20 detects a ~5.2 kb *Hind*III fragment in Xq28 of the normal human X chromosome (Biancalana et al., 1996). OxE20 lies ~600 bp distal to the GCC repeat and within the same 5.2 kb *Hind*III fragment (Knight et al., 1994). A rare polymorphism of the *Hind*III site has been observed and this gives a fragment of ~6.4 kb which is unaltered through successive generations. A *BamH*I digest probed with OxE20 should enable the *Hind*III polymorphism to be delineated from a true *FMR2* expansion (Knight et al., personal communication, 1994). Restriction mapping indicated that the OXE20 probe would not detect the mutation in *EcoR*I digests, since an *EcoR*I site separates the probe sequence from the GCC repeat (Biancalana et al., 1996). Figure 2.7 illustrates the restriction map of the *FMR2* region.

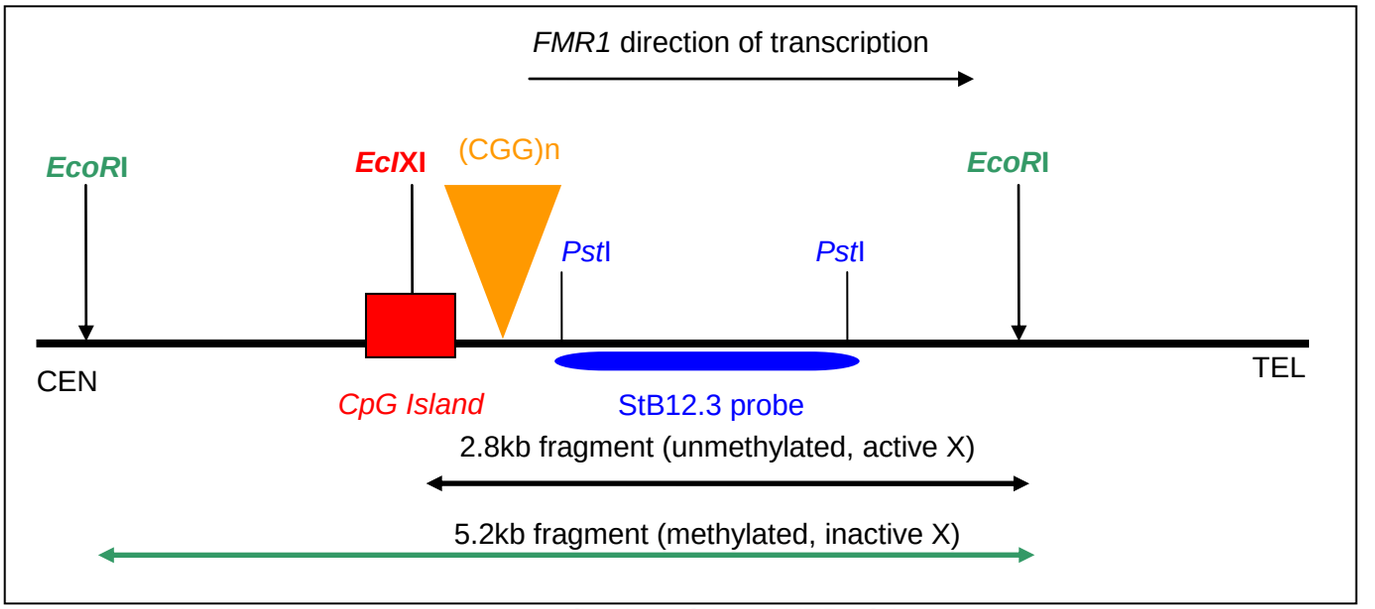


Figure 2.6 This diagram represents a magnification of the 5' UTR of the *FMR1* gene
This gene is subjected to digestion by *Eco*RI and *Ecl*XI for Southern blot analyses. FMR segment includes both the CGG repeat (indicated by the orange inverted triangle), at the proximal portion of exon 1 and the CpG island (indicated by the red box) that is located 250 bp 5' to the CGG polymorphism. The ATG start site is downstream to the CGG polymorphism within exon 1. Restriction sites are indicated by vertical arrows. The StB12.3 probe hybridises to the distal portion of the CGG repeat, contained in the 2.8 kb fragment

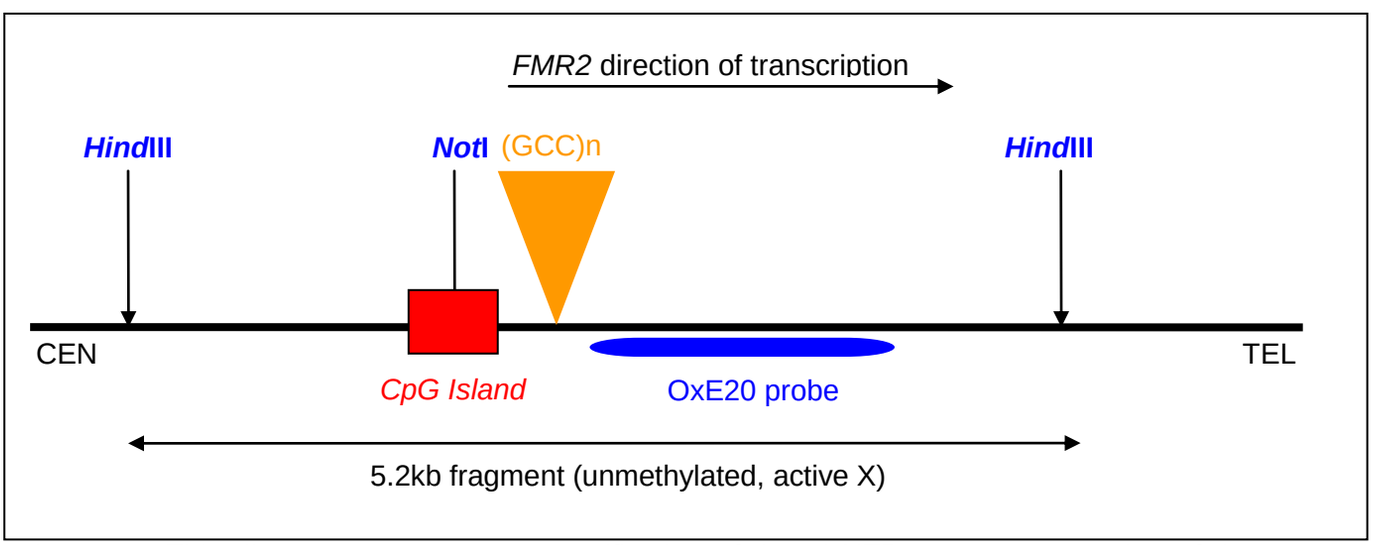


Figure 2.7 This diagram represents a magnification of the 5' UTR or promoter region of the *FMR2* gene.
The gene is subjected to digestion by *Hind*III for Southern blot analyses to detect the GCC expansion at *FMR2*. The exact location of the GCC repeat with respect to the transcription initiation site of the *FMR2* gene is uncertain. Vertical arrows indicate restriction sites. The OxE20 probe hybridises adjacent to but on the same fragment as the GCC repeat, contained in the 5.2 kb fragment (modified from Knight et al, 1994). This FMR segment includes both the GCC repeat (indicated by the orange inverted triangle) and the CpG island (indicated by the red box). Restriction sites are indicated by vertical arrows.

2.12.7 Haplotype analysis in *FMR1* expansion positive family (Family 1)

Two intragenic markers, DXS548 and FRAXAC2, were used for haplotype construction in the *FMR1* expansion positive family. The PCR protocol and detection method for each of these markers is described below. An overview of the procedure is shown in Figure 2.8.

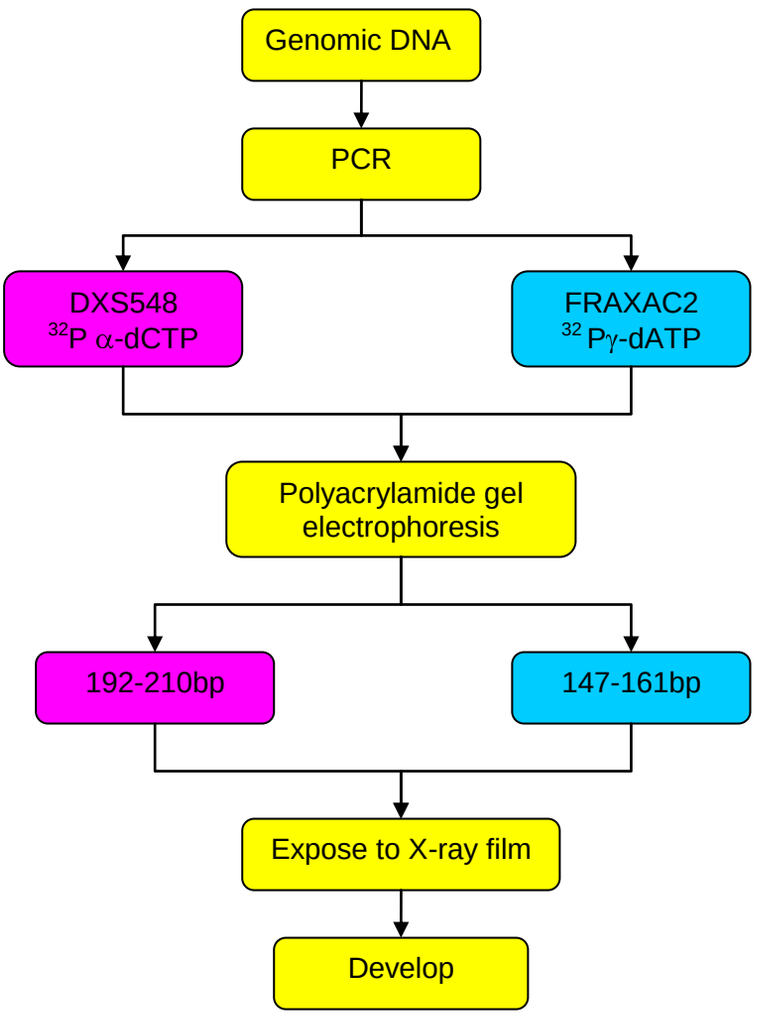


Figure 2.8 An overview of the procedure used for DXS548 and FRAXAC2 marker detection.

2.12.7.1 DXS548 marker

This is a polymorphic repeat marker, 150 kb centromeric to the CGG repeat (Riggins et al., 1991). PCR was used to amplify the repeats and α-^{32}PdCTP radioactivity was incorporated into the PCR reaction to detect the repeat length and corresponding alleles on a vertical PAG with expected fragment sizes between 192 bp and 210 bp. Primer sequences for the DXS548 marker were obtained from Verkerk et al., (1991). The PCR reaction and worksheet for the DXS548 PCR is shown in Appendix D5.

2.12.7.2 FRAXAC2 marker

The FRAXAC2 marker is an Alu-associated polymorphism located in intron 2 of the *FMR1* gene, 10 kb telomeric to the CGG repeat and contains three polymorphic (GT)x(AT)y(T)z subregions (Richards et al., 1991; Zhong, Dobkin and Brown et al., 1993). PCR was used to amplify the repeat and α-^{32}PdCTP radioactivity was incorporated into the PCR reaction to detect the alleles on a vertical PAG with expected fragment sizes between 147 bp and 161 bp. Primer FR (a) (also called PM151) was endlabelled prior to the PCR set up. This was performed using γ-^{32}PdATP as per protocol described by Sambrook, Fritsch, Maniatis et al. (1989). Primer sequences for the FRAXAC2 marker were obtained from Richards et al. (1991). Polynucleotide kinase was used to transfer γ-^{32}PdATP to the 5' end of the primer. The endlabelling reaction and the PCR reaction are shown in Appendix D6.

2.12.7.3 Methodology for DXS48 and FRAXAC2 marker detection

Both the DXS548 and the FRAXAC2 markers are detected using vertical polyacrylamide gel electrophoresis. A mix of 3µl formamide loading dye and 2µl of the PCR product was made and denatured at 95°C for 2 min. The denatured sample was loaded on a 6% denaturing PAG and electrophoresed for 2-6 hrs at ~1500 V (20-25 mA). The gel was transferred from the glass plates onto filter paper (Whatman), covered with cling wrap and vacuum dried in a vacuum oven for ~30-60 min after which it was exposed to an X-ray film (Hyperfilm MP from Amersham) for ~1 hr (FRAXAC2) to 24 hrs (DXS548). The X-ray film was developed using the X-ray developer machine (Axim).

2.13 *XNP* SCREENING

Genomic DNA was extracted (see Section 2.8) and the 300 bp 'hot-spot' region, where the majority of mutations are found (See Section 1.5.3) was sequenced using the ABI PRISM^{TM} 377 Genetic Analyzer to identify any pathogenic mutations described in Appendix C7. An overview of the screening protocol is shown in Figure 2.9.

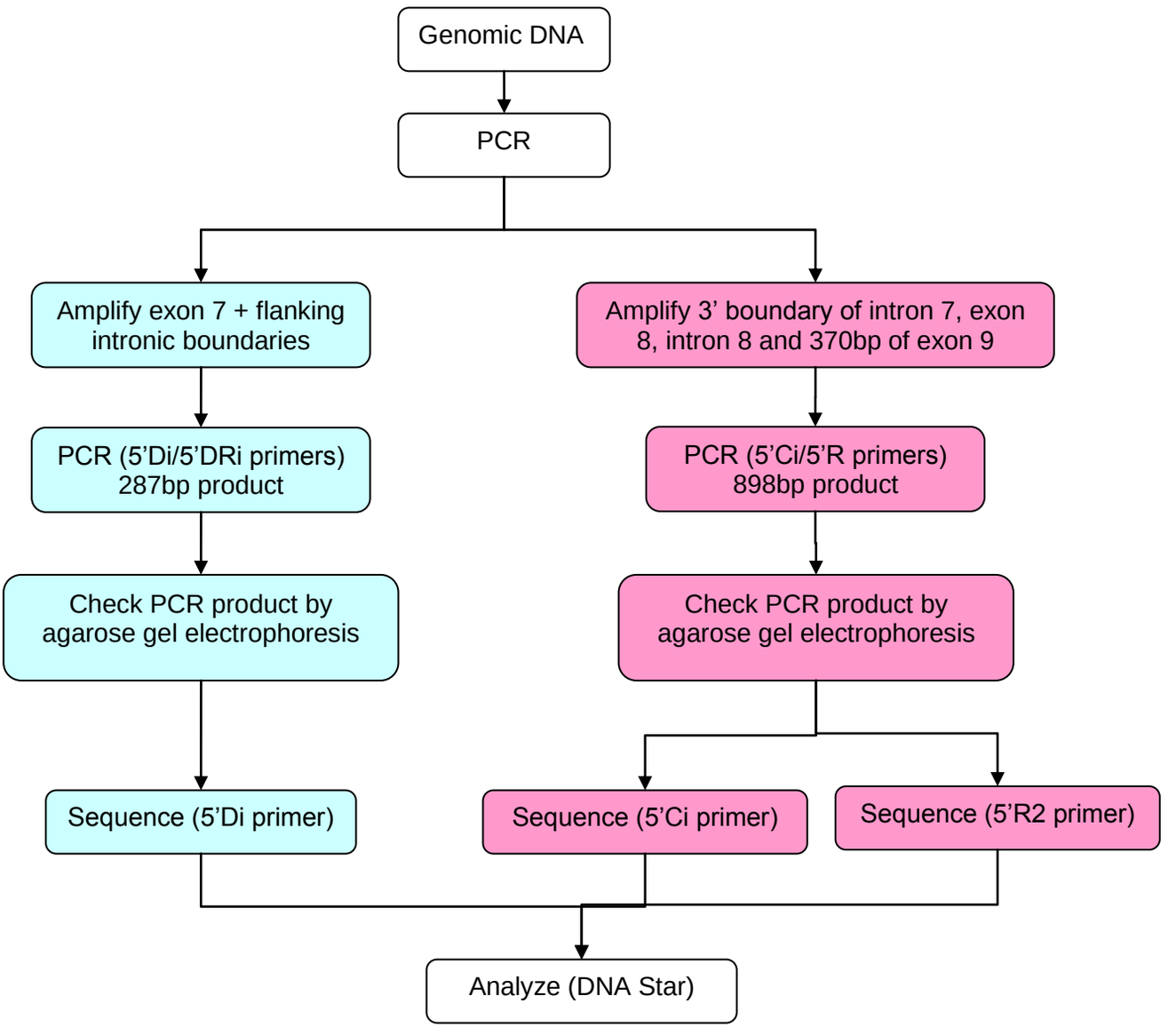


Figure 2.9 An overview of the procedure used for *XNP* mutation screening.

2.13.1 *XNP* sequencing

Two methods exist for rapid DNA sequencing, the chain termination method (Sanger, Nicklen and Coulson, 1977) and the chemical degradation method (Maxam and Gilbert, 1977) but the chain termination procedure is the method of choice for genome sequencing. This is a method in which the sequence of a ssDNA molecule is determined by enzymatic synthesis of complementary polynucleotide chains, these chains terminate at specific nucleotide positions.

The PCR protocol for *XNP* sequencing was taken from Villard et al. (1999a). Two primer pairs were used to amplify and sequence the zinc finger coding region (hotspot) from the *XNP* transcript. For both primer pairs, PCR was performed on the GeneAmp 9700 cycler. Agarose gel electrophoresis with ethidium bromide (EtBr) fluorescence was used to verify the PCR products (see Section 2.11). A volume of 5μl PCR product was loaded on a 3% (for primer set 5'Di/5'DRi) or 0.8% (for primer set 5'Ci/5'R) agarose gel. Unidirectional sequencing was performed for PCR product 1 using the forward primer 5'Di and bidirectional sequencing was performed for PCR product 2 using primers 5'Ci and 5'R2. This method of sequencing was found to be the most cost-effective option which allowed for sequencing of the entire region. A detailed worksheet for the PCR reaction and the amplified regions with primer sequences are shown in Appendix E1 and E2.

DNA was sequenced using the dye terminator labelling method. With this method, each of the four dideoxy terminators (ddNTPs) is fluorescently labelled with a different fluorolabel. Thus the growing chain is simultaneously terminated and labelled with the dye that corresponds to that base. The reaction is carried out in a single tube and each reaction is loaded into just one lane of the polyacrylamide gel. The fluorescent detector can discriminate between the different labels and hence determine if each band represents an A, C, G, or T. The protocol for sequencing analysis on the ABI3130xl Genetic Analyzer is described in Appendix C8.

2.14 *ARX* GENE c. 431-454dup24 MUTATION SCREENING

Genomic DNA was extracted (see Section 2.8) and the 24 bp duplication mutation in exon 2 of the *ARX* gene was screened using conventional agarose gel electrophoresis, followed by DNA sequencing if indicated.

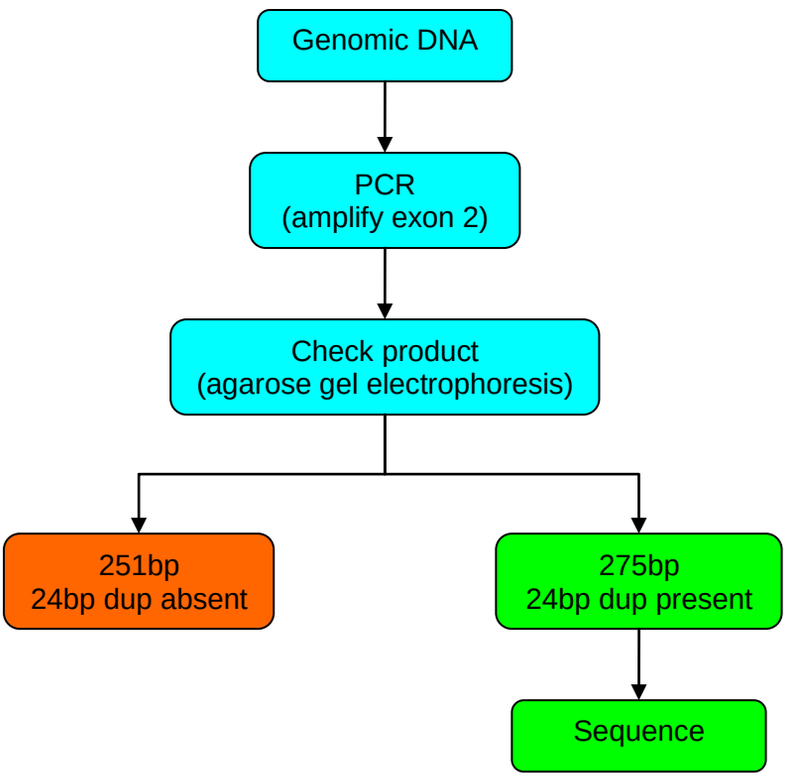


Figure 2.10 An overview of the procedure used for *ARX* mutation screening for the common 24 bp duplication mutation (modified from Gronskov et al. 2004).

2.14.1 *ARX* PCR

Primers amplifying the most common 24 bp duplication mutation in the *ARX* gene were obtained from Gronskov et al. (2004). The primer sequences were verified and validated using the In-Silico PCR application from the UCSC genome browser website (2009). The primers amplified a fragment of 251 bp in an individual without the 24 bp duplication. The presence of the duplication resulted in an expected fragment size of 275 bp (see Figure 2.10).

Gronskov et al. (2004) detected the amplified PCR fragment on the ABI310 Genetic Analyzer using a fluorescently labelled primer (ARX-2F). Due to the high GC-content of the *ARX* region, the primers (GC-content of 70%) and the existence of secondary structures, the laboratory was unable to optimize the assay using the method used by Gronskov et al (2004). Instead, a modified, simpler and cost effective approach was used in this study by using an unlabelled primer (ARX-2F) and agarose gel electrophoresis with ethidium bromide (EtBr) fluorescence to detect the amplified fragment. Any shift observed was sequenced using primer ARX-2F (Appendix F). The ARX-2R primer could not be used as the sequence was unreadable. A detailed worksheet for the PCR reaction showing primer sequences and the amplified region is available in Appendix F.

CHAPTER THREE

3 *FMR1* RESULTS AND DISCUSSION

The Division of Human Genetics, Molecular Laboratory at the NHLS has over the past 13 years taken a keen interest in screening loci associated with XLMR with a view to incorporating common mutation testing into the routine diagnostic service. The *FMR1* PCR assay was optimized as part of my Honours project (Essop, 1999) and is now routinely implemented as a first line diagnostic test for referrals for fragile X MR syndrome.

3.1 RESULTS OF RETROSPECTIVE ANALYSIS OF FRAGILE X A MR SYNDROME REFERRALS

A retrospective analysis was done on probands referred to the Division of Human Genetics for fragile X MR syndrome testing (described in Section 2.1) over a period of 14 years (1992 to June 2009). The results obtained from testing 1862 probands (1736 referred probands and 126 institutionalized male subjects described in Section 2.2.2) are presented below.

3.1.1 Frequency of full mutation in 1862 probands tested

From a total of 1736 unrelated probands referred for routine fragile X MR syndrome testing, 108 probands (6.2%) tested positive for the *FMR1* full mutation (FM) (>200 CGG repeats). Of the 126 institutionalized males tested, the *FMR1* FM was detected in 9 subjects (7.1%). This gives a combined total of 117 positive probands identified in 1862 unrelated probands (6.3%). These consisted of 107 male (5.7%) and 10 female probands (0.5%) who were found to be positive for the *FMR1* full mutation (FM).

The cohort of subjects was classified into 4 different ethnic groups, White, Black, Coloured and Indian. The frequency of the *FMR1* FM was determined in the total cohort of 1862 probands. The *FMR1* FM was found in 46 (2.5%) Black, 38 (2.0%) White, 17 (0.9%) Coloured and 16 (0.9%) Indian probands (summarised in Table 3.1).

The frequency of the *FMR1* mutation was also determined within each ethnic group. From a total of 743 Black probands tested, 46 were found to have the *FMR1* FM. This is a frequency of 6.2% (46/743). Similarly, a frequency of 4.9% (38/783) was observed within the White probands tested, 8.8% (17/194) in the Coloured and 11.3% (16/142) in the Indian probands.

3.1.2 Frequency of premutation and intermediate alleles in probands

PMs (59-200 CGG repeats) accounted for 0.8% (14/1862) of total probands tested, ranging from 0% to 1.4% in the different ethnic groups tested. A total frequency of 1.9% (34/1862) was observed for intermediate alleles (41-58 CGG repeats), ranging from 0.2% to 1.1% in the different ethnic groups tested (summarised in Table 3.1).

3.1.3 Frequency of full and premutation in extended family members

Of the 394 extended family members tested, a FM was found in 50 (25 males, 25 females) (12.7%) subjects and 54 (48 females, 6 males) were found to be PM and/or I carriers (13.7%).

3.1.4 Outcomes of prenatal diagnostic testing

A total of 14 prenatal tests (10 White, 4 Indian, 0 Black and 0 Coloured) were performed on CVS material or amniotic cells. Five of the 14 (36%) fetuses were found to have an *FMR1* FM (4 male fetuses with an EE and 1 female fetus with N/EE) and 9 fetuses (64%) were found to test negative for the *FMR1* FM.

Table 3.1 Results obtained from screening subjects referred for fragile X MR syndrome testing

	***FMR1* GENOTYPE**	**WHITE (n=783)**	**BLACK (n=743)**	**COLOURED (n=194)**	**INDIAN (n=142)**	**TOTAL NO. OF UNRELATED PROBANDS (n=1862)**
Full mutation (FM)						
Males	EE	28	33	15	14	90
Mosaic males	E + EE	2	2	2	1	7
	EE + del	1	0	0	0	1
Females	N/EE	7	2	0	1	10 (0.5%)
Total referred probands		**38**	**37**	**17**	**16**	**108**
Institutionalized males	EE	0	9	0	0	9
GRAND TOTAL		**38**	**46**	**17**	**16**	**117**
FREQUENCY OF FM IN ETHNIC GROUP		**4.9% (38/783)**	**6.2% (46/743)**	**8.8% (17/194)**	**11.3% (16/142)**	**6.3% (117/1862)**
Premutation (PM)						
Males	E	5	2	1	0	8 (0.4%)
Females	N/E	6	0	0	0	6 (0.3%)
TOTAL		**11**	**2**	**1**	**0**	**14**
% OF PM IN ETHNIC GROUP		**1.4% (11/783)**	**0.3% (2/743)**	**0.5% (1/194)**	**0% (0/142)**	**0.8% (14/1862)**
Intermediate allele (I)						
Males	I	14	5	3	3	25 (1.3%)
Females	N/I	6	3	0	0	9 (0.5%)
TOTAL		**20**	**8**	**3**	**3**	**34**
% OF I IN ETHNIC GROUP		**2.6% (20/783)**	**1.1% (8/743)**	**1.6% (3/194)**	**2.1% (3/142)**	**1.9% (34/1862)**
Normal range						
Males	N	586	494	152	108	1340
Females	N/N	128	76	21	15	240
Institutionalised males	N	0	117	0	0	117
TOTAL		**714**	**687**	**173**	**123**	**1697**
% OF N IN ETHNIC GROUP		**91.2% (714/783)**	**92.5% (687/743)**	**89.2% (173/194)**	**86.6% (123/142)**	**91.1% (1697/1862)**

Probands referred for fragile X syndrome (1992 to June 2009), including institutionalized male subjects classified into their different ethnicities.

CGG repeats sizes (GeneReviews website, 2009):

EE (full expansion) = >200
E (premutation) = 59-200
I (intermediate/gray zone) = 41-58
N (normal) = 5-40

3.1.5 Results of distribution of alleles in *FMR1* negative MR male subjects

The distribution of *FMR1* alleles detected in 1184 *FMR1* expansion negative MR males (described in Sections 2.2.1 and 2.2.2) is shown in the histogram below in Figure 3.1. Figure 3.2 illustrates the distribution of common *FMR1* alleles in the different ethnic groups in this cohort.

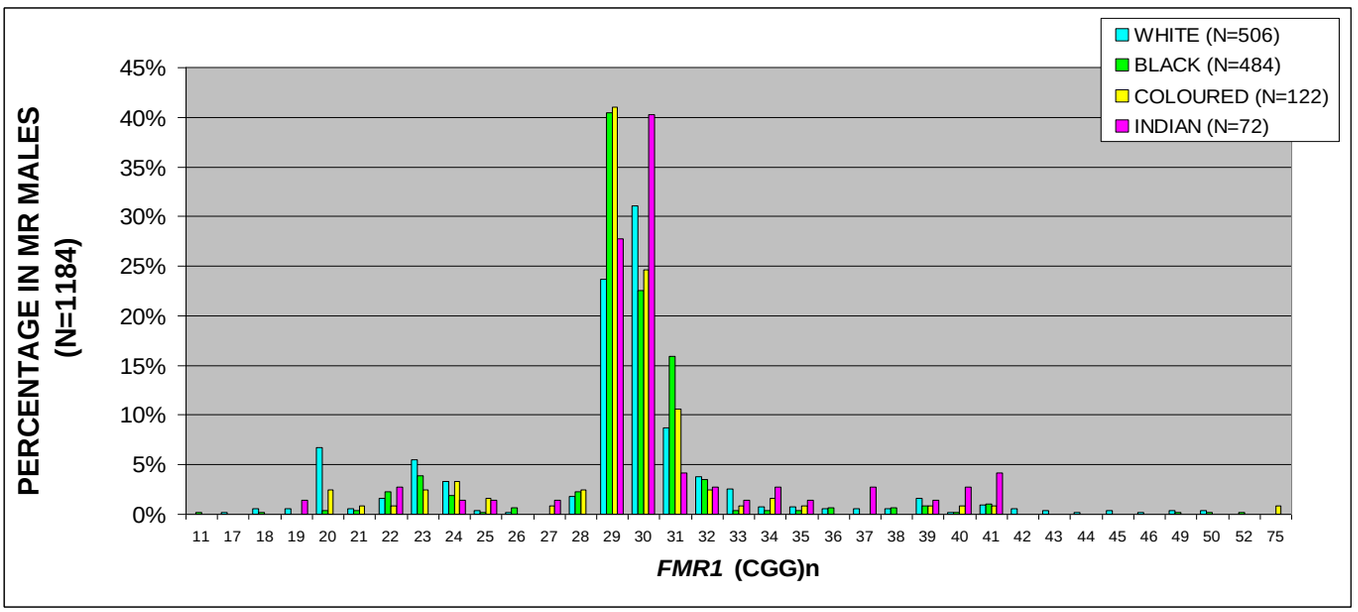


Figure 3.1 Histogram showing the distribution of the *FMR1* CGG repeat in 1184 MR males referred for fragile X MR syndrome testing.

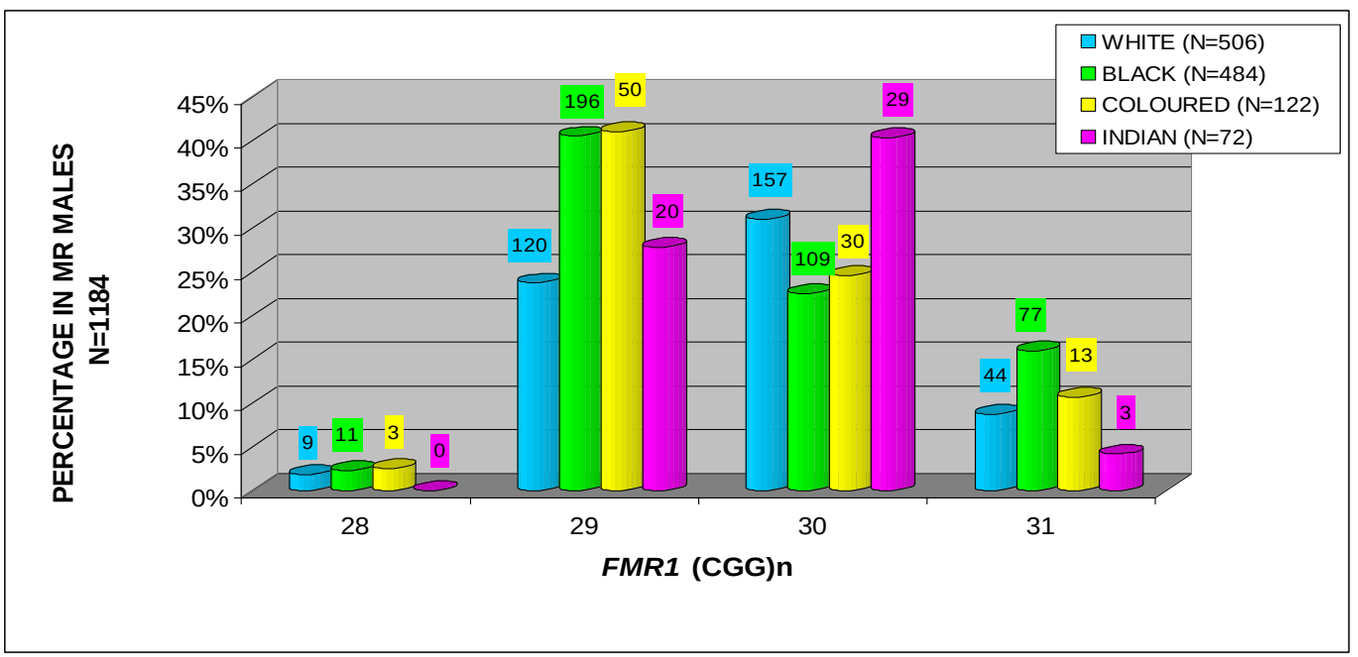


Figure 3.2 Histogram showing the distribution of **common** *FMR1* alleles in the 4 different ethnic groups and their frequencies within each ethnic group tested.

The distribution of the CGG repeat in 1184 *FMR1* expansion negative MR subjects showed that the smallest *FMR1* CGG repeat size detected was 11, while 75 was the largest allele detected on PCR analysis in this cohort (Figure 3.1). The most frequent repeat size observed in the total study cohort of 1184 *FMR1* expansion negative MR males was 29 CGG repeats (32.6%), followed by 30 CGG repeat (27.2%) (Figure 3.1). Allele sizes 20, 23, 29, 30, 31 and 32 CGG repeats together accounted for the majority (82%) of alleles in the study population. The most common allele observed in the Black and Coloured population was 29 CGG repeats (41%) while 30 CGG repeats was common in the White population (31%) and Indian population (40%). The number of CGG alleles identified in each population group can be found in Appendix G.

3.2 DISCUSSION OF RETROSPECTIVE ANALYSIS OF FRAGILE X A MR SYNDROME REFERRALS

As part of an investigation on XLMR loci, this study undertook a retrospective review of all probands referred between 1992 to June 2009 to the Division of Human Genetics, NHLS for fragile X MR syndrome testing. This study is an extension of Goldman et al. (1997), with a focus on a larger cohort of probands in all South African ethnic populations.

3.2.1 Frequency of full mutations

The frequency of fragile X MR syndrome in MR cohorts varies worldwide with estimates reported to range from 0.5% to 5% and with the larger reliable studies reported to be between 1% and 3% (Hofstee, Arinami and Hamaguchi, 1994; Mazurczak, Bocian, Milewski et al., 1996; Turner et al., 1996; Zhong, Ju, Xu et al., 1999; Biancalana et al., 2004).

From a total cohort of 1862 probands tested for fragile X MR syndrome in this study, the *FMR1* full mutation was identified in 117 probands (5.7% male and 0.5% female probands). This represents a frequency of 6.3% (117/1862), which is higher than reported worldwide figures. A recent study undertaken in Iran showed that the frequency of *FMR1* mutations was 6.3% (32/508 families) which is comparable to that found in our study (Pouya, Abedini, Mansoorian et al., 2009).

The frequency of the *FMR1* full mutation was further determined in each ethnic group. Fragile X MR syndrome was previously thought to be rare in the South African Black population. Goldman

et al. (1997) presented the first molecular evidence that fragile X MR syndrome occurs in the South African Black MR population with a frequency of 6.1% (9/148) and that it was severely underdiagnosed in the past due to ascertainment bias. This study reviewed a larger cohort of probands referred to the Division and a frequency of 6.2% (46/743) was observed in all Black subjects tested, which is comparable to that found in Goldman's study. It is noteworthy that this is higher than the frequency of 4.9% (38/783) observed within the White probands tested. This lends further support to the findings by Goldman et al. (1997) that fragile X MR syndrome is not rare in the SA Black population and that it may be a more common cause of MR than in Whites.

The frequency of the full mutation in the Indian population was shown to occur at 11.3%, which is higher than the frequencies observed in the other ethnic groups (4.9% in Whites, 6.2% in Blacks and 8.8% in Coloureds). The majority of these Indian probands were referred from KwaZulu Natal, a region which has a large Indian population base. A possible explanation for this finding could be due to selection bias as testing is expensive and due to restrained health budgets, referrals may be limited to patients with a very high clinical suspicion of fragile X MR syndrome. Studies in the Indian population in the Indian subcontinent report on a frequency of approximately 7% for fragile X MR syndrome due to FMs (Chowdhury, Kabra, Sharma, et al., 2006), lower than the frequency observed in this study but higher than worldwide figures.

The frequency of the full mutation in the Coloured population was shown to occur at 8.8%, which is also higher than the frequency observed in the White and Black ethnic groups (4.9% in Whites, 6.2% in Blacks). This Coloured population in SA comprises of racially mixed individuals of African (Black), European (White) and Asian (Indian) origin.

Cascade screening of extended family members detected an additional 50 subjects with the *FMR1* FM (25 males, 25 females). As the *FMR1* mutation is rarely a new mutation, cascade screening from the probands often detects other affected individuals in a family.

3.2.2 Frequency of premutations and intermediate alleles

The PM frequency in the total MR cohort detected in this study was 0.8% (14/1862) while the intermediate allele was observed at a frequency of 1.9% (34/1862). Both PMs and intermediate alleles were observed to occur at higher frequencies within the White MR population (1.4% and 2.6% respectively) compared to the other MR populations tested in this study. Youings, Murray,

Dennis et al. (2000) was the first report to show a significant excess of intermediate alleles in special education needs' boys compared to normal controls.

The PM is reported to occur in the general population at a frequency of 1 in 260 females (0.4%) and 1 in 800 (0.1%) males, evidently more common than fragile X MR syndrome (1 in 4000 males) (Rousseau, Rouillard, Morel et al., 1995; Dombrowski, Levesque, Morel, et al., 2002; cited in Cornish, Turk and Hagerman, 2008). The frequency of PMs in the normal population was not determined as part of this study. A recent study in Israel performed on 40 000 women showed a PM carrier frequency of 1 in 154 and that there was no difference in the PM carrier frequency observed in women with or without a family history of MR. However, different ethnic groups are reported to have different prevalences, e.g. it is less common in Asian populations and more common in Mediterranean populations (reviewed in Brouwer et al., 2009b).

Studies have shown the presence of neurobehavioural symptoms in children with PMs (Moore, Daly, Schmitz et al., 2004). A study by van Esch (2006) identified several boys who had PMs and presented with learning problems, developmental delay and/or autistic features. She identified four times more PMs in a cohort of MR boys younger than 16 years old who were referred for fragile X MR syndrome testing. This study did not review the age of the subjects tested and a comparison to van Esch's study can not be made. However, there have been a number of studies that have refuted the clinical significance of PMs (Mazzocco, Sonna, Teisl et al., 1997; Mornet, Chateau, Simon-Bouy, et al., 1998; Sherman, Marsteller, Abramowitz et al., 2002).

An average of 3 (394/117) extended family members per positive family was tested. Of the 394 extended family members tested, 54 (48 females, 6 males) were PM and/or I carriers (13.7%). This group included mothers of affected subjects who are expected to be carriers of *FMR1* expansions. There is a need for increased cascade testing of families to identify at-risk members and for early diagnosis in affected members.

3.3 DISCUSSION OF DISTRIBUTION OF ALLELES IN *FMR1* NEGATIVE MR MALE SUBJECTS

The investigation of the normal allele distribution of the *FMR1* CGG repeat in non expansion MR males was assessed. This is an extension of the study undertaken by Goldman et al. (1997). The allele distribution is comparable with other studies in which CGG repeat sizes of 28, 29 and 30 (shown in Figure 3.1) were found to be most common in different populations (Fu et al. 1991; Snow, Doud, Hagerman et al., 1993; Reiss et al., 1994; Goldman et al., 1997). The allele distribution in the normal range in non *FMR1* expansion males is not expected to be significantly different to the distribution in a random population as the normal allele is not associated with MR.

3.3.1 Distribution of *FMR1* alleles in different ethnic groups

The cohort in this study was further classified into different ethnic groups. Goldman et al. (1997) investigated a cohort of South African individuals and showed that the commonest allele identified was 28 CGG repeats (32%) in Negroids (Blacks). In this study the most common allele observed in the Black population was 29 CGG repeats (40.5%), one repeat different to that observed by Goldman et al. (1997). This minor difference could be due to less accurate sizing of CGG repeats previously as newer techniques have allowed for more accurate sizing. Further, a larger cohort of subjects was tested which gives a lower error rate. Hence this study is most likely to reflect the accurate size. It is worth noting that allele sizes scored using this PCR assay corroborate with the mean size of alleles as determined by the College of American Pathologists external quality assurance program which the laboratory participates in biannually.

Goldman et al. (1997) reported on 30 CGG repeats as the common allele in the San and Caucasoids (34% and 39% respectively). In this study, the most common allele observed in the White population was 30 CGG repeats (31%) which correlates with the findings from the Goldman study.

3.4 SUMMARY

The frequency of fragile X MR syndrome in the referred SA MR population was retrospectively reviewed. A frequency of 6.3% was observed, which is consistent with previously reported figures in SA but higher than reported worldwide figures. The analysis of non expansion *FMR1* alleles in MR males shows a similar distribution between the different SA ethnic groups and compares well with other reported studies. This study reinforces the presence of fragile X MR

syndrome in the SA Black population. The frequency of fragile X MR syndrome observed in the Black population and the high frequency in the Indian and Coloured populations may be due to ascertainment bias. With a high pick-up rate of 6.3%, it reinforces and supports the already available diagnostic test offered. Further, it is a relatively simple test to perform and normal alleles are easily detected and the majority of the subjects are excluded on PCR analysis although homozygous normal females and males with an *FMR1* expansion need to have Southern blot analysis done.

CHAPTER FOUR

4 FRAXE/*FMR2* RESULTS AND DISCUSSION

This project investigated the frequency of the *FMR2* expansion causing FRAXE syndrome, together with other XLMR genes, *XNP* and *ARX* in *FMR1* negative South African males with MR of unknown cause. The FRAXE/*FMR2* results are presented and discussed in this chapter.

4.1 RESULTS OF VALIDATION OF *FMR2* IN THE MULTIPLEX PCR ASSAY

One of the main aims of this project was to assess the frequency of the *FMR2* expansion mutation in MR males in the South African population. A multiplex PCR was developed, which could simultaneously detect *FMR2* and *FMR1* repeat sizes in the large numbers of routine referrals for fragile X MR syndrome to the Human Genetics Division at the NHLS. Each primer in the multiplex PCR reaction was labelled with a different fluorescent dye so that detection on the Genetic Analyzer was made possible. All samples were expected to produce a product from either the *FMR1* or the *FMR2* locus or both, thus controlling for failure of the PCR reaction. Peaks were generated and analysed with the GeneScan and Genotyper™ software (ABI/Perkin Elmer) described in Appendix C7.

To validate the multiplex assay and in particular the *FMR2* alleles, subjects with an *FMR1* expansion were tested. In addition, mother-child pairs were tested and an *FMR1* expansion positive fragile X family was used to demonstrate stable transmission of *FMR2* alleles across generations (FRAXA family 2). These results are presented below. The results obtained from screening *FMR1* expansion negative referral patients and MR males from two institutions are also shown.

4.1.1 Screening patients with an *FMR1* expansion

Ten patients with an *FMR1* expansion (FM) were used to validate the consistency of amplification of *FMR2* alleles in the multiplex PCR assay. The *FMR2* allele is expected to amplify in patients with an *FMR1* expansion. As expected, all 8 *FMR1* expansion positive males showed non-amplification of the *FMR1* allele and amplification of the *FMR2* normal allele. The 2 females with an *FMR1* FM showed amplification of one *FMR1* allele and two *FMR2* alleles. These results are summarised in Table 4.1.

Table 4.1 Summary of *FMR2* results obtained from testing 10 *FMR1* expansion positive patients with the multiplex *FMR1*/*FMR2* multiplex PCR assay.

SAMPLE	LAB CODE	GENDER	*FMR1* GENOTYPE	*FMR2* (GCC)n
1.	FRAX728	M	EE	16
2.	FRAX778	F	30/EE	19/20
3.	FRAX809	M	EE	15
4.	FRAX88	M	EE	17
5.	FRAX832	F	30/E	15/18
6.	FRAX854	M	EE	10
7.	FRAX866	M	EE	15
8.	FRAX966	M	EE	18
9.	FRAX974	M	EE	19
10.	FRAX993	M	EE	17

"EE" indicates a full mutation at the *FMR1* locus

4.1.2 Mother-child pairs

Ten normal mother-child (male and female) pairs were also used to validate the PCR assay. The samples were amplified at the *FMR2* and *FMR1* loci and the alleles generated were tracked from mother to child. The child was expected to inherit one stable allele (unchanged) from the mother. The allele that was inherited from the mother to the child is highlighted in blue in Table 4.2 which summarises the results obtained.

Table 4.2 Summary of results obtained from testing mother-child pairs for *FMR2* alleles using the multiplex *FMR1*/*FMR2* multiplex PCR assay.

SAMPLE	LAB CODE	*FMR2* ALLELE(S) IN MOTHER	*FMR2* ALELLE(S) INHERITED BY CHILD	GENDER OF CHILD
1.	FRAX60	15/?	15	M
2.	FRAX63	17/18	18/26	F
3.	FRAX64	16/21	17/21	F
4.	FRAX76	16/20	16	M
5.	FRAX76	18/29	18	M
6.	FRAX83	15/18	15	M
7.	FRAX89	15/20	15	M
8.	FRAX61	15/?	15/?	F
9.	FRAX65	18/18	18	M
10.	FRAX66	13/15	15	M

The *FMR2* allele passed on by the mother and inherited by the child is highlighted in blue

Stable inheritance of alleles was observed in 100% of mother to child pairs. All transmissions of normal alleles were consistent with Mendelian inheritance. This consistency in allele inheritance was sufficient to validate the PCR assay. The PCR was repeated and the results were 100% reproducible.

4.1.3 *FMR1* expansion positive family (FRAXA family 2)

To further validate the PCR assay, the transmission of *FMR2* alleles across generations was assessed in a family. Nineteen members from an *FMR1* expansion positive family were tested to track the *FMR2* allele. The pedigree with the *FMR2* allele amplified is shown in Figure 4.1. As expected, Mendelian inheritance of *FMR2* alleles was observed in all 19 members of the family, further validating *FMR2* in the multiplex PCR assay. The *FMR1* allele was used as a linked marker which assisted in determining the phase of alleles. The *FMR1* expansion in this family tracks with allele 18 at the *FMR2* locus.

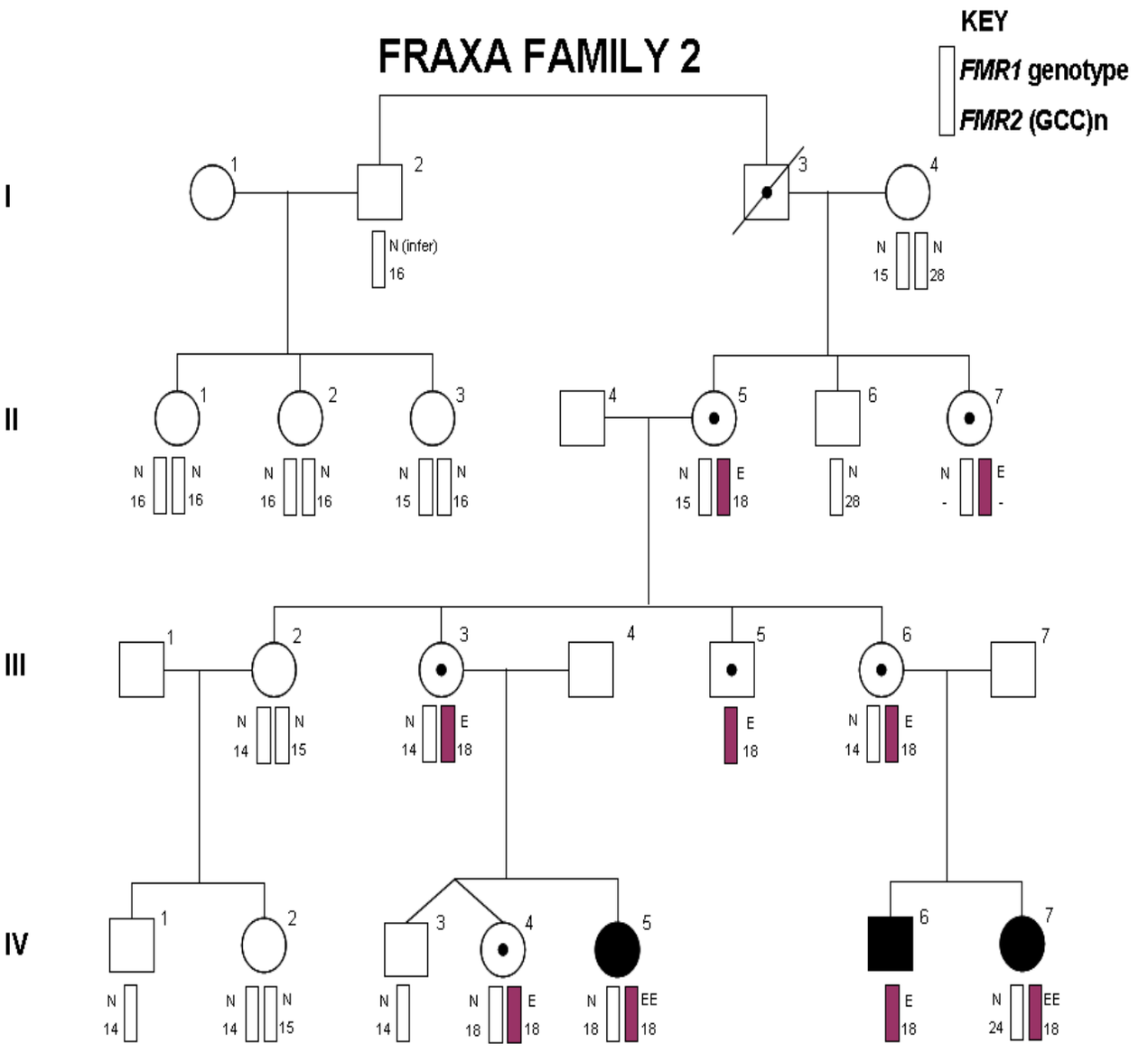


Figure 4.1 Pedigree of *FMR1* expansion positive family (Family 2) tested for the *FMR2* GCC repeat.

The *FMR2* allele was tracked in the family as part of the *FMR1*/*FMR2* multiplex PCR validation process. The *FMR1* genotype is indicated below each of the members tested. 'N' represents a normal *FMR1* allele, 'E' an allele in the premutation range and 'EE' a full expansion. The *FMR2* GCC repeat size is indicated below each of the 19 members tested.

4.2 RESULTS OF SUBJECTS SCREENED FOR *FMR2*

After validating and assessing the reliability and reproducibility of the *FMR1*/*FMR2* PCR assay, 1194 *FMR1* negative MR patients (ranging from mild to severe) were screened for FRAXE syndrome. None of the subjects tested were found to have the *FMR2* expansion mutation, confirming the rarity of FRAXE syndrome. There was a suspicion of an *FMR2* expansion in 2 patients from the cohort as the *FMR2* allele failed to amplify after several attempts on PCR analysis in the mutiplex reaction. Southern blotting (discussed in Section 2.12.6) was performed and the results are presented below. The distribution of *FMR2* non expanded alleles in the 1194 MR male subjects screened is also described below.

4.2.1 *FMR2* Southern blot result

Two samples from the cohort of institutionalised males repeatedly failed to amplify at the *FMR2* locus on PCR analysis, suggesting a possible *FMR2* expansion since the *FMR1* internal control had amplified at each attempt. Southern blotting was performed to clarify the result. In addition, three *FMR1* negative MR males were found to have an allele of 33 *FMR2* repeats in the gray range. Since the amplitude of the peaks was low on PCR relative to control samples, Southern blotting was performed to confirm this result.

On Southern blot analysis, using the OXE20 probe, a normal male and female would be expected to show a 5.2 kb band, corresponding to X chromosomes with GCC repeats in the normal range. A carrier female is expected to show two fragments, corresponding to the normal 5.2 kb fragment and one of increased molecular weight. A summary of expected fragments after Southern blotting is shown in Appendix D2.

Southern blots of *Hind*III-digested genomic DNA from these 5 individuals all showed a normal fragment of 5.2 kb. The autoradiograph of the result is shown in Figure 4.2. The non amplification observed on PCR analysis in the two samples of institutionalized males could have resulted due to poor DNA quality or some factor preventing amplification at the *FMR2* locus, since the *FMR1* allele had amplified at each attempt. This confirms that the *FMR2* GCC repeat expansion is not causative of the non amplification observed on PCR analysis in these subjects. A positive control should always be included in a PCR reaction and on Southern blotting. This also emphasizes the importance of a follow-up test using Southern blotting to confirm whether non-amplification is a result of an expansion.

The *FMR2* expansion positive male control sample (Section 2.2.3) did not amplify as expected on PCR analysis due to the presence of the large expanded GCC repeat. The expanded

fragment could not be sized accurately on the blot since the 1 kb commercial ladder was faintly visible on the X-ray film but was estimated to have >280-300 expanded GCC repeats. As shown in Figure 2.7, the OxE20 detects a ~5.2 kb *Hind*III fragment in Xq28 of the normal human X chromosome and a large amplified fragment of >5.2 kb.

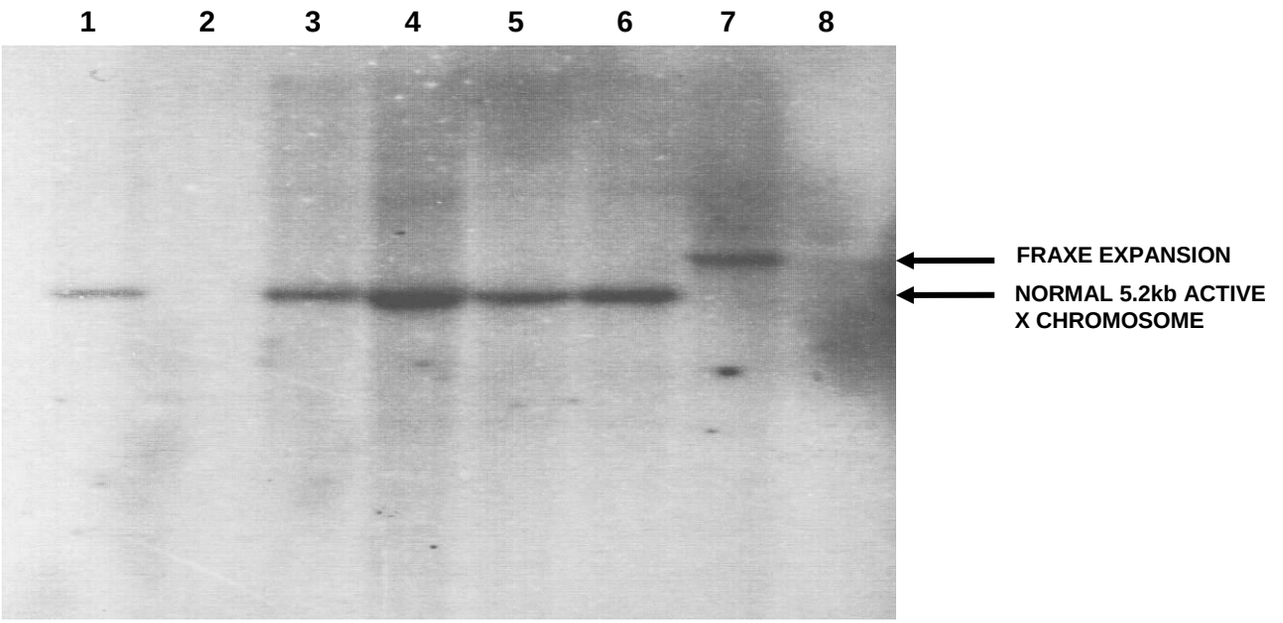


Figure 4.2 *FMR2* autoradiograph produced by probing Southern blots from *Hind*III digests with the OxE20 probe.
Lanes 1, 2 and 3 are males who were shown to have 33 *FMR2* GCC repeats on PCR analysis. No expansion is seen on Southern blot analysis (except the sample in Lane 2 which had no result due to poor DNA quality). Lanes 4 and 5 are two males with normal *FMR1* alleles and who failed to amplify the *FMR2* allele on PCR after several attempts. A normal-sized *FMR2* fragment with no expansion is seen on Southern blot analysis thus excluding FRAXE syndrome. Lane 6 is a normal female (note the active X chromosome and the lyonized, inactive X chromosome are indistinguishable unless subjected to digestion with a methylation sensitive enzyme. Lane 7 is a positive control sample of a male with an *FMR2* expansion
Lane 8 is a 1 kb DNA commercial ladder.

4.2.2 *FMR2* allele distribution in *FMR1* expansion negative male subjects

The distribution of normal alleles observed in the cohort of 1194 male patients (1069 *FMR1* expansion negative MR patients and 125 *FMR1* expansion negative institutionalized patients) of all ethnic groups (described in Section 2.1) was determined. The total distribution of *FMR2* allele sizes in the 1194 MR male study population ranged from 5 to 33. The modal number of 15 GCC repeats was observed in 500 males (41.9%). The second most common allele in the study population was 16 GCC repeats observed in 115 males (9.6%), followed by 14 repeats (9.4%).

4.2.3 *FMR2* allele distribution in ethnic groups tested

The cohort of *FMR1* expansion negative MR male subjects tested for *FMR2* comprised all ethnic groups. The number of GCC alleles identified in each population group can be found in Appendix H. The distribution of *FMR2* alleles identified in each ethnic group is shown in Figure 4.3. The modal number of 15 *FMR2* $(GCC)_n$ repeats is evident in each of the 4 different ethnic groups, observed at 34.1% (173/507) in Whites, 51.7% (255/493) in Blacks, 36.9% (45/122) in Coloureds and 37.5% (27/72) in Indians. The distribution of common *FMR2* alleles identified in each ethnic group is shown in Figure 4.4.

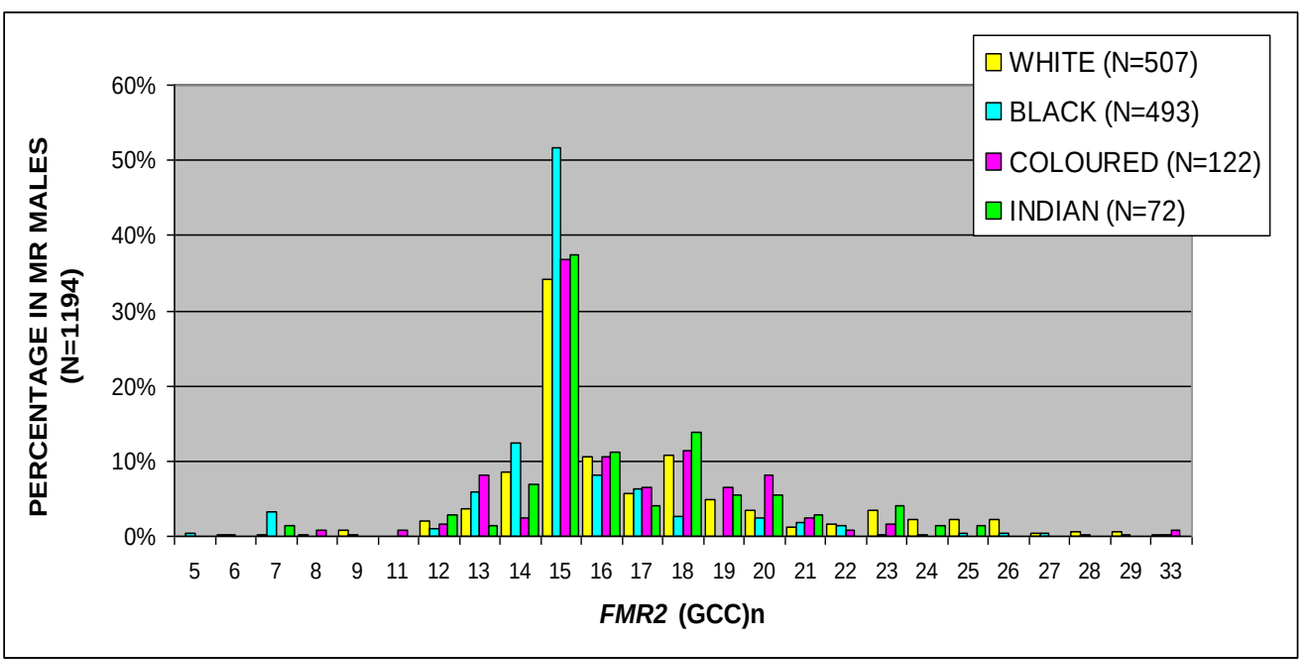


Figure 4.3 Histogram showing the distribution of *FMR2* GCC repeats in the total cohort of 1194 *FMR1* negative MR male patients tested, classified into 4 different ethnic groups.

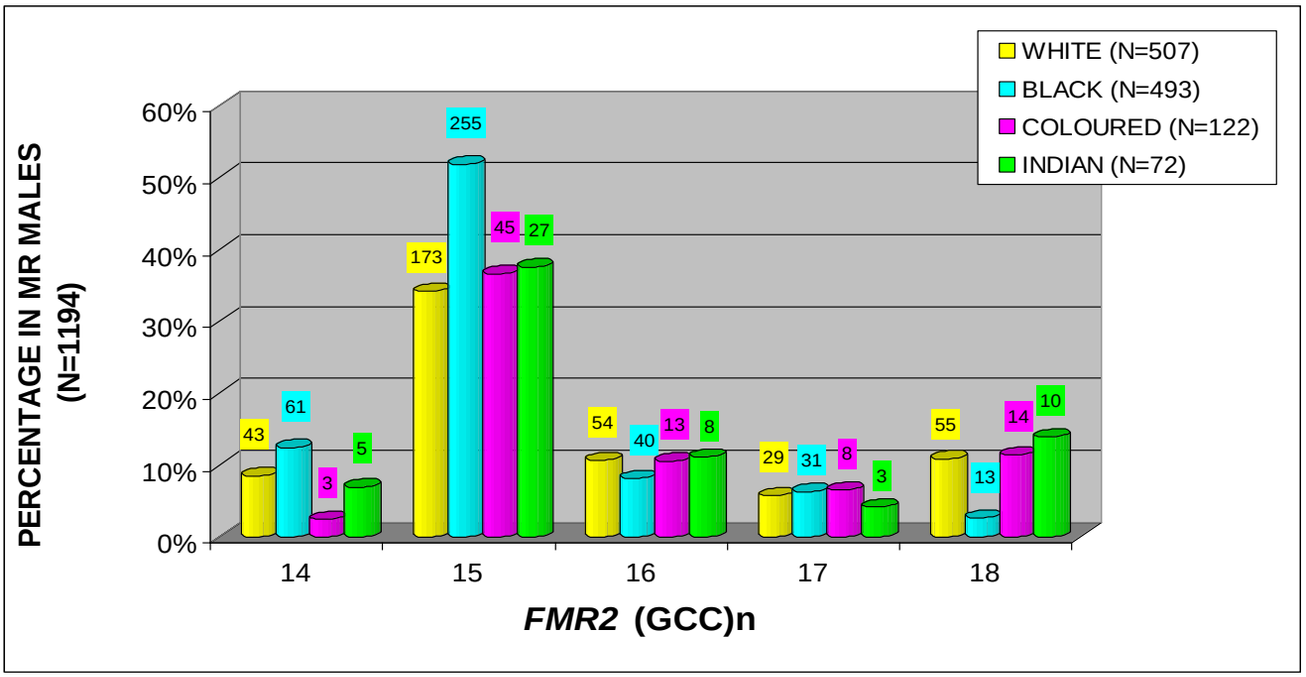


Figure 4.4 Histogram showing the common *FMR2* GCC repeats and its frequency within each ethnic group tested

4.2.4 *FMR2* allele distribution classified into different allele categories

To determine the frequency of alleles in the *FMR2* repeat ranges and assess whether it was significantly different between the different ethnic populations, allele sizes were grouped into 5 categories, Normal rare (<11 repeats), Normal common (11-25 repeats), intermediate (26-30 repeats), gray zone (31-60 repeats), premutation (61-200 repeats) and full mutation (>200 repeats). The distribution of *FMR2* alleles grouped into these categories in the 1194 MR male subjects tested is shown in Table 4.3.

Table 4.3 Distribution of *FMR2* alleles in MR males, grouped into 5 different categories and further classified into different ethnic groups.

CATEGORY	*FMR2* REPEATS	WHITE		BLACK		COLOURED		INDIAN	
Normal rare	<11	7	1.4%	20	4.1%	1	0.8%	1	1.4%
Normal common	11-25	480	94.7%	466	94.5%	120	98.4%	71	98.6%
Intermediate	26-30	19	3.7%	6	1.2%	0	0.0%	0	0.0%
Gray zone	31-60	1	0.2%	1	0.2%	1	0.8%	0	0.0%
Premutation	61-200	0	0.0%	0	0.0%	0	0.0%	0	0.0%
Full mutation	>200	0	0.0%	0	0.0%	0	0.0%	0	0.0%
TOTAL		**507**	100.0%	**493**	100.0%	**122**	100.0%	**72**	100.0%

The majority of alleles in each of the ethnic groups were found in the 11-25 *FMR2* repeat group, classified as the normal common group (94.7% in Whites, 94.5% in Blacks, 98.4% in Coloureds

and 98.6% in Indians). Statistical analysis was performed using Pearson's Chi-square (χ^2) test to compare the distribution of the different categories between the different ethnic populations. A highly significant difference (p value = 0.0019) was observed when the category distribution of the White population was compared with the Black population. There was no significant difference observed between the category distribution of White vs. Coloured (p=0.1959), White vs. Indian (p=0.2296), Black vs. Coloured (p=0.1806), Black vs. Indian (p=0.311) and Coloured vs. Indian (p=0.6932).

4.2.5 Distribution of *FMR2* alleles in institutionalized subjects

Holden, Julien-Inalsingh, Chalifoux et al. (1996a) undertook testing in institutionalized males in Ontario, Canada. A total of 397 individuals from 2 institutions (61.1% being severely to profoundly affected), all of whom were negative for the *FMR1* expansion were tested for the *FMR2* expansion. The distribution of *FMR2* GCC repeat numbers ranged from 5-38 and one profoundly retarded male was found to have a deletion of about 40 bp. These results are shown in Table 4.4 and a comparison is made with the distribution found in the 125 institutionalized MR males (described in Section 2.2.2) tested in this study.

Table 4.4 A comparison of *FMR2* alleles in institutionalized MR male individuals.

***FMR2* ALLELE RANGE**	**HOLDEN STUDY (1996a)**	**THIS STUDY (INSTITIONALIZED MALES)**
5-9	0	1 (0.8%)
10-14	28 (5.6%)	33 (26.4%)
15-19	366 (73.8%)	82 (65.6%)
20-24	74 (14.9%)	9 (7.2%)
25-29	20 (4.0%)	0
30-38	5 (1.0%)	0
>38	0	0

Statistical analysis using Fisher's exact test and Pearson's Chi-square test showed that there was a highly significant difference between the distributions of *FMR2* alleles in the Holden study cohort and this study cohort of institutionalized MR males, with a p value of $2.176e^{-10}$ and $1.629e^{-12}$ respectively.

4.3 DISCUSSION OF VALIDATION OF THE *FMR2* MULTIPLEX PCR ASSAY

The PCR assay can be used as an initial screen to exclude an *FMR2* expansion by demonstrating the presence of a single allele in the normal range in males or heterozygosity within the normal range in females. The limitation with this PCR assay is that it does not

distinguish between a female who is truly homozygous at the *FMR2* locus and a carrier and affected female, who would have one normal sized allele and a second pre- or full mutation sized allele not amplified by PCR.

Normal-full mutation *FMR2* mosaics may give false negative results (Nolin, Glicksman, Houck et al., 1994). PCR is sensitive enough to preferentially amplify the normal alleles in mosaic males even though they may be present in a low percentage of cells. In order to eliminate the small possibility of mosaicism, it is essential to screen all samples with a family history of the disease or a very strong clinical suspicion by Southern blotting. The absence of PCR amplification should never be interpreted as confirming the presence of a full mutation, but confirmation by Southern blot analysis should always be carried out. This is also essential for the diagnosis of female carriers.

4.4 DISCUSSION OF SUBJECTS SCREENED FOR *FMR2*

This is the first study conducted on South African males with MR which provides information on the range of the *FMR2* GCC repeat alleles identified.

4.4.1 FRAXE syndrome prevalence

A total of 1194 male subjects who tested negative for the *FMR1* expansion were screened for FRAXE syndrome. None of these patients were found to have a large *FMR2* expansion. The data obtained in this study suggest that either FRAXE syndrome is very rare or that it is associated with a phenotype not represented in the subjects tested. The latter is unlikely as the cohort of individuals tested comprised of patients who were originally referred for fragile X MR syndrome testing and presented with a range of intellectual or behavioural problems. Thus this population is expected to include a significant number of mild MR and would be representative as *FMR2* is associated with mild MR (Gecz, 2000).

This study is comparable to a number of studies (summarized in Table 4.5) in which FRAXE syndrome was found to be extremely rare. Gecz (2000) in his review mentions that FRAXE syndrome has an estimated population prevalence of 1 in 23 500. This frequency is based on 2 large population based studies undertaken on special education needs cases in an attempt to estimate *FMR2* full mutations. Crawford et al. (1999) found no *FMR2* expansion among a cohort of White and African American subjects (in Atlanta, USA) and Youings et al. (2000) found 1 FRAXE syndrome positive subject among 3731 MR boys. As a result, the authors suggested

that routine FRAXE screening was not warranted due to the rarity of the *FMR2* expansion mutation.

Other smaller studies undertaken worldwide in different ethnic populations also report on the rarity of FRAXE syndrome. A study undertaken in Germany by Holinski-Feder, Chahrokh-Zadeh, Jedele et al. (1996) reports on *FMR1* and *FMR2* screening performed in 737 patients (451 MR males and 276 females who were either MR or 1st degree relatives of MR males). None of the patients tested positive for an *FMR2* expansion. As a result of this finding and the low prevalence of FRAXE syndrome reported in the literature, the authors recommended against routine testing for *FMR2* but suggested follow-up testing only in selected fragile X MR syndrome negative subjects. Routine *FMR2* testing was subsequently discontinued in their laboratory.

The FRAXE screen undertaken in Greece by Patsalis, Sismani, Hettinger et al. (1999) identified no *FMR2* FM in 611 male subjects from a cohort of 866 individuals tested. This study further indicates that FRAXE syndrome is not a common aetiological factor among MR males, or *FMR1* expansion negative males.

In 2002, Pandey, Phadke and Mittal et al. studied 146 Indian patients (118 males, 28 females) with unexplained MR. Three males tested positive for the *FMR1* expansion and no patients were found to have the *FMR2* expansion. Chowdhury et al. (2006) found no cases of FRAXE syndrome in 294 Indian subjects with idiopathic MR.

Lesca, Biancalana, Brunel et al. (2003) reported on a French family with 3 MR individuals having the *FMR2* expansion. Russo, Selicorni, Bedeschi et al. (1998) identified two Italian families (4 FRAXE patients with moderate MR) that had a *FMR2* expansion.

Table 4.5 FRAXE syndrome screening studies and cases identified

AUTHOR	YEAR	POPULATION	SCREEN FOR	GCC ALLELE RANGE	NO. OF INDIV TESTEDϕ	*FMR2* FM
Wang	1993	UK	FRAXA/FRAXE	NA	525	1
Allingham-Hawkins	1995	Canada	FRAXE	7-35	300	None
Chen	1995	Taiwan	FRAXA/FRAXE	NA	273	1
Biancalana	1996	France	FRAXE	NA	NA	3
Holden	1996	USA	FRAXE	5-38	397	None*
Holinski-Feder	1996	Germany	FRAXE	NA	737	None
Meadows	1996	USA	FRAXE	3-35	462	None
Murray	1996	UK	FRAXA/FRAXE	4-39,87	760	None
Zhong	1996	US, China, Finland	FRAXE	4-39	665♣	None
Brown	1997	USA	FRAXA/FRAXE	3-42	953	None*
Mazzocco	1997	USA	*FMR1*/*FMR2*#	NA	1014	None
Mila	1997	Spain	*FMR1*/*FMR2*#	NA	222	1
Chan	1998	China	FRAXA/FRAXE	NA	73	None
Mazzocco	1998	USA	*FMR1*/*FMR2*#	1-43, 66	534	None*
Russo	1998	Italian				4
Crawford	**1999**	**USA**	**FRAXA/FRAXE**	**7-54**	**4779**	**None**
Patsalis	1999	Greece and Cyprus	FRAXA/FRAXE	7-30	866	None
Youings	**2000**		**FRAXA/FRAXE**		**3731**	**1**
Pandey	2002	Indian	FRAXA/FRAXE	13-21	146	None
Lesca	2003	France	FRAXA/FRAXE	NA		3
Chowdhury	2006	Indian	FRAXA/FRAXE		294	None
This study	2009	South Africa	FRAXA/FRAXE	5-33	1194	None

(modified from Gecz, 2000)
*, small alleles indicating deletions within and /or around (GCC)n were found
ϕ including females
♣ including known *FMR1* full mutations
FM, Full mutation
NA, Data not available

The results obtained indicate that FRAXE syndrome is rare in South Africa, consistent with worldwide reports and therefore not included in routine testing at the Division of Human Genetics, NHLS, Johannesburg.

4.4.2 Distribution of normal *FMR2* alleles

A total of 1194 males with MR were screened for FRAXE syndrome and the *FMR2* allele distribution was determined. The most common allele observed was 15 GCC repeats, followed by 16 GCC repeats.

A study undertaken by Allingham-Hawkins and Ray (1995) reported on 7-35 as being the normal range, broader than originally reported by Knight et al. (1993) who reported distributions of 6-25 repeats. The study by Holden et al. (1996a) extended this range from 5-38 repeats.

Gecz (2000) reports that the modal number of 16-18 repeats is found in most populations, with differences likely due to laboratory variation and minor ethnic differences. The modal number of 15 observed in this study is a true allele and unlikely to be an underestimate. The slight variation could be attributed to ethnic differences as the majority of patients tested were SA Whites (43%) followed by SA Blacks (41%). Studies by Sharma, Gupta and Thelma (2001) and Pandey et al. (2002) showed that 15 GCC repeats was the most frequent allele observed in their sample cohort, which is similar to that observed in this study. A study by Zhong et al. (1999) showed that 18 GCC repeats was common in the Chinese population. Youings et al. (2000) undertook a 5 year survey to test for *FMR1* and *FMR2* and found that *FMR2* repeats of 15, 16 or 18 are common. This was also reported by Murray et al. (1996) who tested 992 boys with unexplained learning difficulties.

When comparing the distribution of alleles grouped into the different *FMR2* categories, a significantly shifted distribution was observed between the institutionalized males from Holden et al. (1996a) and this study. A possible explanation could be due to the different ethnic groups tested. The institutionalized MR males are Black individuals while those from the Holden study were presumably predominantly White as they were from Ontario, Canada. This difference is not surprising as a highly significant difference was observed when the category distribution of the White MR population was compared with the Black population (Table 4.3.).

When comparing the distribution of alleles grouped into different categories between each of the ethnic populations tested in this study, no significant difference was observed. However, when the category distribution of the White population was compared with the Black population, a highly significant difference was observed. This shifted distribution observed in our study has been previously observed with other triplet repeat expansion disorders such as DM. Goldman, Ramsay and Jenkins et al., (1995) reported on South African Negroids who have a CTG allelic distribution that is significantly different from that in Caucasoids. Significant differences were found in allele and haplotype distributions in the Caucasoid DM and non-DM chromosomes and Negroid non-DM chromosomes (Goldman, Krause, Ramsay et al., 1996).

4.4.3 Intermediate/gray alleles

Three patients in this study were found to have an *FMR2* repeat size of 33, which is in the gray range. A study undertaken in by Murray et al. (1996) in the MR population of boys from Wessex, England (760 individuals) showed that there was an excess of intermediate/premutation *FMR2* (31-199 repeats) alleles. They suggested that alleles greater than 30 *FMR2* repeats, which accounted for 0.76% of their sample, were rare and should be regarded as potentially unstable. Further, they suggested that these repeats may play some role in MR.

Crawford et al. (1999) tested a diverse population (4779 individuals) of Atlanta, USA. In contrast to the findings of Murray et al. (1996), they found no evidence for an excess of intermediate/PM alleles at the *FMR2* locus. They disputed the association of intermediate alleles with MR and suggested that more studies with larger numbers are needed to resolve this.

It would be recommended to study other related family members of the 3 patients with intermediate alleles to investigate the meiotic stability or instability of this allele. Unfortunately it was not possible as this was a retrospective study.

4.5 SUMMARY

The results obtained from the screening of MR males in South African indicate that FRAXE syndrome, caused by *FMR2* repeat expansions, is not common in South Africa. As a result, diagnostic testing for FRAXE syndrome has not been implemented routinely in the Division of Human Genetics at the NHLS. It is however being used in the multiplex *FMR1*/*FMR2* PCR assay as a control for PCR failure and an indicator for an *FMR1* expansion. If the *FMR2* allele fails to amplify on PCR analysis, further testing could be pursued. Its value is also demonstrated when used as a linked marker in tracking the *FMR1* expansion in families.

CHAPTER FIVE

5 *XNP* AND *ARX* RESULTS AND DISCUSSION

This project was undertaken mainly to investigate the contribution of NS-XLMR genes in the MR population. In addition to the *FMR2* gene, the contribution of 2 other genes, *XNP* and *ARX* to XLMR was investigated in this study. Mutation screening in these genes was undertaken in male subjects with MR who tested negative for the *FMR1* expansion. The results are presented and discussed in this chapter.

5.1 RESULTS OF MUTATION SCREENING OF THE *XNP* HOTSPOT REGION

5.1.1 PCR and sequence analysis

XNP is a large gene (35 exons) but most mutations causing ATR-X syndrome and XLMR are clustered in the zinc finger (ADD) domain (Villard et al., 1997), covering exons 7, 8 and the beginning of exon 9 in which ~40-50% of known *XNP* mutations have been identified (Villard et al., 1999a; Gibbons et al., 2008).

Mutation screening was undertaken in the *XNP* gene in 210 MR male subjects (100 male referrals with MR and 110 Black institutionalized males who tested negative for the *FMR1* expansion (described in Sections 2.2.1 and 2.2.2). No variation or pathogenic mutation was found in the 'hot-spot' region tested. A single nucleotide polymorphism (SNP), c.101786C>T (Reference SNP Id rs35268552) in intron 7 of the *XNP* gene was detected in 108 (51%) subjects. This is a reported SNP with the ancestral 'C' allele reported to occur at a frequency of 87.5% and the 'T' allele at 12.5% in the Yoruba population, which is considered representative of an African population (dbSNP website, 2009).

5.2 DISCUSSION OF MUTATION SCREENING OF THE *XNP* HOTSPOT REGION

This study was prompted by the study undertaken by Villard et al. (1999a) who reported on mutation analysis of the *XNP* gene using direct sequencing of the 300 bp zinc finger coding region, spanning exons 7, 8, and 9. The authors identified 6 mutations (28%) in 21 MR male patients who had facial appearances typical of ATR-X but did not necessarily have urogenital abnormalities or HbH inclusions (see Section 1.5.5.1), features which are commonly found in

patients affected with ATR-X syndrome. They concluded that this method was suitable for screening males who have severe mental retardation associated with hypotonic facies.

As the gene is large, systematic testing of the whole coding region was not practical. As a result, this study concentrated the mutation detection effort in the *XNP* hotspot region. No mutations were identified in 210 MR male subjects tested. These findings show that there is a low frequency of mutations in the hotspot region of the *XNP* gene in South African males with an unknown cause of MR. However, this may not be a true reflection of all the mutations present in other regions of the *XNP* gene as has been reported in the literature where a family with borderline to moderate MR and no obvious facial dysmorphisms was shown to have a missense mutation (c.5069C>T = p.T1621M. Numbering according to GenBank accession number U75653) in exon 18 of the *XNP* gene (Yntema, Poppelaars, Derksen et al., 2002b). The authors suggested screening for *XNP* mutations in males with all degrees of MR and not just patients with severe MR. The authors also suggested that patients with and without typical facial characteristics should be screened for *XNP* mutations. This would allow us to elucidate the function of the *XNP* protein and to identify possible genotype-phenotype correlations.

It must be noted that most mutations in *XNP* have been identified in males who present typically with features of ATR-X syndrome, including profound developmental delay, facial dysmorphism, genital abnormalities, and α thalassaemia. This is supported by our family studies, presented in Chapter 7, where 3 families studied to date have mutations identified in the *XNP* gene. The 210 MR subjects screened in this study were not selected for these clinical criteria, which may further contribute to the lack of mutations identified in our cohort. Gibbons et al. (2008) reported that the proportion of XLMR that can be accounted for by *XNP* mutations has not been fully determined and that further studies are required.

5.3 SUMMARY OF MUTATION SCREENING OF THE *XNP* HOTSPOT REGION

The results obtained from screening MR males for mutations in the ***XNP*** hot-spot region show that this gene is not a significant contributor to NS-XLMR in South African males. Large scale screening is not cost effective in clinical practice. Routine diagnostic testing for mutations in the hotspot region in MR males with an unknown cause has thus not been implemented in the Division of Human Genetics, NHLS. When this study commenced in 2001, the general feeling was that few genes would contribute to a large amount of NS-XLMR cases. With the identification of many genes through the subsequent years, this assumption has been disproved. However, it is important to test patients with clinical features of ATR-X syndrome.

5.4 RESULTS OF *ARX* GENE 24 bp DUPLICATION SCREENING

5.4.1 PCR and sequence analysis

A total of 868 subjects (described in Section 2.4) were screened for the common *ARX* mutation, c.431-454dup24. One White patient (*ARX* patient 1) was found to have the common 24 bp duplication mutation and one Black patient (*ARX* patient 2) from the cohort appeared to have a deletion in this region. Due to technical challenges with sequencing this region as a result of the high GC content, the exact breakpoints of the deletion could not be determined. Figure 5.1 shows the duplication and deletion on an agarose gel. Figure 5.2 illustrates the corresponding sequencing result for *ARX* patient 1 showing the region duplicated.

Due to the high GC-content of the *ARX* region, the primers (GC-content of 70%) and the existence of secondary structures, PCR optimisation proved rather challenging. This appeared to be a common problem experienced by many authors who reported on the poor amplification and sequencing of this region (Stromme et al., 2002; Stepp, Cason, Finnis et al., 2005; de Souza Gestinari-Duarte et al., 2006). The PCR assay was optimized using the recommended Failsafe buffer J (from Epicentre Technologies) used by Gronskov et al. (2004). A screening approach using agarose gel electrophoresis was performed and the presence of the duplication was confirmed on sequencing analysis.

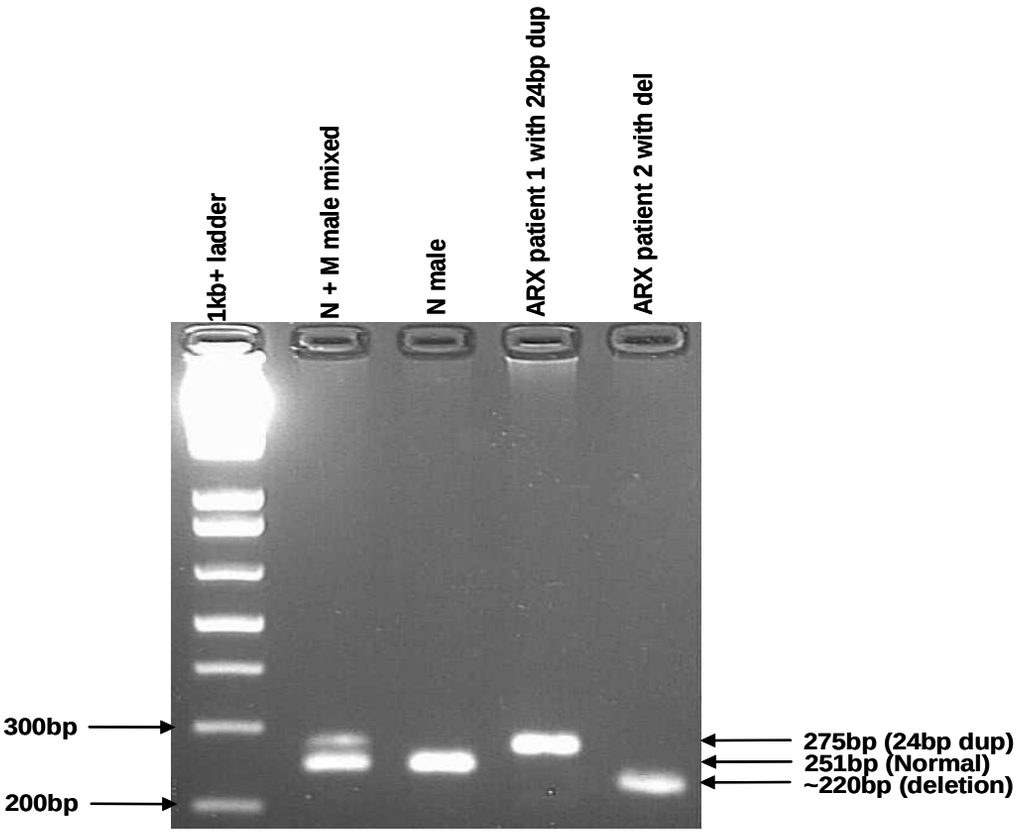


Figure 5.1 A 3% agarose gel showing the 24 bp duplication mutation and the deletion mutation identified in the *ARX* gene.
The normal fragment (N) is 251 bp and the duplicated fragment can be seen as the shifted band, representing a 275bp fragment (M). The deleted fragment is approximately 220bp

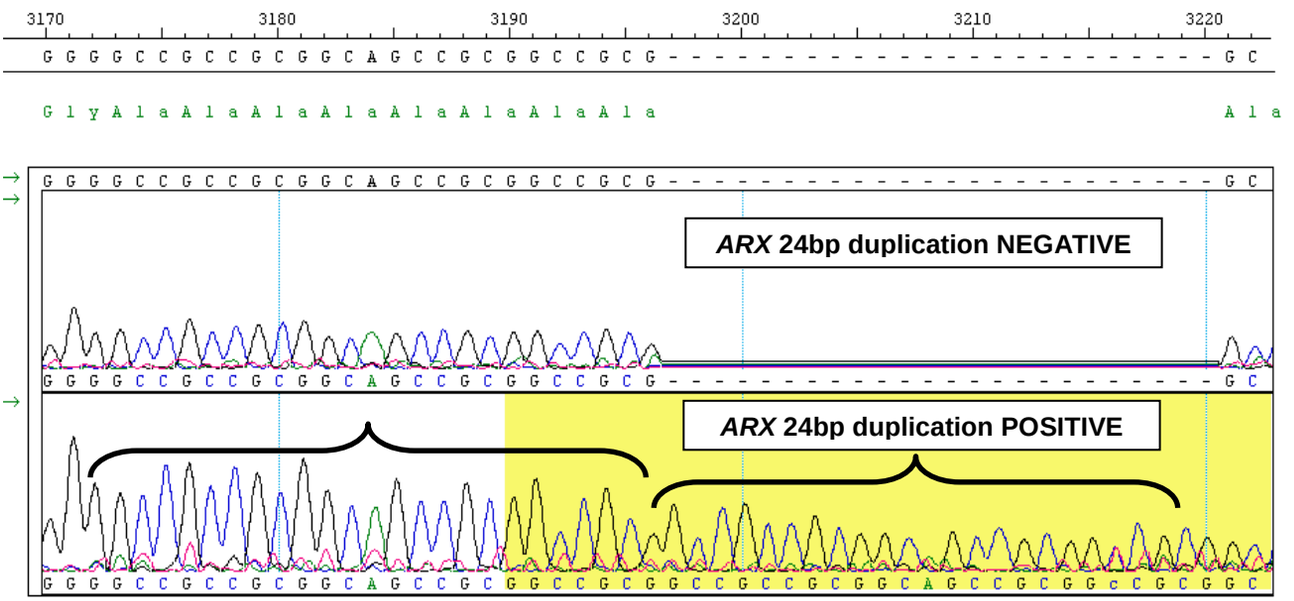


Figure 5.2 Sequencing electropherogram of the (c.431-454 dup) 24bp duplication mutation identified in *ARX* patient 1.
The duplication results in an expansion of a polyalanine tract at amino acid position 144-155 from 12 to 20 alanines.

5.5 DISCUSSION OF *ARX* GENE 24 bp DUPLICATION SCREENING

A total of 868 individuals (described in Section 2.4) were screened for the common *ARX* 24 bp duplication mutation, c.431-454dup24. Only one White patient (*ARX* patient 1) from this cohort was found to have the common 24 bp duplication mutation. This gives a frequency of 0.12% for the duplication mutation. The patient is reported to present with global developmental delay, hypotonia, mild dysmorphism and a family history of MR from the maternal side. A Black patient (*ARX* patient 2) appeared to have a deletion in the amplified region. He was reported to present with autistic features, developmental language delay, behaviour stereotypes and impaired social interaction and functioning. There was no family history of note. A follow up was not made as these 2 patients were distant referrals and were not assessed clinically at the Genetic Counselling Clinic in Johannesburg.

This study undertook to investigate whether the common 24 bp duplication mutation in the *ARX* gene is a common cause of MR in our population as *ARX* mutations have been reported to be the most frequent cause of XLMR patients after fragile X MR syndrome, although the true prevalence remains to be establised (Gecz et al., 2006). Many authors have recommended testing males with NS-XLMR and S-XLMR who have tested negative for fragile X MR syndrome for the 24 bp duplication mutation, especially in the presence of dystonic hand movements, spasticity and epilepsy (Suri, 2005; de Souza Gestinari-Duarte et al., 2006; Nawara et al., 2006). Partington, Turner, Boyle et al. (2004) estimated the frequency of the duplication mutation to be 1 in 12 000 males, or approximately one-third the rate of *FMR1* mutations. Poirier et al. (2006) concluded that *ARX* mutations are found in 9.5% of XLMR families and 2.2% of affected brother pairs.

In contrast, Gronskov et al. (2004) showed that the recurrent mutations in *ARX* appear to be less frequent than previously thought in NS-XLMR cases in Denmark. They recommended that screening is not warranted in a child with MR without any family history. This finding was further supported by a study undertaken by Rujirabanjerd, Tongsippunyoo, Sripo et al. (2007) who undertook *ARX* mutation screening of the *ARX* gene in Thai paediatric patients with delayed development.

Ropers in his review (2006) mentions that the frequency of mutations in the *ARX* gene was much lower (0% to 5%) in smaller families (2.2% in affected brother pairs and 0.13% in sporadic males with MR). Our findings support these studies as we have shown that the frequency of this 24 bp duplication is 0.12% and not as common in our population of MR males as has been observed in other populations. This low frequency may be as a result of sample selection bias. Rujirabanjerd et al. (2007) indicated that this low frequency seen may be explained by the

different ethnic backgrounds in the world which may have different mutations rates. Our sample cohort consisted of 34.4% (299) Whites, 47.6% Blacks (413), 11.3% Coloureds (98) and 6.7% Indians (58). The duplication mutation was identified in a White patient and the deletion mutation in a Black patient. These numbers are too small to make any significant conclusion on the frequency in any particular ethnic group.

5.6 SUMMARY OF *ARX* GENE 24 bp DUPLICATION SCREENING

The results obtained from screening the *ARX* gene for the common 24 bp duplication mutation show that the mutation is rare in South African males with MR of unknown cause. It must be emphasized that this is a small study and one cannot draw conclusions about the frequency of *ARX* in NS-XLMR, given the genetic heterogeneity of this condition. Routine diagnostic testing for this mutation in MR males will not be implemented in the Division of Human Genetics, NHLS. However, males with S-XLMR who have tested negative for fragile X MR syndrome and who present with dystonic hand movements, spasticity and epilepsy or a strong family history, in line with other studies, may be considered for testing.

CHAPTER SIX

6 FRAGILE X MR SYNDROME FAMILY STUDIES

6.1 FRAGILE X MR SYNDROME POSITIVE FAMILY (FRAXA FAMILY 1) RESULTS

A large family positive for the *FMR1* expansion, consisting of 24 members was investigated. The pedigree is illustrated in Figure 6.1 together with results of *FMR1* expansion testing and haplotype analysis. As an incidental finding during a routine investigation for fragile X syndrome, an interesting result was found. Two siblings were found to have two expanded *FMR1* alleles in the intermediate and premutation range respectively. The segregation of this expansion in the family was examined using haplotype analysis. As this is the first South African report of females with 2 expanded *FMR1* alleles and the fifth family reported thus far in the world (Mazzocco and Holden, 1996), it has been presented in this study.

6.1.1 PCR and Southern blot results

The alleles generated from the PCR reaction in the two sisters who were found to be homozygous for the *FMR1* expansion are shown in Figure 6.2. The first female (III-10) was found to be a compound heterozygote for 2 *FMR1* alleles in the intermediate/gray range, 51 and 57 repeats respectively. Her sibling (III-12) was found to have one allele in the intermediate range and one in the premutation range 51 and 69 repeats respectively

The parents of these female siblings were both found to be carriers of *FMR1* expansions in the intermediate/gray range. The father (II-5) carries an allele of 51 repeats and their mother is a carrier of 57 repeats. Both siblings inherited the 51 repeat from their father which did not expand, as expected in a male transmission. The 57 repeat from their mother was inherited stably in III-10 but was shown to have expanded to 69 repeats in III-12 and subsequently expanded to a full mutation in III-12's son, IV-12.

Although III-10 inherited the 57 repeat from her mother stably, the repeat was shown to be unstable in the next generation as it had expanded to 67 repeats in her daughter, IV-6. Her other three children, IV-7, IV-8 and IV-9 were shown to have inherited the 51 repeat which had expanded to 52 repeats. Southern blot analysis was performed which confirmed the PCR results. Figure 6.3 shows the autoradiograph obtained after the blotting procedure.

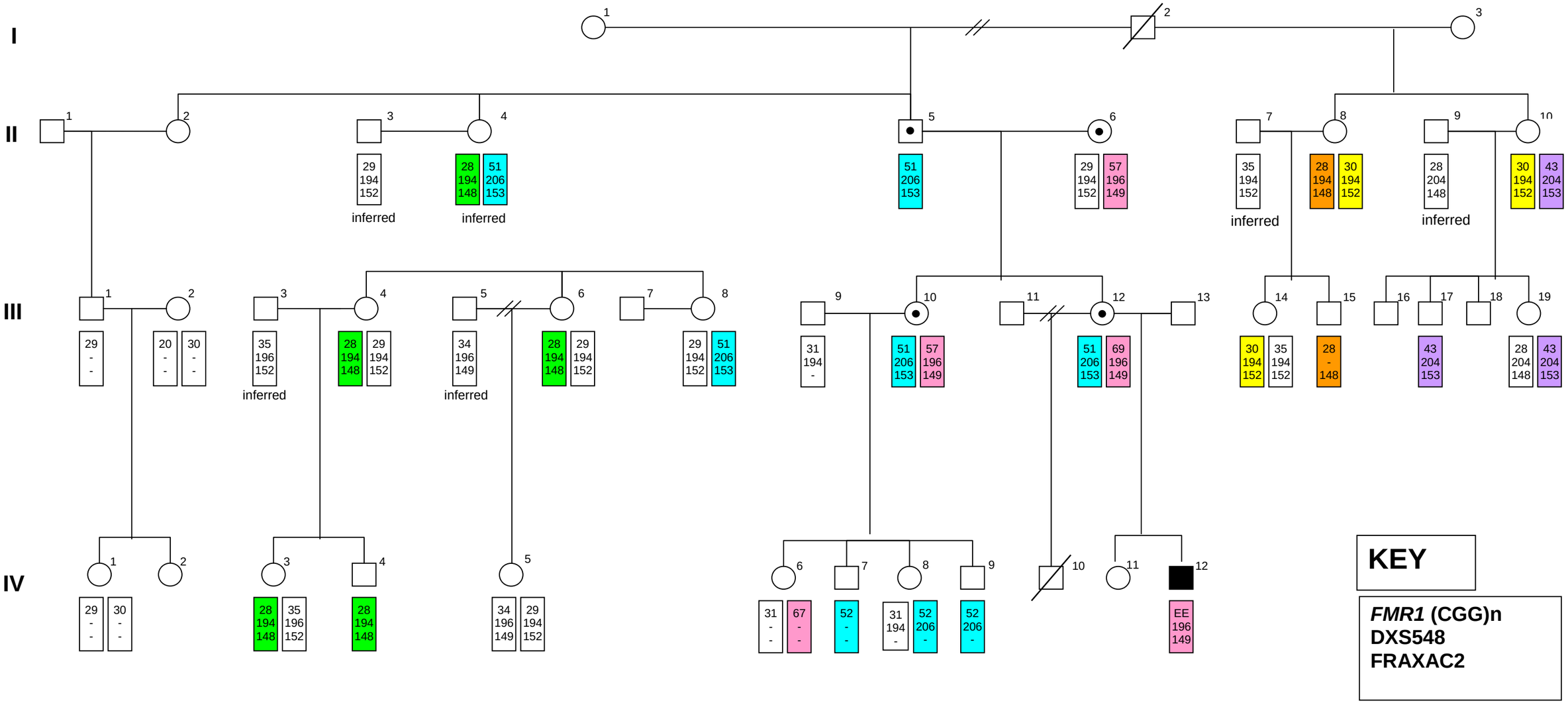


Figure 6.1 Family pedigree of *FMR1* expansion positive family (FRAXA family 2) tested for *FMR1* expansion and at 2 intragenic markers, FRAXAC2 and DXS548.
Haplotype analysis was performed to track the expansion in the family. The *FMR1* CGG allele and haplotype results are indicated below each of the members tested. A dash (-) indicates an unsuccessful result or not tested for.

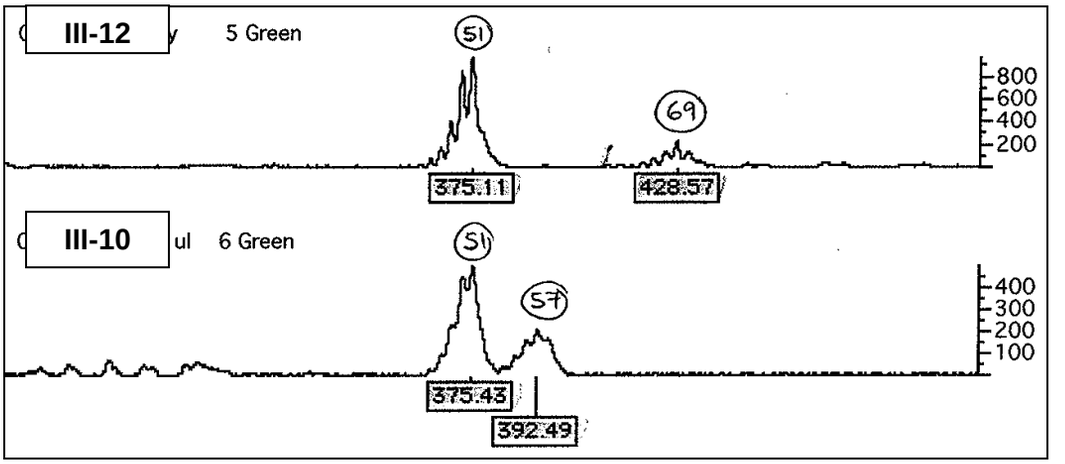


Figure 6.2 *FMR1* alleles on PCR showing two females compound heterozygous for *FMR1* expansions.
III-12 has alleles of 51/69 repeats and III-10 has alleles of 51/57 repeats.

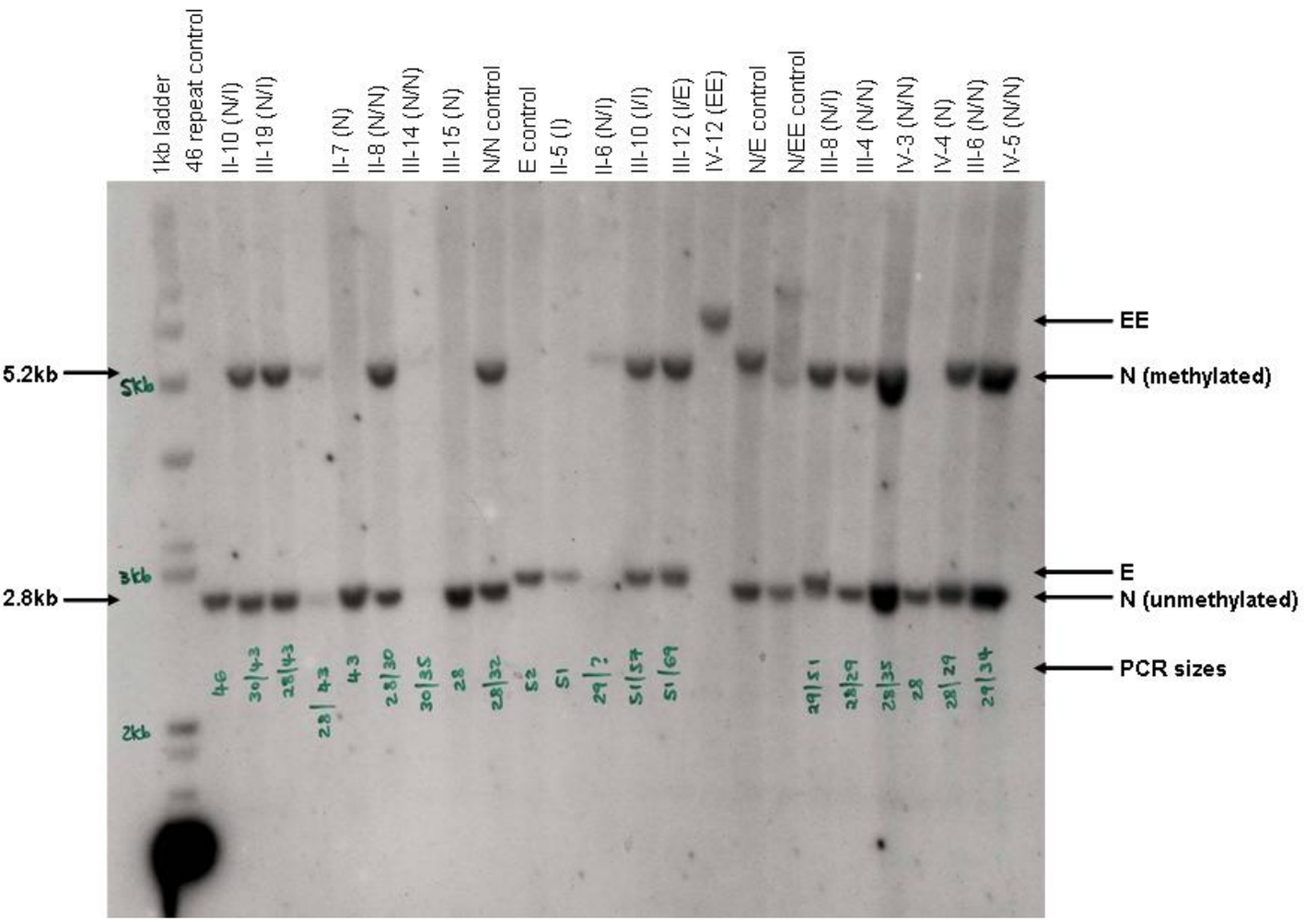


Figure 6.3 Southern blot autoradiograph of *FMR1* expansion positive family 1 produced by probing Southern blots from *Eco*RI and *Ecl*XI digests with the StB12.3 probe.
N (normal) = 5-40
I (intermediate/gray zone) = 41-58
E (premutation) = 59-200
EE (full expansion) = >200 CGG repeats

6.1.2 Haplotype results

Further investigations were undertaken using haplotype analysis to examine the segregation of this expansion. Two intragenic markers, DXS548 and FRAXAC2, previously optimised by Goldman (1997) in the Division of Human Genetics Laboratory, NHLS, were used to track the disease chromosome in the family. The results from the polyacrylamide gels are shown in Figure 6.4 and Figure 6.5 and the haplotypes are shown in the pedigree in Figure 6.1

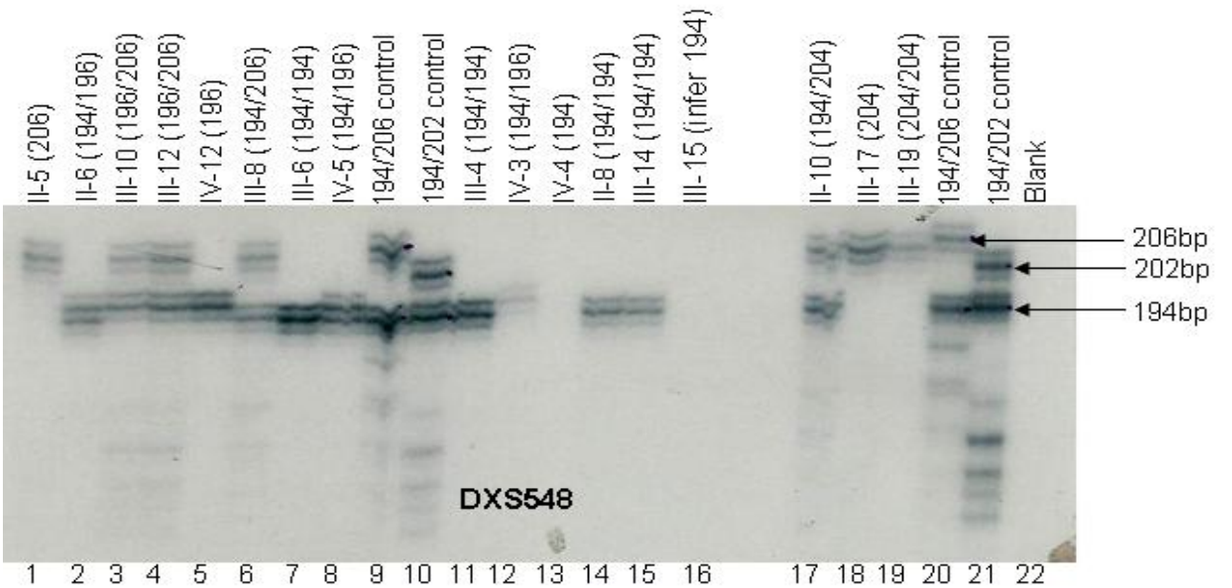


Figure 6.4 Autoradiograph of the DXS548 marker in *FMR1* expansion positive family 1.
The PCR fragment was run on a 6% denaturing polyacrylamide gel and exposed to an X-ray film for 24 hrs.

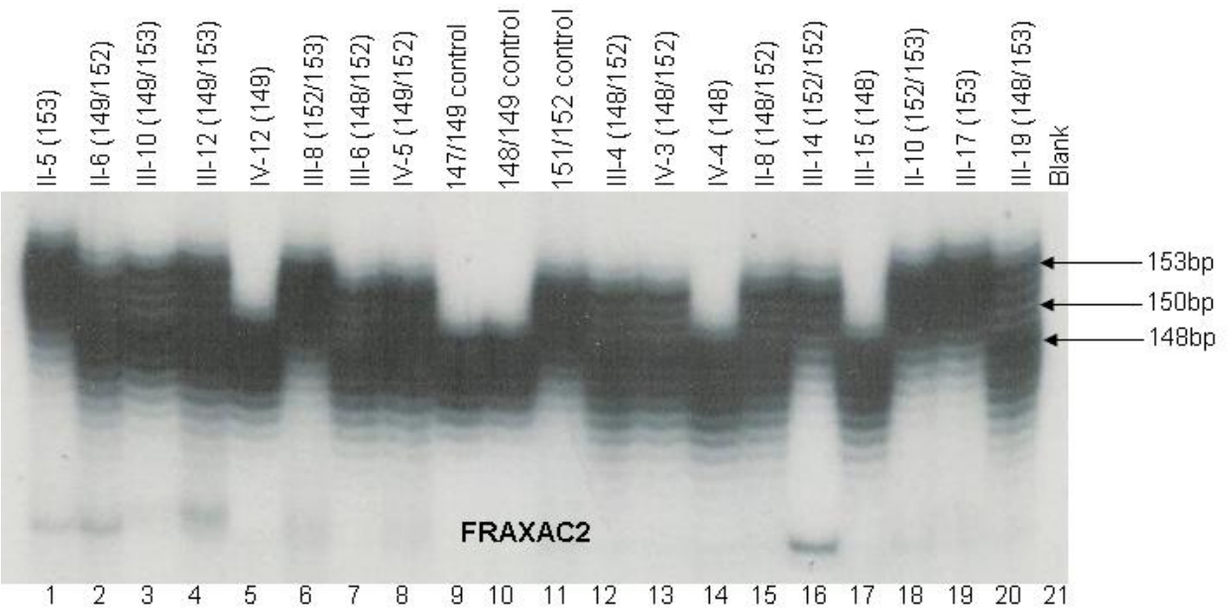


Figure 6.5 Autoradiograph of the FRAXAC2 marker in *FMR1* expansion positive family 1.
The PCR fragment was run on a 6% denaturing polyacrylamide gel and exposed to an X-ray film for ~1 hr.

The *FMR1* full expansion (EE) tracks with allele 196 bp of the DXS548 marker and allele 149 bp of the FRAXAC2 marker as seen in the affected proband IV-12. The X chromosome with the *FMR1* intermediate allele tracks with allele 206 bp of the DXS548 marker and allele 153 bp of the FRAXAC2 marker and appears to be stably inherited in the family.

6.2 *FMR1* EXPANSION POSITIVE FAMILY (FRAXA FAMILY 1) DISCUSSION

This family was referred for fragile X MR syndrome testing and 2 siblings were found to be compound heterozygous for *FMR1* expansions. III-10 was found to have 51/57 CGG repeats and her sibling, III-12 was found to have 51/69 CGG repeats. This is the first such case described in South Africa and the fifth family in the world which has been described (Mazzocco and Holden, 1996).

Li, Malarchok, Martinez et al. (1995) reported on a female homozygous for the PM with no MR or physical features typical of fragile X MR syndrome. Mila, Castellvi-Bel, Gine et al. (1996a) reported on a moderately MR female (IQ 53) with characteristic facial appearance and a short attention span with a PM and a FM. An extended family screen detected 33 normal individuals, 14 carriers of a PM (1 male, 13 females) and 18 individuals with a FM (8 males, 10 females).

Mazocco and Holden (1996) reported on 3 adult sisters who were compound heterozygotes for *FMR1* PMs. Their study aimed to examine whether the sisters manifested a neuropsychological phenotype that was similar to that reported for women affected by fragile X MR syndrome. They concluded that the profiles observed in the 3 females were consistent with reports that the *FMR1* PM does not affect cognitive performance.

Linden, Tassone, Gane et al., (1999) reported on a 15 year old female with fragile X MR syndrome with an *FMR1* FM of 363 repeats and a PM of 103 repeats. She was shown to have inherited *FMR1* repeats of 146 and 98 from her mother and father respectively. The female presented with mild to borderline MR and her behavioural characteristics were typical of females with fragile X MR syndrome.

This study examined the *FMR1* CGG repeat length in a large family. Clinical data were not available but we would expect the 2 sisters not to present with MR. As would be expected, it was established on further investigation that the parents of these female siblings were carriers of the *FMR1* expansion in the intermediate/gray range.

The expansion of the 57 repeat in III-10 to a PM allele of 67 repeats in her daughter IV-6 has implications for this female as she has a 5.3% risk of having children affected with fragile X MR syndrome (see Table 6.1) and an odds ratio of 6.9 of developing POF (Table 6.2). She also has an increased risk for FXTAS, although the precise risk has not yet been defined but it is lower than that reported for males (Hagerman and Hagerman, 2004; Biancalana, Toft, Le Ber et al., 2005).

Her other 2 sons, IV-7 and IV-9 and her daughter IV-8 were shown to have inherited the 51 repeat which had expanded to 52 CGG repeats. The clinical significance of carrying an allele in this intermediate size range is unclear. There is an increased risk of expansion compared to normal alleles, but expansion into the full mutation range is very rare (Morris et al., 1995). The number of "pure" CGG repeats without interrupting AGG repeats is a good predictor of repeat instability, especially in intermediate alleles at the upper end. This was not determined in these individuals. Eichler et al. (1994) reported on longer pure repeats, especially those with more than 35 uninterrupted CGG repeats to become more unstable.

The smallest repeat known to expand to a full mutation in a single generation was 59 repeats (Nolin et al., 2003). The odds ratio of POF in IV-8 is estimated to be 2.5 (Table 6.2) although there appears to be no consensus for estimating an absolute risk for POF.

Table 6.1 Risks for expansion from a maternal premutation to a full mutation when transmitted to offspring.

NUMBER OF MATERNAL PREMUTATION CGG REPEATS	TOTAL MATERNAL TRANSMISSIONS	EXPANSIONS TO FULL MUTATIONS (%)
55-59	27	1 (3.7%)
60-69	113	6 (5.3%)
70-79	90	28 (31.1%)
80-89	140	81 (57.8%)
90-99	111	89 (80.1%)
100-109	70	70 (100%)
110-119	54	53 (98.1%)
120-129	36	35 (97.2%)
130-139	18	17 (94.4%)
140-200	19	19 (100%)

(GeneReviews website, accessed online September 2009).

Table 6.2 Odds ratio for POF by intermediate and premutation size.

INTERMEDIATE/PREMUTATION SIZE IN CGG REPEATS	ODDS RATIO FOR POF
41-58	2.5
59-79	6.9
80-99	25.1
>100	16.4

(GeneReviews website, accessed online September 2009).

PM carriers are reported to have increased *FMR1* mRNA levels in leucocytes and this toxic RNA gain-of-function mechanism has been proposed for the pathogenesis of FXTAS and may also be a cause of POF (reviewed in Brouwer et al., 2009b). Female PM carriers are less likely to develop FXTAS and tend to present with a less severe phenotype compared to males with a PM, most probably due to the presence of a normal *FMR1* allele. Both female siblings in this study (III-10 and III-12) were found to be compound heterozygous for the *FMR1* expansion with no normal *FMR1* transcript, it is unknown if this increases the risk of FXTAS and POF.

Anticipation is demonstrated to occur in this family as seen in IV-12 who has inherited the larger PM which has expanded to a full mutation. In compound heterozygous females, it is expected that the larger allele is more unstable than the smaller allele.

Haplotype analysis was performed at the *FMR1* locus and the high-risk X chromosome was determined. Haplotype studies performed previously by Goldman et al. (1997) showed that 2 haplotypes 194-154 DXS548-FRAXAC2 and 204-153 DXS548-FRAXAC2 accounted for 61% of Caucasoid disease chromosomes. The haplotype in the study family is 196-149 DXS548-FRAXAC2 and is not one of the common haplotypes observed in Caucasoid populations.

6.3 SUMMARY

A large family positive for an *FMR1* expansion causing fragile X MR syndrome was investigated and 2 females were found to be compound heterozygotes for 2 *FMR1* alleles. Extended family members were tested and their FRAXA status was determined. As a result of this investigation, females at risk for POF and FXTAS have been identified. The risk is uncertain in compound heterozygotes, but could be increased. Unexpected results in family analysis can sometimes alter the risk.

CHAPTER SEVEN

7 ATR-X SYNDROME FAMILY STUDIES

In addition to screening the *XNP* mutation hotspot in MR males, extensive molecular investigations were undertaken on three patients suspected clinically to have ATR-X syndrome and extended family members. The results obtained from these investigations are presented below followed by a discussion of these cases. For the naming of the mutations, mRNA accession no. NM_000489.3 and protein accession no. NP_000480.2 were used (36 exons with exon 7 spliced out). The cDNA number of +1 corresponds to the A of the ATG translation initiation codon in the reference sequence.

7.1 ATR-X PATIENT 1 RESULTS

Figure 7.1 illustrates the family pedigree of patient 1. Patient 1 (II-5) reportedly had a phenotype similar to MR brother, II-2 who had died. Patient 1 was developmentally delayed with features consistent with ATR-X syndrome. He had marked short stature, was microcephalic and entered puberty at 20 yrs of age. He had a long face, a big jaw, a cows-lick to the frontal hairline, bilateral epicanthic folds and had a soft palate cleft. He had inverted nipples, fine digits and flat feet. He had bruxism and drools. His physical appearance is shown in Figure 7.2. Haematological analysis performed at Johannesburg Hospital, special haematology laboratory showed a haemoglobin of 14.0g/dL (N=14.3-18.3), a RCC of $4.87x10^{12}$/L (N=4.89-6.11), MCV of 89.1fL (N=79.1-89.9), MCH 29.2pg (N=27.0-32.0), a haematocrit of 0.434 (N=043-0.55). “Golf balls” (HbH inclusions) were seen on the incubated reticulocyte preparation which was done at the Johannesburg Haematology Laboratory and confirmed in the UK (Dr Richard Gibbons Laboratory, University of Oxford), in keeping with the clinical diagnosis of ATR-X syndrome.

7.1.1 Screening the *XNP* hotspot region

DNA from patient 1 was sent to France (Dr L Villard, INSERM, Marseille, France) to sequence the hotspot region in the *XNP* gene. Molecular testing was undertaken concurrently in our laboratory. No mutation was identified in the hotspot region of *XNP* in exons 7, 8 and 9.

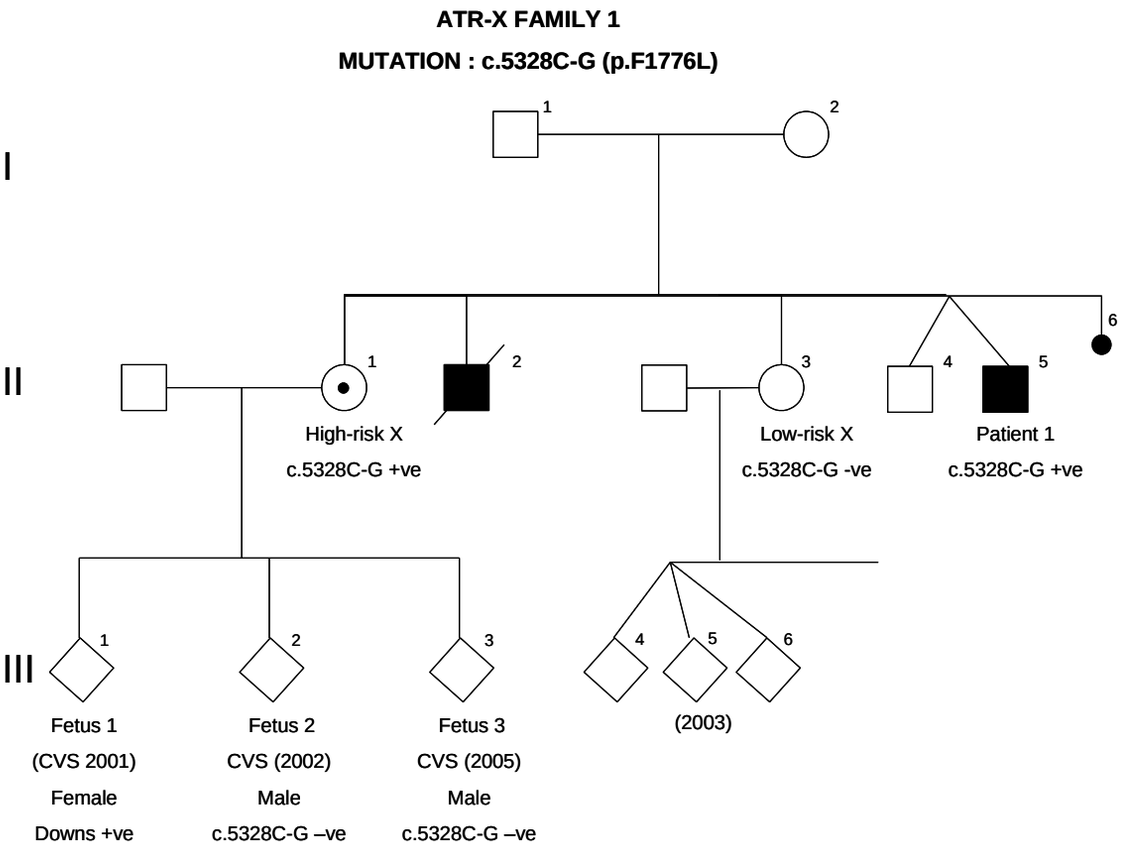


Figure 7.1 Family pedigree of patient 1 (II-5) affected with ATR-X syndrome.

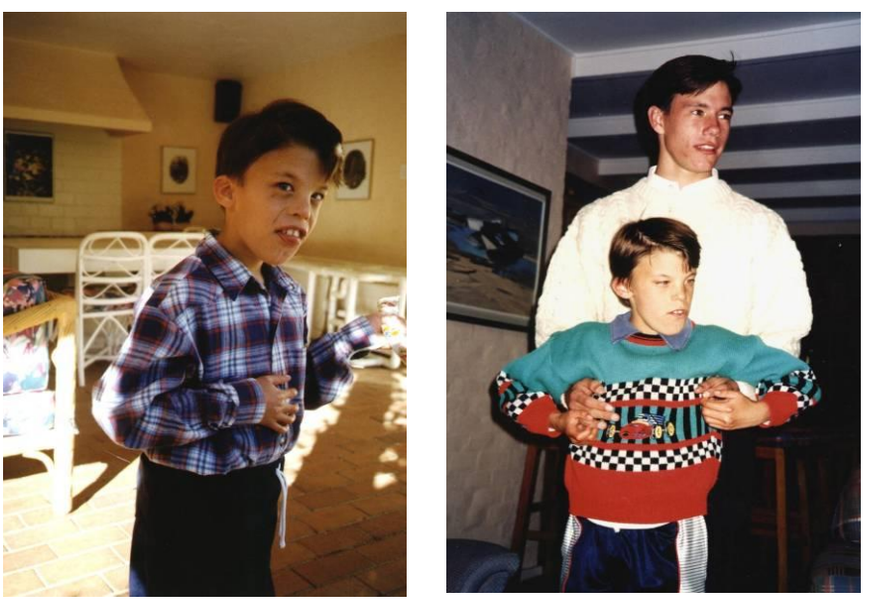


Figure 7.2 Patient 1 showing clinical features of ATR-X syndrome.
The photograph on the right shows the normal twin of the patient.

7.1.2 Testing for fragile X MR syndrome

Patient 1 was tested for fragile X MR syndrome and shown to have 30 *FMR1* CGG repeats on PCR analysis thus excluding a diagnosis of fragile X MR syndrome.

7.1.3 Prenatal investigation 1 using linked marker analysis (Fetus 1, CVS 2001)

Prenatal diagnosis was requested for the sister of ATR-X patient 1 (II-1). Since the ATR-X causing mutation had not been identified at the time, linked marker analysis was undertaken on the family in 2001 at the Kennedy-Galton Centre in England to determine the carrier status of II-1. She was found to carry the high-risk X chromosome and her sister II-3 was shown to carry the low-risk X chromosome. Chorionic villus sampling (CVS) was performed and the fetus, III-I was shown to be female on PCR testing at the SRY and amelogenin-Y (AMEL-Y) Y-chromosome specific loci. Further testing for ATR-X was therefore not indicated for the fetus. The fetus was however found to have Down syndrome and the pregnancy was subsequently terminated.

7.1.4 Mutation identification

Mutation analysis on a research basis was subsequently carried out in the UK (Dr Richard Gibbons Laboratory, University of Oxford) on ATR-X patient 1 (II-5). The mutation in the family was identified as a novel missense mutation, c.5328C>G (p.F1776L) in exon 22 of the *XNP* gene, in the C-terminal region corresponding to the helicase domain (Figure 7.3). The mutation was confirmed by restriction enzyme (RE) analysis using *Mnl*I and direct carrier testing in the patient's sisters, II-1 and II-3 was performed using the RE assay in order to confirm the results obtained by linked marker analysis. The mother of the index case was confirmed to be a carrier. Further, II-1 was confirmed to be a carrier and II-3 was confirmed not to carry the causative mutation, consistent with the linked marker results. This is shown in Figure 7.5. The primer sequences to detect the family mutation were obtained from Dr Gibbons (UK). Diagnostic testing by sequence analysis was performed to confirm the mutation in Patient I and his mother (I-2) (Figure 7.4).

ttggtaattcacttgtagatcagacttataaccatgtttattctatacttagcttgatattctttctttatagcttaattctaatgcatgtgtttttgaca
tgagcatttcattggggaatttttctaagtctttttaacgattgttaatgttgttaatgaataatgtcttctctcttagATCATTGTATGG
TTAATTTTATCAAGGAAAATTTACTTGGATCCATTAAGGAGTT**C**AGGAATAGATTTATAAATC
CAATTCAAAATGGTCAGTGTGCAGATTCTACCATGGTAGATGTCAGAGTGATGAAAAAACG
TGCTCACATTCTCTATGAGATGTTAGCTGGATGTGTTCAGgtacaaatatgttccatagtaataaaatattctt
actttttatataacaatcccaacatattttctgagcttaaagtttaataa**ttttgtgttttctttcccgc**tctttcagtagatagttttacagttata
tctccttgcgttgtcttaaaaaatttctgctgcattcaacttttaaa

Figure 7.3 Sequence of exon 20 of the *XNP* gene showing the site of the novel c.5328C>G (p.F1776L) mutation identified in Patient 1 affected with ATR-X syndrome.
The base which is mutated is highlighted in red and the primers are highlighted in blue. The lowercase letters represent the intronic region and the uppercase letters represents the exon.

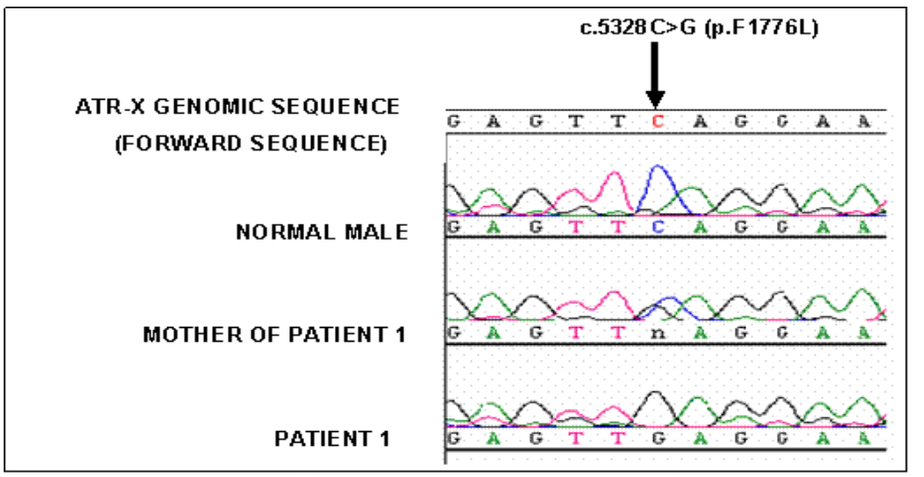


Figure 7.4 Sequence analysis in Patient 1 affected with ATR-X syndrome and his mother compared to a negative control sample.
The mutation identified in the patient is a novel missense mutation, c.5328C>G (p.F1776L) in exon 20 of the *XNP* gene.

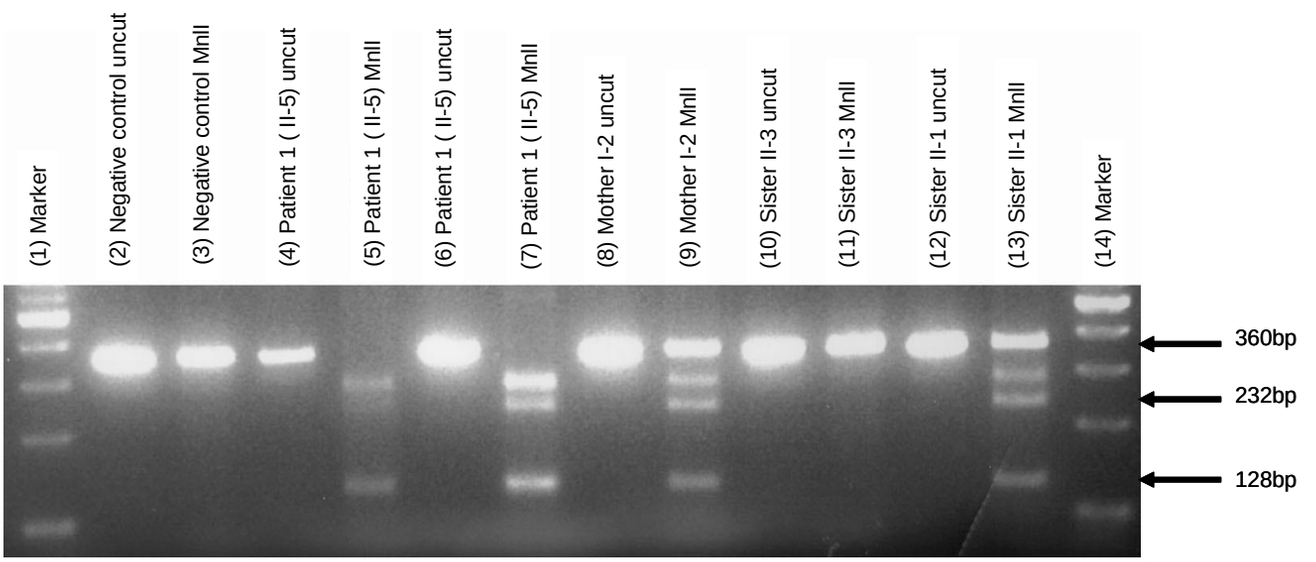


Figure 7.5 Agarose gel picture showing PCR products from patient 1 and extended family members digested with *Mnl*I enzyme to detect the 5328C>G (F1776L) mutation.
The enzyme creates a restriction site in the presence of the mutation as seen in lanes (5), (7) and (9). Lanes (2), (4), (6), (8), (10) and (12) show PCR products that were not restricted with *Mnl*I. Patient I (II-5) was set up twice, using 2 different concentrations of DNA template, as shown in lanes (5) and (7).

7.1.5 Prenatal investigation 2 (Fetus 2, CVS 2002)

A second prenatal investigation for II-1 was performed in 2002 in the Molecular Genetics Laboratory, NHLS by me. Further, the RE assay using *Mnl*I was used to confirm the prenatal investigation.

Prenatal testing was undertaken on CVS material. The fetus, III-2 was found to be male on PCR analysis at the SRY and AMEL-Y Y-chromosome specific loci and thus ATR-X syndrome testing was indicated. The fetus tested negative for the c.5328C>G (p.F1776L) mutation on RE analysis. The result from this investigation is shown in Figure 7.6.

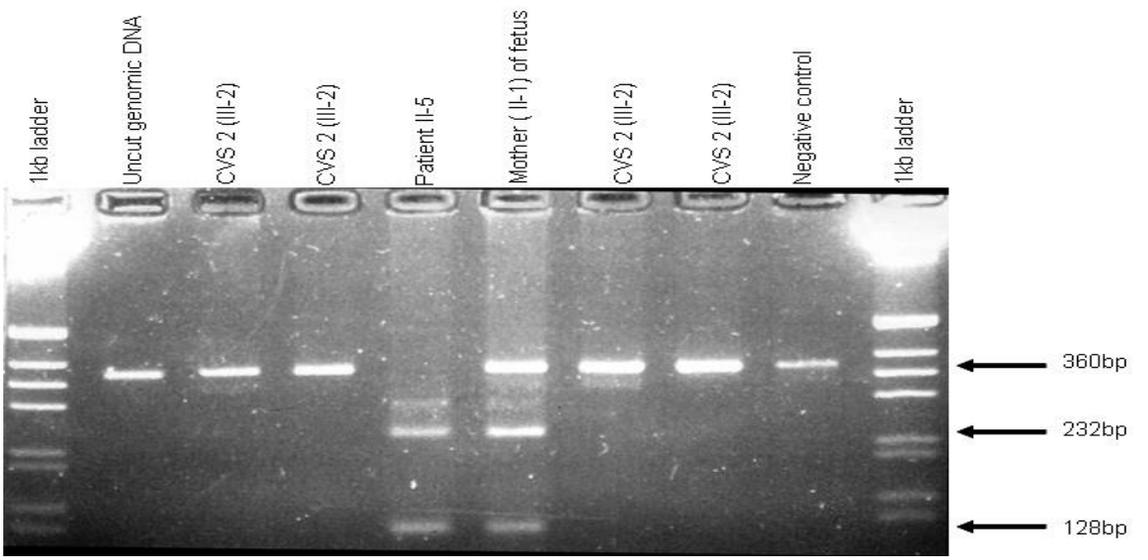


Figure 7.6 Agarose gel picture showing the result from the second prenatal investigation workup undertaken on CVS material obtained from the sister (II-1) of Patient 1 who was found to be a carrier of the c.5328C>G (p.F1776L) mutation.
Genomic DNA was digested with MnlI enzyme.

7.1.6 Prenatal investigation 3 (Fetus 3 CVS 2005)

A third prenatal investigation for II-1 was performed in 2005 in the Molecular Genetics Laboratory, NHLS under my supervision. Prenatal testing was undertaken on CVS material. The fetus, III-3 was found to be male on PCR analysis at the SRY and AMEL-Y Y-chromosome specific loci and thus ATR-X syndrome testing was indicated. The fetus tested negative for the c.5328C>G (p.F1776L) mutation on RE analysis.

7.2 ATR-X PATIENT 2 RESULTS

This patient presented with extreme hypotonia at birth, a persistently weak cry, hypoglycaemia, severe global delay, failure of speech development, microcephaly (HOC = 46 cm), brisk reflexes and mild dysmorphism comprising: spiky, thick hair, low-set rotated ears, protruding tongue, high-riding testes, mild mid-facial hypoplasia, carp-shaped mouth. Figure 7.7 shows the family pedigree and Figure 7.8 shows the clinical features of Patient 2.

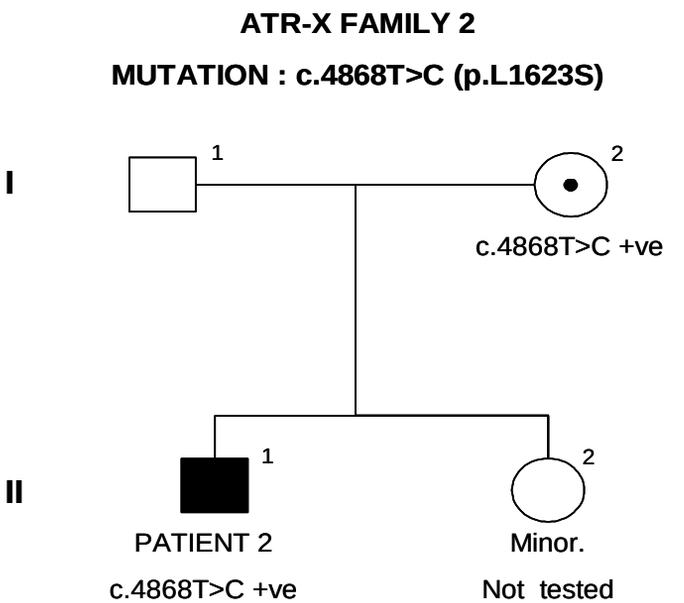


Figure 7.7 Family pedigree of patient 2 (II-1) affected with ATR-X syndrome.



Figure 7.8 ATR-X Patient 2 showing clinical features of ATR-X syndrome.

7.2.1 Testing for fragile X MR syndrome

This patient was tested for fragile X MR syndrome and shown to have 46 *FMR1* CGG repeats on PCR analysis. This is in the intermediate range and is not thought to be causative of the features in the patient (see Section 1.4.2.2).

7.2.2 Screening the *XNP* hotspot region

XNP screening of the 300 bp hotspot region was undertaken in the Molecular Genetics Laboratory, NHLS and a mutation was not identified. Mutation analysis undertaken in the UK (Dr Richard Gibbons Laboratory, University of Oxford) identified a point mutation, reported as c.4868T>C (p.L1623S) in exon 19 of the *XNP* gene (see Figure 7.9). This was a novel mutation and was confirmed to be in a highly conserved region in the helicase domain of the gene and was thus likely to be pathogenic. The mutation was subsequently confirmed in the Molecular Genetics Laboratory, NHLS on sequencing analysis as shown in Figure 7.10.

tgatacatcatctagttgaactttggcattttactatctctttccctgtgccttgcctggtttggaaattctggccgtttattgtttaagcaaaaca
aagctataatattgtaagattgcttaatttattaaatttttttgtggatatttaaacgaaggtgacaatctgttttcttatagGTGGTAAGTT
TTCTTCATACAGTTCTTTTGTGTGACAAACTGGATTTCAGCACGGCGTTAGTGGTTTGTCCT
CTTAATACTGCTTTGAATTGGATGAATGAATTTGAGAAGTGGCAAGAGGGATTAAAAGATGA
TGAGAAGCTTGAGgtaacttattttaaatgttttctattattaaaatagtcacatgaaattcatatagtaaactatttttaatttttttaatt
tttaatgttttttaaccagtgatgcatatttcagtgggaatatttaatattcacatgtttttagtagctacttaaaaaaatctttatttccccacaaa
a

Figure 7.9 Sequence of exon 19 of the *XNP* gene showing the site of the c.4868T>C (p.L1623S) mutation identified in Patient 2 (II-1).
The base which is mutated is highlighted in red. The lowercase letters represent the intronic region and the uppercase letters represents the exon.

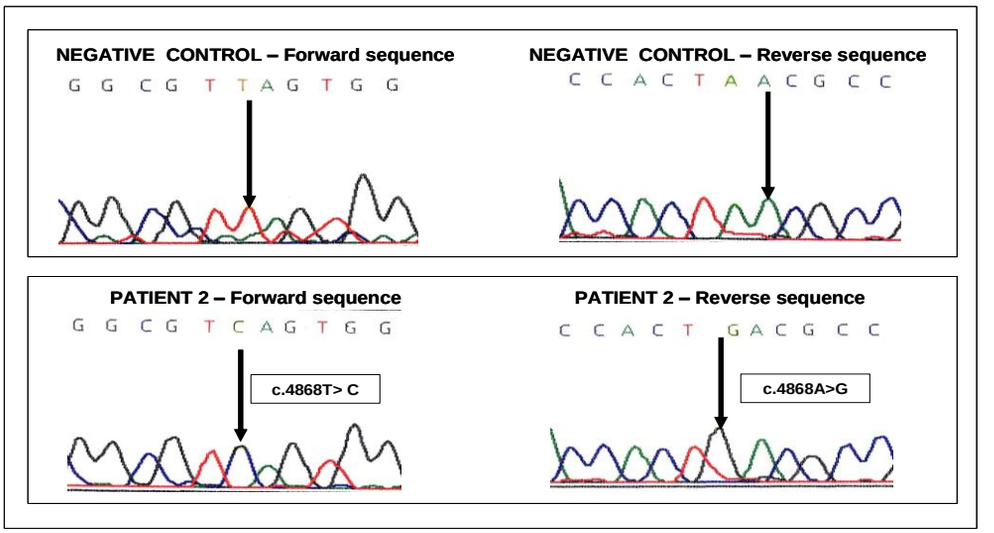


Figure 7.10 Sequence analysis in patient 2 affected with ATR-X syndrome compared to a negative control sample.
The mutation identified in the patient is c.4868T>C (p.L1623S) in exon19 of the *XNP* gene. The reverse sequence shows an A to G transition.

7.2.3 Carrier testing in mother of patient 2

DNA analysis was undertaken in the Molecular Genetics Laboratory, NHLS under my supervision in the patient's mother I-2, and she was shown to be a carrier of the mutation. The result from sequencing analysis performed in the mother can be seen in Figure 7.11. The sister (II-2) of patient 2 was not tested as she is a minor. Carrier testing will be offered to her in the future.

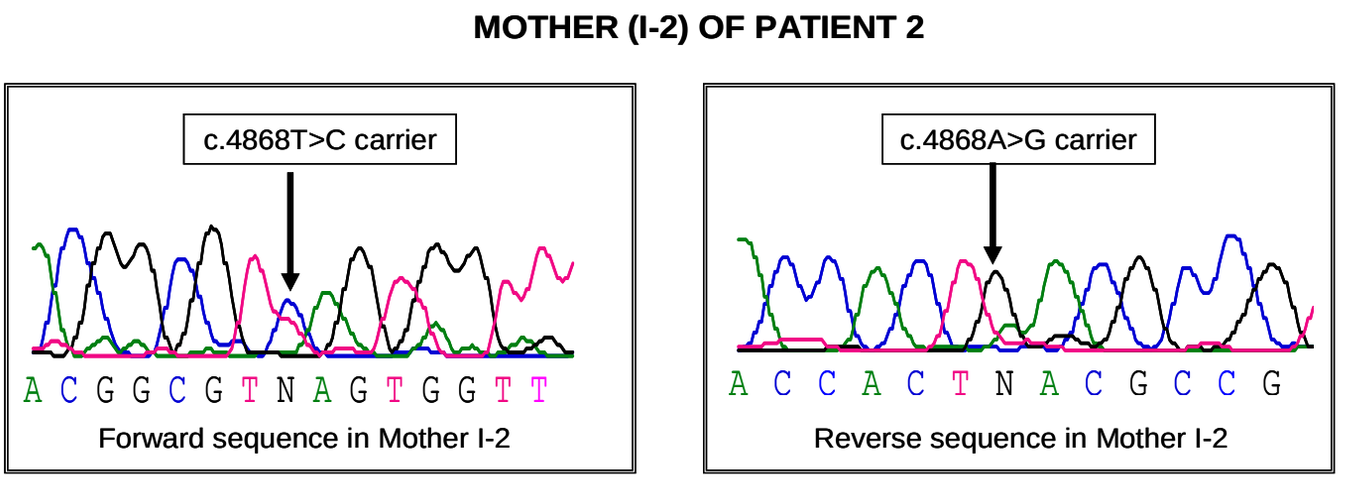


Figure 7.11 Sequence analysis in the mother of patient 2 affected with ATR-X syndrome.
The mother was confirmed to be a carrier of the c.4868T>C (p.L1623S) mutation in exon19 of the *XNP* gene as seen in the forward sequence and confirmed in the reverse sequence.

7.3 ATR-X PATIENT 3 RESULTS

This patient presented with marked hypotonia at birth and on clinical examination was shown to have brachycephaly, upsweep (‘cowlick’) of the anterior hairline, bilateral epicanthic folds, conjunctivitis of the right eye and undescended left testes and shawl scrotum, clinical features compatible with ATR-X syndrome. On neurological examination he was found to be quite hyperactive and made frequent hand flapping movements. Figure 7.13 shows the clinical features of Patient 3. A possibility of an XLMR syndrome was considered as there was some history of two other males on the maternal side of the family with developmental delay (Figure 7.12). The mother’s brother, II-5 is reported to have had Down syndrome but not confirmed and died at age 24 years. Her mother has 2 sisters – one has a son (II-7) with developmental delay and the other has a daughter who had a son (III-2) with developmental delay who is now deceased. The developmental delay was apparently put down to rubella in one case and birth asphyxia in the other. There is no other significant family history.

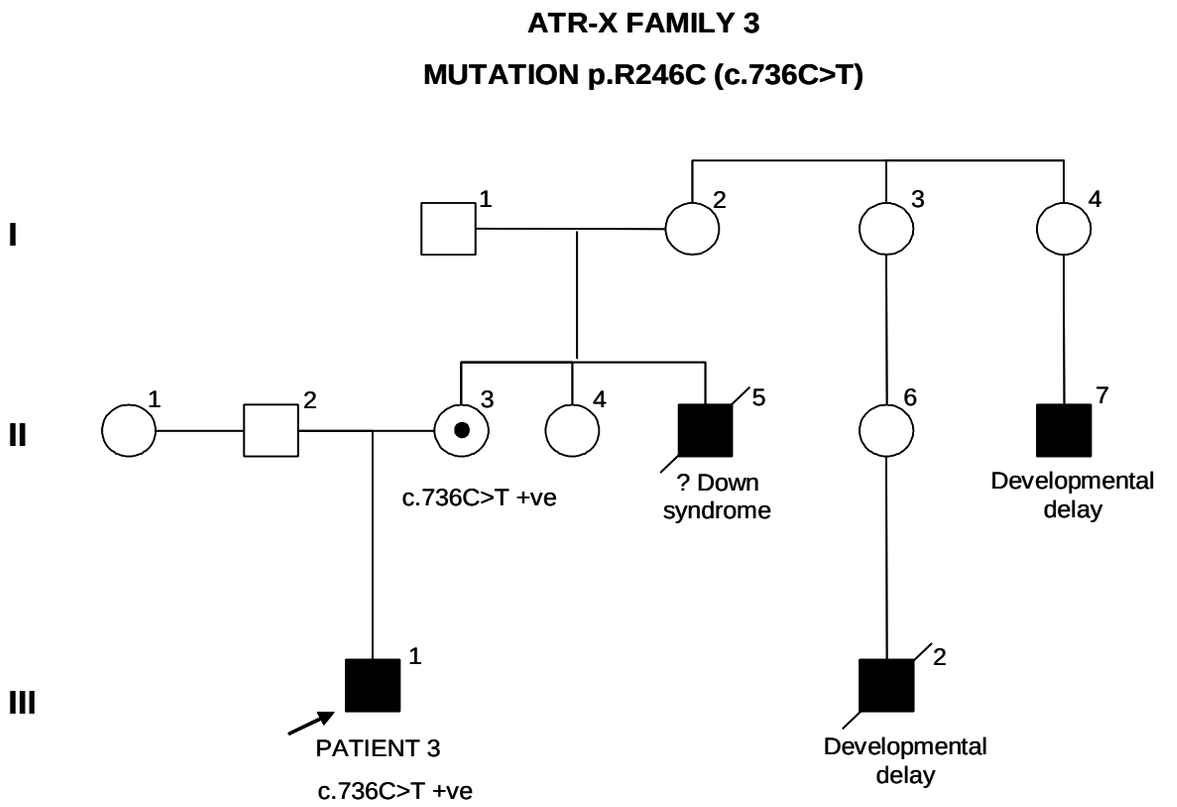


Figure 7.12 Family pedigree of patient 3 (III-1) affected with ATR-X syndrome.

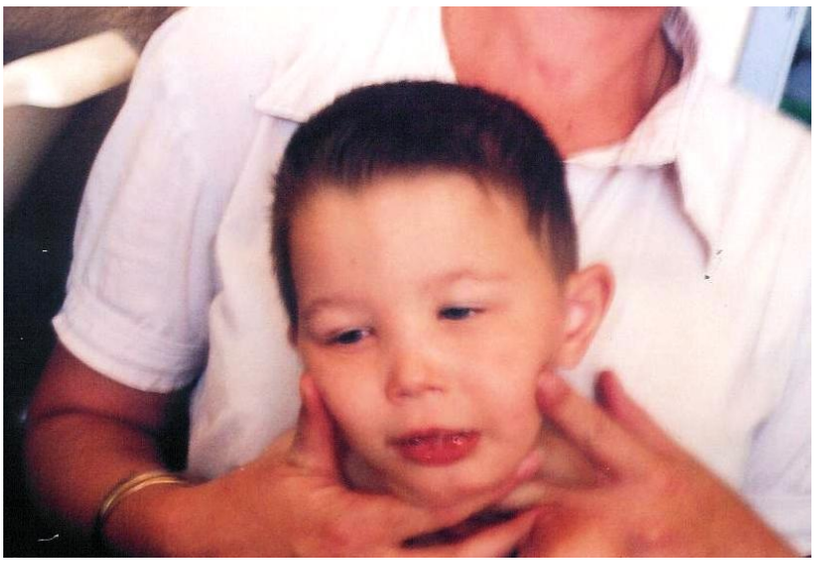


Figure 7.13 ATR-X Patient 3 showing clinical features of ATR-X syndrome.

7.3.1 Testing for fragile X MR syndrome

In view of the XLMR history, this patient was tested for fragile X MR syndrome. He was shown to have 29 *FMR1* CGG repeats and 15 *FMR2* GCC repeats thus excluding a diagnosis of fragile X MR syndrome and FRAXE syndrome.

7.3.2 Screening the *XNP* hotspot region

As he was suspected clinically of having ATR-X syndrome, DNA testing was undertaken in the Molecular Genetics Laboratory, NHLS under my supervision. Mutation screening in the *XNP* 300 bp hotspot region was undertaken by sequencing analysis (Figure 7.14) and a common missense mutation, c.736C>T (p.R246C) changing an arginine to a cysteine, was identified in exon 10 in the zinc finger domain. The sequencing electropherogram is shown in Figure 7.15.

acatatcttttcagtttcctgaaagaagggaataatgttttaatctttaaaacatttcagGTGGTGTGCGGAAGGTGGAAAC
TTGATTTGTTGTGACTTTTGCCATAATGCTTTCTGCAAGAAATGCATTCTACGCAACCTTGG
TCGAAAGGAGTTGTCCACAATAATGGATGAAAACAACCAATGGTATTGCTACATTTGTCACC
CAGAGCCTTTGTTGGACTTGGTCACTGCATGTAA

Figure 7.14 Sequence of exon 10 of the *XNP* gene showing the site of the c.736C>T (p.R246C) mutation identified in Patient 3.
The base which is mutated is highlighted in red. The lowercase letters represent the intronic region and the uppercase letters represents the exon.

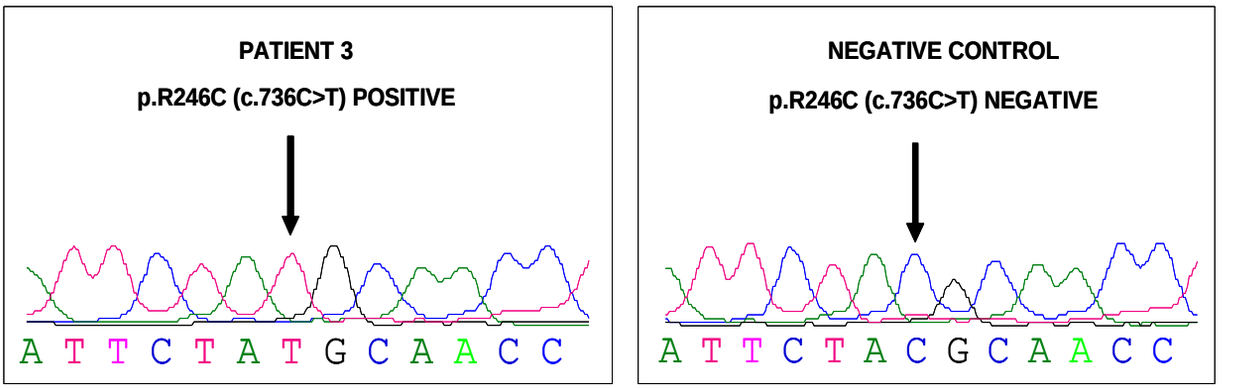


Figure 7.15 Sequence analysis in patient 3 affected with ATR-X syndrome compared to a negative control sample.
The mutation identified in the patient is c.736C>T (p.R246C) in exon 10, the zinc finger domain of the *XNP* gene.

7.3.3 Carrier testing in mother of patient 3

DNA analysis undertaken in the patient's mother II-3 showed her to be a carrier of the mutation. The result from sequencing analysis performed in the mother can be seen in Figure 7.16. The family pedigree is shown in Figure 7.12. As there are male relatives affected with intellectual disability, the possibility exists they may have, or had, ATR-X.

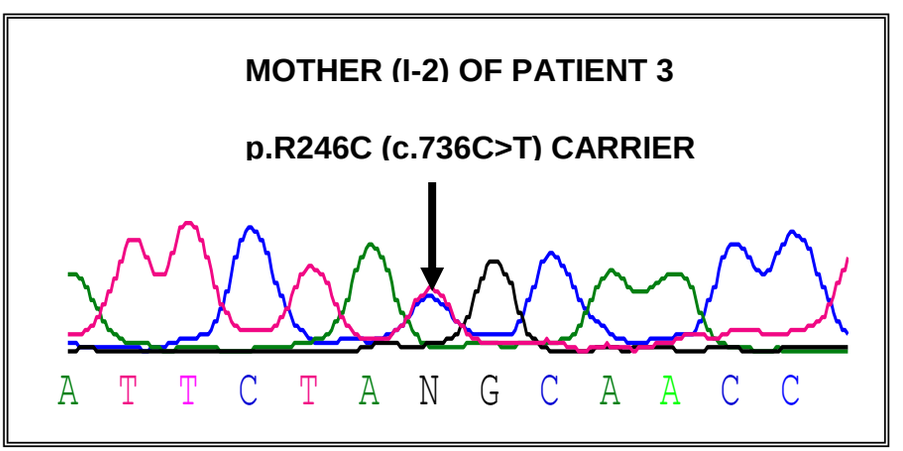


Figure 7.16 Sequence analysis in the mother of patient 3.
The mother was confirmed to be a carrier of the c.736C>T (p.R246C) in the zinc finger domain of the *XNP* gene.

7.4 DISCUSSION OF ATR-X FAMILY STUDIES

Three families were investigated for ATR-X syndrome and a mutation in the *XNP* gene was identified in each family. A mutation was found in all 3 patients with a clinical suspicion of the condition. This allowed for accurate carrier and prenatal testing in these families using direct mutation analysis. All the mothers were found to be carriers and in one family, 2 extended family members were also confirmed to be carriers. There is a high chance of finding a mutation in clinically suspected patients as has been shown in the 3 patients discussed in this study. In all 3 cases, the mutation was not *de novo*. This has important family implications.

7.4.1 Diagnostic strategy for ATR-X syndrome

Due to its large size, comprehensive mutation analysis of the *XNP* gene is time-consuming and expensive. Gibbons (Gibbons Laboratory website, 2008) proposed the following methods for diagnostic testing of ATR-X syndrome:

- Detection of α-thalassaemia (presence of HbH inclusions). This is a technically difficult test to perform, especially in laboratories unfamiliar with HbH inclusions. There are many false positive and false negative results.
- Sequence analysis of the *XNP* gene
  - Mutation detection in the zinc finger domain (exons 7, 8 and proximal area of exon 9) and helicase region (exons 17-20) by sequencing analysis can detect disease-causing mutations in ~90% of affected individuals. Approximately 50-60% of known ATR-X mutations are clustered in the zinc finger domain region and 30% of mutations are located in the helicase domain
  - Screening exons 35 and 36 in patients with severe genital abnormalities
  - Testing for the c.109C>T (p.R37X) mutation in families with individuals who have mild to moderate MR
- Protein studies to determine the level of ATRX protein
- X-inactivation studies to determine the carrier status of at-risk women if a mutation is not identified

The strategy for mutation detected suggested above is a sensible and cost-saving approach and is only of value when there is accurate clinical direction as *XNP* mutations are rare in undefined MR cases. The Division of Human Genetics, Molecular Laboratory can perform mutation testing in the *XNP* hotspot region. If no mutation is identified, further screening is available in the UK. The patients in this study were clinically suggestive of ATR-X syndrome and initially screened for mutations in the ADD region. One patient (Patient 3) was found to have the

common c.736C>T, p.R246C mutation which accounts for 20% of *XNP* mutations identified (Gibbons et al., 2008). The other 2 patients (Patients 1 and 2) had a mutation in the next most commonly affected region, exons 17-20 of the helicase region. Gibbons et al. (2008) reported on more than 200 mutation positive individuals in 182 families with 113 different mutations. The majority of disease-causing mutations are single base changes, most of which are missense mutations.

ATR-X syndrome is an X-linked recessive disorder. Not all mothers of affected individuals are carriers of the mutation. New mutations are extremely frequent and no data are available on the frequency of *de novo* gene mutations in ATR-X syndrome (Stevenson et al., 2000). In sporadic cases resulting from *de novo* mutations, the mother is expected to have an 85% risk of being a carrier (Bachoo and Gibbons, 1999; Gibbons et al., 2008). If mutations are not detected in peripheral blood leucocytes, germline mosaicism cannot be excluded. The mothers of the patients investigated in this study were all found to be carriers of ATR-X syndrome. This has important implications for the family as females who are confirmed to be carriers have a 25% risk of having an affected child with every pregnancy. If the mutation is known, there is an opportunity for carrier detection and prenatal diagnosis.

7.5 SUMMARY OF ATR-X FAMILY STUDIES

In collaboration with the UK lab, the causative mutation in the ATR-X families has been identified. As a result of this, carrier testing to siblings and other at-risk family members and accurate prenatal testing has been offered to one family. Since the workups were undertaken locally, the cost of the test was considerably reduced and a result was available sooner than would be expected if undertaken overseas. Clinically suspected ATR-X can be referred to the Division of Human Genetics, Molecular Laboratory and will be investigated in collaboration with the UK laboratory, with mutation testing in the *XNP* hotspot region performed locally and if negative, mutation screening of the *XNP* gene performed in the UK.

CHAPTER EIGHT

8 FINAL DISCUSSION AND CONCLUSION

8.1 FINDINGS FROM THIS STUDY

This study focused on 4 XLMR genes, *FMR1, FMR2*, *XNP*, and *ARX* and their contribution to MR in the South African population. The results obtained have shown the *FMR1* XLMR gene, which was reported previously to be significant was confirmed in this study and is therefore an important gene to screen in patients presenting with MR. The *FMR2*, *XNP* and *ARX* genes investigated were shown not to contribute significantly to XLMR in the South African population and therefore will not be incorporated into the routine diagnostic testing for MR in the Division of Human Genetics, NHLS, Johannesburg. Previous studies undertaken in the Division on genes involved in XLMR also showed that the *HOPA* gene, a dodecamer duplication is not a major cause of MR and not a significant aetiological factor in XLMR (Friez et al., 2000).

When this study commenced in 2001, the general feeling was that mutations in a few genes would contribute to a large amount of NS-XLMR cases and screening for the *FMR2* and the *XNP* gene in males with MR of unknown cause was considered a viable approach. The approach taken in this study was to screen mutation hotspots as a first target for screening MR in patients. However through the subsequent years, many genes have been identified, none of which has been reported to contribute substantially to NS-XLMR. The number of XLMR genes involved is estimated to be >100 and present data show that the diagnostic contribution of each of the genes involved in XLMR is a maximum of 0.5% (Yntema et al., 2002a; Gronskov et al., 2004; Mandel and Chelly, 2004; reviewed in Gecz et al., 2009). This low probability of finding a mutation in an XLMR gene in MR male patients makes routine screening of XLMR genes in MR males very expensive and of little value. Thus it is difficult to generate a specific protocol for screening MR males to identify the underlying cause. It seems sensible and cost effective to only test those genes that are more frequently causally involved in MR, such as the *FMR1* gene.

The testing approach used for the diagnosis of fragile X MR syndrome since 1994 at the Division of Human Genetics (NHLS) was Southern blotting. Prior to this, the diagnostic test was based exclusively on cytogenetic detection of the FRAXA fragile site. As of 2000, the PCR assay was implemented as an initial screen for the routine diagnosis of fragile X MR syndrome. This has improved and refined the diagnostic testing for fragile X MR syndrome by accurately

determining the *FMR1* CGG repeat size, which was not possible with Southern blotting. The current approach used to test for fragile X MR syndrome at the Division of Human Genetics (NHLS) is first line PCR screening. Southern blotting is done to confirm all expansions. Together, Southern blot assays and the PCR-based assay complement each other to provide reliable results for routine analysis of specimens referred for fragile X MR syndrome testing. The PCR assay is reliable, relatively cheap, and an efficient test for a primary screen for fragile X MR syndrome. Cytogenetic detection of FRAXA is not reliable alone as a diagnostic test for fragile X MR syndrome and should no longer be done. However, routine cytogenetic analysis should complement the DNA test to rule out any other chromosomal abnormalities, a practice which occurs routinely in the Division of Human Genetics, NHLS, Johannesburg.

8.2 LIMITATIONS OF STUDY

There are a number of limitations to this study. The poor clinical definition in the routine referrals, many with minimal clinical information limits further investigation. Also, the study has spanned a considerable period of time over which basic principles to mutation screening have changed. With the availability of newer techniques and testing approaches, the current study would not be undertaken. An obvious limitation to such a study is the cost and time with performing large screens. Data handling is important and one has to be cautious with analysis and interpretation.

8.3 CURRENT DIAGNOSTIC APPROACH

Now with so many other genes implicated in XLMR, there is no simple diagnostic approach and test. Not only is it costly but inefficient and laborious. Ropers and Hamel (2005) in their review emphasize the huge costs of current methods and their unreliability to justify mutation screening of all known NS-XLMR genes for routine diagnostic testing. Diagnostic testing approaches will have to be improved upon and the utilization of newer molecular tools and techniques has to be cheaper and more robust if it is to be incorporated into clinical practice. Currently the technology is focused on cost-effective, efficient, and reliable methods. The uses of genomic microarray technology or related methods that assay gene function have revolutionized the study of human copy number variation (CNV) in patients with unexplained MR. It appears to have become the current method of choice in routine clinical practice and is likely to eventually replace DNA-based mutation screening. However, this comes with technical challenges of its own, precluding widespread implementation of this analysis, e.g. CNVs will not detect point mutations. Currently 17 CNVs have been reported on the X chromosome (Koolen et al., 2009).

Ropers (2006) proposes the development of re-sequencing chips to simultaneously screen many genes for mutations in one assay and their laboratory (Max-Planck Institute for Molecular Genetics, Germany) has developed a chip for diagnostic purposes in NS-XLMR families.

Recently through the efforts of the IGOLD project, large-scale systematic resequencing using Sanger sequencing was undertaken on the coding exons, covering approximately 1 Mb of the X-chromosome and 720 genes, in 208 families with XLMR. As a result of this study, 12 new XLMR genes were identified using high-throughput sequence analysis (Tarpey, Smith, Pleasance et al., 2009). Further testing using array CGH is being used to identify whole exon deletions and duplications in the 16% of the cohort that did not have any significant sequence variant detected on the high-throughput sequence analysis. The authors proposed this method as a future strategy for identifying disease-causing variants although cautioned on the interpretation of the data and the need for more careful analysis. Interestingly, more than 1% of truncating variants which is the predominant disease-causing class of mutations in XLMR genes were found to be non pathogenic. One of the other major challenges faced with this type of strategy is distinguishing rare sequence variants from pathogenic mutations (Raymond, Whibley, Stratton et al., 2009).

The contribution of ARMR also has to be considered. The use of high-resolution array CGH and MLPA has allowed genome wide screening for submicroscopic deletions and duplications in patients with MR of unknown cause. This is currently being offered as a routine diagnostic test in many laboratories. Our laboratory is currently assessing the clinical utility of MLPA testing. The limitation with this technique however is that point mutations are not detected.

A cheaper but effective alternative screening approach has to be taken in this country due to the limited resources available. There is a need for rapid, cost-effective methods for detection of mutations in XLMR genes and with the new generation of sequencing technology, this seems possible. A strict clinical screening approach has to be taken and collaborative ventures need to be set up with laboratories overseas. Frints et al. (2002) suggested that a correct sub classification into S-XLMR and NS-XLMR should be the first differential diagnostic step in MR males, with or without other clinical/neurological findings, or dysmorphic features, normal karyotype and normal *FMR1* repeat length. It is important to use the clinical information to direct the S-XLMR type. The possibility of using linkage sets in an environment with limited resources should be considered.

In those undiagnosed cases with a positive familial history, Genetic counselling can be provided. Individuals with a known or a suspected history of XLMR or MR of unknown cause are strongly encouraged to seek genetic counselling. The NHLS offers a comprehensive counselling service through the Genetic Counselling Clinics attached to the Division of Human Genetics. A confirmed diagnosis leads to improved support for newly diagnosed patients and allows identification of all carriers in the family. Prenatal diagnosis can be offered to 'at-risk' individuals and allow couples to make informed choices about their future.

8.4 FUTURE PROSPECTS

The genetic causes of XLMR are heterogeneous and despite the great collaborative efforts that have allowed the elucidation of many genes involved in these conditions, it remains complex and challenging to identify a causative mutation in an individual or family. Sustained efforts are required to identify the remaining genes in XLMR. As additional genes involved in the development of intellectual and cognitive functions are identified, a more reliable molecular diagnosis for MR in general and XLMR specifically can be provided. This will not only improve our understanding of the molecular and cellular bases underlying cognitive impairment, but also improve our understanding, management, counselling, prevention and ultimately perhaps even treatment of MR.

Further studies can be proposed. An interesting study would be to determine the frequency of the *FMR1* premutations in the normal South African population. There is controversy around whether or not premutation males and females have subtle behavioural or learning difficulties. A number of studies have refuted the clinical significance of PMs (Mazzocco, Sonna, Teisl et al., 1997; Mornet, Chateau, Simon-Bouy, et al., 1998; Sherman, Marsteller, Abramowitz et al., 2002). It may be an interesting study to undertake in the South African population.

9 APPENDICES

APPENDIX A: ETHICS CLEARANCE CERTIFICATE

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

COMMITTEE FOR RESEARCH ON HUMAN SUBJECTS (MEDICAL)
Ref: R14/49 Krause/Essop

CLEARANCE CERTIFICATE **PROTOCOL NUMBER** M5 (M000105)

PROJECT Molecular Aspects of X-Linked Mental Retardation

INVESTIGATORS Dr/Ms A/F Krause/Essop

DEPARTMENT Dept of Human Genetics, SAIMR

DATE CONSIDERED 00/28/01

DECISION OF THE COMMITTEE *

Approved unconditionally

DATE 00/02/28 **CHAIRMAN** .....(Professor P E Cleaton-Jones)

* Guidelines for written "informed consent" attached where applicable.

c c Supervisor: Dr A Krause
Dept of Dept of Human Genetics, SAIMR

Works2\ia\n0015\HumEth97 wdb\M 5

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10001, 10th Floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee.

DATE 7/3/00 SIGNATURE .....

PROTOCOL NO.: M 5

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX B: SOLUTIONS

- **Alconex solution (1%) to clean vertical plates for ABI377 Genetic Analyzer**
  1g alconex crystals dissolved in 100ml ddH_2O

- **Ammonium persulphate (10% APS) for ABI377 Genetic Analyzer Gel mix**
  1g APS dissolved in 10ml ddH_2O
  Store away from light at 4°C

- **Chloroform : Isoamylalcohol (24:1)**
  960ml Chloroform + 40ml Isoamylalcohol
  Store in glass bottle at room temperature

- **Deionized formamide**
  Mix 50ml of formamide and 5g of AG501 X8 ion-exchange resin.
  Ensure that the pH is >7.0
  Allow the beads to settle to the bottom of the beaker.
  Remove the supernatant (formamide)
  Dispense into 1ml aliquots and store for up to 3 months at -15°C to -25°C

- **Denaturing solution for Southern Blotting**
  87.66g of 1.5M $NaCl_2$
  20g of 0.5M NaOH
  Ensure that pH of solution is basic.
  Make up to 1L with ddH_2O and store at room temperature

- **Dextran-Formamide loading dye (ABI377 Genetic Analyzer loading dye) (20mg/ml)**
  20mg blue dextran
  1ml deionized formamide
  Aliquot 1ml into 1.5ml eppendorfs and store at 4°C

- **Deoxyribonucleic triphosphate (dNTP) 10x working stock solution (1.25 mM)**
  125µl each of 10 mM Na-salt dATP, dGTP, dTTP and dCTP
  Add 500µl ddH_2O to make up to 1ml
  Make 100µl aliquots and store at -20°C. Avoid excessive freezing and thawing. Final concentration in PCR reaction is 0.125 mM (125µM).

- **Ethidium Bromide (EtBr) (10mg/ml)**
  Commercially available from Sigma as an aqueous solution (10mg/ml)
  Caution: EtBr is a powerful mutagen. Always wear gloves while handling gels or solutions containing the dye.

- **Ethylenediamine tetra-acetic acid (EDTA) (0.5M) for DNA extraction**
  93.06g EDTA disodium salt crystals (Note : MW=372.24g/l)
  pH to 8.0 with 5M NaOH to allow crystals to dissolve completely
  Make up to 500ml with ddH_2O. Autoclave and store at room temperature

- **Ethanol (70%) for DNA extraction**
  70ml 100% Ethanol + 30ml water
  Store at room temperature

- **Ficoll-bromophenol blue loading dye (100ml)**
  50g of 50% sucrose crystals
  0.1ml 0.5M EDTA (pH 7.0)
  0.1g 0.1% bromophenol blue dye
  10g 10% ficoll powder
  Make up to 100ml with ddH_2O. Aliquot into 1.5ml eppendorf tubes. Store at 4°C

- **1 kb Molecular Weight DNA marker (GIBCO BRL; 1μg/μl)**
  250μl ladder (0.25μg/μl stock)
  125μl bromophenol loading dye
  2.1ml 1xTE
  Load 10μl onto each gel

- **$MgCl_2$ (1M) for DNA extraction**
  20.33g $MgCl_2$
  Make up to 100ml with dH_2O
  Autoclave. Store at room temperature. Note: MW=203.31g/l

- **Neutralising solution (pH 7.2) for Southern blotting**
  87.6g of 1.5M $NaCl_2$
  60.6g of 0.5M Tris Base
  0.292g of 0.01M EDTA
  ~42ml HCl_2 (Adjust with more HCl_2 until pH = 7.2). Store at room temperature

- **4.3% Polyacrylamide gel (100ml) for ABI377 Genetic Analyzer**
  10.6 ml of 40% Acrylamide : Bisacrylamide (19:1)
  20ml of 10xTBE
  36g Urea
  Water to make up to 100ml
  Stir until urea has dissolved and contents are translucent. Filter to remove any solid particles, which may fluoresce. Store at 4°C in a dark bottle for up to two weeks.

Polyacrylamide Gel Mix for ABI377 Genetic Analyzer	36cm Plate	12cm Plate
4.3% Polyacrylamide gel	40ml	20ml
APS	200μl	100μl
Temed	24μl	12μl Temed

- **Prehybridisation buffer (modified Church and Gilbert) (250ml)**
  12.78g Na_2HPO_4
  9.75g $NaH_2PO_{4.2}H_2O$
  17.5g SDS
  5ml of 0.5M EDTA
  Water to make up to 250ml
  pH to 7.2. Filter sterilize. Store at room temperature. Heat at 65°C to dissolve crystals.
  Use ~30 ml / 200 cm^2 blot

- **Proteinase-K Mix (500μl) for 16 DNA extractions (2mg/ml)**
  850μl of 10% SDS
  34μl of 0.5M EDTA
  1700μl of 10mg/ml proteinase-K
  ddH_2O to make up to volume. Use 500μl per sample.

- **Saline Solution (0.9%) (Isotonic) for DNA extraction**
  4.5g $NaCl_2$
  Make up to 500ml with dH_2O
  Autoclave. Store at room temperature

- **Saturated NaCl for DNA extraction**
  Add 40g $NaCl_2$ to 100ml sterile ddH_2O until absolutely saturated. Prior to use agitate solution and allow $NaCl_2$ to precipitate out. Store at room temperature

- **Sephadex™ G-50 fine (5% solution) (SIGMA) for removing unincorporated nucleotides**
  Add 5g Sephadex powder to 100ml 1x TE buffer (pH 8.0). Leave solution overnight before use. Store at room temperature

- **Sodium citrate solution (SSC) (20x)**
  350.7g of 0.15M $NaCl_2$
  176.5g of 0.15M) Trisodium citrate
  Make up to 2L with ddH_2O. No need to autoclave. Store at room temperature

- **Sodium dodecyl sulphate (SDS) (10%) for DNA extraction**
  Add 100g SDS powder to 1L autoclaved ddH_2O
  NB! Weigh out powder in fume hood. Store at room temperature

- **Sucrose-Triton X Lysing Buffer for DNA extraction**
  10ml 1M Tris-HCl (pH8.0)
  5ml of 1M $MgCl_2$
  10ml Triton-X 100
  Make up to 1L with distilled water. Autoclave
  Add 109.5g sucrose just before use. Stir until dissolved and keep solution chilled at 4°C

- **TEMED (N,N,N',N'-tetramethylenediamine)**
  Commercially available from Stratagene. Store at 4°C in the dark.

- **Tris Borate EDTA (TBE) (10x)**
  109.02g Tris Base
  55.64g Boric acid
  7.44g NaEDTA
  pH solution to 8.3 with HCl and make up to 1L with distilled water. Dilute tenfold before use. Store at room temperature

- **Tris-EDTA (TE) Buffer (1x) for DNA extraction**
  10ml of 1M Tris-HCl pH8.0
  2ml of 0.5M EDTA
  pH with HCl. Make up to 1L with ddH_2O. Autoclave and store at room temperature

- **T20E5 for DNA extraction**
  20ml of 1M Tris-HCl pH8
  10ml of 0.5M EDTA
  pH to 8.0
  Make up to 1L with ddH_2O. Store at room temperature

- **Tris-HCl pH 8.0 (1M) for DNA extraction**
  121.1g Tris base
  pH with 1M HCl
  Make up to 1L with ddH_2O. Autoclave and store at room temperature

APPENDIX C: TECHNIQUES

APPENDIX C1 - Salting out procedure for extracting DNA from human nucleated cells

Miller, Dykes and Polesky et al., 1988

DAY 1

1) Collect 5-10ml blood into ACD or EDTA vacutainer tubes
2) Decant no more than 10ml of whole blood into a 50ml polypropanol tube, i.e. 2 ACD's/EDTA's per tube. For volumes below 10ml the solution volumes are halved. Freeze at -20°C until ready to extract then thaw
3) Fill each tube to the 45/50ml mark with Sucrose-Triton-X Lysing Buffer, which should be kept cold during the procedure
4) Invert the tube several times to mix
5) Centrifuge for 10 minutes at 1000g at 4°C (±2300rpm) to pellet cells
6) Discard the supernatant fluid
7) Wash the pellet in 20-25ml Sucrose-Triton-X Lysing Buffer
8) Leave on ice for 5 minutes
9) Centrifuge for 5 minutes at1000g at 4°C (±2300rpm)
10) Discard supernatant fluid
11) Add 3ml T20E5, 0.2ml (200μl) 10% SDS and 0.5ml (500μl) Proteinase-K mix
12) Mix well by inversion.
13) Incubate at 42° to 50°C overnight (no need to agitate).

DAY 2

14) Add 1ml saturated NaCl
15) Agitate vigorously for 15 seconds by inversion
16) Leave at -20°C for 5 minutes
17) Centrifuge for 30 minutes at 4°C (2500rpm)
18) A white pellet should be visible, which consists of protein pecipitated by salt. If no pellet is visible centrifuge again
19) Transfer the supernatant fluid containing the DNA to a new 50ml polypropanol tube.
20) Add 2 volumes of absolute ethanol kept at room temperature
21) Agitate gently and spool, fish or precipitate DNA. If precipitating, do not centrifuge for too long to avoid salts and proteins precipitating out as well
22) Wash the DNA in 70% ice-cold ethanol
23) Air dry the DNA.
24) Resuspend the DNA in an appropriate volume of 1XTE Buffer (~200μl-1000μl)

APPENDIX C2 – DNA extraction protocol using the High Pure PCR Template Preparation Kit from Roche Diagnostics

Protocol for isolating DNA from 200 µl mammalian blood, buffy coat, or cultured mammalian cells (pack insert available at www.roche-applied-science.com)

Note: Before starting the purification, warm the Elution Buffer to +70°C.

High Pure PCR Template Preparation Kit workflow

1) To a nuclease-free 1.5 ml microcentrifuge tube:
    - Add 200µl of sample material
    - Add 200µl Binding Buffer
    - Add 40µl Proteinase K (reconstituted)
        - Mix immediately and incubate at +70°C for 10 min
2) Add 100µl Isopropanol and mix well
3) Insert one High Pure Filter Tube into one Collection Tube
    - Pipette the sample into the upper buffer reservoir of the Filter Tube
    - Insert the entire High Pure Filter Tube assembly into a standard table-top centrifuge
    - Centrifuge 1 min at 8,000 × *g*
4) After centrifugation
    - Remove the Filter Tube from the Collection Tube; discard the flow through and the Collection Tube
    - Combine the Filter Tube with a new Collection Tube
    - Add 500µl Inhibitor Removal Buffer to the upper reservoir of the Filter Tube
    - Centrifuge 1 min at 8,000 × *g*
5) Remove the Filter Tube from the Collection Tube; discard the flow through and the Remove Collection Tube
    - Combine the Filter Tube with a new Collection Tube
    - Add 500µl Wash Buffer to the upper reservoir of the Filter Tube
    - Centrifuge 1 min at 8,000 × *g* and discard the flow through
6) Remove the Filter Tube from the Collection Tube; discard the flow through and the Collection Tube
    - Combine the Filter Tube with a new Collection Tube
    - Add 500 µl Wash Buffer to the upper reservoir of the Filter Tube
    - Centrifuge 1 min at 8,000 × *g* and discard the flow through
7) After discarding the flow through:
    - Centrifuge the entire High Pure assembly for an additional 10 s at full speed

The extra centrifugation time ensures removal of residual Wash Buffer

    - Discard the Collection Tube
8) To elute the DNA:
    - Insert the Filter Tube into a clean, sterile 1.5 ml microcentrifuge tube
    - Add 200 µl pre-warmed Elution Buffer to the upper reservoir of the Filter Tube
    - Centrifuge the tube assembly for 1 min at 8,000 × *g*
9) The microcentrifuge tube contains the eluted, purified DNA, which can be used directly or stored at +2 to +8°C or +15 to +25°C for later analysis. For details on adding an optional RNase digestion, see related procedures in the pack insert

APPENDIX C3 – The polymerase chain reaction (PCR)

The Polymerase Chain Reaction (PCR) (Mullis and Faloona, 1987) is a technique that involves the use of specific oligonucleotide primers (18 to 30 mer in length) that flank a DNA sequence of interest to amplify it. The PCR procedure involves denaturing the template DNA at a high temperature in the presence of the primers, dNTPs (dATP, dGTP, dCTP, dTTP), and a heat stable DNA polymerase (*Taq* polymerase) with its corresponding buffer. The primers are then allowed to anneal to their target sequence by decreasing the temperature. This is followed by an extension of the primers, with the help of the DNA polymerase and nucleotides. The cycle of denaturation, annealing and extension is repeated to produce a large number of copies of the DNA region of interest. An exponential increase in product yield is obtained, which can be viewed under UV light on an agarose gel or using an automated Genetic Analyzer e.g. the ABI377 or ABI3130xl electrophoresis instruments.

APPENDIX C4 – Agarose gel electrophoresis (AGE)

This technique is used to separate DNA molecules by base composition, size and conformation. Ethidium bromide (EtBr), (10mg/ml), a fluorescent dye that intercalates between the DNA bases) makes it possible to visualise the product under ultraviolet (UV) light.

A vertical gel-casting tray was cleaned with 70% ethanol and an appropriate comb was positioned in the gel former. A 3% agarose gel was made by adding 3g agarose to 100ml 1x TBE buffer. This was heated in a microwave until all the particles were completely in solution (~400ml per 20 x 20 cm gel tray was used). The gel solution was allowed to cool before adding ~12μl EtBr (10mg/ml) solution to the gel. The cooled gel was swirled gently to mix and poured carefully into the former. The gel was allowed to set for at least 1 hour and stored at 4°C until ready to use.

5μl of PCR product was mixed with 5μl ficoll dye and loaded on the gel. The agarose gel was run for ~30 min to 1 hr at 120 V in 1xTBE running buffer.

APPENDIX C5 – Microsatellite detection using the ABI377 Genetic Analyzer

The procedure below was followed for GeneScan analysis of *FMR2* and *FMR1* alleles, as well as the preparation of a sequencer gel for detection of *XNP* sequencing products on the ABI377 Genetic Analyzer.

Plate Cleaning

A set of 12 cm (36 cm for sequencing) glass plates were washed with a 1% Alconox® (Labretoria) solution by gently rubbing the surface of the plates to remove any particles (dust, acrylamide, powder) that may fluoresce. A 'well-to-read' length (the distance the fragment migrates before being detected by the laser) of 12 cm was found to be sufficient for a good separation of *FMR2* alleles. The longer 36 cm plates are required for a good resolution of sequencing products. The plates were placed upright in a rack and allowed to drip-dry for at least 30 min. No siliconizing treatment was necessary.

Preparation of the gel-pouring apparatus and gel casting

The 0.2 mm spacers were aligned into position and the plates were assembled in the gel apparatus and clamped. The gel-pouring device with an attached syringe was clamped securely to the gel frame. A 4.3% PAG mix (see Appendix B) was prepared. A volume of 100μl APS, which acts as a polymerizing agent, was added to the gel mix and the polymerization was catalyzed by the addition of 12μl TEMED solution. The acrylamide mix was poured gently into the syringe and the plates were tapped to ensure that the gel front was running evenly and that no bubbles were formed. A casting comb was inserted, taking care not to trap any air bubbles. The gel was left for at least 1 hr to polymerize.

Preparing the gel for loading

The gel pouring device and the comb were removed and the area flushed with ddH_2O. The plates were cleaned with tissue paper moistened in ddH_2O, ensuring that the area scanned by the laser is free of all debris. The bottom buffer chamber was attached and the frame containing the gel was placed into the Genetics Analyzer. The ABI PRISM™ 377 collection software was selected. A new sample sheet was generated and saved.

A new GeneScan run was set up. The GeneScan software converts fluorescent signals to different coloured peak intensities by comparing the signals detected with Matrix standards. Matrix standards are obtained by loading the Genetic Analyzer with each of the different fluorochromes. The electrophoresis parameters for the gel were chosen. A run module, GS 12A-2400 was selected. This corresponds to a gel separation distance, or a 'well-to-read' distance of 12 cm, using a virtual filter set A with a scan rate of 2400/ hr. A GeneScan pre-run module, PR GS 12A-2400 was chosen to match the run module. A matrix file was selected. This adjusts for the spectral overlap between the fluorescent dyes used on the ABI PRISM^{TM} 377 Genetic Analyzer. The operator, run time and the virtual filter set corresponding to the dyes being used was defined. The sample sheet was imported into the GeneScan run.

Polyacrylamide gel electrophoresis

A plate check was done to determine gel quality prior to adding the running buffer and loading since this allowed easy removal of the plates if they needed to be re-cleaned. The buffer chambers were assembled and 1x TBE running buffer was poured into the top (~500ml) and bottom (~1l) buffer chambers until the levels just reached those indicated on the chamber. A pre-run for ~10 min was done to allow the gel to reach the required temperature and voltage output of 51°C and 3.00kV.

The samples were prepared whilst the gel was pre-running. A mix of 1μl PCR product, 0.3μl Rox-500 (an internal size standard) and 2.2μl dextran formamide loading dye was prepared. The samples were placed on a 95°C heating block for ~2 min and cooled on ice to quench the reannealing of the single stranded (ss) DNA. The pre-run was stopped and the wells were flushed with the running buffer, using a disposable syringe and hypodermic needle. 1.7μl of the denatured samples was loaded into every odd lane, since alternate loading alleviates contamination arising from leakage of wells. The pre-run was resumed for ~10 min, after which the even lanes were loaded and the run was started on the Genetic Analyzer. The gel was run for at least 2 hours at a voltage of 3.00kV, ~15mA current, 200W electrophoresis power and a laser power of 40.0mW. The progress of the gel was intermittently viewed by monitoring the gel image and following the migration of the ROX standard.

On completion of the run, the gel was removed from the frame and the running buffer in the top and bottom chambers was siphoned out. The gel was discarded and the plates and attachments used were thoroughly cleaned. The sample sheet was transferred into the run folder, which was then transferred to the Macintosh computer used to analyse the raw data.

GeneScan analysis

The data were analyzed using the software programmes such as GeneScan™ (ABI/Perkin Elmer) and Genotyper™ (ABI/Perkin Elmer). The GeneScan software automatically sizes and quantitates DNA fragments by using the internal size standard to create a size calibration curve for each lane. The PCR product size is compared with this calibration curve. The size standard also serves as a positive control for scanner analysis, especially if the PCR amplification has failed. A gel file was created, which allowed the results to be analyzed. The gel image was regenerated and the gel contrast was adjusted to visualize the gel better. Each lane was tracked manually to mark the channel that exhibits the strongest fluorescent signal. The tracked lanes were extracted and a sample file for each lane was created. The peaks of the GeneScan 500 size standard (or Rox 500, which is displayed as a red, fluorescent dye) were defined. Size standards are DNA fragments of known sizes labelled with ABI prism dyes (in this case, Rox). GeneScan 500 is a size standard with different peaks, corresponding to different sizes of the fragments in the size standard. This marker is useful for sizing fragments between 35 and 500 base pairs. The ROX ABI Prism dye is detected as a red colour using the virtual filter set A.

The software used this information, plus other analysis parameters such as the size calling method, analysis range of the gel, minimum peak height (a minimum of 50 was chosen and adjusted accordingly) and the colour of the fluorescent molecule to define all unknown fragments. The sample files were examined by displaying the fragments as peaks on an electropherogram. The gel results were analyzed and saved.

Genotyper analysis

The data that were analyzed with GeneScan were imported into Genotyper™ software (ABI/Perkin Elmer). This software converts size and quantitative data produced by GeneScan into genotypes by assigning fragment sizes to the peaks. The alleles were sized by selecting the correct colour of the fluorescent dyes, followed by running a macro. The macro is a programme that is designed specifically for the mutation being analyzed. A range in which peaks are detected is specified in the macro.

Interpretation of results

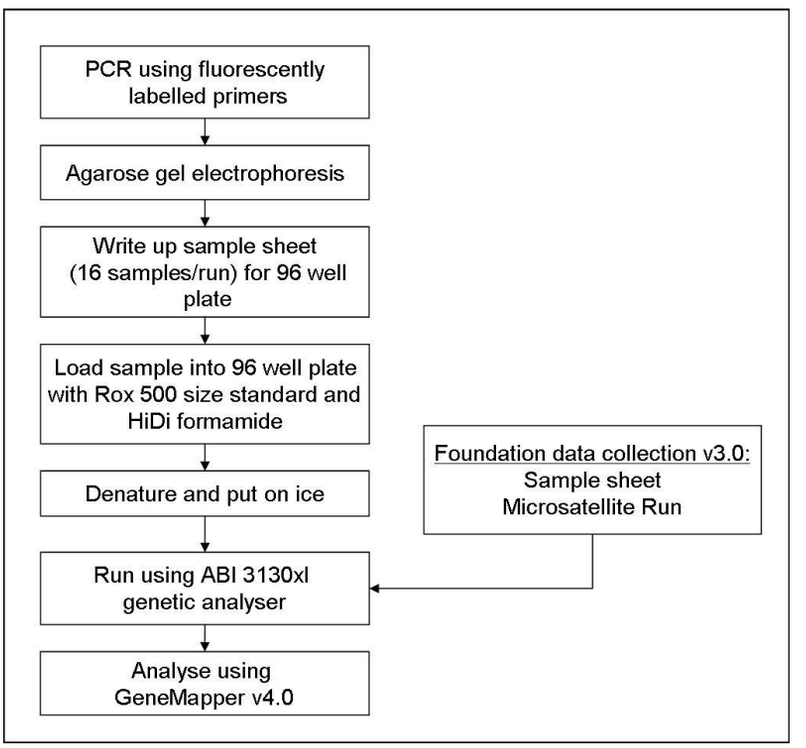
Each *FMR2* peak was analysed and scored as an allele after analysing and verifying the controls. All peaks were scored as reliable alleles if a characteristic pattern was noted. Any peak that did not have the characteristic shape (Figure 2.5) of an allele was disregarded. *FMR1* alleles were required to have an amplitude >1000. This ensured reliability determined from the

previous validation performed. The results were saved and printed. These sizes are converted to the number of GCC repeats using a formula based on primer length and distance. Since the *FMR2* primer is labelled with a blue fluorescent dye (5'FAM) a blue peak is observed and similarly a green peak is observed (HEX) for the *FMR1* allele.

APPENDIX C6 – Microsatellite detection using the ABI3130xl Genetic Analyzer

The following protocol is designed to demonstrate the procedure of microsatellite detection on the ABI 3130xl genetic analyser. (ABI instruction manual)

OVERVIEW OF TECHNIQUE



An overview of the procedure for automated genotyping of microsatellites

PCR using fluorescently labelled primers and agarose gel electrophoresis

1) One of the PCR primers must be fluorescently labelled
2) Perform PCR using specific protocol.
3) Verify PCR products on agarose gel to identify if the PCR has worked, was not contaminated and to identify whether the sample needs to be diluted before running on the 3130xl.

Sample sheet for the ABI3130xl genetic analyser

1) Create a plate record
    a. GA Instruments → ga3130xl → Plate Manager
    b. Click on the NEW icon
    c. Choose Microsatellite as type of plate
    d. A new window will open. Fill in the following information:
    e. Name name the plate (the saved name is case sensitive manner)
    f. Description (optional)

   g. Application *GeneMapper-ABI_3130XL* (or *GeneMapper-Generic*)
   h. Plate type *96-well*
   i. Owner name *molecular*
   j. Operator name *your name*
   k. Click OK
2) Add the sample names. NB!:
   a. The 96-well plate is divided into 12 columns (1-12 across) and 8 rows (A-G down) and so that each well has a unique number, e.g. G5
   b. One run consists of 16 samples (columns 1&2, 3&4, 5&6, 7&8, 9&10 or 11&12)
   c. One sample sheet can be created for more than one run. The runs will only be processed one at a time
   d. A sample sheet can start anywhere on the plate (e.g. columns 5&6) but a full run must be filled with HiDi so all 16 capillaries have liquid passing through them.
   e. Make sure that sample identification is clear e.g. patient name, disease testing for, name of microsatellite or linked marker
3) Select the following parameters in the sample sheet:
   a. Comment: (optional)
   b. Priority: *100*
   c. Sample type: *Sample*
   d. Size Standard: *GS500(-250)*
   e. Panel: own panel
   f. Analysis method: *Microsatellite Default* or own analysis method
   g. SNP set:
   h. Study:
   i. User-Defined 1:
   j. User-Defined 2:
   k. User-Defined 3:
   l. Results Group 1: *Molecular_Diagnostics* (GeneMapper-ABI_3130XL)
   m. *Molecular-Genetics_Diagnostics* (GeneMapper-Generic)
   n. Instrument Protocol 1: *microsatellites*
4) Save and close the sample sheet

Prepare the samples for analysis

1) Add 1-2μl of PCR product (depending on how strong/weak the product is) to 8-9μl Hi-Di™ Formamide (highly deionised formamide) mixed with Rox 500 size standard (for 16 samples – 1 run – use 146μl HiDi and 7μl Rox 500).
2) Denature the plate at 95ºC for 2 minutes
3) Place on ice until ready to load into the machine

Preparing the Microsatellite Run

1) Verify whether there is sufficient polymer, buffer and water
   a. If the polymer needs to be changed: follow the steps of the Polymer change wizard
   b. The buffer and water needs to be changed every 3 days or every 12 runs
2) Place the 96-well plate into the machine
   a. Press the TRAY button on the 3130xl genetic analyser
   b. When the tray has moved to the front of the machine, open the door
   c. Place the 96-well plate onto the correct sample tray (either tray A or B)
3) Link the correct plate to the correct tray position
   a. GA Instruments → ga3130xl → 3130XL → Run Scheduler → Plate View
   b. Find all plates with the status "pending" (click the "Find all" icon)
   c. Link the correct plate to the Autosampler positions (tray A or B) (click the plate/file name, followed by the correct tray, the "in use" tray image icon will turn green)
4) Verify that the runs schedules match the correct wells on the plate

   a. GA Instruments → ga3130xl → 3130XL → Run Scheduler → Run View
   b. Verify that each run scheduled matches the correct positions on the plate
5) Verify if the spectral is correct for the fluorescent label used.
   a. GA Instruments → ga3130xl → 3130XL → Spectral Viewer
      (do not move from this screen or the spectral will automatically change back to Spectral F. Just move on to Step 5).
6) Initialise the run
   a. Click the green "play arrow" icon on the top tool bar of the Navigator window
   a. The run will start. The "instrument status" window will automatically open to give details on the run in progress. Otherwise go to GA Instruments → ga3130xl → 3130XL → Instrument Status

Analysing the Microsatellite Results

Use the GeneMapper v4.0 (Applied Biosystems) to analyse the raw data.
Open the program by selecting the GeneMapper v4.0 icon on the desktop

1) Log into the program
   a. User Name: *gm*
   b. Password: *Password* (case sensitive)
2) Open a new project (if one isn't already open)
   a. File → New Project → choose Generic or Microsatellite (depending on what you used for your sample sheet)
3) Add sample files to be analysed
   a. Select File → Add Samples to Project
   a. Select the folder containing the sample files (My Computer → Local Disk (E) → Applied Biosystems → UDC → DataCollection → data → select your file
   b. Click the "Add to List" button
   c. Click the "Add and Analyse" button
4) The SAVE PROJECT AS window opens. Name your project and click the "OK" button. Your project will now be analysed. The SQ column of analysed samples should contain a green square or a yellow triangle to pass analysis. If a red circle is present, highlight the sample row and click on the "Size Match Editor" button (the button with 3 red peaks on the top toolbar). Once this comes up, adjust the sizes on the peaks if needed, to fit the size standard. If changes were made, highlight the samples with changed size standard and select Analysis → Analyse selected samples → OK
5) To view results:
   a. Highlight the sample and click the "Display Plots" button (the button with the different coloured peaks on the top toolbar).
   b. Choose the colour of the fluorescent label (click off the other colour buttons)
   c. To zoom into the plot, move your cursor onto the axis so the magnifying glass ion appears. Click and drag the cursor over the area to be zoomed in. Double click on the axis to zoom out to full view.
   d. If a peak is not sized, click on the peak
   e. If it is highlighted, right click and select "Add allele call"
   f. If it is not highlighted, right click and select "Add peak". Right click again and select "Add allele call"
6) To print display plots, File → Print → choose printer

APPENDIX C7 – Gel-based DNA sequencing using the ABI377 Genetic Analyzer

The method for **automated sequencing** using the 377 Genetic Analyzer is described below

Gel purification of PCR products

It is essential that the PCR product be purified to obtain a pure sequencing template. The PCR product was electrophoresed on an agarose gel in 1x TBE buffer (120V, 85mA). The SAP:Exonuclease method was used to purify the PCR products. The NucleoSpin® Extract II Cleanup method is currently used. The NucleoSpin® Extract II Cleanup kit removes excess primers and contaminants such as salts from PCR reactions in preparation for cycle sequencing reactions. DNA can be purified from either TAE/TBE agarose gels or directly from PCR products. When verifying PCR products on an agarose gel, the gel was visualised with a Variable-intensity Transilluminator 312 Ultraviolet Model TVC-412A (Spectroline) and the band containing the fragment to be purified was cut out with a clean scalpel and allowed to melt in the buffer, NT.

DNA purification was carried out as follows: the PCR product or molten gel containing the PCR product are loaded into the NucleoSpin® Extract II column in the presence of the NT buffer and centrifuged. DNA binds to a silica membrane within the column in the presence of chaotropic salts. Simple washing steps with ethanolic buffer, NT3, removed contaminants while the DNA fragments stayed bound to the silica membrane. A slightly alkaline buffer, NE facilitated the elution of DNA under low ionic strengths into a clean microcentrifuge tube (NucleoSpin® Extract II Cleanup kit manual).

Cycle sequencing

The ABI PRISM^{TM} Dye Terminator Cycle Sequencing Ready Reaction Kits (v3.1) with AmpliTaq DNA polymerase, FS (from Applied Biosystems), combine the unique properties of this enzyme (it has no 5' to 3' nuclease activity) for dye terminator sequencing with reagents that are premixed. The terminator premix contains A-Dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, dGTP, dATP, dCTP, dTTP, Tris-HCl (pH 9.0), $MgCl_2$, thermal stable pyrophosphatase and AmpliTaq DNA polymerase, FS (ABI User Manual).

A cycle sequencing PCR reaction was set up as suggested by the manufacturers. The reaction consisted of 5-8μl purified DNA (the quantity of product required for the sequencing reaction was also estimated from the intensity observed on the EtBr-stained agarose gel), 3.3pm/μl primer

(either forward or reverse), 4μl of BigDye® terminator FS-kit (Applied Biosystems/Perkin Elmer) and ddH2O to make up to a total volume of 20μl. The PCR was carried out in the GeneAmp thermocycler with denaturing at 96°C for 30 sec, annealing at 50°C for 15 sec and extension at 60°C for 4 min for 25 cycles.

Qiagen DyeEx Cleanup for purification of PCR products

The cycle sequencing reaction contains a large quantity of unincorporated dye-labelled terminators as well as labelled extension products that could interfere and contaminate the sequence. The removal of these contaminants requires the use of a spin column purification method. The DyeEx (Applied Biosystems) dye-terminator removal system removes unincorporated primers, deoxy- and dideoxynucleosides from cycle sequencing reactions.

The procedure involves the use of a simple spin column containing a gel filtration system, made up of spheres with uniform pores allowing the separation of molecules according to molecular weight. The cycle sequencing products were added to the column. This was tapped gently to allow the gel material to settle to the bottom of the column. A volume of 800μl ddH_2O was loaded onto the column. This was vortexed briefly to mix and left on the bench for at least 30 min to allow the resin to equilibrate and hydrate. The column was placed in a residual tube and allowed to drain. The column was spun for 2 min (3000xg) and thereafter the residual tube was discarded. A centrifugation step removes the storage buffer from the column and the cycle sequencing products are loaded.

A second centrifugation step removes unincorporated dye terminators. Dye-terminator molecules are small enough to diffuse into the pores and are retained within the gel while DNA fragments are excluded and can therefore be recovered from the collection tube (Qiagen DyeEx dye-terminator system manual). About 20μl of the cycle sequenced PCR product was loaded on top of the column, which was placed into an eppendorf collection tube. The column was placed in the same orientation as it was for the first spin. This was important since the gel is at an angle in the column after the spin. The purified cycle sequencing products were collected after centrifuging the column for 2 min (3000xg). The sample was vacuum-dried for 40 min-1 hr using a Concentrator 5301 vacuum drier (eppendorf). The dried samples were stored in a black bag at 4˚C.

Polyacrylamide gel preparation for ABI377 Genetic Analyzer

The gel was prepared as described in Appendix C7. However, a 36 cm gel was used. The sequence sample was selected from the ABI Collection Software options and a new sample sheet was generated. A new Sequence run was chosen and all the essential analysis parameters were defined. A plate check and a pre-run were done as described in Appendix C5 (See Appendix C6 for detection method using the ABI3130xl capillary Genetic Analyzer)

Preparation of samples for loading

The dried samples were resuspended in 3μl of dextran/formamide mix and denatured at 100°C for 2 min and placed on ice.

Gel electrophoresis

The bands in the electrophoresis gel move past a laser which detects which ddNTP is present in each band. This information is passed on to the imaging system.

Analysis of sequencer gel

Two software programmes exist for sequence analysis: one is for data collection and one for data analysis as described in Appendix C5. The gel file was tracked and sample files were generated.

Interpretation of sequencing results

The electropherogram was printed and analysed. The sequence is represented by a series of peaks, one for each nucleotide position. A green peak represents an 'A', blue 'C', black 'G' and red 'T'. The letters above the peaks are coloured to represent the appropriate bases. An N above a peak means that the software could not confirm that base (ABI User Manual).

Sequence data analysis

The DNAstar software program (DNASTAR, Inc.) was used to compare the sequence generated to a reference sequence obtained from the UCSC genome browser website (2007) using the SeqMan II ™ module of DNAstar. The sequences were aligned to the reference sequence and analysed and mismatches, if any, were highlighted.

APPENDIX C8– Capillary DNA sequencing using the ABI3130xl Genetic Analyzer

The method for **automated sequencing** using the 3130xl Genetic Analyzer is described below (Applied Biosystems 3130/3130xl Genetic Analyzers: Getting started guide, 2004):

1) Add 10 µl Hi-Di™ Formamide (Applied Biosystems) directly to the dried DNA samples and resuspend the DNA.
2) Add 10 µl of each of these purified cycle sequencing products into a MicroAmp® 96-well reaction plate (Applied Biosystems). Since the Genetic Analyzer utilises 16 capillaries simultaneously, samples need to be loaded either in multiples of 16 or by adding 10 µl Hi-Di™ Formamide to empty wells to make the sample number a multiple of 16
3) Cover the MicroAmp® 96-well reaction plate with a MicroAmp® optical 96-well plate septa (Applied Biosystems) and centrifuge the plate using the CS 6R Centrifuge (Beckman).
4) Insert the MicroAmp® 96-well reaction plate into the GeneAmp PCR System 9700 (Applied Biosystems) and allow the samples to denature for 2 min at 95 °C. Immediately place the MicroAmp® 96-well reaction plate on ice
5) Press the “Tray” button on the Genetic Analyzer to allow the autosampler to move to the front. Load the MicroAmp® 96-well reaction plate into a plate retainer and insert the plate retainer onto the plate base of the autosampler
6) Press the “Tray” button once again to allow the autosampler to move towards the back of the machine
7) Verify whether the buffer and water reservoirs are filled. If not fill the reservoirs with the respective solutions. Verify whether there are any bubbles present in the Genetic Analyzer machinery
8) Open Foundation Data Collection version 3.0 (Applied Biosystems) on the computer linked to the Genetic Analyzer. Prepare a sample sheet. The instrument protocol, KB_3130_POP7_BDTv3.mob is used to analyse the samples.
9) The sample sheet is linked to the relevant plate and the green play button is pressed to start sequencing
10) A program called Sequencing Analysis v5.2 (Applied Biosystems) is used to analyse the data generated by Foundation Data Collection v3.0. Select the samples to be analysed, click on “Add selected samples” and press the green play button to analyse the data. Each sample can be accessed individually, edited and printed.

APPENDIX D: FRAGILE X MR SYNDROME

APPENDIX D1 – Worksheet for *FMR1*/*FMR2* multiplex PCR

Modified from Crawford et al., 1999

PCR COMPONENTS	STOCK	FINAL CONC	VOLUME	X MIX
DNA	100ng		1μl	
dNTPs mix	10X	1X	0.75μl	
7-deaza dGTP	10X	1X	0.75μl	
Expand Long Template Buffer 1 (with 17.5 mM $MgCl_2$)	10X	1X	1.5μl	
DMSO	100%	10%	1.5μl	
$MgCl_2$	25mM	0.25mM	0.15μl	
FRAXA fxf primer	10pmol/μl	5pm	0.5μl	
FRAXA fxc primer *(HEX labelled)	10pmol/μl	5pm	0.5μl	
FRAXE 603	10pmol/μl	2pm	0.2μl	
FRAXE 598 *(FAM labelled)	10pmol/μl	2pm	0.2μl	
Expand Long Template Enzyme (Roche)	3.5U/μl	1.05U	0.3μl	
Water			7.65μl	
TOTAL VOLUME			**15μl**	

dNTPs MIX	LOT/BATCH NO	STOCK	1μL	100μL MIX
dATP		100mM	0.05μl	5μl
dCTP		100mM	0.05μl	5μl
dTTP		100mM	0.05μl	5μl
Water			0.35μl	35μl

PCR CONDITIONS: Thermocycler:GeneAmp9700 File: frax: elt

95°C – 10 minutes	1 cycle
95°C – 2.5 sec 65°C – 1 min 72°C – 2.5 min	5 cycles
95°C – 1.5 sec 55°C – 1 min 72°C – 2.5 min	25 cycles
72°C – 5 minute	1 cycle

FMR1 (CGG)n = (*FMR1* product – 221 bp) ÷ 3 *FMR1* = GREEN
FMR2 (GCC)n = (*FMR2* product – 288 bp) ÷ 3 *FMR2* = BLUE
Add 2.3μl dextran formamide dye + 0.2μl ROX 500 size standard to 1μl of undiluted sample and load on ABI377 Genetic Analyzer

NO.	SAMPLE	GENDER	DNA CONC	FAMILY NO (CODE)	*FMR1* $(CGG)_n$	*FMR2* $(GCC)_n$
1						
2						
3						
4						
5						
6						

FMR1 AND *FMR2* FRAGMENT SIZES WITH THEIR CORRESPONDING CGG/GCC REPEAT NUMBERS

FMR1 (CGG)n = (*FMR1* PCR PRODUCT – 221 bp) ÷ 3
FMR2 (GCC)n = (*FMR2* PCR PRODUCT – 288 bp) ÷ 3

***FMR1* (bp)** (HEX = GREEN)	(CGG)n	***FMR2* (bp)** (FAM = BLUE)
224	**1**	291
227	**2**	294
230	**3**	297
233	**4**	300
236	**5**	303
239	**6**	306
242	**7**	309
245	**8**	312
248	**9**	315
251	**10**	318
254	**11**	321
257	**12**	324
260	**13**	327
263	**14**	330
266	**15**	333
269	**16**	336
272	**17**	339
275	**18**	342
278	**19**	345
281	**20**	348
284	**21**	351
287	**22**	354
290	**23**	357
293	**24**	360
296	**25**	363
299	**26**	366
302	**27**	369
305	**28**	372
308	**29**	375
311	**30**	378
314	**31**	381
317	**32**	384
320	**33**	387
323	**34**	390
326	**35**	393

***FMR1* (bp)** (HEX = GREEN)	(GCC)n	***FMR2* (bp)** (FAM = BLUE)
326	**35**	393
329	**36**	396
332	**37**	399
335	**38**	402
338	**39**	405
341	**40**	408
344	**41**	411
347	**42**	414
350	**43**	417
353	**44**	420
356	**45**	423
359	**46**	426
362	**47**	429
365	**48**	432
368	**49**	435
371	**50**	438
374	**51**	441
377	**52**	444
380	**53**	447
383	**54**	450
386	**55**	453
389	**56**	456
392	**57**	459
395	**58**	462
398	**59**	465
401	**60**	468
404	**61**	471
407	**62**	474
410	**63**	477
413	**64**	480
416	**65**	483
419	**66**	486
422	**67**	489
425	**68**	492
428	**69**	495

APPENDIX D2 – FRAXE/*FMR2* Southern blot working sheet

DATE:_____
BLOT NO:_____

NO.	NAME	FAMILY NO.	DNA AMT	PCR RESULT	BLOT RESULT	COMMENTS
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						

DIGEST: (37°C overnight)

	STOCK	1 X MIX	FINAL CONC
Genomic DNA		10µl	5-10µg
Spermidine	0.1M	1µl	
*Hind*III (Roche)	10 units/µl	3µl	30 units
Buffer B	10 x	5µl	1x
Water		31µl	
TOTAL		**50µl / reaction**	**50µl / reaction**

Run digest on a 0.8% agarose gel (HGT) over 1 night
Label OXE20 probe with Megaprime kit
Hybridise over 2 nights at 65°C in the rotisserie oven

Post-hybe washes: 2 X SSC, 0.1% SDS 2 X 10min @ room temp
1 X SSC, 0.1% SDS 1 X 30min @ 65°C
0.1 X SSC 3 X rinses

Expose blot to X-ray film for ~3-10 days

Expected fragment sizes corresponding to the *FMR2* GCC repeat ranges after hybridisation of the OxE20 probe to DNA digested with *Hind*III on Southern blot analysis:

PHENOTYPE	(GCC)n	FRAGMENT DETECTED BY OXE20 PROBE (kb)
Normal male (N)	6-25	~ 5.2 kb
Normal female (N/N)	6-25	~ 5.2 kb
Premutation carrier male (E)	>30	5.2 kb +
Premutation carrier female (N/E)	>30	5.2 kb +
Full mutation male (EE)	>200	5.2 kb ++
Full mutation female (N/EE)	>200	5.2 kb ++

APPENDIX D3 – *FMR1* Southern blot working sheet

DATE:_____
BLOT NO:_____

NO.	NAME	FAMILY NO.	DNA AMT	PCR RESULT	BLOT RESULT	COMMENTS
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						

DIGEST: (37°C overnight)

	STOCK	1X MIX	FINAL CONC
Genomic DNA			5-10µg
Spermidine	0.1M	1µl	
Buffer B	10X	5µl (1X)	1X
EclX I (Roche)	10units/µl	3µl	30units
ddH_2O		µl	
TOTAL		**47µl**	

Add *EcoR* I (30u = 3µl/reaction) next day. Digest at 37°C for ~6 hrs.
Run digest on a 0.8% agarose gel (HGT) over 1 night

Label StB12.3 probe with Megaprime kit
Hybridise over 2 nights at 65°C in the rotisserie oven

Post-hybe washes: 2 X SSC, 0.1% SDS 2 X 10 min @ room temp
1 X SSC, 0.1% SDS 1 X 30 min @ 65°C
0.1 X SSC 3 X rinses

Expose blot to X-ray film for ~3-10 days

APPENDIX D4 – Procedure for labelling probe for Southern blotting

PROBE LABELLING REACTION USING THE MEGA-PRIME LABELLING REACTION KIT (AMERSHAM BIOSCIENCES).

The Megaprime systems allow DNA from a variety of sources to be labelled *in vitro* to high specific activity with ^{32}P and other radionuclides.

Labelling reaction mix

COMPONENTS	**1 X REACTION**
Probe DNA	5µl - 15µl (depending on DNA concentration)
Primer	5µl
ddH_2O	adjust as needed
Mix 1	**26µl**

Incubate reaction at 95°C in the heating block for 5 min

Place on ice to avoid renaturation. Make hole in the lid of the eppendorf tube (To prevent the lid from popping and spilling radioactivity)

Add the following reagents to mix 1:

COMPONENTS	**1 X REACTION**
*dATP, dGTP and dTTP	12µl (4µl each)
*Reaction buffer (10x concentrated buffer)	5µl
α^{32}PdCTP	5µl
* DNA polymerase 1 Klenow fragment (1 unit/µl)	2µl
Final reaction volume	**50µl**

Reaction buffer containing Tris/HCl pH7.5, 2-mercaptoethanol and $MgCl_2$

DNA polymerase 1 Klenow fragment cloned in 100 mM potassium phosphate pH6.5, 10 mM 2-mercaptoethanol and 50% glycerol

*supplied in Mega-prime labelling kit

Incubate the reaction @ 37°C for 20 – 30 min.

Stop the reaction with 5µl of warm 0.2M EDTA.

Add 50µl 1XTE buffer (pre-warmed at 37°C) to make up to 100µl volume.

SPIN COLUMN PREPARATION

Items required: 1ml syringe, Glass wool, 1 falcon tube, 1.5ml eppendorf tube
Sephadex G50 saturated in TE buffer and 1X TE buffer

- Remove plunger from the syringe and plug the bottom of syringe with the glass wool up to 0.1ml mark. Fill syringe with Sephadex
- Place labelled syringe with plug in a 15ml falcon tube and centrifuge @ (2700rpm) for 5 min to compact the Sephadex beads
- Discard TE buffer collected at the bottom of the falcon tube
- Top up syringe with Sephadex and centrifuge for 5 min.
- Repeat top up process of Sephadex until the column is packed to approx 0.9ml.
- If needed, store at 4°C, covered with cling wrap to minimise evaporation until ready to use.

PROBE PURIFICATION (FOR THE REMOVAL OF UNINCORPORATED NUCLEOTIDES)

- Cut the lid off an eppendorf tube and place it in the falcon tube under the column
- Load the probe-mix onto the column and centrifuge @ (2700rpm) for 5 min
- Load another 100ul pre-warmed 1XTE buffer into the column and centrifuge @ (2700rpm) for 5 min
- Verify the amount of probe eluted. If less than 200µl, add another 100µl 1X TE buffer and spin again.
- Retrieve the eppendorf containing the purified labelled probe
- Transfer the labelled probe into another eppendorf (punch a hole in lid)
- Add 1XTE buffer to purified labelled probe to make up to 1ml
- Denature 1ml of labelled probe @ 95°C for 5 min in the heating block
- Put on ice for 7 min
- Pour the pre-hybridization solution (from the Hybaid bottle with the membrane in it in the 65°C oven) into a new 50ml NUNC tube and add the denatured probe to the buffer
- Pour back the Prehyb solution and the labelled probe into the bottle and hybridize for 2 to 3 nights at 65°C in the Hybaid rotisserie oven.

APPENDIX D5 – DXS548 PCR working sheet

DATE:_____

PCR REACTION:

PCR Components	Volume	Final conc	X Mix
Genomic DNA	1-3µl (100ng-600ng)		
dNTPs mix	2.5µl	0.125Mm	
Taq polymerase buffer	2.5µl	1x	
Primer 83 or Dx (a) (20pm/µl)	0.5µl (10pm)	10pm	
Primer 84 or Dx (b) (20pm/µl)	0.5µl (10pm)	10pm	
Taq polymerase enzyme	0.2µl (1 unit)	1U	
Water	9.8µl		
TOTAL	16µl		
Add 1/40dil^{n} ^{32}P α-dCTP	5µl	0.125uCi	
TOTAL	**21µl**		

PCR CONDITIONS: HYBAID file

DENATURATION	ANNEALING	EXTENSION	CYCLES
94°C, 30 sec	57°C, 15 sec		30
72°C, 10 min			1
15°C			HOLD

Size: approx 192 bp- 210 bp
Load product on a long 6% polyacrylamide gel. Run light blue dye 30 cm from well
Expose O/N

NO.	SAMPLE	CODE	RESULT

APPENDIX D6 – FRAXAC2 PCR working sheet

DATE:_____

ENDLABELLING REACTION:

Components	Volume	X Mix
Primer: PM151 or FR (a)	2.5μl (50pm)	
Buffer	2μl	
Water	11.5μl (1U)	
Kinase enzyme (10u/μl)	2μl (20U)	
^{32} P γ-dATP	2μl	
TOTAL	20μl	

Incubate the reaction at 37°C for 1 hour.
Use 0.5ul, 1ul or 2ul of the labelled primer per PCR reaction (0.5μl usually works best)

PCR REACTION:

PCR Components	Volume	Final conc	X Mix
Genomic DNA	1-3μl (100ng-600ng)		
dNTPS mix	1.5μl	0.125mM	
Taq polymerase buffer	1.5μl	1x	
Primer PM151*-or FR(a) end labelled	0.5μl *	10pm	
Primer PM152 or FR(b) (20pm/ul)	0.5μl	10pm	
Taq polymerase enzyme	0.2μl	1U	
Water	10.8μl		
TOTAL	15μl		

PCR CONDITIONS: CETUS PCR cycler

DENATURATION	ANNEALING	EXTENSION	CYCLES
94°C, 4 min			1
94°C, 1 min	65°C, 1min	72°C, 1min	30
72°C, 10 min			1
15°C			HOLD

PCR product size range: ~147 bp to 161 bp
Load PCR product on a long 6% polyacrylamide gel. Run light blue dye 32 cm from well
Expose for 1 hr

NO.	SAMPLE	CODE	RESULT

APPENDIX E: *XNP* PCR WORKING SHEETS

APPENDIX E1 – Di, DRi Primer pair 1

DATE:_____

PCR COMPONENTS	STOCK	VOLUME	FINAL CONC
DNA	200-500ng	1μl	
Taq Buffer (Roche) (with 17.5mM $MgCl_2$)	10X	2.5μl	1x
dNTPs	10X (=250μM)	2.5μl	0.25μM
5'Di primer	10pm/μl	0.5μl	0.2pm
5'DRi primer	10pm/μl	0.5μl	0.2pm
Taq polymerase (Roche)	3.5U/μl	0.2μl (1 unit)	0.03U
Water		17.8μl	
TOTAL VOLUME		**25μl / reaction**	

PCR CONDITIONS: GeneAmp File: xnp

94°C – 50 sec 58°C – 50 sec 72°C – 1 minute	32 cycles
15°C – hold temperature	

PCR fragment 1 using primers 5'Di and 5'DRi (Villard et al., 1999b) = 287 bp
Primers used for sequencing: 5'Di



ggattatgaggtatttcatgtctaatacaaaagttacaaaatgctgactaaagtaagtatctttttctgtgtataaatgtgtgaataatagcc

actcctttctcctagAAGATGGGCTTCATGGGATTGTGAGCTGCACTGCTTGTGGACAACAGGTCA

ATCATTTTCAAAAAGATTCCATTTATAGACACCCTTCATTGCAAGTTCTTATTTGTAAGgtatgta

atgatctgttgatatttcataccgtaaatatttgaatgcttaccatataccaggcctttgag 5'DRi

Load 5μl PCR product on 3% agarose gel to verify the product size

NO.	SAMPLE	CODE	RESULT

APPENDIX E2 – 5'Ci, 5'R Primer pair 2

DATE:_____

PCR COMPONENTS	STOCK	VOLUME	FINAL CONC
DNA	200-500ng	1µl	
Taq Buffer (Roche) (with 17.5mM $MgCl_2$)	10X	2.5µl	1x
dNTPs	10X (=250µM)	2.5µl	0.25µM
5'Ci primer	10pm/µl	0.5µl	0.2pm
5'R primer	10pm/µl	0.5µl	0.2pm
Taq polymerase (Roche)	3.5U/µl	0.2µl (1 unit)	0.03U
Water		17.8µl	
TOTAL VOLUME		**25µl / reaction**	

PCR CONDITIONS: GeneAmp File: xnp

94°C – 50 sec 58°C – 50 sec 72°C – 1 minute	32 cycles
15°C – hold temperature	

PCR fragment 2 using primers 5'Ci and 5'R (Villard et al., 1999b) = 898 bp
Primers used for sequencing: 5'Ci and 5'R2

5'Ci

cagtgtcctggagattttccccagtgtttttaattacttaaaaaaataaagtttttgtcctttatgaatgttatcaattttgaattaaatttccccttc
ctctttttcttttaatagAATTGCTTTAAGTATTACATGAGTGATGATATTAGCCGTGACTCAGATGGAAT
GGATGAACAATGTAGgtaggtaatatagatgagctaacattcagtgtttcctacttttgggaggaattgttacataatcatttatg
aggtttgtctttcatagtatcgtcattgatgtctttaatgaaaggttgttcttttccataaaaccctggatttatgtctagtttaagtttctaataaag
agtaagcagatgacctaaattaccactaaaattactggcagtaaaatttaaattgcattataacaaagagattaaaatgagttttgttgg
gaataatgtttatttttattttttacatatcttttcagtttcctgaaagaagggaataatgttttaatctttaaaacatttcagGTGGTGTGC
GGAAGGTGGAAACTTGATTTGTTGTGACTTTTGCCATAATGCTTTCTGCAAGAAATGCATTC
TACGCAACCTTGGTCGAAAGGAGTTGTCCACAATAATGGATGAAAACAACCAATGGTATTG
CTACATTTGTCACCCAGAGCCTTTGTTGGACTTGGTCACTGCATGTAACAGCGTATTTGAG
AATTTAGAACAGTTGTTGCAGCAAAATAAGAAGAAGATAAAAGTTGACAGTGAAAAGAGTAA
TAAAGTATATGAACATACATCCAGATTTTCTCCAAAGAAGACTAGTTCAAATTGTAATGGAG
AAGAAAAGAAATTAGATGATTCCTGTTCTGGCTCTGTAACCTACTCTTATTCCGCAC

5'R2

5'R

Load 5µl PCR product on 0.8% agarose gel to verify the product size

NO.	SAMPLE	CODE	RESULT

APPENDIX F: *ARX* 24 bp DUPLICATION PCR WORKSHEET

Sequence analysis of Exon 2

DATE:_____

PCR COMPONENTS	STOCK CONC	1X MIX	X MIX
DNA	200 ng/μl	0.5μl	-
Failsafe PCR 2X PreMix J	2X	5μl (1X)	μl
Primer ARX-2F	20pmol/μl	0.5μl (10pm)	μl
Primer ARX-2R	20pmol/μl	0.5μl (10pm)	μl
ddH2O	-	3.4μl	μl
Expand Long Template Enzyme	3.5U/μl	0.1μl (0.35 U)	μl
TOTAL		10μl	9.5μl /rxn

Include a control sample for each PCR run. Refer to PCR SOP (CGEN/CMGT00012).

PCR CONDITIONS: Thermocycler: GeneAmp9700 Program: arx (~2.5 hour run)

95°C – 5 minutes	1 cycle
95°C – 1 minute 64°C – 1 minute 72°C – 1 minute	30 cycles
72°C – 5 minute	1 cycle
15°C – hold temperature	

Primers ARX-2F (also called 2563-F) and ARX-2R (also called 2794-R) (Gronskov et al., 2004)
PCR fragment = 251 bp.
Mutation c.431-454dup (24 bp) = 275 bp

The sequence in bold represents the region duplicated in the presence of the common 24 bp duplication mutation in exon 2 of the *ARX* gene.

ARX-2F

CCCAAGCTGCGGCGCCTGTACGGCCCGGGCGGGGGCCGCCTCCTTCAGGGTGCGGCAG
CGGCGGCGGCGGCGGCGGCGGCGGCGGCGGCAGCGGCCGCCACGGCCACGGCGGGT
CCACGCGGGGAGGCCCCTCCGCCGCCACCGCCAACCGCGCGGCCCGGGGAACGGCCG
GACGGCGCAGGG**GCCGCCGCGGCAGCCGCGGCCGCG**GCCGCCGCGGCCTGGGACACG
CTCAAGATCAGCCAGGCGCCGCA

ARX-2R

Load 2 μl PCR product on a 3% agarose gel to verify the product size

NO.	SAMPLE	CODE	RESULT

APPENDIX G: *FMR1* ALLELES IN 1184 NON EXPANSION MR MALES

CATEGORIZED BY ETHNICITY									
FMR1 **(CGG)n**	**WHITE (N=506)**		**BLACK (N=484)**		**COLOURED (N=122)**		**INDIAN (N=72)**		**GRAND TOTAL**
11	0	0.0%	1	0.2%	0	0.0%	0	0.0%	**1**
17	1	0.2%	0	0.0%	0	0.0%	0	0.0%	**1**
18	3	0.6%	1	0.2%	0	0.0%	0	0.0%	**4**
19	3	0.6%	0	0.0%	0	0.0%	1	1.4%	**4**
20	34	6.7%	2	0.4%	3	2.5%	0	0.0%	**39**
21	3	0.6%	2	0.4%	1	0.8%	0	0.0%	**6**
22	8	1.6%	11	2.3%	1	0.8%	2	2.8%	**22**
23	28	5.5%	19	3.9%	3	2.5%	0	0.0%	**50**
24	17	3.4%	9	1.9%	4	3.3%	1	1.4%	**31**
25	2	0.4%	1	0.2%	2	1.6%	1	1.4%	**6**
26	1	0.2%	3	0.6%	0	0.0%	0	0.0%	**4**
27	0	0.0%	0	0.0%	1	0.8%	1	1.4%	**2**
28	9	1.8%	11	2.3%	3	2.5%	0	0.0%	**23**
29	**120**	23.7%	**196**	40.5%	**50**	41.0%	**20**	27.8%	**386**
30	157	31.0%	109	22.5%	30	24.6%	29	40.3%	**325**
31	44	8.7%	77	15.9%	13	10.7%	3	4.2%	**137**
32	19	3.8%	17	3.5%	3	2.5%	2	2.8%	**41**
33	13	2.6%	2	0.4%	1	0.8%	1	1.4%	**17**
34	4	0.8%	2	0.4%	2	1.6%	2	2.8%	**10**
35	4	0.8%	2	0.4%	1	0.8%	1	1.4%	**8**
36	3	0.6%	3	0.6%	0	0.0%	0	0.0%	**6**
37	3	0.6%	0	0.0%	0	0.0%	2	2.8%	**5**
38	3	0.6%	3	0.6%	0	0.0%	0	0.0%	**6**
39	8	1.6%	4	0.8%	1	0.8%	1	1.4%	**14**
40	1	0.2%	1	0.2%	1	0.8%	2	2.8%	**5**
41	5	1.0%	5	1.0%	1	0.8%	3	4.2%	**14**
42	3	0.6%	0	0.0%	0	0.0%	0	0.0%	**3**
43	2	0.4%	0	0.0%	0	0.0%	0	0.0%	**2**
44	1	0.2%	0	0.0%	0	0.0%	0	0.0%	**1**
45	2	0.4%	0	0.0%	0	0.0%	0	0.0%	**2**
46	1	0.2%	0	0.0%	0	0.0%	0	0.0%	**1**
49	2	0.4%	1	0.2%	0	0.0%	0	0.0%	**3**
50	2	0.4%	1	0.2%	0	0.0%	0	0.0%	**3**
52	0	0.0%	1	0.2%	0	0.0%	0	0.0%	**1**
75	0	0.0%	0	0.0%	1	0.8%	0	0.0%	**1**
TOTAL	**506**	100.0%	**484**	100.0%	**122**	100.0%	**72**	100.0%	**1184**

APPENDIX H: *FMR2* ALLELES IN 1194 *FMR1* EXPANSION NEGATIVE MR MALES

CATEGORIZED BY ETHNICITY									
***FMR2* (GCC)n**	**WHITE (N=507)**		**BLACK (N=492)**		**COLOURED (N=123)**		**INDIAN (N=72)**		**GRAND TOTAL**
5	0	0.0%	2	0.4%	0	0.0%	0	0.0%	**2**
6	1	0.2%	1	0.2%	0	0.0%	0	0.0%	**2**
7	1	0.2%	16	3.2%	0	0.0%	1	1.4%	**18**
8	1	0.2%	0	0.0%	1	0.8%	0	0.0%	**2**
9	4	0.8%	1	0.2%	0	0.0%	0	0.0%	**5**
11	0	0.0%	0	0.0%	1	0.8%	0	0.0%	**1**
12	10	2.0%	5	1.0%	2	1.6%	2	2.8%	**19**
13	19	3.7%	29	5.9%	10	8.2%	1	1.4%	**59**
14	43	8.5%	61	12.4%	3	2.5%	5	6.9%	**112**
15	173	**34.1%**	255	**51.7%**	45	**36.9%**	27	**37.5%**	**500**
16	54	10.7%	40	8.1%	13	10.7%	8	11.1%	**115**
17	29	5.7%	31	6.3%	8	6.6%	3	4.2%	**71**
18	55	10.8%	13	2.6%	14	11.5%	10	13.9%	**92**
19	25	4.9%	0	0.0%	8	6.6%	4	5.6%	**37**
20	18	3.6%	12	2.4%	10	8.2%	4	5.6%	**44**
21	6	1.2%	9	1.8%	3	2.5%	2	2.8%	**20**
22	8	1.6%	7	1.4%	1	0.8%	0	0.0%	**16**
23	18	3.6%	1	0.2%	2	1.6%	3	4.2%	**24**
24	11	2.2%	1	0.2%	0	0.0%	1	1.4%	**13**
25	11	2.2%	2	0.4%	0	0.0%	1	1.4%	**14**
26	11	2.2%	2	0.4%	0	0.0%	0	0.0%	**13**
27	2	0.4%	2	0.4%	0	0.0%	0	0.0%	**4**
28	3	0.6%	1	0.2%	0	0.0%	0	0.0%	**4**
29	3	0.6%	1	0.2%	0	0.0%	0	0.0%	**4**
33	1	0.2%	1	0.2%	1	0.8%	0	0.0%	**3**
TOTAL	**507**	100.0%	**493**	100.0%	**122**	100.0%	**72**	100.0%	**1194**

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