ASCERTAINMENT, DIAGNOSTIC EVALUATION AND GENE MAPPING OF SOUTH AUSTRALIAN FAMILIES WITH POSSIBLE X-LINKED MENTAL RETARDATION

By

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Chapter One: Introduction

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1.1 Literature review

1.1.1 Mental Retardation

Mental retardation (MR) is a common neuropsychiatric disorder that affects children and adults throughout the world. Yet it has proved difficult to find appropriate terminology for the disorder and an agreed definition that can be used for clinical and research use.

Terms that have been used to describe persons with cognitive impairment include idiot, mentally retarded and intellectually disabled. Learning disability is another term that is used and for many years was distinguished from MR based on level of measured intellectual functioning, but as years passed this distinction became less valid. Polloway et al. (1997) discussed both of these categories and some related conceptual issues, noting that significant overlap exists between them and that they can coexist. The distinction between these two terms has limited value for deciding which service and support systems to apply.

1.1.1.1 Definition and Terminology

The definition of MR has been the subject of debate for many years. The most recent definition issued by the American Association on MR at www.aamr.org, states that it is "*a disability which originates before the age of 18 and is characterized by significant limitations both in intellectual functioning and in adaptive behaviour as expressed in conceptual, social, and practical adaptive skills"*. There are 5 assumptions that are essential to apply this definition:

1) Limitations in present functioning must be considered within the context of community environments typical of the individual's age peers and culture.

2**)** Valid assessment considers cultural and linguistic diversity as well as differences in communication, sensory, motor, and behavioural factors.

3**)** Within an individual, limitations often coexist with strengths

4) An important purpose of describing limitations is to develop a profile of needed supports.

5) With appropriate personalized supports over a sustained period, the life functioning of the person with MR generally will improve.

Wehmeyer (2003) analysed this definition and suggested that attention be paid to it when considering the types and intensities of support people need to succeed in different contexts. The term 'mentally retarded' has been associated with negative attitudes and reduced expectations. Therefore, a new term has been sought to substitute. Panek et al. (2005) assessed the terms used to describe MR using three factors: evaluation, activity and potency. 284 participants from different Midwestern USA populations completed assessments of several terms used to describe MR. The results showed that the term mentally challenged was the most potent, active and positively evaluated. On the other hand, mental retardation (MR), intellectual disability (ID) and developmental disability (DD) were viewed as similar terms. Therefore, the term mentally challenged can be a good substitute for the term mentally retarded but this need to be supported by similar assessment studies in populations in other parts of the world.

1.1.1.2 Classification

Classification of the severity of MR has been the subject of debate. There have been various classifications but the most widely used is based on the level of severity as determined by performance on standardized cognitive tests –the Intelligence Quotient (IQ test. Based on this approach MR is classified into:

Borderline retardation IQ 70-85

Mild retardation	IQ 50-69
Moderate retardation	IQ 35-49
Severe retardation	IQ 20-34
Profound retardation	IQ <20

Many epidemiological studies combine moderate, severe and profound into one category - severe MR (Leonard et al., 2003). As an example of an alternative approach, the new revised classification of the American Association on Mental Retardation defines severity in terms of intensity of support needed and includes the categories intermittent, limited, extensive, and pervasive.

1.1.1.3 Prevalence

There is wide variability in the published prevalence of MR in different populations. This variability has been attributed to differences in study location, inclusion criteria and case ascertainment methods. Leonard et al. (2003) used record linkage to ascertain West Australian children with MR between 1983 and 1992 and estimated the overall prevalence of MR to be 14.3 per 1000. Boyle et al. (1996) presented data based on the Metropolitan Atlanta Surveillance Program – the overall estimate of prevalence of MR was 8.7 per 1000 children 3-10years old. In another study, Bhasin et al. (2006) estimated the prevalence of MR in 8 year-old children between the years 1996 and 2000. In this study the overall prevalence was 15.5 per 1000 in 1996 and 12.0 per 1000 in 2000.

The exact prevalence of MR in South Australia is not known. However, it is not expected to differ significantly from the prevalence of MR in Western Australian children, which is estimated to be 14.3 per 1000 (Leonard et al., 2003).

Knowing the prevalence of MR in a population, and how it changes overtime, is essential for planning services for the affected individuals and their families.

It is well recognized that the prevalence of MR varies with gender, age, social class, ethnicity, and level of severity.

Variability of prevalence according to gender:

Studies have shown that the prevalence of MR varies with gender. Leonard et al. (2003) demonstrated increased prevalence among males compared to females with a ratio of 1.6. Other studies have shown similar results (Boyle et al., 1996; Bhasin et al., 2006).

Variability of prevalence according to age:

Boyle et al. (1996) demonstrated that the prevalence of MR varies with age from 5.2 per 1000 in 3-4 year old children to 12.3 per 1000 in 9-10 year olds. This increased prevalence with increased age was noticed in those with mild to moderate MR while the prevalence was constant in those with severe or profound MR. This was attributed to the fact that many children with mild disabilities are not identified until they attend school.

Variability of prevalence according to social class:

Drews et al. (1995) demonstrated that the prevalence of MR is associated with low socio-economic status. In a Norwegian study representing 30,037 births, children of parents with lower socio-economic status were at increased risk of mild MR but not severe MR (Strømme et al., 2000).

Variability of prevalence according to ethnicity:

Boyle et al. (1996) demonstrated that the rates of MR for black males was 3.1 times higher than for of white females, 2.4 times higher than for white males and 1.7 times higher than for black females. Bhasin et al. (2006) reported that the prevalence was twice as high for black children as for white children. Furthermore, Leonard et al. (2003) found that the prevalence of MR was found to be greater in children of Aboriginal origin compared to those of Caucasian origin with a ratio of 2.3.

Variability of prevalence according to severity:

Bhasin et al. 2006) estimated the prevalence for mild MR among 8 year old children in the year 2000 to be 7.3 per 1,000 while for moderate to profound MR it was estimated to be 3.3 per 1000. On the other hand, Leonard et al. (2003) estimated the prevalence for mild and moderate MR to be 10.6 per 1,000 while for severe MR it was 1.4 per 1000. In those cases where the severity was unspecified the prevalence was 2.3 per 1,000.

1.1.1.4 Causes

The aetiology of MR can be established in 60-75% of cases of severe MR but only in 38-55% of cases of mild MR (Inlow et al., 2004). There have been several approaches to classification of the causes of MR but mostly are based on the timing of insult to the central nervous system. The American Association on MR also followed this approach classifying causes into prenatal, perinatal and postnatal while the Finnish classification method divided them into six groups as illustrated in Table 1.1 (Wilska and Kaski. 1999).

Table 1.1 Aetiology of intellectual disability based on the time and mechanism of the injury to the CNS

Divisions and groups	**Examples**
1. Genetic disorders	
-Chromosomal	Trisomy 21,13,18,Angelman, Prader-Willi
-Genetic mutations	Tuberous sclerosis, PKU, Tay Sachs, Fragile X
-Multifactorial	Neural tube defects
2. Malformations	
-CNS malformations	Lissencephaly, holoprosencephaly
-Multiple malformation syndromes	Goldenhaar
3. External prenatal disorders	
-Maternal infections	Rubella, CMV, HIV
-Medicines/toxins	Alcohol/hydantoin
-Nutrition/IUGR/prematurity	Placental insufficiency, toxaemia
-Other	Diabetes, radiation, trauma
4. Perinatal disorders	
-Infections	Meningitis, herpes
-Delivery problems	Asphyxia, trauma
-Other newborn complications	Hypoglycaemia, hyperbilirubinemia
5. Postnatal disorders	
- Infections	Meningitis, encephalitis
-Other CNS damage	Toxic agents, tumours, hypoxia
-Psychosocial problems	Deprivation
-Psychosis, non-familial	
6. Unknown causes	
-Pure non-familial	No history/no finding
-CNS symptoms	ID, cerebral palsy/ID, epilepsy/ID, autism/ID
-Not classified	

The distribution of the causes of MR varies according to the severity, selection of cases, age, classification and ascertainment methods, and it is therefore difficult to compare different studies. Curry et al. (1997) presented a general distribution by causation in different reported populations as illustrated in Table 1.2.

Table1.2 Causes of MR in literature surveys

Cause	%
Chromosome abnormalities	4-28 %
Recognizable syndromes	3-7 %
Known monogenic conditions	3-9 %
Structural central nervous system abnormalities	7-17 %
Complications of prematurity	2-10 %
Environmental/teratogenic causes	5-13 %
Cultural familial MR	3-12 %
Provisionally unique, monogenic syndromes	1-5 %
Metabolic /endocrine causes	1-5 %
Unknown	30-50%

In another study, Rauch et al. (2006) found that the most common cause of MR was Down syndrome (9.2%) followed by microdeletion 22q11.2 (2.4%), Williams-Beuren syndrome (1.3%), Fragile X Syndrome (1.2%), Cohen syndrome (0.7%) and monosomy 1p36.3 (0.6%).

1.1.2 X-Linked Mental Retardation

Epidemiological studies in various populations around the world have shown that MR is more prevalent in males than females. This excess in males has suggested that X-linked MR is an important contributor to MR.

1.1.2.1 Definition

The term XLMR describes MR which shows an X-linked inheritance pattern and is attributed to mutations in genes on the X chromosome. Lehrke. (1972) was the first to suggest that genes for coding intellectual function might be on the X chromosomes followed by Turner (1974,1996) and Inlow et al. (2004) who suggested that the concentration of MR causing genes may be higher on the X chromosome compared to the autosomes.

Recognition of X-linked recessive inheritance

X-linked (XL) inheritance is based on pedigree characteristics which allow certain phenotypes caused by mutations in genes on the X chromosome to be distinguished from those caused by autosomal genes. The difference is determined by the presence of a single X chromosome in males and two X chromosomes in females. The characteristics of X-linked recessive inheritance are:

1. Only males are affected. Female carriers may manifest milder symptoms due to skewed X inactivation (Plenge et al., 2002) or due to the effect of differential expression of X linked genes in males and females (Skuse 2005)
2. There is no male to male transmission
3. All daughters of affected males are carriers
4. Sons of carrier females have a 50% risk of being affected while daughters of carrier females have a 50% risk of being carriers.

1.1.2.2 Prevalence:

XLMR has been estimated to be present in 1.83/1,000 males (Herbst et al., 1980) and to account for 20-30% of all learning disability.

1.1.2.3 Classification

XLMR can be classified as either syndromic or non-syndromic. Syndromic XLMR is referred to as MRXS while non-syndromic XLMR is referred to as MRX where S stands for specific or syndromic. Sequential MRX numbers beginning with MRX1 have been given to families satisfying MRX criteria i.e. the families whose only consistent clinical manifestation is MR and that have significant linkage (LOD score >2) to the X chromosome.

However, this distinction is becoming more blurred as, for several XLMR genes, mutations in the one gene have been found capable of causing both MRX and MRXS (Frints et al., 2002).

The most common cause of XLMR is fragile X syndrome, a form of syndromic XLMR which has a prevalence of 1 in 4,000-6,000 (De Vries et al., 1997). It was also found to be responsible for mild to moderate MR in 1 in 700-1,000 females. The estimated prevalence of nonsyndromic XLMR is 0.9-1.4/1,000 males (Kerr et al., 1991).

1.1.2.3.1 Syndromic XLMR (MRXS)

MRXS is XLMR accompanied by other features like malformations, dysmorphic features, neurological dysfunction or behavioural disturbances. The syndrome is often named on the basis of the most distinctive clinical feature or after the dysmorphologist who described it. The gene, FMR1, involved in the most common form of XLMR, the fragile X syndrome, was identified in 1991. It accounts for 15-20% of all XLMR.

1.1.2.3.2 Non syndromic or non specific XLMR (MRX)

MRX is XLMR occurring as an isolated phenomenon with absence of consistent somatic, neurological, biochemical or behavioural manifestations.

1.1.2.4 Causes of XLMR

It has been observed for many decades that MR is more common in males compared to female with an excess of about 30%. In addition, many families have been reported in which MR is inherited in an X-linked pattern. These two facts contributed to recognition of the importance of the genes on the X chromosome in the cause of MR. Researchers are

continuously publishing new papers to describe recent progress in this field (Ropers et al., 2005).

Although the use of positional cloning and candidate gene approaches has led to the identification of many XLMR genes, linkage analysis and mutation screening of known XLMR genes indicate that the contribution of each of the so far identified genes to the overall prevalence of XLMR is small <1%. It has been estimated that 30-50 additional genes remain to be identified (Ropers 2006).

To date, more genes have been found on the X chromosome than on any similarly sized segment of an autosome. This might be due to the greater ease of identifying genes on the X chromosome. Chiurazzi et al. 2004) provided a listing of known XLMR conditions; there were 201 entries (136 MRXS and 65 MRX), 107 localized by linkage (53 MRXS and 54 MRX) and 45 defined by a cloned gene (35 MRXS and 10 MRX). The listing was updated recently, with categorization into three groups based on clinical presentation: syndromes, neuromuscular disorders and non-specific conditions (MRX) (Chiurazzi et al., 2008). There are 215 entries: 149 with specific clinical features (98 syndromes and 51 neuromuscular conditions) and 66 with non-specific MRX. Ninety-seven conditions have been localized by linkage: 47 with specific clinical features (31 syndromes, 16 neuromuscular conditions) and 50 with non-specific MRX forms. Eighty-two conditions were defined by a cloned gene: 66 with specific clinical features (38 syndromes, 28 neuromuscular conditions) and 16 with MRX.

Mutations in at least 11 genes have been found to cause both MRXS and MRX, with considerable variation in intra-familial and inter-familial phenotypic expressivity. These are OPHN1, SLC6A8, ARX, MECP2, ATRX (XNP), RPS6KA3 (RSK2), FGD1, PQBP1, JARID1C/ SMCX, SLC16A2/MCT8 and AP1S2.

The XLMR gene listing has been updated by regular searching of the published literature. There are some Internet resources which are updated regularly to provide useful information for researchers. The following figures show the localization of all the currently known cloned XLMR genes (Figure 1.1), MRXS conditions (Figure 1.2) and MRX conditions (Figure 1.3), respectively http://xlmr.intrfree.it/home.htm.

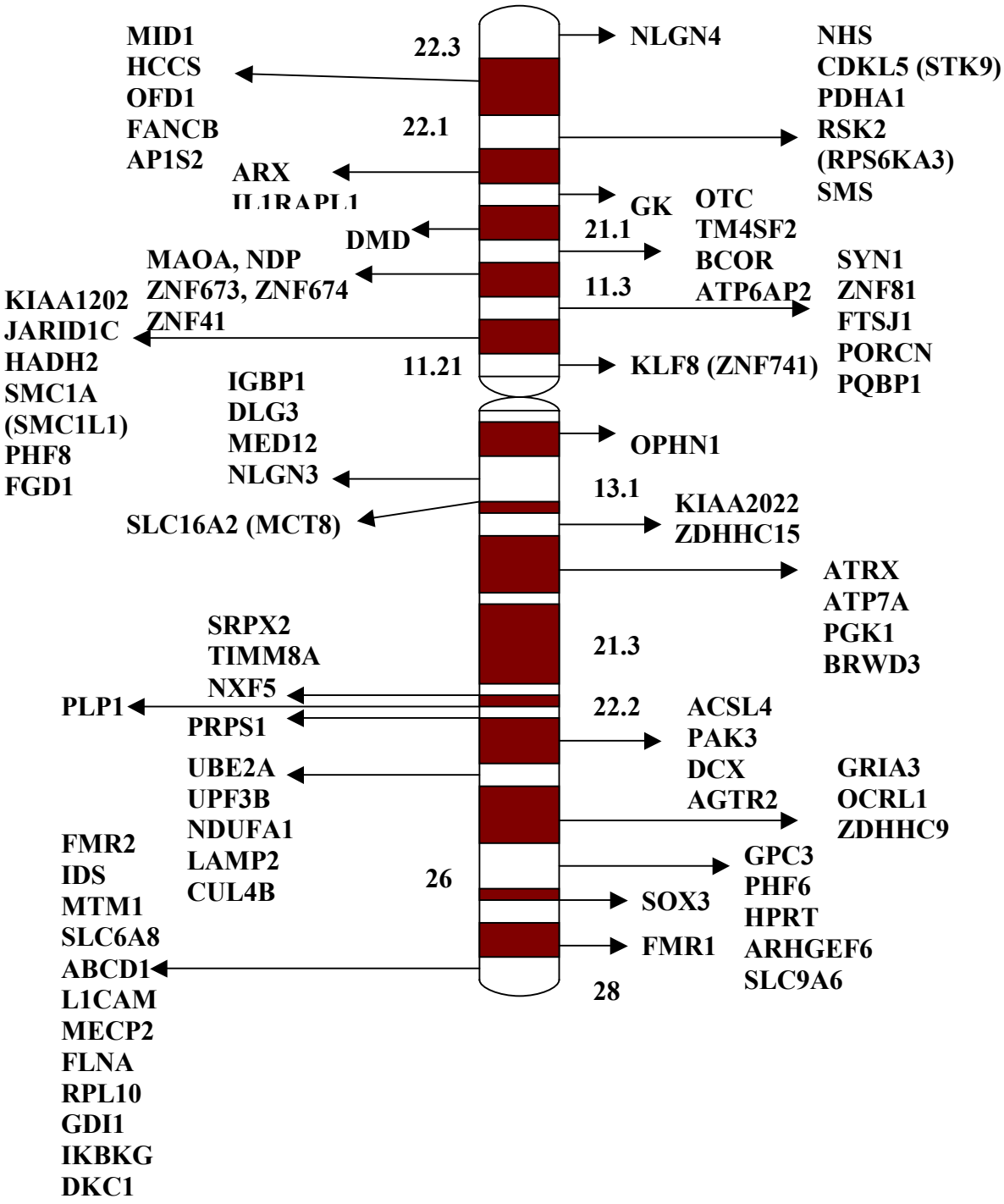


Figure 1.1 Localization of currently known cloned XLMR genes

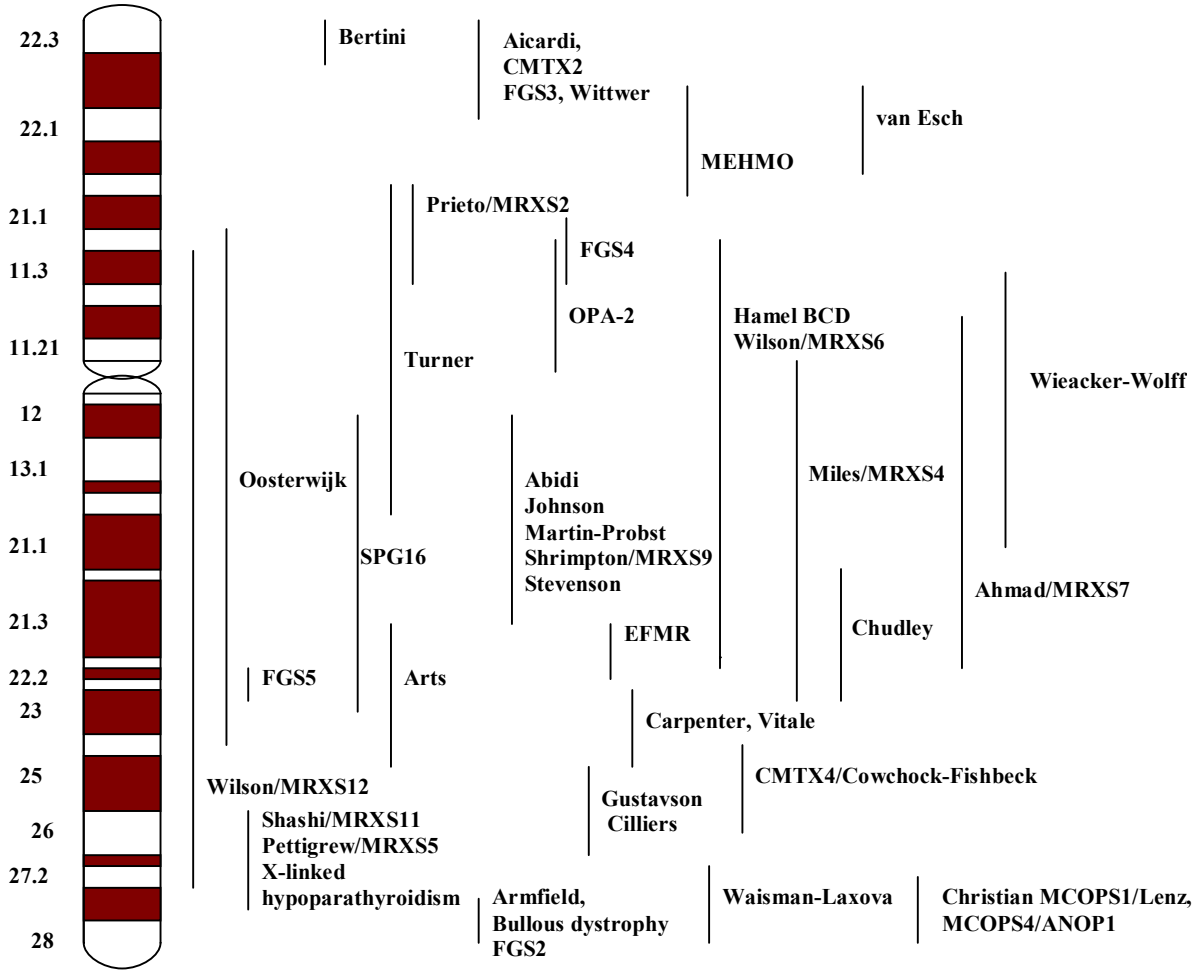


Figure 1.2 The MRXS conditions. Vertical bars indicate the linkage / cytogenetic intervals

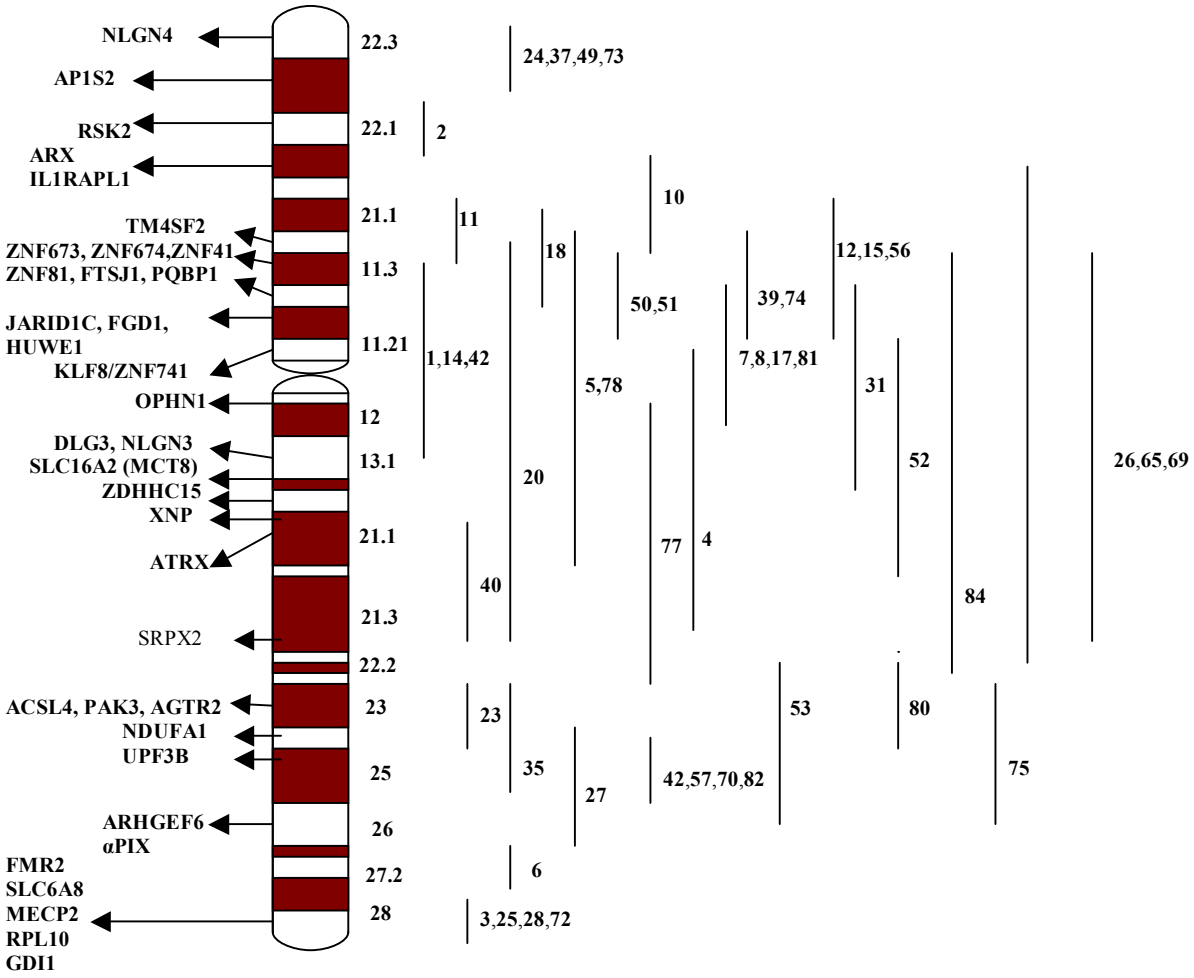


Figure 1.3 The MRX conditions. Vertical bars indicate the linkage / cytogenetic intervals. The numbers correspond to individual MRX families.

1.1.2.5 Diagnosis and investigation

Determining the cause of MR can be challenging, especially when it is mild. Curry et al. (1997) pointed out that there is no right or wrong approach and there is no single universal approach that can be applied to every patient in every situation. Van Karnebeek et al. (2005), Shaffer (2005) and Moeschler et al. (2006) have provided useful approached to the evaluation of the child with MR.

The benefits (for patients, parents and pediatricians) of providing an etiological diagnosis for MR are shown in Table 1.3 (Curry et al., 1997; Moeschler et al., 2006).

Table 1.3 Benefits of evaluation of MR

Benefits of evaluation of MR
For patients: -Identification of appropriate medical and non medical treatments -Identification of indicated medical intervention/referrals -Presymptomatic screening for associated complications/functional disabilities -Educational planning -Elimination of unnecessary testing and evaluations
For parents: -Education and clarification of aetiology, prognosis, genetic mechanism, complications, prevention, recurrence risks, treatment options -Referral to appropriate medical and social service agencies -Referral to support groups -Reproductive counseling, carrier testing, prenatal diagnosis -Family networking
For paediatricians: -Clarification of aetiology, prognosis, genetic mechanism, recurrence risks, treatment and prevention options and implications for other family members -Avoidance of unnecessary tests

-Information regarding management or surveillance and family support
-Research/treatment protocols
-Co-management of appropriate patients

One recently documented benefit of making a diagnosis is that it can restore reproductive confidence as exemplified by families with XLMR. Turner et al. (2007) demonstrated the effect of learning carrier status on the reproductive behaviour of women in XLMR families. The women from the different XLMR families were divided into 3 groups according to their carrier status - at risk, not carriers and carriers as shown in Table 1.4. After counselling and before identifying the causal mutation most women were not reassured by the risk expressed as a probability even if it was as low as 2% by linkage, resulting in fewer children being born. The number of offspring per year fell from the New South Wales average of 1 in 11 to 1 in 27. However, after finding the causal mutation and defining the carrier status the number of offspring rose above the NSW average. It made no difference whether the carrier status was confirmed or excluded. Finding the causal mutation restored the reproductive confidence of these women.

Table 1.4 The effect of learning carrier status on the reproductive behaviour of women in XLMR families

Carrier status	**No of Women**	**Women with children (%)**	**Women with no children (%)**	**Sons(No of affected)**	**Daughters**	**Offspring /reproductive year**
At risk	48	16 (33)	32 (66)	8 (2)	17	1 in 27
Not a carrier	23	18 (78)	5 (21)	11 (0)	7	1 in 6
Carrier	19	16 (84)	3 (16)	11 (2)	13	1 in 7

There are several key components of the genetic evaluation of a person with MR:

1-Clinical history

This includes the prenatal, natal and postnatal history. The prenatal history should assess adverse events and exposure to potential teratogens such as alcohol, medications or drugs. In addition, the presence of previous miscarriages, unexplained stillbirth or infant death can indicate chromosomal rearrangements. It is also important to know the developmental progress, education and schooling, and any history of seizures, autism, specific behaviours or loss of skills. A full medical history including a systemic review may give valuable information about the underlying cause.

Van Karnebeek et al. (2002) highlighted the role of clinical assessment in diagnosis of MR. The aetiology of one third of the cases was resolved by history and examination alone, another one third was resolved using clues provided by history and examination with confirmation by additional studies, and the last third was resolved by laboratory studies alone.

2-Family history (with construction of multi-generation pedigree)

This includes educational and medical history of the siblings, parents, uncles, aunts, grandparents and even other distal relatives with significant medical history.

3-General physical examination

This includes general and systemic examination looking for clues to the diagnosis.

4- Dysmorphologic examination

This focuses on dysmorphic features, minor anomalies and presence of any malformations.

Van Karnebeek et al. (2005) reported in a study based on the results of other studies the yield of six major diagnostic investigations and evaluated their variability with setting, gender and MR severity. These include cytogenetic studies, fragile X screening, metabolic investigations, neurological examination, neuro-imaging studies and dysmorphic examination. The results showed that the yield for dysmorphological studies was the highest. It varied between 39-81%.

5- Neurological examination:

This focuses on neurological abnormalities, such as the presence of features of cerebral palsy.

6- Investigations

There is no established evidence based guideline for diagnostic studies in MR patients. But the following publications provide useful guidance: Van Karnebeek et al. (2005), Shaffer (2005) and Moeschler et al. (2006). Some of the investigations used to reveal the aetiology of MR are:

a) Chromosome studies

(i) High resolution karyotyping:

Routine chromosomal analysis - karyotyping – has been used in investigating MR patients for many years. It detects numerical as well as structural chromosome aberrations, such as deletions, duplications, inversions and translocations. Deletions and duplications can be detected as long as they are at least 5-10 Mb in size – smaller pathogenic chromosomal imbalances will usually go undetected. High resolution analysis is needed to detect many abnormalities at the limit of resolution of the light microscope.

The mean yield of chromosome aberrations using classical cytogenetics was 9.5% (van Karnebeek et al., 2005). This varied between 5.4% in school populations to 13.3% in

institutionalised populations. It also varied according to the severity of MR from 4.1% in borderline-mild MR to 13.3% in moderate-profound MR. More structural anomalies were noted in females than males.

Macayran et al. (2006) reported a diagnostic yield of less than 1% for chromosome abnormalities in patients with MR of unknown aetiology who had no dysmorphic features, which is lower than previously described.

However, Battaglia et al. (1999) found in their study that 10.2% of retarded individuals had normal physical findings and abnormal chromosomes and recommended performing a karyotype even if dysmorphic features are absent.

(ii) Studies for microdeletions / microduplications by FISH or MLPA

Targeted FISH or MLPA (eg. For Williams syndrome or deletion 22q syndrome) can detect submicroscopic (<5Mb) alterations that can not be detected using cytogenetic analysis. They rely on the use of location specific probes and are used when the phenotype suggests a particular disorder for which there is a specific probe.

(iii) Studies for subtelomere rearrangements by FISH or MLPA

The subtelomeric regions are prone to rearrangements and are gene rich. Since the ends of chromosomes lack distinctive G-bands, it is difficult to detect small rearrangements at these regions by cytogenetic analysis. The presence of repetitive sequences within the telomeric regions, shared by chromosomes, precludes the use of chromosome-specific telomeric probes for FISH analysis. However, the development of unique subtelomere probes has permitted analysis of the regions approximately 300kb proximal to the repetitive telomere region.

Subtelomere FISH

Knight et al. (1999) reported a frequency of 7.4% of subtelomere abnormalities in moderate to severe MR patients. Van Karnebeek et al. (Artigas-Pallares and other (2005) estimated a median yield of subtelomeric FISH studies of 4.4% with female predominance.
Similarly, Rooms et al. (2005) reviewed multiple studies which used multiprobe FISH to detect subtelomeric rearrangements in MR patients and found that the detection rate varied between 1.4 and 16.3% with a higher percentage among moderate to severe MR patients.

Subtelomere MLPA

Koolen et al. (2004) demonstrated that subtelomere MLPA identifies a subtelomeric alteration in 6.7% of the cases. Abnormalities with clinical relevance were observed in 6.3%, 5.1% and 1.7% of mildly, moderately and severely retarded patients, respectively, indicating the importance of this test in mildly retarded patients.

(iv) Copy number imbalance by array CGH (Molecular karyotyping)

Molecular karyotyping by array-based comparative genomic hybridisation is a recently introduced technique used to detect submicroscopic genomic imbalances (deletions or duplications). It involves the labelling of test and normal reference DNA samples with two different-colored fluorochromes, mixing of the DNA samples and hybridisation on an array of mapped sequences. Array can be based on BACs, oligonucleotides or single nucleotide polymorphism (SNPs). The presence of copy number changes at a particular locus is suggested by a deviation from the expected 1:1 fluorescence intensity ratio between the test and normal samples at that chromosomal locus.

Clinical studies utilizing array CGH with BAC clones spaced at 1-1.4 Mb intervals across the genome have detected potentially pathogenic copy number changes in 10-17% of patients with MR and normal chromosomal analysis (Vissers et al., 2003; Shaw-Smith et al., 2004;

Schoumans et al., 2005; Menten et al., 2006; Tyson et al., 2005; Miyake et al., 2006; Rosenberg et al., 2006). In patients with MR, array CGH screening for anomalies has been reported using 1Mb resolution full genome arrays (Vissers et al., 2003; Shaw-Smith et al., 2004; Schoumans et al., 2005; Menten et al., 2006), full coverage X chromosome arrays (Bauters et al., 2005) and full genome tiling path arrays (De Vries et al., 2005).

It was found that molecular karyotyping by CGH doubled the pathogenic chromosomal imbalances detection rate by increasing the resolution level from 5Mb using the conventional karyotype to as low as 100kb by array technology.

De Vries et al. (2005) showed that the diagnostic yield of molecular karyotyping by array CGH was at least twice as high as that of standard karyotyping.

Bauters et al. (2005) developed and validated full coverage high resolution X chromosome array-CGH for fast and easy detection of submicroscopic copy number changes in patients with possible XLMR

Chromosome specific arrays have been developed and, for example, Kousoulidou et al. (2007) demonstrated that an X-chromosome specific MAPH platform can be a valuable tool for screening XLMR patients.

Table 1.5 illustrates the different reported studies using molecular karyotyping for MR.

Table 1.5 Summary of reported whole genome array genomic hybridisation studies for submicroscopic CNVs in idiopathic MR patients

Reference	**Selection criteria**	**Resolution**	**Pathogenic CNVs (%)**	**Positive/total patients**
Vissers et al. (2003)	MR/dysmorphism	1Mb 3,500BACs	10	5/20
De Vries et al. (2005)	MR	100kb 32,447BACs	10	10/100
Shaw-Smith et al. (2004)	LD/dysmorphism	1Mb 3,431BACs	14	12/50
Schoumans et al. (2005)	MR/dysmorphism	1Mb 2,600BACs	10	4/41
Menten et al. (2006)	MR/MCA	1Mb	13.6	28/140

		3,431BACs		
Rosenberg et al. (2006)	MR/MCA	1Mb 3,431BACs	16	20/81

b) Fragile X screening

In the review by van Karnebeek et al. (2005), molecular screening for fragile X syndrome showed a yield of 2.0%. It showed a higher yield in moderate-profound MR compared to borderline - mild MR.

c) Metabolic studies

These may include screening of urine amino acids, organic acids, oligosaccharides, and mucopolysaccharides and targeted metabolic tests based on clinical features like 7-dehydrocholesterol for Smith-Lemli-Optiz syndrome, transferrin isoforms for carbohydrate-deficient glycoprotein syndrome and uric acid for Lesch-Nyhan syndrome.

In the review by van Karnebeek et al. (2005), the mean yield for metabolic investigations was 1.0%.

d) Targeted neuroradiologic studies

This includes screening for intracranial abnormalities by magnetic resonance imaging (MRI) scan, computed tomography scan or ultrasound.

In the review by van Karnebeek et al. (2005), neuro-imaging studies identified some form of brain abnormality in 30.0% of cases but provided a specific aetiological diagnosis in only 1.3%.

In the American College of Medical Genetics consensus conference report, Curry et al. (1997) stated that neuro-imaging by CT or MRI in normocephalic patients without focal neurologic signs should not be considered standard practice.

e) X linkage with molecular testing of XLMR genes

Linkage analysis has proved to be a useful strategy for mapping MR genes. Linked genes are those located close enough to each other so that an expected crossover rate within the genetic material separating them at meiosis is less than 50%. Linkage analysis refers to the application of statistical algorithms that assess evidence of linkage and is a process that is most often used to map traits to particular chromosome locations. If a trait is mapped to a chromosome location it is assumed that there is a gene in that location contributing to the expression of the trait. A successful linkage analysis is followed by fine mapping to better localize and possibly identify the putative trait gene.

Linkage analysis has been used in large families with XLMR. A number of polymorphic markers along the X chromosome are studied and linkage is determined by a minimum lod score of +2 between the MR locus and at least one of these markers. Once a region has been delineated by linkage analysis candidate genes within this region are identified and some if not all are screened for mutations.

f) Overall yield from investigation of MR

The yield of aetiological evaluation of children with MR has shown a wide variation. This is attributed to many factors like differences in study populations, extent of diagnostic evaluation and improving diagnostic technologies over time. Table 1.6 displays the diagnostic yields of some of the mentioned investigations from different studies.

Table 1.6 Overview of the diagnostic yield from investigation of MR

The Diagnostic Investigation	Yield (%)	Study
Chromosomal aberrations	4.1-13.3% <1%	van Karnebeek et al. (2005) Macayran (2006)
Subtelomeric studies ST MLPA ST FISH	4.4% 1.3% 6.7% 1.4-16.3%	van Karnebeek et al. (2005) Rauch et al. (2006) Koolen et al. (2004) Rooms et al. (2005)
Fragile X molecular studies	2.0%	van Karnebeek et al. (2005)
Metabolic screening -Urine amino and organic acids	1.0% 1.1%	van Karnebeek et al. (2005) Poplawski et al. (2002)
Neuro imaging studies -For abnormalities -For finding a diagnosis based solely on the study	 30.0% 1.3%	van Karnebeek et al. (2005)
Dysmorphic examination	39-81% 40%	van Karnebeek et al. (2005) Rauch et al. (2006)

The American College of Medical Genetics consensus conference report (Curry et al., 1997) recommended that chromosomes be performed in any patient with MR. It also reported a low yield for unselected metabolic screening and concluded that metabolic testing should be selective and targeted at certain disorders based on history and examination. The American Academy of Paediatrics (Moeschler et al., 2006) supported this statement and suggested that any test that is positive in 1% of patients is appropriate. Fragile X testing in unexplained MR was recommended by both van Karnebeek et al. (2005) and Moeschler et al. (2006).

In another retrospective study Hunter (2000) evaluated 411 children with MR referred to a university based genetic center between 1986 and 1997. The author stated that certain factors

were associated with making a diagnosis. These included referral from a paediatrician or neurologist, absence of cerebral palsy, presence of more than three minor anomalies and/or an unusual appearance and a recognizable gestalt or key anomaly. It was found that half of the diagnoses made were based on the initial gestalt or key malformation.

In a more recent study, Rauch et al. (2006) analysed the diagnostic yield of various investigations in patients with MR referred to their cytogenetic laboratory and genetic clinic. These included conventional karyotyping, subtelomere screening, molecular karyotyping, X-inactivation studies and dysmorphological evaluation. Table 1.7 shows the diagnostic yields from each.

Table 1.7 Overview of diagnostic yield from cytogenetic, molecular genetic and clinical genetic assessment in intellectual disability

Assessment	Diagnostic yield (%)
Numerical chromosome abnormality	11.3
Array abnormality	6.6
Microdeletion syndrome	5.3
Monogenic disorder	4.8
Segmental chromosome abnormality	4.7
Mendelian disorder (pedigree)	2.7
Known syndrome without known cause	1.3
Environmental/acquired	1.3
Subtelomere abnormality	1.3
Balanced de novo chromosome abnormality	0.6
Mosaic trisomy	0.6
Uniparental disomy	0.3
Unknown	59.3

1.1.2.6 Recurrence risks:

Caring for a mentally retarded child places great emotional, physical and economic burdens on families. Providing an accurate estimate of recurrence risk of MR in these families is important. A population based study of the recurrence of developmental disabilities in

Metropolitan Atlanta identified all children born to the same mother in the period 1981-1991 and estimated that the risk for having a subsequent child with isolated MR was 8.4% if the first affected child had isolated MR (Braun et al., 2005). The risk of having a second child with isolated mild MR was 7.1% if the first affected child had isolated mild MR which was greater than the 4.7% recurrence risk estimate if the first child had isolated severe MR. This study also showed that the recurrence risk estimates overall and by severity level did not differ significantly by the sibling gender in families with two affected children. The calculated offspring risk for the intellectually normal siblings of a single affected male were 1-2% for the offspring of a normal brother and 2-5% for the offspring of a normal sister. Raymond et al. (2006) gave a summary of published recurrence risks for MR by sex of proband (Table 1.8).

Table 1.8 Published recurrence risks for MR by sex of proband

Reference	Affected Male			Affected Female		
	Brother (%)	Sister (%)	All Siblings (%)	Brother (%)	Sister (%)	All Siblings (%)
Turner et al			2-9			3.5-4.0
Bundey et al	6.7	3.2	5.0	4.4	6.3	5.4
Herbst et al	6.0	2.3	4.3	2.9	5.6	4.2
Bundey et al	10.0	5.0	7.5			
Costeff et al	14.0	14.0	14.0	9.6	9.6	9.2
Van Naarden et al			8.4			8.4

1.1.1.7 Prevention

A variety of preventive interventions have been developed depending on the disease mechanism causing MR. These include prenatal diagnosis, newborn screening, dietary supplementation or restriction, hormone replacement, vaccination, and immunotherapy. Crawfurd (1982) suggested that prevention can be divided into three levels. The first level was the prevention of the conception of affected embryos or development of disease in the fetus. This can be achieved by genetic counselling or avoidance of teratogens like alcohol and teratogenic drugs. The second level was the prevention of manifestation of an established

defect in the baby once born. A good example for this is the early detection of congenital hypothyroidism, phenylketonuria and other inborn errors of metabolism. The third level was the prevention of the birth of an affected baby by termination of pregnancy after prenatal diagnosis which can be established either by ultrasound, amniocentesis or chorionic villus sampling. However this might not be an option for many families based on religious or conscientious grounds.

Finally, preimplantation diagnosis can provide an option for some couples who cnnot accept termination of pregnancy.

1.1.3 X-linked mental retardation with Marfanoid body build (Lujan Fryns syndrome)

This syndrome is described separately because the research involved screening a cohort of South Australian males with MR and Marfanoid body build for mutations in genes associated with Lujan Fryns syndrome.

1.1.3.1 Definition

Lujan Fryns syndrome is a syndromal form of X-linked mental retardation characterised by mild to moderate MR, Marfanoid habitus, dysmorphic facial features and behavioural problems.

1.1.3.2 Prevalence

It affects males predominantly but the exact prevalence is not known.

1.1.3.3 Clinical features

The Marfanoid features of this syndrome include tall stature, long extremities and long thin hyperextensible fingers and toes. The Marfanoid stature becomes evident after puberty and

adult height is tall but still in the normal range. Pectus excavatum and joint hyperextensibility may be present. Some patients may have seizures but the no lens dislocation has been reported.

The facial features include long narrow face, prominent forehead, maxillary hypoplasia, high arched palate, thin upper lip, small mandible and long nose with narrow and high nasal bridge. Behavioural features have been observed in 80% of cases. These include extreme shyness, aggression, emotional instability, hyperactivity and autistic behaviour (Williams 2006). Other psychiatric problems like schizophrenia have also been observed. However, Donders et al. (2002) presented a patient who did not demonstrate any of these psychiatric features. In addition, Wittine et al. (1999) reported a patient and his maternal uncle with subaortic ventricular septal defect and aortic root dilation and suggested that this may implicate a mutation in a structural connective tissue gene. Lacombe et al. (1993) determined the frequency of MR in Lujan Fryns syndrome to be 100%.

1.1.3.4 Diagnosis and investigations

The diagnosis is based on the clinical features. Other differential diagnoses should be ruled out by cardiac examination, ultrasound, ophthalmic evaluation, chromosomal and biochemical analysis. Schwartz et al. (2007) identified a novel missense mutation in the MED12 (Mediator of RNA Polymerase II Transcription Subunit 12 gene) in the original family with Lujan Fryns syndrome. Raymond et al. (2007) identified mutations in ZDHHC9 in Lujan Fryns syndrome. ZDHHC9 (Zinc finger DHHC domain-containing protein 9) is a palmitoyltransferase that catalyses the posttranslational modification of NRAS and HRAS. On the other hand, Stathopulu et al. (2003) reported a young man with Lujan Fryns syndrome with a subtle terminal deletion of the short arm of chromosome 5. Recently, Tarpey et al. (2007) identified

protein truncating mutations in UPF3B, a member of the nonsense-mediated mRNA decay complex in some patients with the syndrome.

1.2 The GOLD SA Project (Genetics of Learning Disability South Australia)

As part of its research into the genetic causes of intellectual disability, Professor Jozef Gecz's team at the Women's and Children's Hospital , Adelaide has developed a strong relationship with the pioneering GOLD New South Wales (GOLD NSW) service. The GOLD NSW service was established in 1986 and has helped more than 300 families with fragile X syndrome. GOLD NSW has also made contact with many families with other types of intellectual disability and about 50 families have had the gene causing the problem identified. In January 2007 a donation allowed the team to establish a GOLD program for South Australia (GOLD SA). It aims to help families with inherited learning disability by identifying the cause of the problem and providing genetic counseling. GOLD SA is a collaboration between Women's and Children's Hospital and the Women's and the Women's and Children's Health Research Institute. The present research constituted a part of the GOLD SA project.

1.3 Aim of the research:

Mental retardation is a clinical condition which affects 14.3 per 1000 Australian children (Leonard et al., 2003). There are many causes for MR but, in spite of detiled investigation 50% of cases remain with unknown aetiology. Many genes have been identified to cause MR and many are yet to be identified. There are many South Australian families with MR where the cause is unknown. The aim of this study was:

1- The ascertainment of South Australian families with possible X
2- The diagnostic evaluation of these families
3- Linkage analysis with a view to gene mapping in large families

The study was carried out in the Department of Genetic Medicine of Women's and Children's Hospital. It has been approved by the Women's and Children's Hospital's Human Research Ethics Committee (REC 786/07/2008).

There are projects elsewhere in the world, for example in Italy and the EuroMRX consortium, similar to the GOLD SA and GOLD NSW projects.

Chapter Two: Materials and Methods

Chapter Two: Materials and Methods

2.1 Materials

The reagents used in this project were derived from many sources and these are stated in brackets wherever possible. All solutions were made with distilled water and all the tubes and disposable pipette tips were autoclave sterilised. Gloves were used at all times.

2.2 Methods

2.2.1 Clinical Methods

2.2.1.1 Ascertainment

2.2.1.1.1 South Australian XLMR families

This research was carried out in the Department of Genetic Medicine at Women's and Children's Hospital. The South Australian Clinical Genetics Service's database (Kintrak) was searched for individuals and families with a diagnosis of MR. To prevent missing any individuals all categories of MR diagnosis were searched including MR, X-linked MR and familial MR according to the MR diagnosis codes on the database. The files of all the identified families were reviewed to determine the ones that could possibly have XLMR. The pedigrees of potential XLMR families were then drawn using (Progeny software) which is linked to the main database (Kintrak). These families were divided by size into three categories depending on the likelihood of successful gene mapping: mappable, borderline and not mappable. The Department of Genetic Medicine's molecular genetics laboratory database was searched to determine the investigations already done on these families and the

availability of stored DNA samples. The amount of DNA available was determined for those with stored DNA. Family members whose DNA was required for linkage or other studies were identified. These included affected individuals and some of the informative unaffected individuals. An XLMR data collection form was created for each affected individual to record the identifying details of the patient, the family history, clinical information of the affected individual and an investigation sheet. A database was created to store all the collected data. This database was linked to Progeny and Kintrak to ensure that all data will be stored for future research and/or clinical use. A consent form was created for research participants and an information sheet setting out the aims of the project, what participation involves and other details. All potential XLMR families were contacted and invited to participate in the research project.

2.2.1.1.2 South Australian males with MR and Marfanoid habitus (Lujan Fryns syndrome):

The South Australian Clinical Genetics Service's database (Kintrak) was searched for males with a diagnosis of MR and Marfanoid body build according to the diagnosis codes on the database. The files of all these individuals were reviewed. After case selection based on clinical features, the pedigrees were drawn using the Progeny program which is linked to the main database (Kintrak). Then the Department of Genetic Medicine's molecular genetics laboratory database was searched for the investigations which had been done on these families and the availability of previously stored DNA samples.

2.2.1.2 Diagnostic evaluation and gene mapping

Appointments were arranged with families agreeing to be involved in the study for obtaining written consent and documentation of clinical information. In the appointments, the medical

history of the affected individuals was taken including prenatal and birth history, family history with construction of a multigenerational pedigree (or updating an existing one) and physical, dysmorphological and neurological examinations were carried out. A clinical geneticist was present for some but not in all of the clinical assessments. Family members were given laboratory request forms for DNA extraction and storage. Lymphoblastoid cell lines, routine chromosomes, Fragile X molecular testing and ST MLPA were requested for at least one affected member in each family if not done earlier. In an EDTA tube, 20 ml of venous blood was collected for DNA extraction/storage, 10 ml for lymphoblastoid cell line set up and 5ml for ST MLPA. In addition, 5ml of venous blood was collected in a lithium heparin tube for chromosomes.

2.2.1.3 Patient samples

Blood samples were collected at the Women's & Children's Hospital in Adelaide, through the person's general practitioner, or one of the blood collection centres located near to the patient's residence. The samples were sent to the Department of Genetic Medicine where the DNA was extracted and stored. For family members living outside Adelaide arrangements were made for the samples to be collected locally and transferred to Women's and Children's Hospital in Adelaide. Family members who were not willing to give a blood sample were provided with DNA self collection kits (Oragene) for extraction of DNA from saliva.

2.2.2 Laboratory methods

2.2.2.1 General methods

The methods and protocols used in this project are routinely used in the Department of Genetic Medicine at Women's and Children's Hospital in Adelaide.

2.2.2.1.1 DNA isolation

Genomic DNA was extracted from whole blood lymphocytes using Qiagen QIAamp maxi kits. Disposable gloves were worn at all times.

The water bath was preheated to 70˚C. Meanwhile the DNA number and the patient's name were checked and recorded. Two clean 50ml centrifuge tubes and a QIAamp Maxi column (provided in kit) were labeled with DNA laboratory number and tube number. 500 µl QIAGEN protease was pipetted into the bottom of the first labeled 50ml centrifuge tube and 10 ml of blood was added to it. 12 ml of Buffer AL was added and mixed thoroughly by vortexing at least three times for 30 seconds each time. The sample was then left at room temperature for 30 minutes. The sample was incubated at 70 ˚C for 10 minutes. 10ml ethanol was added and mixed by vortexing.

Half of the solution was poured onto the labeled Maxi Column held within a 50ml centrifuge tube. The cap was closed and the column centrifuged at 3000 rpm for 3 minutes. The maxi columns were removed from the tubes keeping the columns identified and ordered at all times. The filtrate was discarded into the blood waste bottle in a fume-hood and the thread of the centrifuge tube was wiped. The column was placed back into the centrifuge tube. The remainder half of the solution was loaded onto the column. The cap was closed and the column centrifuged at 3000 rpm for 3 minutes. The Maxi column was removed and the filtrate discarded into the blood waste bottle in the fume-hood. Any spillage was wiped from the thread of the centrifuge tube and the Maxi column was placed back into the tube.

5ml Buffer AW1 was added to the Maxi column. The cap was closed and the column centrifuged at 5000 rpm for 1minute. 5ml Buffer AW2 was added to the Maxi column without discarding the filtrate. The cap was closed and the column centrifuged at 5000 rpm for 15 minutes. The Maxi column was removed from the waste tube and placed into a clean labeled

50ml centrifuge tube (provided in the kit). 800 µl of Buffer AE was added directly onto the membrane of the Maxi column and the cap was closed. It was incubated at room temperature for 30 minutes and then centrifuged at 5000 rpm for 5 minutes. The filter was removed and the eluate containing the DNA was reloaded directly onto the membrane of the Maxi column. The column was placed back into the tube and the cap was closed. It was then incubated at room temperature for 5 minutes and then centrifuged at 5000rpm for 5 minutes. To remove remaining AE buffer from the column the tube was turned 180˚ within the centrifuge and respun for 5 minutes. The column was discarded and the eluate was transferred into a clean screw top tube clearly labeled with the laboratory number and patient initials.

The DNA was quantitated as per 'DNA quantification' MG-M-007 and placed in the appropriate DNA box in the DNA fridge.

2.2.2.1.2 RNA isolation and cDNA synthesis

Blood from probands was used to establish lymphoblastoid cell lines (Ms Lucianne Vandeleur) and cell pellets from these lines were used for RNA or DNA isolation and cDNA synthesis. This DNA and / or cDNA were used subsequently for specific gene tests. RNA isolation was performed using Trizol and QIAGEN RNeasy Mini kit. While keeping the tissue frozen, the sample was weighed using a pre-zeroed balance and left in dry ice to estimate the amount of Trizol required, using 1ml / 50-100mg of tissue. Then the pre-measured Trizol was added to the tissue immediately before homogenisation to prevent RNA degradation. Thorough homogenisation was ensured and the sample was left at room temperature for 5 min. 200µl of chloroform was added per 1ml Trizol used and shaken vigorously for 1min. The sample was left for 2-3 minutes at room temperature and then centrifuged for 15 minutes at max rpm at 4˚C. The upper aqueous phase containing the RNA was transferred to a fresh tube and 600 µl of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. 700µl of

the sample, including the precipitate that had formed, was applied to an RNeasy mini column placed in a 2 ml collection tube both of which were supplied with the kit. The tube was then gently closed and centrifuged for 15s at max rpm. The flow through was discarded.
350µl buffer RW1 was pipetted into the RNeasy mini column and centrifuged for 15s at max rpm to wash. The flow through was discarded. Then, 10µl DNase I stock solution was added to 70µl buffer RDD and mixed by gently inverting the tube. The DNase I incubation mix (80µl) was then pipetted directly onto the RNeasy silica-gel membrane and placed on bench top at room temperature for 15 minutes. Later, 350 µl buffer RW1 was pipette into the RNeasy mini column and centrifuged for 15s at max rpm. The flow through was discarded. Next, the RNeasy column was transferred into a new 2ml collection tube which was supplied with the kit. Then 500µl buffer RPE was pipetted to the RNeasy column. The tube was closed gently and centrifuged for 15s at max rpm to wash the column. The flow through was discarded. Another 500µl buffer RPE was added to the RNeasy column. The tube was closed gently and centrifuged for 2 minutes at max rpm to dry the RNeasy silica-gel membrane. The RNeasy column was transferred into a new 2 ml collection tube and the old collection tube was discarded with the flow through. The tube was then centrifuged in a microcentrifuge at max rpm for 1min. To elute, the RNeasy column was transferred to a new 1.5 ml collection tube. Then 30-50µl RNase-free water was pipetted directly onto the RNeasy silica-gel membrane. The tube was then gently closed and centrifuged for 1min at max rpm to elute. 1-5µg was then run on a 1% agarose gel to check the integrity of the RNA.
2 µg of RNA was used for the Reverse Transcription PCR (RT-PCR) reaction. The following reagents were added to each tube: Hex Random primers (1 µl), RNA (2 µg) and water (to 12 µl). Duplicate was made for each sample, 1 for +RT and 1 for –RT reaction. The samples were heated at 70^{o}C for 10 minutes and recovered on ice for 2 minutes. This step allows denaturing of RNA and binding of Hex primers. Meanwhile, the master mix was made for the

reactions using 5X first stranded buffer (4 µl), 0.1 M DTT (2 µl) and 10mM dNTPs (1 µl). Then, 7 µl of master mix was aliquotted to each sample, +/–RT. 1 µl of Super Script RT II (200u/µl) was added to each +RT reaction and 1 µl of water was added to each –RT reaction. It was then incubated at 42°C for 1h. The enzyme was heat inactivated at 70°C for 15 minutes. 30 µl of water was added to dilute samples. cDNA was tested with *ESD* PCR.

2.2.2.1.3 Polymerase chain reaction (PCR)

All PCR's were performed in 50µl reactions using 10x reaction buffer (Roche), 10mM dNTP mix (Fisher) and Taq polymerase (Roche). Each reaction contained the following unless indicated otherwise:

Table 2.1 The PCR components

Component	**Volume**	**Final Concentration**
DNA (100ng/µl)	1.0 µl	2 ng/µl
10x reaction buffer	5.0 µl	1x(1.5mM $MgCl_2$)
10mM dNTP mix	1.0 µl	200µM
Forward primer(50µM)	1.0 µl	1µM
Reverse primer (50µl)	1.0 µl	1µM
Taq polymerase(5U/ul)	0.5 µl	0.5 U/µl
dH_2O	To 50µl	

The cycling conditions and primers for each PCR are discussed for each gene tested in the relevant section below.

2.2.2.1.4 Agarose gel electrophoresis:

Agarose gel electrophoresis is a technique for the separation, identification and purification of DNA fragments. The size of the fragments to be separated determines the shape, size and concentration of the gel to be used. Ethidium bromide is a known mutagen. Care was taken

when handling it or any materials known to have come into contact with it. Gloves were worn at all times.

TBE buffer was prepared as a 5x concentrate and diluted to 1X TBE. 4g of agarose (Scientifix) was dissolved in 400ml of 1x TBE. The agarose solution was melted to a uniform liquid by heating it in the microwave while checking every minute until all was dissolved. Then it was allowed to cool. Whilst the gel solution was cooling, a gel tray was selected with a comb of suitable size for analysis of samples (i.e. at least the number of samples plus the ladder (Biolabs)). A 1Kb plus DNA ladder was used which consists of 12 bands from 1,000 bp to 12,000 bp in exact 1,000 increments. In addition to these 12 bands, the ladder contained 8 bands ranging in size from 100 bp to 1,650 bp.

The open ends of the gel tray were taped with autoclave tape and the comb positioned appropriately in the tray. The agarose was poured into it and left to set for about 15 minutes. The samples were prepared for loading onto the gel by mixing 10µl of sample and 1µl of loading dye (Fermentas). When the gel had solidified completely, the comb and the tape from the ends of the tray were removed. An appropriate gel tank was filled with 1xTBE and the gel was placed in the tank with the wells toward the black electrode. A black strip was placed under the tank below the wells to enable easier visualization of sample addition. Then the samples were loaded with the marker into the wells. The lid was placed on the tank, matching the terminals (Glasson and others). The leads were then connected to a power pack, again matching red to red and black to black. The power pack was turned on and the power adjusted as appropriate. The rate and time for the gel to be run depended on the size of the gel, the agarose concentration and the size of the fragments. 110 Volts for approximately 30 minutes were appropriate for most situations. Most agarose gels were made with, and run in, 1x TBE buffer. On completion of electrophoresis, the power pack was switched off and the gel was removed from the tank. The GeneSnap software (Syngene) was then used to capture the

electrophoretic gel images. Once the images were captured the gel was discarded. Then the PCR products were cleaned.

2.2.2.1.5 PCR products purification

The PCR products were cleaned up using QIAquick PCR purification kit. Five volumes of buffer PB1 were added to 1 volume of the PCR sample and mixed. A QIAquick spin column was placed in a provided 2ml collection tube. The sample was applied to the QIAquick column and centrifuged for 30-60s to bind DNA. 0.75 ml buffer PE was added to the QIAquick column to wash, and centrifuged for 30-60s. The flow-through was discarded and the QIAquick column was placed back in the same tube. This step was then repeated using 0.50 ml buffer PE. After discarding the flow-through the column was then centrifuged for an additional 1min. Then the QIAquick column was placed in a clean 1.5ml microcentrifuge tube. To elute DNA, 50µl water was added to the center of the QIAquick membrane and the column was centrifuged for 1min. The concentration of the cleaned up products was then checked using the NanoDrop ND-1000 Spectrophotometer.

2.2.2.1.6 Sequencing

Dye terminator sequencing was performed. To each tube the following reagents were added: dye terminator (2µl), dye buffer (2µl), purified PCR product (~90ng), (1-3µl), primer (1µl), and water (to 20µl). Then the sequencing program was run.

2.2.2.1.7 Cleaning up the sequencing products

Cleaning up the sequencing products was performed using precipitation. To each tube, 60µl of 100% isopropanol and 20µl of water were added. The sample was vortexed and spun briefly. The sample was then left covered on the bench for 15 minutes followed by centrifugation at 13k for 20mins. All liquid was removed from tube without disturbing the

invisible pellet. 250µl of 75% isopropanol was added, vortexed briefly and centrifuged at 13k for 10mins. All liquid was removed from tube. Vacuum spinning was done for 10mins to dry the pellet. The samples were then sent to the Institute of Medical and Veterinary Science (IMVS) for sequencing

2.2.2.1.8 Sequence analysis

DNA sequence analysis (i.e. comparison to the reference genome) was done through NCBI web page http://www.ncbi.nlm.nih.gov. In addition, all genomic sequences and characterised gene sequences were downloaded from the nucleotide database division and were imported into the EditSeq program (Lasergene software package, DNASTAR, USA) for further analysis. The results were analysed with the Seqman module.

2.2.2.2 Specific methods

2.2.2.2.1 X-tiling path micro array analysis

The X-tiling path array developed by the Flanders Interuniversity - Institute of Biotechnology (VIB) Leuven, Belgium was used. The array is a full coverage X-chromosome array-CGH. It consists of approximately 1850 clones, each analyzed in duplicate. The array was validated by male versus female hybridizations to confirm X-derived clones and hybridizations with known duplications or deletions versus pooled male or female controls. In all cases, known deletions or duplications were detected in both male and female patients by the VIB array.

2.2.2.2.2 Linkage analysis

DNA samples of each individual from respective families (~5 µg) were sent to the Australian Genome Research Facility (AGRF) Ltd, Genotyping section, Walter and Eliza Hall Institute,

Melbourne. The data was then analysed by Dr Kathie Friend. Genotype analysis of 48 microsatellite markers spanning the X chromosome at 5cM intervals (210 Mb / cM) was performed at the AGRF.

2.2.2.2.3 PCR amplification of the PHF6 gene for sequencing

Direct sequencing of the candidate gene PHF6 from an affected member of the families GOLD SA 1 and GOLD SA 7 was used to try to identify a disease causing mutations. The PCR was performed using cDNA instead of genomic DNA as per section 2.2.2.1.2. Since PHF6 is expressed in lymphocytes, cDNA was preferred because it is easier and less time consuming because 2 instead of 11 exons are tested. Table 2.2 lists the primers used for PHF6 sequencing (Invitrogen).

Table 2.2 The PHF6 primers

Primers	Sequence
PHF6 cDNA F1	CATTTCTTGAGACTTAAAGTGG
PHF6 cDNA F2	AATGAACATGAACTGGAGC
PHF6 cDNA R1	GTTCCATGGGAGGATGTGG
PHF6 cDNA R10/11	TCTGTCCCATGAACCTAGTT

The cycling conditions used were:

94 °C 2 minutes

94 °C 30 seconds

55 °C 30 seconds

72 °C 30 seconds } X 35

72 °C 2 minutes

2.2.2.2.4 PCR amplification of the UPF3B gene for sequencing

Individuals with suspected Lujan Fryns syndrome were tested for UPF3B gene mutations by direct sequencing. The PCR was performed using the genomic DNA as per section 2.2.2.1.2. Tables 2.3 and 2.4 list the primers (Geneworks) used for UPF3B.

Table 2.3 The UPF3B primers

Primer	Sequence
UPF3B genomic exon 1F	TAAAACCTTTGCACTGGGAAA
UPF3B genomic exon 1R	ATTTCGGGTGAATGGATGAA
UPF3B genomic exon 2F	AGCATTGGGCTGCAGAAG
UPF3B genomic exon 2R	AAAGCGATCCCTGAACAAAA
UPF3B genomic exon 3F	GAAAGGGGTAAATGAAAAAGGTT
UPF3B genomic exon 3R	GGTTTAAGCCAGGGCAAA
UPF3B genomic exon 4F	TGAATTTGTGGCTCCTTTTGT
UPF3B genomic exon 4R	GAATGAAATGCCCTAGCCAA
UPF3B genomic exon 5F	ACCTTGTGGGTATGGTTTGG
UPF3B genomic exon 5R	CCTTATCAGTCATCGAACTTTTTG
UPF3B genomic exon 6F	GGTGTACATGTTTTCCCACCT
UPF3B genomic exon 6R	TACCACCGTTATCCCCACTC
UPF3B genomic exon 7F	TGGTCTTCATTGTCTTTCGTTTT
UPF3B genomic exon 7R	TTTTATTGGATCACTTCCCCC
UPF3B genomic exon 8F1	GTTTTTGTCATTGAAAATGTTGG
UPF3B genomic exon 8F2	GTAAATGAGGGCTTAGGGCTG
UPF3B genomic exon 8R1	GCAGCCAGTTATGAGAAGCA
UPF3B genomic exon 8R2	TGTGAGAGGGAAAGGCACAT
UPF3B genomic exon 9F	GCAGAGATAGGGGGCTTTGT
UPF3B genomic exon 9R	TGAGGTTGCACTTTTAGAGCAG
UPF3B genomic exon 10F1	GGGTTTTGTTCCTTCTTAAAACAC
UPF3B genomic exon 10F2	GAAGAAGAGCGCCGTAGG
UPF3B genomic exon 10R1	CACTTGCAGGAAAAGGGAAA
UPF3B genomic exon 10R2	GTTGCAGATGATTAAAGTTCCC
UPF3B genomic exon 11F1	TGGTTGCTTTTCTTTGGAGA
UPF3B genomic exon 11F2	TTTACCGTGCCCTCACATTT
UPF3B genomic exon 11R1	CTGATCAGATTCCTGCTTTGA
UPF3B genomic exon 11R2	TGTAGAAATTGCAAAAGCAATGA

Table 2.4 The UPF3B primers

Exon	Forward primers	Reverse primers	Length (bp)	Temperature (˚C)	Extension Time
1	gEx 1 F	gEx 1 R	500	56	30
2	gEx 2 F	gEx 2 R	500	56	30
3	gEx 3 F	gEx 3 R	500	58	30
4	gEx 4 F	gEx 4 R	500	58	30
5	gEx 5 F	gEx 5 R	500	56	30
6	gEx 6 F	gEx 6 R	500	58	30
7	gEx 7 F	gEx 7 R	500	56	30
8	gEx 8 F1	gEx 8 R1	499	58	30
9	gEx 9 F	gEx 9 R	500	56	30
10	gEx 10 F1	gEx 10 R2	566	58	30
11	gEx 11 F1	gEx 11 R1	500	58	30

The cycling conditions used were:

94 °C	2 minutes	
94 °C	30 seconds	} X 35
55/58 °C	30 seconds	
72 °C	30 seconds	
72°C	2 minutes	

2.2.2.2.5 PCR amplification of the GRIA3 gene for sequencing

Direct sequencing of GRIA3 was used to try to identify a disease causing mutations in family GOLD SA 1. The PCR was performed with genomic DNA using the same reagents as per section 2.2.2.1.2 but instead of using 1.5mM $MgCl_2$ the concentration was adjusted according

to the primers as well as the PCR cycles. Table 2.5 illustrates the primers used for GRIA3 sequencing (Invitrogen).

Table 2.5 The GRIA3 primers

Exon	Forward primer	Reverse primer	Product size (bp)	[$MgCl_2$]	Annealing temperature (°C)
1	GTTGCGCCCATGCTCTTGTC	GGGAGAGCCTGGACCGACG	188	1.5	60
2	CAATTCCTAACTGGCCCTAGTG	ACAAGAAAGATGCAAATGAGGTC	283	1.5	60
3	AAGGACCATGGAGCTATTGAATC	AACACAATTACATGAATATCTAAAG	335	3.5	48
4	CAGTAGAATCTTTAATACCTACAG	ATATGGGAGCTGACTTAGGTTG	321	1.5	60
5	GAAAATGTCCTTCTTGAATAAGC	TAAATAGGATATATTGCCCAGGG	145	3	60
6	CATACAACTTCCCACAATCATG	GGTTTTTTACATGCCATGGAAC	232	1.5	60
7	GAGGGTATCATTTATCATGATATC	TATGTCTTTATAAGTTTCTTCCAC	265	2.5	50
8	ACCTCATCCCAGAGACTTAG	CCAAATGCTGTCCTCTTTCAAG	235	1.5	60
9	GGGAAAAATAGGCATCCAGG	AACGGAAGTCTTGCTGGACAG	200	1.5	60
10	ATGTATAGGAGTAATCCTTTTATC	TAATTGGAAAGCACAGAATAAATG	297	2.5	60
11	CAAATAACTGATTAAAGACTGTCTC	CTGCTGACTAGGAAAAGAACTAC	289	5	60
11(2)	GGCTTATGAAATCTGGATGTGC	GAATGGAGTATGACCATAAAATG	281	5	60
12	CCCTCTGGGTCTGGATTTG	GATAAAATAAATATCAGCAGGCTC	282	5	60
13	CCAGACTGAATATCTGGCAG	CACATATGGCACCATAACTTGGC	330	5	60
14	CTAAGCATTTGTGCATTTTCTTAC	GATTACTCGTCACTAAGGGTAC	210	3.5	60
15	GCTCAGGTTGCCTGCAGTG	AAAGACCTTCAGCGACTGAC	278	2.5	60
16	TATTATAATGTTCCATTGCCTTTG	CTGATTAGTTTACTAGACCTTAAC	339	5	48

The cycling conditions used were:

94 °C	2 minutes	
94 °C	30 seconds	} X 35
48/50/60 °C	30 seconds	
72°C	30 seconds	
72°C	2 minutes	

Chapter Three: Results

Chapter Three: Results

3.1 Ascertainment

3.1.1 South Australian XLMR families

The South Australian Clinical Genetics Service's database (Kintrak) was searched for individuals and families with possible XLMR. A total of 40 families were found. Six of these families had already been mapped or had an existing diagnosis. The remaining 34 families were then classified into syndromic or non-syndromic XLMR. Their pedigrees were drawn using Progeny software. The families were then divided into categories depending on the likelihood of successful gene mapping - mappable (6 families), borderline (8 families) and not mappable (13 families). Seven families were excluded for various reasons including the presence of only one affected member or some uncertainty about the X-linked pattern of inheritance. Table 3.1 summarizes the families with their categories.

Table 3.1 Categories and numbers of XLMR families

Category	No. of families	% of families
Mapped/diagnosed	6	15.0
Mappable	6	15.0
Mappable/borderline	8	20.0
Not mappable	13	32.5
Excluded	7	17.5
Total	40	100

Then the Department of Genetic Medicine's molecular genetics laboratory database was searched to document the investigations that had been done previously on the 27 included families. The availability of previously stored DNA samples was determined and the amount of available DNA was checked. Table 3.2 summarizes the results.

Table 3.2 Availability of DNA samples from XLMR families

	With DNA	Without DNA	Total
Syndromic	7	0	7
Non syndromic	13	7	20
Total	20	7	27

It was then determined which family members were to be contacted to ask for a blood sample. All the families were contacted and invited to participate in the research project and appointments were arranged for obtaining written consent and documentation of clinical information. Seven of the families declined for various reasons. A total of 109 DNA samples were collected from the families for the study and stored.

3.1.2 South Australian males with MR and Marfanoid habitus (Lujan Fryns Syndrome):

The South Australian Clinical Genetics Service's database (Kintrak) was searched for males with a diagnosis of MR and Marfanoid body build according to the diagnostic codes on the database. A total of 14 individuals were found .The files of these individuals were reviewed and the Department of Genetic Medicine's molecular genetics laboratory database was searched for the investigations which had been done previously on these individuals. The availability of previously stored DNA samples, and the amount of available DNA was checked.

3.2 Diagnostic evaluation

3.2.1 South Australian XLMR families

Appointments were arranged with the 27 families involved in the study. Some of the families were contacted initially by the geneticists who had seen them previously to introduce the

research to them. Seven of the families had SXLMR and 20 had NSXLMR. Unfortunately, 7 of the 27 families declined to participate in the study, mainly for social reasons. The following is a description of the families involved in the research.

The large mappable families

Family GOLD SA 1

The family consists of 80 members, 7 of whom have NSXLMR of moderate severity. The informant was III-2, who had approached the genetic service in 1994 when she was pregnant and wanted to know the risk of having a child with MR given the family history of learning difficulties in males. The affected family members were three of her step brothers, two nephews, one maternal uncle and one great maternal great uncle. No information could be obtained from her about the perinatal and childhood periods of the affected individuals. There was no history of consanguinity. Two of the step brothers (III-7 and III-15) were seen and examined. A third brother lives in Perth. III-7 was 47 years old at the time of the assessment. He had no other medical problems except myopia, for which he wears glasses. He had no dysmorphic features and his growth parameters including, weight, height and head circumference were within the normal range. Patient III-15 was 53 years old at the time of the assessment. He lived in a hostel, could not read or write, was unable to manage money but otherwise was able to manage independently. He had been diagnosed two years previously as having diabetes mellitus type II and was on insulin. None of the obligate female carriers had learning difficulties. In 1979, III-7, III-15, III-2 and III-13 had had a chromosome test which showed no abnormalities and no fragile X. In 1994, cytogenetic and molecular studies for fragile X were done and were negative. Blood samples were collected from 5 affected males, 4 obligate carrier females and 3 normal males in this family as shown in pedigree 1. The DNA

was extracted and stored. Several tests were carried out including repeat chromosomes and ST MLPA and both were normal. A lymphoblastoid cell line was prepared and cell pellets were obtained. Linkage mapping was done and the results are shown in section 3.4 in detail, after which the family was tested for mutations in GRIA3 and PHF6. The results are discussed in section 3.5.1.

Family GOLD SA 2

The family consists of 84 members, 16 of whom have NSXLMR of borderline to mild severity. The initial contact was with individual II-3, who had 5 affected sons and one affected grandson. She also had 7 brothers, one nephew and one maternal uncle who were also affected but unfortunately she had lost contact with them. As shown in pedigree 2 many of the affected individuals had married and had children.

III-13 was 20 years old at the time of the assessment. The pregnancy and the perinatal period were uneventful except for gastroesophageal reflux in the neonatal period. His general physical health was good. Individual IV-16 was 11years old at the time of the assessment. He was born 3.5 weeks post term. He had been diagnosed with attention deficit syndrome; his physical health was good.

III-6 was 31 years old at the time of the assessment. He was born 12 weeks premature and suffered from lung maturity problems. He had been diagnosed with schizophrenia and bipolar disorder. His physical health was otherwise good.

III-10 was 30 years old at the time of the assessment. He had gastroesophageal reflux in infancy. He wore glasses and had hearing loss in one ear.

Chromosomes and fragile X molecular testing were done and showed no abnormalities. Three of the brothers had married and had children (III-6, III-7, and III-10). Some of those children

had learning disabilities (IV-3, IV-7, IV-13, and IV-14). DNA samples were collected from 6 affected males, 7 obligate carrier females and 2 normal males.

Family GOLD SA 3

The family consisted of 32 members, 6 of whom had NSXLMR of moderate severity. II-1 was keen to help but unfortunately was unable to contact the rest of the family. The rest of the family could not be contacted by other means.

Family GOLD SA 4

The family consisted of 72 members 9 of whom had NSXLMR of moderate severity. Unfortunately the family declined to participate in the research.

Family GOLD SA 5

The family consisted of 42 members, 5 of whom had NSXLMR of moderate severity. Individual IV-4 was 12 years old boy at the time of the assessment. He was born at term without complications. He started to have epilepsy at the age of 4 months. He had an abnormal behavioural characteristic – rubbing his knuckles on his teeth. He had left amblyopia secondary to strabismus. Weight, height and head circumferences were within the normal range. He had dysmorphic features including blue sclerae, sloping forehead, brachycephaly, large ears, slightly upslanting palpebral fissures and mild micrognathia with malocclusion. He also had limited extension at the elbows and proximal interphalangeal joints of the little and ring fingers.

There was a family history of intellectual disability and epilepsy in three maternal uncles and a maternal great uncle, consistent with X-linked inheritance. The patient's immediate family was happy to participate but unfortunately other relatives were not willing to participate and

linkage mapping was not possible. However, the patient was tested individually for chromosomes, molecular testing for fragile X, and ST MLPA, all of which gave normal results.

Family GOLD SA 6

The family consisted of 25 members, 5 of whom had NSXLMR of moderate severity. The proband's family was in New South Wales and family members had already been investigated and found to have a duplication on the X chromosome.

Mappable/borderline families

Family GOLD SA 7

This was a family of 3 brothers with syndromic XLMR. All had moderate intellectual disability, hypotonia, poor coordination, obesity and mildly dysmorphic craniofacial appearance. The obesity was greatest over the lower abdomen, buttocks and thighs. They had minor dysmorphic features including upslanting palpebral fissures, deep set eyes, full lips and large mouth. All three had a mild thoracic kyphosis with lumbar lordosis, hyperextensible joints with genu valgum and mild gynecomastia. Assessment by an endocrinologist did identify a cause for the obesity and gynaecomastia but revealed hypogonadism in one of the brothers.

III-12 was 26 years old at the time of the assessment. He had testes measuring at least 25 ml, with a normal sized penis, stage 6 pubic hair and some beard growth. His free androgen index was below the normal adult male range suggesting mild/moderate hypogonadism. His FSH and LH, which were low normal, were compatible with partial hypothalamic-pituitary insufficiency or possibly markedly delayed puberty. However, the test results were surprising

as clinically he showed large testes and his sexual and body hair development also suggested a reasonable level of virilisation.

III-13 was 28 years old at the time of the assessment. His testes were approximately 25 ml in volume and he had a normal sized penis, stage 6 pubic hair and moderate beard growth. Endocrine studies showed a normal free androgen index.

III-8 was 12 years old at the time of the assessment. He had also been seen by an endocrinologist and found to have normal sized testes and penis; no further tests were recommended for him. However, he was overweight with a BMI of 34 and was referred to a gastroenterologist who tested his HbA1c, glucose, liver function, vitamin A and E, cholesterol, HDL cholesterol, urea, electrolytes creatinine and C-reactive protein, all of which gave normal results.

A number of investigations were carried out on the three brothers. These include chromosomes, urine amino and organic acids, urine mucopolysaccharides and oligosaccharides, maternal urine amino and organic acids, leukocyte lysosomal enzymes, electron microscopy of skin, skeletal survey, molecular studies for fragile X (A) and (E) syndromes, a search for haemoglobin H bodies on two occasions, 7-dehydrocholesterol and transferrin isoforms. The possibility of X-linked alpha thalassemia – mental retardation syndrome (ATRX) was also raised even though no haemoglobin H bodies had been found. Mutation screening of the most commonly affected regions of the ATRX gene had been performed in 1999; no abnormality was found but the presence of a mutation had not been excluded. ST MLPA, ST FISH, sequencing of part of the PQBP-1 gene and methylation studies for Prader-Willi syndrome all gave normal results.

Family GOLD SA 8

The family consisted of 51 members, 6 of whom had NSXLMR of mild severity.

III-7 was 44 years old at the time of the assessment. He had a long face with bulbous nose and brachycephaly. He also had asthma, nasal polyps and obesity.

III-12 was 38 years old at the time of the assessment. He had a long narrow face, brachycephaly, and hypoplastic ala nasi and upslanting palpebral fissures.

Chromosomes, molecular testing for fragile X, ST MLPA and sequencing of part of PQBP1 all gave normal results. DNA samples were collected from 2 affected males, 1 obligate carrier females and 2 normal males.

Family GOLD SA 9

The family consisted of 38 members, 5 of whom had NSXLMR of mild severity.

III-3 agreed to participate in the research. She had mosaic Turner syndrome but had no problem with her fertility. Her fragile X test was normal. Three of her sons (IV-1, IV-2 and IV-4) and one of her brothers (III-7) had mild MR. Chromosomes of III-7 and IV-1 were normal. Unfortunately, the affected individuals could not attend the clinic as they lived in rural South Australia; information about them was obtained from the family's medical records.

Family GOLD SA 10

The family consisted of 48 members, 5 of whom had NSXLMR of borderline to mild severity.

III-8 had approached the genetic service regarding IVF because of the family history of NSXLMR. The affected individuals were not seen in the clinic as the family declined, with some of them living in Victoria. They preferred to give a saliva sample for DNA extraction instead of giving a blood sample. DNA samples were collected from 1 affected male, 3 obligate carrier females and 3 normal males.

Family GOLD SA 11

The family consisted of 26 members, 4 of whom had NSXLMR of borderline to mild severity. III-5 and III-6 had approached the genetic service for preimplantation genetic diagnosis for sex selection to avoid the possibility of having a male affected by X-linked intellectual disability. III-6 had 2 brothers and 2 cousins with NSXLMR.

III-10 was 31 years old at the time of the assessment. He had mild hypotelorism, a prominent jaw, posteriorly sloping forehead, slightly anteverted ear lobes, mild brachydactyly of fingers and a high arched palate.

Chromosomes, urine amino and organic acids, urine mucopolysaccharides, plasma uric acid, molecular testing for fragile X and ST MLPA were all normal. DNA samples were collected from 4 affected males, and 2 obligate carrier females.

Family GOLD SA 12

The family consisted of 65 members, 11of whom had NSXLMR of borderline to mild severity.

IV-6 was 37 years old at the time of the assessment. There were no neonatal problems except for mild jaundice. He had attended special classes at school and was working in a sale yard. The rest of the family members declined participation in the research. III-19 had a 5q deletion which was also present in his father and uncle.

Chromosomes, molecular testing for fragile X syndrome and ST MLPA were all normal for IV-6.

Family GOLD SA 13

The family consisted of 45 members, 4 of whom had NSXLMR of borderline to mild severity. Family members who were contacted declined to participate and said they had lost contact

with the rest of the family. There were previously stored DNA samples from 2 affected males and 1obligate carrier female.

Family GOLD SA 14

The family consisted of 33 members, 7 of whom had NSXLMR of borderline to mild severity. This family was related to family GOLD SA 5. Unfortunately the family declined participation in the research.

The small not mappable families:

Family GOLD SA 15

This was a family with two brothers with NSXLMR of borderline to mild severity. Both had microcephaly, moderate intellectual disability, epilepsy and height at the lower end of the normal range. There was no history of consanguinity and no additional family history of similar problems.

III-1 was 29 years old and had been seen and investigated in 1981; no cause was found.

III-2 was 16years old at the time of the assessment. His head circumference was below the second centile, height was on the third centile and weight was between the 75th and the 90th centile. He had a sloping forehead with closely set eyes, relatively long nose, short and relatively smooth philtrum and nine café au lait patches, most of which were less than 1.5 cm in diameter. Physical examination was normal otherwise.

The following investigations gave normal results: skull X-ray, EEG, cerebral MRI and CT scan, TORCH screen, immunoglobulins and urine amino and organic acids, chromosomes, ST MLPA, molecular testing for fragile X syndrome and testing for the common duplication in ARX. DNA samples were collected from both affected males for future studies.

Family GOLD SA 16

This was a family with three brothers with SXLMR of borderline to mild severity.

III-10 was 17 years old at the time of the assessment. He had large central incisors, widely set eyes, small mouth and ridged metopic sutures.

III-8 was 23 years old at the time of the assessment. He had widely set eyes, small mouth, large central incisors and upslanting palpebral fissures. He also had a submucous cleft palate, velopharangeal insufficiency and short stature.

III-9 was 22 years old at the time of the assessment. He had widely set eyes, upslanting palpebral fissures, hypospadias and unilateral renal agenesis.

Chromosomes, molecular testing for fragile X syndrome, deletion 22q studies, thyroid function tests, 7- dehydrocholesterol and haemoglobin H bodies were negative. DNA samples were collected from the three affected males.

Family GOLD SA 17

This was a family with two brothers with SXLMR of borderline to mild severity.

III-6 was 8 years old at the time of the assessment. He had severe developmental delay, microcephaly and spastic quadriplegia. He also had undescended testes and chronic constipation. His MRI brain scan showed pachygyria, hypoplastic corpus callosum and mild dilatation of the ventricular system. There was evidence of immaturity of myelination and an anomalous cisterna magna.

Chromosomes, molecular testing for fragile X syndrome, TORCH screen, thyroid functions tests, 7- dehydrocholesterol were normal. DNA samples were collected from both affected males.

Family GOLD SA 18

The family consisted of 41 members, 3 of whom had SXLMR of moderate severity. DNA samples were collected from 3 affected males, and 2 obligate carrier females.

IV-7 and IV-8 were originally requested seen to discuss preimplantation genetic diagnosis for sex selection to avoid the risk of having another son with intellectual disability.

IV-8 had a brother and maternal uncle with moderate intellectual disability and the same characteristic dysmorphic features. Following genetic counseling regarding the potential risk to offspring, she elected to have a spontaneous pregnancy and unfortunately her first child was a boy with the same dysmorphic features and developmental delay, indicating that he is affected.

V-5 was born by caesarean section (breech). At the age of 2 years 6 months psychological assessment showed that he had moderate developmental delay. He had an abnormal behaviour - head banging. He had brachycephaly, plagiocephaly, microcephaly, dysmorphic facial features, bilateral clinodactyly of the little fingers and proximally placed thumbs. The dysmorphic features were a high forehead, low set ears, epicanthic folds, depressed nasal bridge and a small nose. He appeared to have the same syndromic form of X-linked intellectual disability as was present in his uncle and great uncle. IV-10 was 34 years old at the time of the assessment. He was born prematurely at 34 weeks. He was long sighted in one eye and short sighted in the other. He had a square jaw, flat ears, stiff finger joints, dystrophic toe nails with overriding toes and bilateral clinodactyly of the fifth fingers.

Chromosomes, molecular testing for fragile X, urine amino and organic acids, urine mucopolysaccharides, 7- dehydrocholesterol, transferrin isoforms, FISH for Smith Magenis syndrome and deletion 22q11.2, ST MLPA methylation testing for Angelman and mutation screening of the MECP2 gene (Rett syndrome) gave normal results.

Family GOLD SA 19

This was a family with two brothers with SXLMR of borderline to mild severity.

V-5 was 18 years old at the time of the assessment. He was born prematurely at 32 weeks. He had developmental delay and had been diagnosed to have ADHD, Tourette syndrome and bipolar disorder. He had had generalized tonic-clonic seizures until 15 years of age. Other features were microcephaly and pes planus.

V-6 was 20 years old at the time of the assessment. He was born prematurely at 32 weeks. He had developmental delay. He had had generalized tonic-clonic seizures which had changed to absence seizures at 13 years of age. Other features were short stature and microcephaly. Chromosomes, molecular testing for fragile X, urine amino and organic acids, urine mucopolysaccharides, thyroid function test, MRI brain, molecular testing for the common duplication in ARX and ST MLPA were normal. DNA samples were collected from both affected males.

Family GOLD SA 20

This was a family with two step brothers with NSXLMR of borderline to mild severity.

II-2 was 14 years old at the time of the assessment. He had a dysmorphic appearance with slight facial asymmetry, a broad nose, prominent nasal bridge, thick lips and bifid uvula. He also had striae on the right side of his back and upper thighs. He had been treated for arthritis between 7 and 10 years of age; recovery was nearly complete. His height was 173.3 cm (90^{th} centile), weight was 49.1 kg (50^{th} centile) and head circumference was 57.5 cm (98^{th} centile).

II-1 was 20 years old at the time of the assessment. He was tall, had epilepsy and was obese with striae on the proximal limbs. Chromosomes, molecular testing for fragile X, and ST and 22q MLPA were normal. DNA samples were collected from both affected males.

Family GOLD SA 21

This was a family with two brothers with NSXLMR of borderline to moderate severity.

II-1 was 15 years old at the time of the assessment. . He had epilepsy. His development had been slow; he walked at around the age of 2 years and had significantly delayed speech. His ability to read and write was limited. He also had some behavioural difficulties with occasional violent outbursts. He was a large boy with slightly unusual facial features.

II-2 was 12 years old at the time of the assessment. . He was a small, very active boy, with a diagnosis of ADHD and epilepsy. He was not dysmorphic. Chromosomes, molecular testing for fragile X, FISH for Smith Magenis syndrome, thyroid function tests, CK, urine amino and organic acids and urine mucopolysaccharides gave normal results. MRI brain scan showed asymmetrical temporal horns. DNA samples were collected from both affected males.

Family GOLD SA 22

This was a family with two brothers with SXLMR of borderline to mild severity.

II-2 was 10 years old at the time of the assessment. . He was unable to read or write. He had some dysmorphic features including a prominent frontal cow lick, protruding teeth, large ear lobes, upturned nose, a small thin upper lip and smooth philtrum. He had truncal obesity and inverted nipples.

II-1 was 16 years old at the time of the assessment. He was unable to read or write. He had poor concentration and some violent outbursts. A diagnosis of ADHD had been made. He had the same protruding teeth as his brother and long eye lashes and straight eyebrows. His dysmorphic features were not as marked as those of his brother. Both boys had tapering fingers and some slight joint laxity. Chromosomes and molecular testing for fragile X were normal. DNA samples were collected from both affected males.

Family GOLD SA 23

This was a family with two step brothers and their uncle with mild to severe SXLMR.

III-1 was 7 years old at the time of the assessment. . At birth was noted to have a dysmorphic appearance with small low set ears and a prominent jaw. He also had a small supra-umbilical hernia. He had severe intellectual disability with only a few single words of speech. He was being treated for epilepsy. He had tantrums and would hit his head with frustration. Glasses had been prescribed for myopia. On examination, his weight was 23.9 kg (50^{th} centile), his height was 121 cm (25^{th} centile) and his head circumference was 52 cm (50^{th} centile). He had an unusual facial appearance with a tall broad forehead, long face, hypotelorism, downward slanting palpebral fissures, speckled irides, bulbous nasal tip and brachycephaly. He had shortish fingers. His weight, height and head circumference were all above the 97^{th} centile. I II-2 was 9 years old at the time of the assessment. He had been born by a Caesarean section because of cephalopelvic disproportion resulting from a large head. He walked at 18 months and attended a special school. He had better speech development than his brother. His weight was 60.8 kg, height was 160 cm and head circumference was 57.7 cm (all above the 97^{th} centile). He also had a long face, mildly downward slanting palpebral fissures, a prominent jaw and round nasal tip. His palate was high arched. He had joint laxity especially involving thumbs, fingers and knees. He had bilateral hallux valgus and short left 3^{rd} and 4^{th} toes. II-4 was 39 years old at the time of the assessment. He lived alone and had a carer twice a week for 7-8 hours. He was not working but was involved in a number of activities such as ten pin bowling, and attended an adult disabled group. He had attended a special school. Chromosomes, molecular testing for fragile X, FISH for deletion 22q11, methylation test for Angelman syndrome, thyroid function test, transferrin isoforms, urine amino and organic acids, urine mucopolysaccharides and CT brain scan were normal. EEG was abnormal with

bicentral sharp and slow wave discharges. DNA samples were collected from all the affected males.

Family GOLD SA 24

This was a family with four brothers with XLMR of borderline severity. 2 of the affected brothers had a Marfanoid body build while the other 2 did not.

III-5 was 15years old at the time of the assessment and was considered to have Asperger syndrome.

III-6 was 17 years old at the time of the assessment. He had borderline intellectual abilities, Marfanoid habitus, microcephaly, kyphoscoliosis and keratoconus. His height was 172.5 cm (25^{th} - 50^{th} centile), his weight was 58.1kg (25^{th} - 50^{th} centile) and head circumference was 52 cm (3^{rd} centile). He was dynsmorphic with an asymmetric face, prominent ears, prominent nose, flat mid-face, high arched palate, small chin and deep set eyes. He had lax finger joints. Span to height ratio was 1.06 and upper to lower segment ratio was 0.88. He had positive thumb and wrist signs and his elbows were in a valgus position. He had a mild thoracic kyphoscoliosis, lumbar lordosis and pes cavus. X-ray of his hips (for protrusio acetabulae) and an MRI scan of the lumbosacral spine looking for dural ectasia were both normal. Echocardiography showed a normal aortic root diameter and heart valve function. He did not meet the criteria for a diagnosis of Marfan syndrome.

III-1 was a 7 year old boy at the time of the assessment. He had developmental delay and microcephaly. He had been assessed to have borderline Asperger/autism spectrum. He tended to chew things, including his fingers which became infected, and had anxiety with panic attacks. His height was 122.5 cm (50^{th} – 75^{th} centile), weight was 20.4 kg (25^{th} centile) and head circumference was 49 cm (2^{nd} centile).

Investigations included chromosomes, FISH for Smith-Magenis and deletion 22q11.2 deletion syndromes, subtelomere MLPA ,urine amino acids / organic acids / purines and pyrimidines / mucopolysaccharides, and molecular testing for fragile X syndrome. All of these investigations gave normal results. DNA samples were collected from all 4 affected males, one normal male and one female. Individuals III-1 and III-6 were also involved in section 3.2.2 as patients (13,14) because of the suspicion of Lujan Fryns syndrome.

Family GOLD SA 25

This was a family with two brothers with NSXLMR of borderline to mild severity.

V-1 was 6 years old at the time of the assessment. His pregnancy was complicated by substantial marijuana and alcohol use. He was born at 37 weeks by normal vaginal delivery with Apgar scores of 8 at 1 minute and 9 at 5 minutes and birth weight 2740 gm, length 48 cm, head circumference 33.5 cm. he had significant developmental delay. At one year of age he was not crawling, had poor head control and was not vocalising. He was hypotonic with joint hyperextensibility. He had some behavioural features including head banging, self biting, eye gouging and poor sleeping. He had acrocephaly, brachycephaly, single palmar creases and a depigmented patch on his right calf. Previous investigations had all been normal including chromosomes, CK, thyroid function tests, urine metabolic screen and brain MRI scan (which was normal apart from prominence of ventricles and subarachnoid spaces).

V-2 was 4 years old at the time of the assessment. His development was less delayed than his brother. He walked between 18 and 24 months and had speech delay. He was enrolled with Disability SA. He had some dysmorphic features with a low hair line, protuberant ears and down slanting palpebral fissures.

Subtelomere MLPA, 22q MLPA and 7- dehydrocholesterol level gave normal results. DNA samples were collected from both affected males

Family GOLD SA 26

This was a family with two brothers with NSXLMR of borderline to mild severity.

IV-4, IV-7 were diagnosed to have hypertrophic obstructive cardiomyopathy (HOCM) soon after birth and this was associated with mild intellectual disability. IV-7 had a left hemiparesis and epilepsy. Other members of the family had also had HOCM without intellectual disability. The family declined participation in the research.

Family GOLD SA 27

This was a family with two cousins with NSXLMR of borderline to mild severity. The family declined participation in the research.

3.2.2 South Australian males with MR and Marfanoid habitus (Lujan Fryns syndrome):

The South Australian Clinical Genetics Service's database (Kintrak) was searched for males with a diagnosis of XLMR and Marfanoid body build according to the diagnosis codes on the database. A total of 14 individuals were found. After identifying all the individuals, the pedigrees were drawn using the Progeny program. The following is a description of the individuals involved in the study.

Patient 1

The patient's early development was delayed and he had recurrent febrile convulsions. He had significant learning difficulties at school and was in a special class from year 8. He had angry outbursts and lack of motivation. His TORCH screen, plasma amino acids, urine amino acids/urine organic acids/mucopolysaccharide screen, thyroid function test, chromosomes and EEG were normal. His chromosomes were repeated at the age of 16 and molecular studies for fragile X syndrome were performed – both gave normal results. Marfan syndrome was

considered because of his lean build and high arched palate. Ophthalmologic review revealed an early cortical cataract and his echocardiogram showed a bulbous and slightly dilated aortic root at 3.1-3.2 cm (upper limit of normal for his age being 3 cm).

There was no family history suggestive of Marfan syndrome and no one else in the family had the same lean body build. There was a family history of intellectual disability that included his mother, who attended a special class at high school and is microcephalic, and his maternal uncle, who also attended a special class at school. The maternal grandmother and some of his cousins also had learning difficulties.

On examination, his height was 175.5 cm (50^{th} centile) and his weight was 48.5 kg (3^{rd} - 10^{th} centile). He had microcephaly with a head circumference of 50.5 cm (< 2^{nd} centile). His arm span/height ratio was 1.03. His upper segment/lower segment ratio was 0.85. The thumb sign was positive on the left and there was minimal chest wall asymmetry.

Patient 2

He was first seen by a geneticist at the age of 15 when referred by an orthopaedic surgeon because of severe scoliosis, Marfanoid habitus, unusual facial appearance and learning difficulties. He wore glasses for long sightedness. He had a sister who died at the age of 19 months due to a congenital heart defect.

On examination, his height was 180.5 cm (50^{th} centile) while wearing his spine brace, weight was 53.4 kg (3^{rd} centile) and head circumference was 53.2 cm (10^{th} centile). His arm span/height ratio was 1.0. He had a long face and prominent ears. His palate was high arched and his uvula was normal. He had lax finger joints. He had a wide sandal gap between the first and second toes and three café au lait patches. His Beighton score was 2/9.

He had many investigations (all normal) including routine chromosomes, FISH for Sotos syndrome and deletion 22q 11.2, subtelomere MLPA, total plasma homocysteine and an eye

assessment by an ophthalmologist. He had had two normal echocardiograms. His DNA was sent to Sydney for FBN1 and TGFBR1 mutation screening.

Patient 3

The patient had mild intellectual disability (IQ 68), hypermetropia, a lean body build and a past history of complex partial seizures with focal EEG features at around 9 years of age. His mother and two maternal cousins had significant learning difficulties at school. His sister had epilepsy. His paternal grandfather had had an abdominal aortic aneurysm at 78 years of age.

On examination, his height was 169.5 cm (75^{th} centile), weight was 37.5 kg (3^{rd} - 10^{th} centile), head circumference was 57.0 cm (90^{th} centile), span /height ratio was 1.0 and upper segment/lower segment ratio was 0.86. He had slightly upslanting palpebral fissures and a flattish midface. He had finger arachnodactyly and a positive thumb sign. His chest was long and narrow. The spine was straight.

His chromosomes, urine amino acid/organic acid/mucopolysaccharide screen, CT head scan, molecular studies for fragile X (A) and (E) syndromes and subtelomere FISH were normal. Echocardiogram showed a little bowing of the mitral valve but no prolapse and a normal aortic root diameter (2.2 cm).

Patient 4

The patient was first seen by a geneticist at the age of 9years. He had mild intellectual disability, Asperger syndrome, Marfanoid body build and dysmorphic craniofacial features. He had a past history of undescended testis and hypospadias. He had a right sided aortic arch and mitral valve prolapse. His chromosomes, plasma homocystinuria, ST FISH, urine amino acid/organic acid/mucopolysaccharide screen, cerebral CT scan and molecular testing for fragile X syndrome test results were normal.

On examination, he had upslanting palpebral fissures, flat mid face, short philtrum, small jaw and a high palate. His height was 186.6 cm (75^{th} centile), weight 57 kg (5^{th} centile) and head circumference was 56.4 cm (75^{th} centile). His arm span / height ratio was 1.0. His upper segment/lower segment ratio was 0.87. He was tall (after correction of his height for scoliosis) with lean body build, scoliosis, anterior chest wall deformity, arachnodactyly, long narrow feet, pes planus and a few cutaneous striae. His repeat echocardiogram was normal. TGFBR1 and TGFBR2 mutation screens were also normal.

Patient 5

He was one of five brothers. He had moderate intellectual disability and was in the care of his nephew. He had chronic atrial fibrillation and a dilated aortic root (4.7cm). His height was 195 cm (> 97^{th} centile). His head circumference was 61 cm (90^{th} centile). He had a high palate. There was no scoliosis, chest wall deformity, arachnodactyly, joint hypermobility or striae.

His chromosomes, molecular studies for fragile X syndromes and urine amino acid/organic acid/mucopolysaccharide screen were normal.

Patient 6

He was one of three brothers. Development was delayed by the age of 1year. He developed tics in his early teenage years including head turning, facial grimacing, and shoulder and arm movements and was diagnosed to have Tourette syndrome.. His mother had borderline intellectual disability, hypertelorism and short palpebral fissures.

He had a triangular facies, broad forehead, telecanthus with short palpebral fissures, high palate, poorly folded ears, high nasal bridge, long nose, short philtrum, small mouth, narrow high arched palate, micrognathia and smooth velvety skin. His height was 187 cm (90^{th} - 97^{th} centile), his weight was 79 kg (75^{th} centile) and his head circumference was 59.8 cm (98^{th}

centile). His arm span /height ratio 0.94 and his upper segment/lower segment ratio was 1.00. He had mild ulnar deviation at both wrists. He also had increased carrying angle bilaterally, narrow shoulders with long thorax and mild thoracic kyphoscoliosis,

His echocardiogram showed an aortic root on the 95th centile and mitral valve prolapse. His urine amino acid/organic acid/mucopolysaccharide screen was normal. Subtelomere FISH, FISH for Sotos, deletion 22q11.2 and deletion 4p syndromes were normal. His EEG showed frontal slow wave activity but no electrographic seizures.

Patient 7

This patient had mild MR with special difficulties in the areas of planning and coordination, conceptual development memory and receptive and expressive language. His verbal IQ was 62, performance IQ was 75, and full scale IQ was 66 at the age of 12 years. He had some dysmorphic features including upslanting palpebral fissures and notched ears. His palate was normal. His height was around the 97th centile, weight and head circumference were around the 50th centile and arm span /height ratio was 1.1. He had a negative thumb sign and positive wrist sign. There were 3 café au lait spots > 1.5cm on his lower back but no axillary or inguinal freckling.

His urine amino acid/organic acid/mucopolysaccharide screen, molecular studies for fragile X syndrome and CT brain scan were normal.

Patient 8

The patient had borderline intellectual abilities. His mother had used thioridazine and abused alcohol during pregnancy. She had been seen many years earlier because of concern that she might have had Marfan syndrome because of her Marfanoid habitus. Eye and heart

examinations were normal, however, and there was no documented family history of Marfan syndrome.

The patient had borderline working memory and significant behavioural and learning difficulties. He had a slim build, mild dolichocephaly, prominent ears, deep-set eyes, long face and nasal bridge. His height was between the 25th - 50th centile, weight was < 3rd centile, head circumference was on the 2nd centile, arm span /height ratio of 0.96. He had scoliosis, positive wrist signs, arachnodactyly, reduced elbow extension bilaterally and bilateral pes planus.

His pelvic X-rays, spinal MRI scan, slit lamp eye examination and echocardiogram were normal. His chromosomes, subtelomere FISH, FISH for deletion 22q11.2, plasma homocysteine level, blood lead level, thyroid function test, urine amino acid/organic acid/mucopolysaccharide screen and CT head were normal.

Patient 9

At 4 yr 4m of age the patient was assessed as having mild intellectual disability, with speech and language being more delayed than other areas. His speech was nasal. There were light coloured hair patches bilaterally, a triangular facies resulting from a relatively broad forehead and narrow chin, everted ears, prominent eyes, flat mid face, short philtrum and a small mouth. There were multiple cutaneous naevi on the trunk and scalp, 0.2 - 0.5 cm in diameter, purple-brown in colour and slightly raised. He also had a few dark brown macules/lentigines. He had a lean body build, his height was 173.3 cm (25th centile), weight was 49 kg (< 3rd centile), arm span /height ratio was 1.00 and upper segment /lower segment ratio was 0.86. He had kyphosis of the thoracic spine associated with the X-ray appearance of Scheuermnn's disese, cavus feet with claw toes and slender calves. His wrist sign was positive, thumb sign was negative and there was no joint laxity.

His chromosomes, subtelomere MLPA of chromosomes, 22q MLPA and blood homocysteine level were normal.

Patient 10

The patient had intellectual disability, nocturnal frontal lobe epilepsy, hypotonia, tall stature with Marfanoid body build, severe kyphosis and mild scoliosis. He had a myopic left eye and amblyopic right eye. He had cervical vertebral fusions and a past history of left inguinal hernia and left undescended testis.

On examination, he had a long narrow face with upslanting palpebral fissures and eyes that appeared small but were normally set. He had a short neck and a low posterior hairline. The mouth was small and the palate was high arched. He had missing lower incisors and a solid chin. He was tall with a lean Marfanoid body build.. He had long hands and fingers. The thumbs were broad distally with short nails tended to be held adducted and could not be fully abducted. His feet were narrow and flat. He did not have joint hypermobility (Beighton score was 2/9) or striae. His head circumference was on the 50^{th} centile, weight was on the 10^{th} centile, height was on the 75^{th} centile. There was severe kyphosis and mild scoliosis.

He had an IQ of 55-69 and obsessive compulsive behaviours. His EEG was normal in the awake state. His echocardiogram, subtelomere MLPA and MRI head scan were normal. MRI scan of the cervical and thoracic spine showed marked upper thoracic kyphoscoliosis centered upon T4 convex to the right with no spinal canal narrowing. His chromosomes, FISH for deletion 22q and subtelomere rearrangements, urine amino acid/organic acid/mucopolysaccharide screen, thyroid function tests, lactate, 7-dehydrocholesterol and molecular studies for myotonic dystrophy were all normal.

Patient 11

The patient was an apparently normal boy until around 12 months of age when his development appeared to slow down. He was subsequently shown to have intellectual disability, with a Marfanoid body build. He had pain and swelling of multiple joints, especially in winter, and was suspected to have juvenile arthritis. He also had Raynaud's phenomenon in winter. There was no family history of comparable problems. He had a triangular face resulting from a small chin, relatively smooth philtrum, micrognathia, a high arched palate and dental malocclusion. His height was 176 cm (75^{th}-90^{th} centile), weight was 47.9 kg (10^{th} - 25^{th} centile), head circumference was 54 cm (2^{nd}-50^{th} centile), arm span /height ratio was 0.99 and upper segment/lower segment ratio was 0.85. The thumb and wrist signs were positive and he had arachnodactyly. The spine was straight and there was no chest wall deformity or joint hypermobility (Beighton score 0/9). He did not have striae or pes cavus but there was mild eversion at the left ankle.

His routine chromosomes, subtelomere MLPA and plasma homocysteine were normal. Urine amino acids showed a small increase in arginosuccinate and its metabolites which was unusual but the patient did not have any other abnormalities in his blood or urine to suggest a specific metabolic disorder. The test was repeated after a meal rich in protein but the results were the same with a normal blood ammonia level.

Patient 12

This is a patient with mild MR who was diagnosed with autism at around the age of 2 years. There was no other family history of autism. At 24 years of age he worked 2 days a week packing medical kits. He had problems with social interaction and obsessive hand washing, was fond of pacing and often hit one hand against the other or against his head.

He was a tall thin young man with Marfanoid body build. His height was 192cm (90^{th} - 97^{th} centile), weight was 62.4 kg (10^{th}- 25^{th} centile) and arm span/ height ratio was 0.97. He had a long face, long slim fingers, hammer toes and flat feet.

His chromosomes, subtelomere MLPA screening, urine amino acid/organic acid/mucopolysaccharide screen, molecular testing for fragile X and testing for deletion 22q11 gave normal results.

Patients 13 and 14

This was a family with four brothers with intellectual disability of borderline severity.

III-5 was 15 years old and thought to have Asperger syndrome.

III-6 was 17 years old at the time of assessment. He had borderline intellectual abilities and a Marfanoid habitus, IQ assessment at 6 years of age had shown a borderline IQ. On examination, he had an asymmetric face, prominent ears, deep set eyes, high arched palate, flat midface, prominent nose and small chin. His height was 172.5 cm (25 - 50^{th} centile), his weight was 58.1kg (25^{th} - 50^{th} centile) and head circumference was 52 cm (< 3^{rd} centile). His arm span/ height ratio was 1.06 and upper/lower segment ratio was 0.88. He had lax finger joints, a positive thumb and wrist sign and valgus elbows. There was a mild thoracic kyphoscoliosis, lumbar lordosis and pes cavus.

Echocardiogram showed a normal heart. X-ray of his hips and MRI scan of the lumbosacral spine were both normal. He had bilateral keratoconus. His clinical features did not meet the criteria for a diagnosis of Marfan syndrome.

III-1 was 7 years old at the time of assessment. He was delayed in his speech development and slow in learning to read and write. He had been assessed to have “borderline Asperger/autism spectrum. He tended to chew things, including his fingers, and had anxiety

with panic attacks. On examination, he was not dysmorphic, his height was 122.5 cm (50^{th} - 75^{th} centile), weight was 20.4 kg (25^{th} centile) and head circumference was 49 cm (2^{nd} centile). His arm span/ height ratio was 0.99 and upper segment/lower segment ratio of 0.99. He had no scoliosis or chest deformity.

Investigations included chromosomes, FISH for Smith-Magenis and deletion 22q11.2 syndromes, subtelomere MLPA, urine amino acid/organic acid/mucopolysaccharide screen, urine purines/pyrimidines screen, molecular testing for fragile X syndrome; all were normal.

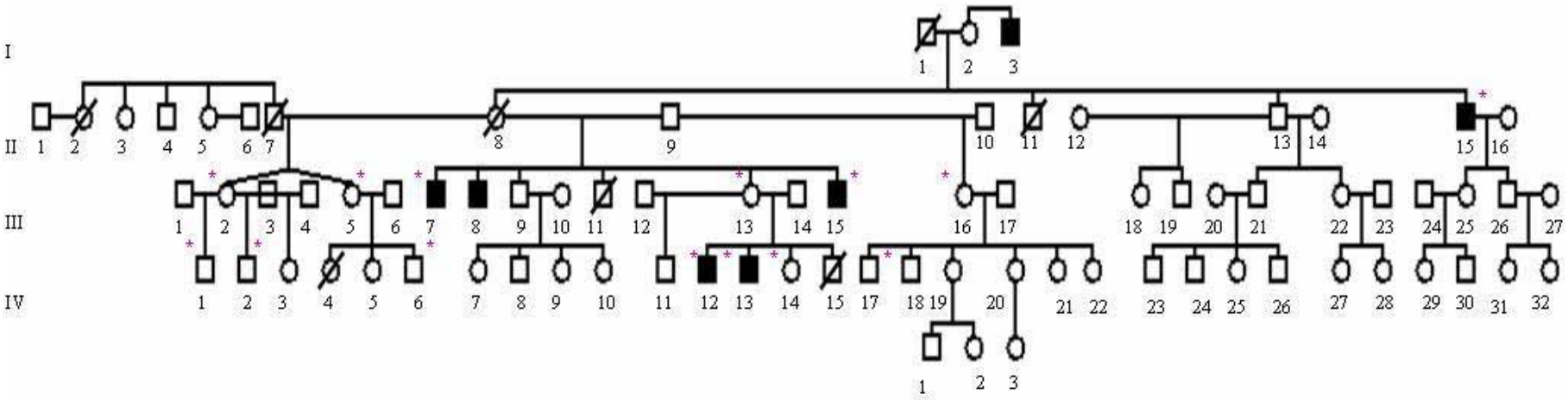
This family is included among the South Australian XLMR families in section 3.2.1 as family GOLD SA 24 due to the presence of MR in 4 brothers. DNA samples were collected from all 4 affected males, one normal male and one female.

3.3 Linkage mapping

3.3.1 Linkage mapping of family GOLD SA 1

The family included 9 affected males in 4 generations (pedigree 1- Family Gold SA 1). There were no other consistent clinical manifestations in the affected individuals other than moderate MR. Segregation of this phenotype was consistent with X-linked inheritance. The family was classified as having moderate non syndromic XLMR.

Pedigree 1 – Family GOLD SA 1



Whole blood was collected from 17 family members: 6 affected males, 7 females (including obligate carriers) and 4 unaffected males (as indicated in figure 1). Genomic DNA was isolated from lymphocytes using the QIAamp DNA blood maxi kit (Qiagen Pty Ltd. Doncaster, VIC, Australia).

3.3.1.1 Linkage interval

Genotype analysis of 48 microsatellite markers spanning the X chromosome (at approximately 5cM intervals) was performed at the Australian Genome Research Facility (Melbourne, VIC, Australia) using Linkage Mapping set v2.5 (Applied Bios stems, Foster City, CA, USA. Two point linkage analyses of these markers were carried out using Linkage program (MLINK) (Lathrop and Lalouel version 3.1). X-linked recessive inheritance with complete penetrance was assumed, with the frequency of the disease allele set at 0.0001. Allele frequencies for all microsatellite markers were set as equal The maximum two point LOD score (at $\theta = 0$) was 2.16 at DXS1001 (Table 3.3). Recombination events detected in two affected males, II-5 and II-7, localised the disease gene between the markers DXS8067 and DXS1062 (Figure 3.1). The linkage interval between DXS8067 and DXS1062 is approximately 16 cM.

Table 3.3 Two point LOD scores with 48 markers spanning the X chromosome in Family GOLD SA 1

Order	Markers	θ=0.0	θ=0.01	θ=0.05	θ=0.1	θ=0.2	θ=0.3	θ=0.4
1	DXS1060	-infini	-4.57	-2.49	-1.63	-0.82	-0.40	-0.16
2	DXS1223	-infini	-4.18	-2.12	-1.27	-0.53	-0.20	-0.04
3	DXS8051	-infini	-1.37	-0.67	-0.38	-0.13	-0.04	-0.01
4	DXS7108	-infini	-3.56	-1.58	-0.82	-0.22	-0.00	-0.05
5	DXS1224	-infini	-0.46	0.13	0.30	0.34	0.25	0.13
6	DXS987	-infini	-5.41	-2.72	-1.64	-0.70	0.27	-0.06
7	DXS8019	-infini	-4.06	-2.04	-1.23	-0.51	-0.18	-0.03
8	DXS7593	-infini	-0.47	0.12	0.29	0.32	0.24	0.13
9	DXS1226	-infini	-2.16	-0.85	-0.36	0.01	0.11	0.09
10	DXS1061	-infini	-4.05	-2.00	-1.16	-0.43	-0.12	0.01
11	DXS1214	-infini	-3.24	-1.64	-0.95	-0.37	-0.13	-0.03
12	DXS8102	-infini	-4.15	-2.12	-1.30	-0.58	-0.25	-0.08
13	DXS8090	-infini	-4.36	-2.32	-1.49	-0.75	-0.38	-0.16
14	DXS1068	-infini	-5.76	-3.04	-1.94	-0.94	-0.45	-0.17
15	DXS8015	-infini	-2.75	-1.40	-0.86	-0.39	-0.18	-0.06
16	DXS993	-infini	-5.76	-3.04	-1.94	-0.94	-0.45	-0.17
17	DXS8080	-infini	-4.44	-2.37	-1.50	-0.72	-0.33	-0.12
18	DXS8083	-infini	-1.36	-0.68	-0.41	-0.17	-0.07	-0.02
19	DXS1055	-infini	-2.75	-1.40	-0.85	-0.39	-0.18	-0.07
20	DXS1039	0.21	0.20	0.17	0.14	0.08	0.04	0.01
21	DXS991	0.26	0.26	0.23	0.20	0.13	0.07	0.03
22	DXS1216	-infini	-1.05	-0.40	-0.17	-0.01	0.03	0.02
23	DXS986	-infini	-1.34	-0.64	-0.35	-0.08	0.02	0.04
24	DXS1196	-infini	-3.48	-1.91	-1.20	-0.53	-0.21	-0.05
25	DXS1217	-infini	-2.16	-0.87	-0.39	-0.05	0.04	0.04
26	DXS990	-infini	-2.75	-1.39	-0.84	-0.36	-0.14	-0.03
27	DXS8077	0.26	0.26	0.23	0.20	0.13	0.07	0.03
28	DXS8020	-infini	-3.86	-1.85	-1.07	-0.40	-0.13	-0.01
29	DXS1106	-infini	-1.20	-0.54	-0.28	-0.07	0.00	0.01
30	DXS1059	-infini	-2.17	-0.90	-0.45	-0.13	-0.03	-0.00
31	DXS8088	-infini	-1.86	-0.58	-0.12	0.18	0.21	0.14
32	DXS8055	0.28	0.28	0.28	0.25	0.18	0.10	0.04
33	DXS8064	0.63	0.62	0.61	0.57	0.46	0.32	0.16
34	DXS8067	-infini	-0.76	-0.13	0.07	0.17	0.15	0.08
35	DXS1001	2.16	2.13	1.98	1.79	1.38	0.94	0.48
36	DXS8009	0.57	0.56	0.50	0.43	0.29	0.16	0.07
37	DXS1047	1.03	1.02	0.97	0.88	0.67	0.45	0.22
38	DXS1062	-infini	0.13	0.71	0.85	0.81	0.61	0.34
39	DXS984	-infini	-1.97	-0.66	-0.18	0.17	0.23	0.16
40	DXS1205	-infini	-0.46	0.13	0.30	0.34	0.25	0.13
41	DXS1227	-infini	0.13	0.71	0.85	0.81	0.61	0.34
42	DXS8106	-infini	-0.56	0.06	0.25	0.32	0.25	0.14
43	DXS8043	-infini	-1.91	-1.09	-0.72	-0.38	-0.20	-0.09
44	DXS8045	-infini	-0.46	0.14	0.32	0.36	0.28	0.15
45	DXS998	-infini	-3.85	-1.83	-1.02	-0.33	-0.06	0.03
46	DXS8091	-infini	-1.70	-0.91	-0.57	-0.28	-0.14	-0.06
47	DXS8069	0.97	0.94	0.83	0.71	0.48	0.29	0.13
48	DXS1073	-infini	-2.68	-1.63	-1.13	-0.63	-0.36	-0.16

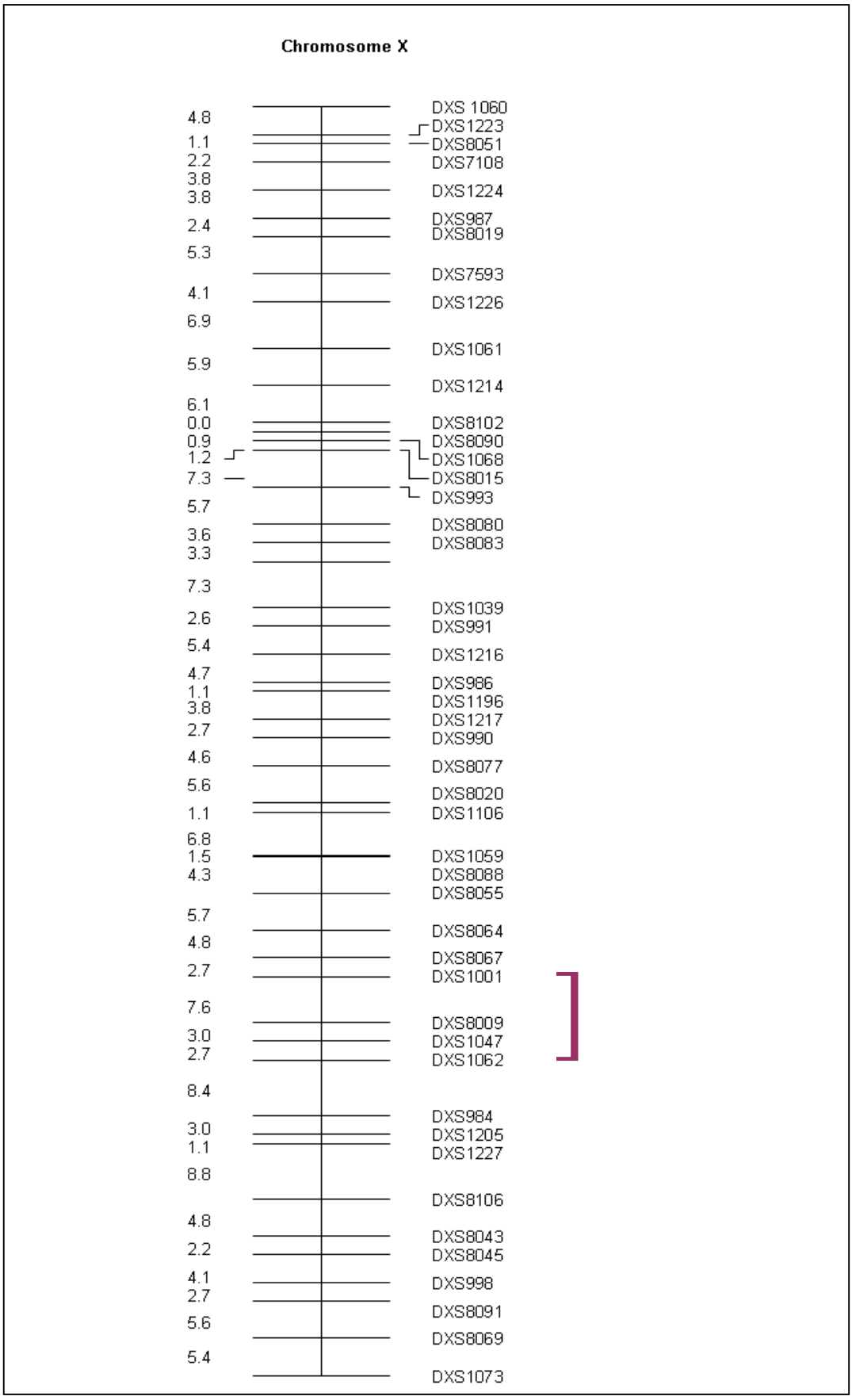


Figure 3.1 Order of the 48 microsatellite markers on the X chromosome

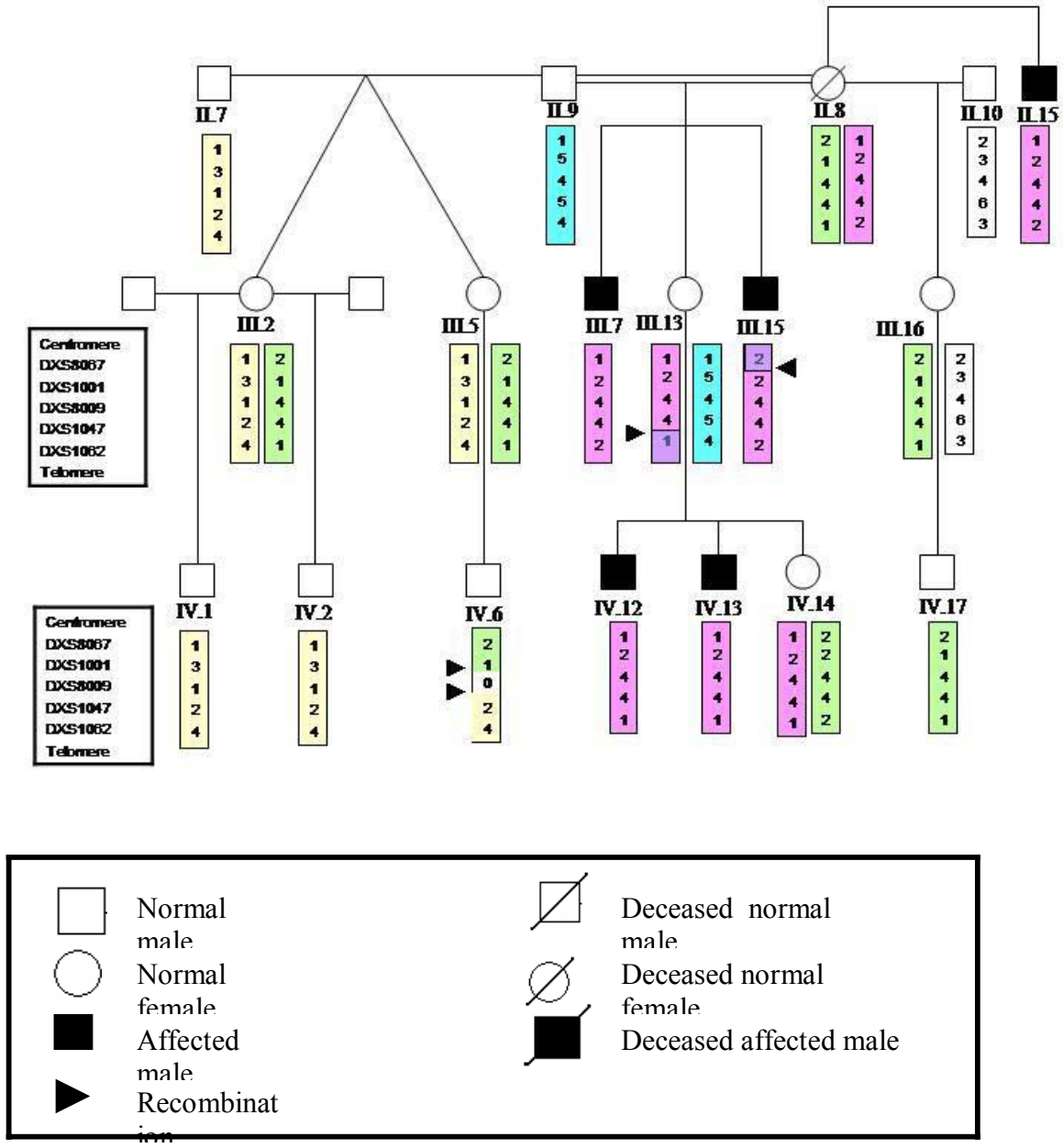


Figure 3.2 Partial pedigree of the GOLD SA family 1. The boxed numbers of different colours represent individual haplotypes. The pink haplotype is the most likely one to carry the responsible mutation with maximum LOD score of 2.16 at DXS1001. The arrows on individuals III-13 and III-15 indicate crucial cross overs which defined the minimal region of linkage.

3.3.1.2 Candidate gene selection

The physical distance of the minimal linkage interval between DXS8067 and DXS1062 is based on the Ensembl map (www.ebi.ac.uk/emnsembl) about 17.88Mbp, which is about 12% of the X-chromosome. This region contains about 90 genes, 9 of which have previously been associated with XLMR. These are ARHGEF6, CUL4B, GPC3, GRIA3, HPRT, LAMP2, OCRL1, PHF6 and ZDHHC9 (Figure 3.3).Table 3.4 lists all genes located within the linkage interval and highlights the currently known XLMR genes.

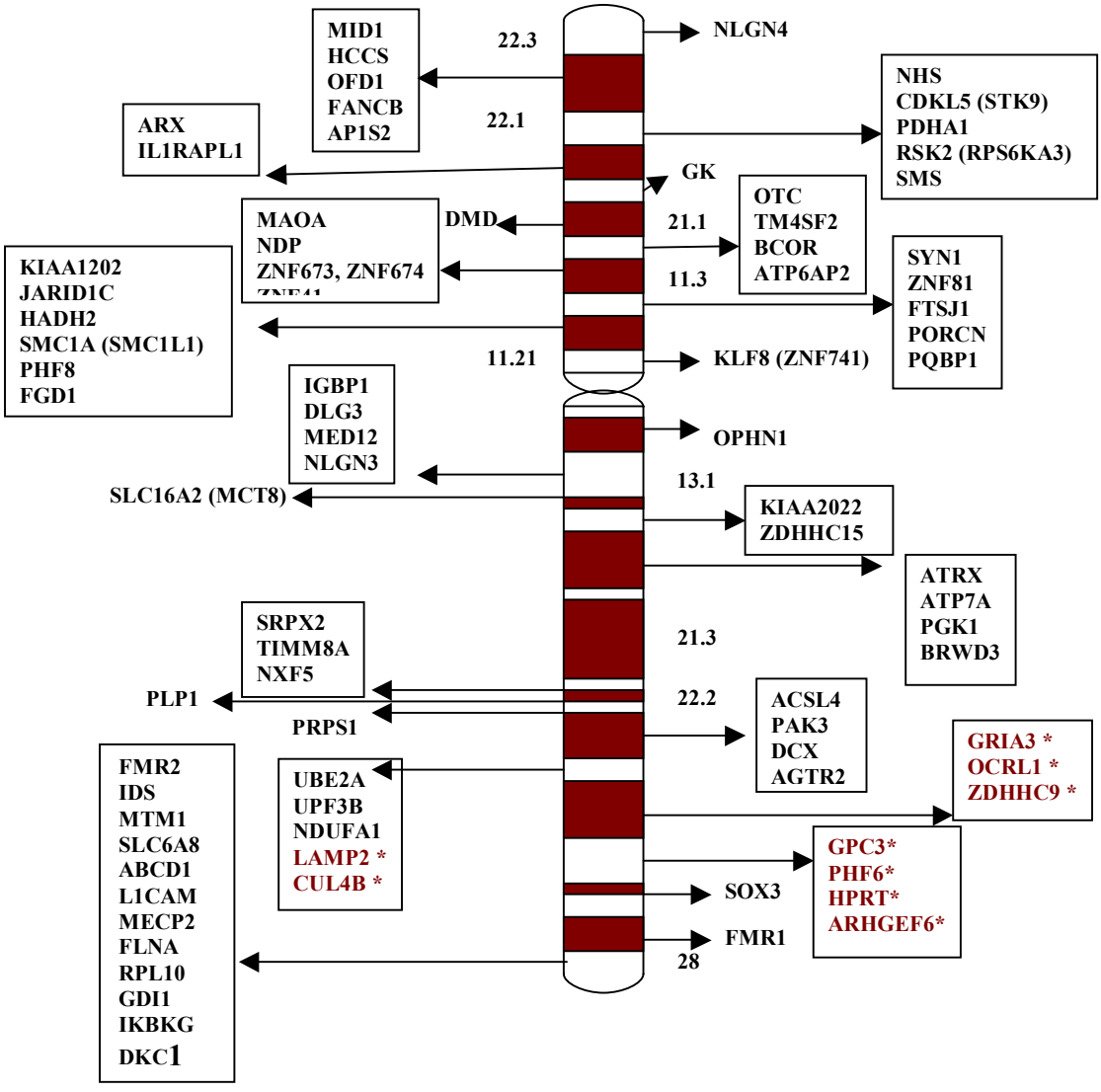


Figure 3.3 The XLMR genes within the linkage interval of family GOLD SA 1. The 9 genes are highlighted.

Table 3.4 All genes within the linkage interval of family GOLD SA 1; known XLMR genes are highlighted in purple.

Ensembl Gene ID	MIM Gene Accession	External Gene ID
ENSG00000131721	300447	RHOXF2
ENSG00000177485	300329	ZBTB33
ENSG00000125355		FAM70A
ENSG00000101892		ATP1B4
ENSG00000005893	309060	LAMP2
ENSG00000158290	300304	CUL4B
ENSG00000101898		MCTS1
ENSG00000212749		Q8NHH5_HUMAN
ENSG00000171155	300611	C1GALT1C1
ENSG00000203983	300592	CT47B_HUMAN
ENSG00000213505	300592	CT47A_HUMAN
ENSG00000203981	300592	CT47A_HUMAN
ENSG00000213504	300592	CT47A_HUMAN
ENSG00000213503	300592	CT47A_HUMAN
ENSG00000197443	300592	CT47A_HUMAN
ENSG00000203979	300592	CT47A_HUMAN
ENSG00000203978	300592	CT47A_HUMAN
ENSG00000203976	300592	CT47A_HUMAN
ENSG00000213499	300592	CT47A_HUMAN
ENSG00000203977	300592	CT47A_HUMAN
ENSG00000203975	300592	CT47A_HUMAN
ENSG00000182890	300144	GLUD2
ENSG00000174264		Q4TT42_HUMAN
ENSG00000125675	305915	GRIA3
ENSG00000207655		hsa-mir-220
ENSG00000125676		THOC2
ENSG00000101966	300079	BIRC4
ENSG00000101972	604359	STAG2
ENSG00000183918	300490	SH2D1A
ENSG00000009694	300588	ODZ1
ENSG00000198354		WDR40C
ENSG00000198889		WDR40B
ENSG00000183631		Q5JQE8_HUMAN
ENSG00000214979		PNPLA10P
ENSG00000123165	300487	ACTRT1
ENSG00000102038	300012	SMARCA1
ENSG00000122126	300535	OCRL
ENSG00000171388		APLN
ENSG00000122121	300145	XPNPEP2
ENSG00000122122	300441	CXorf9
ENSG00000188706	300646	ZDHHC9

ENSG00000156697	300508	UTP14A
ENSG00000085185		BCORL1
ENSG00000102034		ELF4
ENSG00000156709	300169	AIFM1
ENSG00000134594	300333	RAB33A
ENSG00000056277		SUHW3
ENSG00000102078	300242	SLC25A14
ENSG00000147262	300513	GPR119
ENSG00000134597		RBMX2
ENSG00000203956		FAM45B
ENSG00000165675	300282	ENOX2
ENSG00000147256		RHGXX_HUMAN
ENSG00000147255		IGSF1
ENSG00000171054		OR13H1
ENSG00000134602	300547	MST4_HUMAN
ENSG00000165694	300628	FRMD7
ENSG00000123728		RAP2C
ENSG00000212748		Q9Y6A3_HUMAN
ENSG00000076770	300413	MBNL3
ENSG00000171004	300545	HS6ST2
ENSG00000134588	300309	USP26
ENSG00000183434		TFDP3
ENSG00000076716	300168	GPC4
ENSG00000147257	300037	GPC3
ENSG00000208034		hsa-mir-92-2
ENSG00000207812		hsa-mir-19b-2
ENSG00000207710		hsa-mir-20b
ENSG00000207602		hsa-mir-106a
ENSG00000156531	300414	PHF6
ENSG00000165704	308000	HPRT1
ENSG00000199132		hsa-mir-450-1
ENSG00000207755		hsa-mir-450-2
ENSG00000207784		hsa-mir-542
ENSG00000165705		Q96IN8_HUMAN
ENSG00000208005		hsa-mir-503
ENSG00000199097		hsa-mir-424
ENSG00000170965	300296	PLAC1
ENSG00000156504		FAM122B
ENSG00000156500		FAM122C
ENSG00000101928		MOSPD1
ENSG00000184785		Q96HG1_HUMAN
ENSG00000212747		FAM127C
ENSG00000134590		FAM127A
ENSG00000203950		FAM127B
ENSG00000196972		Q86V52_HUMAN
ENSG00000203331		Q3ZM63_HUMAN
ENSG00000169551		CXorf48
ENSG00000203329		Q3ZM63_HUMAN

ENSG00000186376	314997	ZNF75
ENSG00000173275	300627	ZNF449
ENSG00000178947		Q86V52_HUMAN
ENSG00000165359		DDX26B
ENSG00000187267		NP_001017417.1
ENSG00000203948		NP_689795.3
ENSG00000213444		NP_001017435.1
ENSG00000203947		NP_001017438.1
ENSG00000213441		NP_001007552.1
ENSG00000203946		NP_001017438.1
ENSG00000181433	300359	SAGE1
ENSG00000169446		TMEM32
ENSG00000203945		Q5JPQ1_HUMAN
ENSG00000198689	300231	SLC9A6
ENSG00000022267	300163	FHL1
ENSG00000129680		MAP7D3
ENSG00000156920		GPR112
ENSG00000102239	300107	BRS3
ENSG00000102241	300346	HTATSF1
ENSG00000102243		VGLL1
ENSG00000203941		Q5JVP7_HUMAN
ENSG00000102245	300386	CD40LG
ENSG00000129675	300267	ARHGEF6
ENSG00000147274	300199	RBMX
ENSG00000206979		SNORD61
ENSG00000197152		Q96NB7_HUMAN
ENSG00000165370	300393	GPR101
ENSG00000156925	300265	ZIC3

3.3.1.3 Candidate gene screening

After careful examination and assessment we concluded that the affected individuals from family GOLD SA 1 did not closely resemble any families known to be associated with the 9 known XLMR genes from the linkage interval (Table 3.5). However, we decided to test at least some of these known XLMR genes for mutations in this family. XLMR gene mutation pleiotropy is a well described phenomenon and thus it was possible that different mutations in any of these genes could yield slightly different, non-dysmorphic phenotype. So far the proband from this family was tested for mutations in the coding regions of two of the 9 genes - PHF6 and GRIA3 (see below for results and further detail of this screening). The families have not yet been informed about the linkage results, as the project is still going on as a part of the GOLD SA project.

Table 3.5 Summary of the cardinal clinical features other than MR reported for the 9 XLMR genes from the minimal linkage interval of family GOLD SA 1.

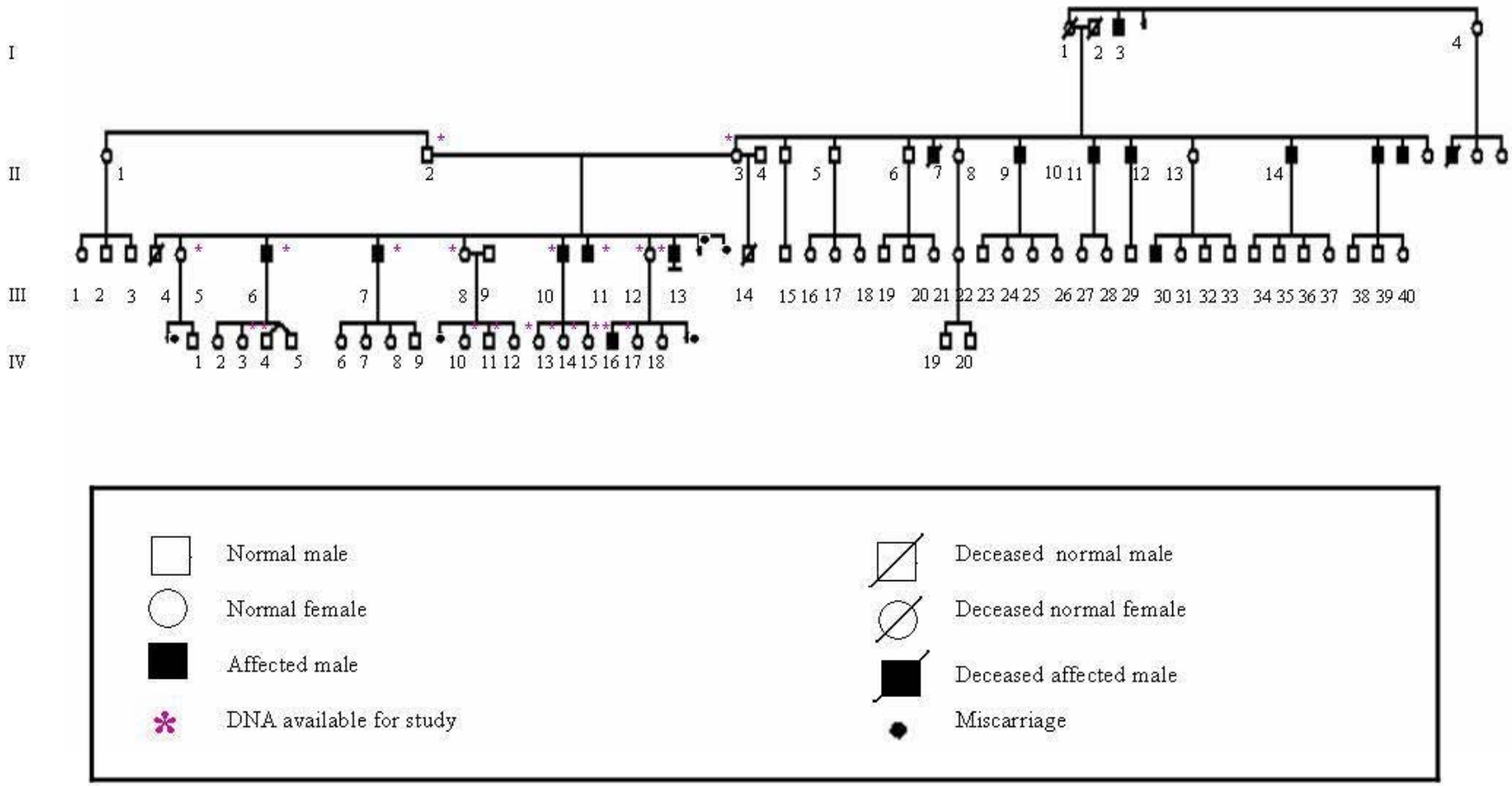
Gene	OMIM	Clinical features
LAMP2	300257 309060	Vacuolar hypertrophic cardiomyopathy, proximal muscle weakness, scapuloperoneal muscular dystrophy, myopia
CUL4B	300304 300354 300639	Short stature, restlessness, seizures, muscle wasting, tremor, obesity, hypogonadism, large mouth with prominent lips short thumbs
GRIA3	305915	Bipolar disorder, epileptic seizures in infancy
OCRL1	300535	Hydrophthalmia, cataract, vitamin D-resistant rickets
ZDHHC9	300646	Hypotonia, Marfanoid habitus, pronounced language handicap
GPC3	300037 312870	Macrosomia, “coarse” face, polydactyly, extra nipples, heart defects

PHF6	300414 301900	Obesity, hypogonadism, round face, narrow palpebral fissures, epilepsy
HPRT	308000 300322	Cerebral palsy, choreoathetosis, self-destructive biting
ARHGEF6	300267 300436	Mild dysmorphic features, sensorineural hearing loss

3.3.2 Linkage mapping of family GOLD SA 2

The family included 16 affected males in 4 generations (pedigree 2- Family Gold SA 2). There were no consistent clinical manifestations in the affected individuals other than borderline to mild MR. Segregation of this phenotype is consistent with X-linked inheritance, and as such the family is classified as having borderline to mild non syndromic XLMR.

Pedigree 2 – Family GOLD SA 2



Blood was collected from 20 family members with samples from 6 affected males, 11 females (including obligate carriers) and 3 unaffected males (as indicated in pedigree 2- Family GOLD SA 2). Genomic DNA was isolated from lymphocytes using the QIAamp DNA blood maxi kit (Qiagen Pty Ltd. Doncaster, VIC, Australia).

Genotype analysis of 48 microsatellite markers spanning the X chromosome (at approximately 5cM intervals) was performed at the Australian Genome Research Facility (Melbourne, VIC, Australia) using Linkage Mapping set v2.5 (Applied Bios stems, Foster City, CA, USA. Two point linkage analyses of these markers were carried out using Linkage programme (MLINK) (Lathrop and Lalouel version 3.1). X-linked recessive inheritance with complete penetrance was assumed, with the frequency of the disease allele set at 0.0001. Allele frequencies for all microsatellite markers were set as equal. The family's linkage analysis has not yet been completed because of uncertainty about the clinical classification of some individuals.

Table 3.6 Two point LOD scores with 48 markers spanning the X chromosome in Family GOLD SA 2

Order	Markers	θ= 0.0	θ=0.01	θ= 0.05	θ=0.1	θ= 0.2	θ= 0.3	θ=0.4
1	DXS1060	-infini	-0.1	0.48	0.62	0.58	0.39	0.17
2	DXS1223	-infini	-4.08	-2.05	-1.23	-0.50	-0.17	-0.03
3	DXS8051	-infini	-0.1	0.50	0.65	0.63	0.45	0.20
4	DXS7108	-infini	-0.1	0.50	0.65	0.63	0.45	0.20
5	DXS1224	-infini	-0.1	0.50	0.65	0.63	0.45	0.20
6	DXS987	-infini	-2.68	-1.32	-0.77	-0.30	-0.10	-0.02
7	DXS8019	-infini	-2.09	-0.78	-0.30	0.05	0.13	0.1
8	DXS7593	0.43	0.42	0.39	0.36	0.28	0.20	0.11
9	DXS1226	-infini	-2.09	-0.78	-0.30	0.03	0.08	0.02
10	DXS1061	-infini	-0.1	0.50	0.65	0.63	0.45	0.20
11	DXS1214	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12	DXS8102	-infini	-infini	-infini	-infini	-infini	-infini	-infini
13	DXS8090	-infini	-2.09	-0.78	-0.30	0.05	0.13	0.1
14	DXS1068	-infini	-0.39	0.23	0.43	0.47	0.35	0.15
15	DXS8015	-infini	-2.38	-1.04	-0.52	-0.1	0.04	0.06
16	DXS993	-infini	-3.79	-1.77	-0.97	-0.30	-0.03	0.05
17	DXS8080	-infini	-0.1	0.49	0.63	0.61	0.42	0.17
18	DXS8083	-infini	-0.1	0.49	0.63	0.61	0.42	0.17
19	DXS1055	0.12	0.12	0.11	0.10	0.08	0.05	0.03
20	DXS1039	-infini	-0.1	0.49	0.63	0.61	0.42	0.17
21	DXS991	-infini	-0.1	0.49	0.63	0.61	0.42	0.17
22	DXS1216	-infini	-2.39	-1.06	-0.55	-0.13	0.02	0.05
23	DXS986	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00
24	DXS1196	0.12	0.12	0.11	0.10	0.08	0.05	0.03
25	DXS1217	0.12	0.12	0.11	0.10	0.08	0.05	0.03
26	DXS990	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00	-0.00
27	DXS8077	-infini	-0.39	0.23	0.43	0.47	0.35	0.15
28	DXS8020	0.30	0.30	0.28	0.26	0.20	0.15	0.08
29	DXS1106	-infini	-2.09	-0.78	-0.30	0.03	0.08	0.02
30	DXS1059	0.43	0.42	0.39	0.36	0.28	0.20	0.11
31	DXS8088	0.12	0.12	0.11	0.10	0.08	0.05	0.03
32	DXS8055	-infini	-3.79	-1.77	-0.97	-0.30	-0.03	0.05
33	DXS8064	-infini	-2.39	-1.06	-0.55	-0.13	0.02	0.05
34	DXS8067	-infini	-0.1	0.50	0.65	0.63	0.45	0.20
35	DXS1001	-infini	-0.1	0.50	0.65	0.63	0.45	0.20
36	DXS8009	-infini	-2.68	-1.32	-0.77	-0.30	-0.10	-0.02
37	DXS1047	-infini	-0.39	0.22	0.40	0.43	0.30	0.12
38	DXS1062	-infini	-2.10	-0.80	-0.34	-0.01	0.07	0.06
39	DXS984	0.43	0.42	0.39	0.36	0.28	0.20	0.11
40	DXS1205	-infini	-4.08	-2.05	-1.23	-0.50	-0.17	-0.03
41	DXS1227	-infini	-0.1	0.49	0.63	0.61	0.41	0.14
42	DXS8106	0.43	0.42	0.39	0.36	0.28	0.20	0.11
43	DXS8043	-infini	-3.79	-1.77	-0.97	-0.30	-0.03	0.05
44	DXS8045	-infini	-0.68	-0.04	0.17	0.27	0.20	0.07

45	DXS998	-infini	-2.09	-0.79	-0.32	0.02	0.1	0.07
46	DXS8091	-infini	-1.58	-0.89	-0.60	-0.32	-0.17	-0.07
47	DXS8069	-infini	-3.79	-1.77	-0.97	-0.30	-0.03	0.05
48	DXS1073	0.43	0.42	0.39	0.36	0.28	0.20	0.11

3.4 Candidate gene screening

3.4.1 South Australian XLMR families

After the linkage mapping of family GOLD SA 1, proband DNA was screened for mutations in two genes in the linkage interval.

1. The GRIA3 gene

Mutations in the Glutamate receptor ionotropic AMPA 3 (GRIA3) gene have been associated with XLMR (Wu et al. 2007). Glutamate receptors are responsible for most of the excitatory neurotransmission in the brain and are involved in synaptic plasticity and learning and memory efficacy. Gecz et al. (1999) cloned the GRIA3 cDNA of 5,894bp. The gene has 17 exons. It is highly expressed in the brain except in substanita nigra, occipital lobe and the spinal cord. The gene maps to Xq25-q26. Apart from MR inactivation of the gene was associated with bipolar disorder and epileptic seizures in infancy, neither of which were present in family GOLD SA 1. The proband of family GOLD SA 1 was screened for mutations in this gene. DNA sequence analysis was done through NCBI web page http://www.ncbi.nlm.nih.gov. In addition, all genomic sequences and characterized gene sequences were downloaded from the nucleotide database division and were imported into the EditSeq program for further analysis. The results were analyzed with the Seqman module. No pathological mutations were found. However, 3 polymorphic changes that had been described

previously in the database were identified in exons 1, 6 and 9. These are illustrated in the following figures (3.4, 3.5 and 3.6)

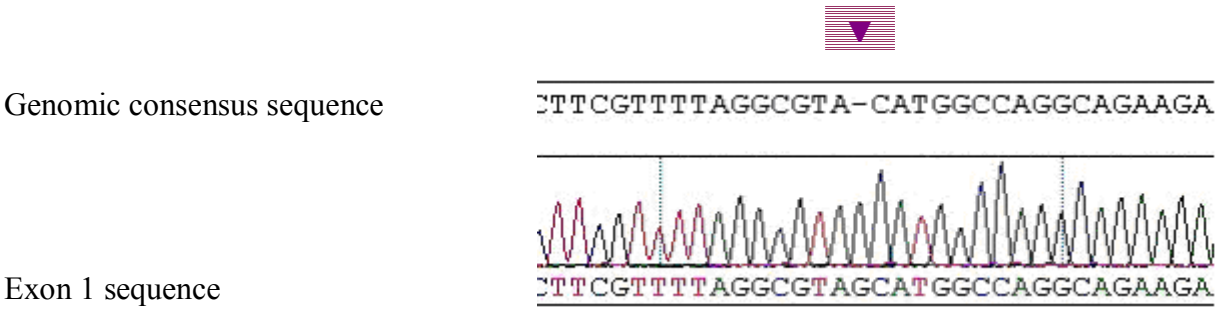


Figure 3.4 The polymorphic change in Exon 1

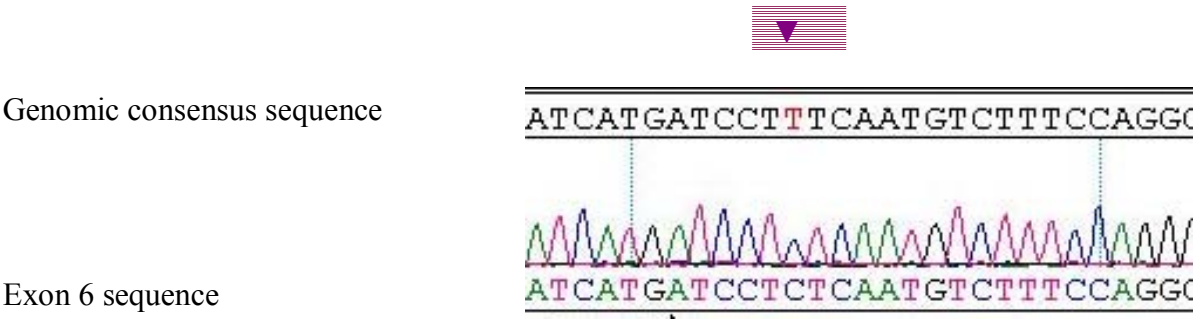


Figure 3.5 The polymorphic change in Exon 6

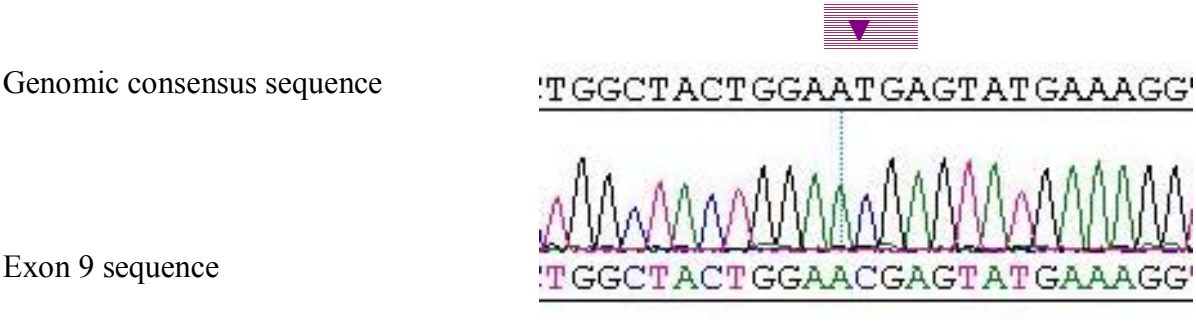


Figure 3.6 The polymorphic change in Exon 9

2. The PHF6 gene

Mutations in the plant homeodomain like finger gene (PHF6) are associated with Borjeson-Forssman-Lehmann syndrome. In mouse embryos PHF6 is highly expressed in the central nervous system and remains present in the adult brain at very low levels. The human PHF6 gene consists of 11 exons and was identified as the BFLS gene by Lower et al. (2002). They also showed that when PHF6 was tagged with green fluorescent protein (GFP) it showed diffuse nuclear accumulation, suggesting that it has a role in transcription. Although the affected individuals did not show any signs of Borjeson-Forssman-Lehmann syndrome, the proband of family GOLD SA 1 was screened for mutations in this gene because it was within the linkage interval and it is still possible that some mutations of PHF6 will cause NSXLMR but no mutations were found. The clinical review of the affected individuals of family GOLD SA 7 did show some features of the BFLS syndrome and were also screened for a mutation in this gene, but no mutation was found.

3.4.2 South Australian males with MR and Marfanoid habitus (Lujan Fryns syndrome):

DNA from these individuals was screened for mutations in the UPF3B gene.

UPF3B gene

Mutations in the UPF3 regulator of nonsense transcripts homolog B (yeast) (UPF3B) have been associated with SXLMR and NSXLMR. UPF3 protein facilitates the export of spliced mRNAs by recruiting mRNA export proteins. Kim et al. (2001) proposed that UPF3 is a key factor in nonsense mediated messenger RNA decay (NMD) an important process of selective degradation of mRNAs that contain premature stop codons. The binding of UPF3 to mRNAs before export as part of the complex that assembles near exon-exon junctions, present it as a link between splicing and NMD in the cytoplasm. The gene maps to Xq25-q26. It consists of

11 exons and is associated with XLMR with Marfanoid body build (Lujan Fryns syndrome) (Tarpey et al. 2007). All the 14 males with MR and Marfanoid body build described earlier were tested for mutations but no UPF3B mutations were found.

3.5 Array CGH

DNA samples from 13 families were sent to New South Wales to be tested with a chromosome X specific tiling BAC CGH array (Van Esh et al. 2005). The rest of the samples will be sent later as part of the GOLD SA project. Briefly, DNA of one patient, labeled with one dye was hybridized with another patient's DNA, labeled with another dye and subsequently the mixture applied to the BAC array to detect gains or losses. Only regions with gain or loss of greater than one contiguous clone were scored as either a gain or loss of material. This relates to a theoretical resolution of 150 kbp for this particular array. 8 of the 13 DNA samples showed no disease related deletions or duplications. These were from affected individuals of families (2, 5, 7, 13, 16, 17, 20 and 22). The results of the rest of the families are pending.

Chapter Four: Discussion

Chapter four: Discussion

4.1 South Australian XLMR families

Mental retardation is a highly heterogeneous condition. Over the last decade remarkable progress has been made in the unraveling of its causes. One of the most intensively studied regions of the human genome is the X chromosome. This is for several reasons.

1- The increased prevalence of MR among males compared to females (Leonard et al., 2003; Boyle et al., 1996; Bhasin et al., 2006), which might be explained by excess of X-chromosome linked genes causing MR.
2- Simplicity of the X-chromosome inheritance and considerable reduction of the effort to about 5% (proportional share of the human X chromosome).
3- The availability of many families segregating MR in an X-linked pattern

The identification of the molecular cause of MR in various patients is challenging even in the presence of specific recognizable clinical features. This is because of vast genetic heterogeneity (multiple genes & mutations underlying the same or similar clinical phenotypes) and mutation pleiotropy (one gene & mutation giving rise to multiple, clinically distinct phenotypes) underlying mental retardation. The presence of consistent, syndromic features does increase the chance of mutation detection in a known gene. There are no generally accepted rules, just guidelines on how to investigate patients with MR. Different approaches are being used by different centers. Similar to GOLD SA and GOLD NSW projects, other projects were created in other parts of the world. The EuroMRX consortium, for example, which is a collaboration of several institutes undertaking research into MR, was established in 1996 to identify mutations in known and candidate XLMR genes and to study the biological function of these genes. The EuroMRX family cohort consists of 400 families with NSXLMR

and 200 families with SXLMR (de Brouwer et al., 2007) in which all probands had normal karyotypes. This a larger number compared to this study which included 27 families 4 SXLMR and 27 NSXLMR. After excluding Fragile X syndrome, lymphoblastoid cell lines were established from at least one proband of each family. The same approach was used in this study but in addition to testing the probands for Fragile X syndrome, ST MLPA was also done. In 141 families linkage interval on the X chromosome has been determined and a lod score LOD>2.0 was established for 61 of these families. This is a bigger number compared to the current study in which linkage studies were done for only 2 families. Probands from selected families were tested for mutations in the coding region of 12 XLMR genes compared to 3 genes in our study because of lack of resources and limitation of time. In addition 78 candidate XLMR genes were analyzed by direct sequencing after prescreening using denaturing high performance liquid chromatography (dHPLC), single strand conformation polymorphism (SSCP) or denaturing gradient gel electrophoresis (DGGE) in a number of cases. The candidate gene selection was based on their position in the linkage interval, their physiological function and/or expression in the brain which is the similar criteria used in the current study. Patients with a linkage interval including the respective gene were screened for mutation then the mutation analysis was expanded to include all available EuroMRX patients with a comparable phenotype which is ideal but in the current study this was not possible partly because of lack of resources and time limitation. Array CGH was carried out on some of the families looking for deletion or duplications and X-inactivation profiles was determined in obligate carrier females. Similarly array CGH was done for the families in the current study but due to lack of resources X-inactivation profiles were not determined.

A similar project was set up in Italy. It started in 2003 and collected detailed clinical information and biological samples from a large number of MR patients (Pescucci et al., 2007). It collected all sporadic and familial MR males negative for Fragile X syndrome,

chromosomal and subtelomeric rearrangements. This is different from the families included in this study which were mainly collected because of the suspicion of X-linked pattern of inheritance. Mutation analysis was performed on all patients for all genes in study (MECP2, OPHN1, FMR2, NLGN3/NLGN4, DCX, ARX, IL1RAPL1, AGTR2, ATRX, PQBP1 and RSK2) which is different from the current study in which mutation analysis was done for some of the genes which are present in the linkage interval of the mapped families only. Linkage analysis for families with at least three affected individuals in two generations and mapping for families were performed. Array CGH was performed in all enrolled patients to investigate segmental submicroscopic aneuploidies.

In our study, a similar approach was used to investigate XLMR patients as in those projects. LCL lines were established from at least one affected MR patient. Karyotyping, molecular test for fragile X syndrome, ST MLPA and array CGH was done. Linkage mapping was done with a LOD score of >2.0 for the large families and mutation analysis were done using the same selection criteria i.e. based on the position of the genes in the linkage interval, their physiological function and/or expression in the brain.

Our study gives an overview of the number of (available/ascertained) XLMR families in South Australia and their clinical manifestations. Among the 27 families involved in the study 7 families declined due to social reasons. On contacting those families and interviewing them it was obvious that having a history of individuals with XLMR within the family was a sensitive matter and was faced with denial and avoidance. In certain numbers of families the parents of the affected individuals were willing to participate but the rest of the relatives were not while in others there were many problems within the family which lead to loss of contact and prevented the individuals concerned from contacting the rest of the family. Despite these issues with some families and individuals, 109 DNA samples were collected from various participating families.

Similar to other studies, we selected 6 large mappable families who were the main focus of our study because there were many affected individuals within the family and they were suitable for linkage mapping only two families agreed to participate in the study. Linkage analysis was done for the first family while the other is currently being finalized and will be followed up under the GOLD SA Project.

Linkage mapping of the first family GOLD SA 1 localized the disease gene between the markers DXS8067 and DXS1062. Based on the Ensembl map (www.ebi.ac.uk/emnsembl) the physical distance contains 9 genes associated with XLMR. These are ARHGEF6, CUL4B, GPC3, GRIA3, HPRT, LAMP2, OCRL1, PHF6 and ZDHHC9. The proband was tested for mutations in the coding regions of two of them, PHF6 and GRIA3. These were the best candidates since the family's clinical manifestations did not correspond with the clinical features associated with mutations in the other genes (Table 3.4). As with the other projects the same selection criteria for the genes involved was used i.e. based on their position in the linkage interval, their physiological function and/or expression in the brain but due to lack of resources only two genes were tested.

DNA sequence analysis was done through the NCBI web page http://www.ncbi.nlm.nih.gov. In addition, all genomic sequences and characterized gene sequences were downloaded from the nucleotide database division and were imported into the EditSeq program for further analysis. There were 3 polymorphic changes identified in GRIA3 gene, all of which had been described previously. No changes were found in the PHF6 gene.

Possible explanations for the absence of mutations in these genes are:

1- Their low mutation frequency.
2- The mutation in this family could be in exons that have not been annotated yet.
3- The mutation in this family could be in other XLMR genes in the region.
4- The mutation affects transcript levels.

5- The mutation results in defective splicing.

6- The mutation could be a copy number change not detectable by sequencing.

While no pathological mutations were found in this family we were able to reveal the carrier status of the females, which will help them in making reproductive decisions in the future. It will be worth while to test the probands for mutations in the other 7 known XLMR genes from the linkage interval before embarking on screening of the rest of the ~ 90 genes. Possibly soon a novel technology of massive parallel sequencing will become available and families like this one will benefit from it by having the responsible mutation localized to a relatively small region of the human genome.

In the four SXLMR families no specific syndrome was suspected, except for family GOLD SA 15, in which affected individuals had some features of Borjeson-Forssman-Lehmann syndrome – intellectual disability, microcephaly, obesity, gynecomastia and hypogonadism. They were tested for a PHF6 mutation but no mutation was found. No PHF6 mutations have been found as yet in the NSXLMR families.

All families will be followed as part of the GOLD SA project. Some of these are currently being analysed at the Wellcome Trust Sanger Institute in Cambridge (UK) using novel technologies of exon capture (Albert et al., 2007) and massive parallel sequencing of the entire human X-chromosome . The challenge facing this project is the assignment of the significance to the changes found and confirmed given that many of them, and in particular those from the linkage interval will segregate with the disease status, but might not be causative of the associated intellectual disability. It will depend on the nature of the DNA change, its predicted molecular outcomes and further functional experimentation on cell and/or animal models of the disease.

4.2 South Australian males with XLMR and Marfanoid habitus (Lujan Fryns Syndrome):

Tarpey et al. (2007) identified protein truncating mutations in UPF3B in some patients with Lujan Fryns Syndrome. There were 14 individuals with suspected Lujan Fryns Syndrome in this study but none of them had any mutations in the gene. Raymond at al. (2007) identified one frame shift mutation, one splice site mutation, and two missense mutations in highly conserved residues in ZDHHC9 in 4 of 250 families with XLMR. In 3 of the families MR was associated with Marfanoid habitus. On the other hand, Schwartz et al. (2007) found a novel missense mutation in MED12 in the original family with Lujan syndrome and a second family which was considered to have Optiz-Kaveggia syndrome. Therefore, these two genes are good candidates for future tests.

The absence of mutations in the UPF3B gene is possibly because of its low mutation frequency. It can be due to its presence in a new XLMR gene or one of the other two genes that are associated with Lujan Fryns syndrome - MED12 and ZDHHC9 (Schwartz et al.,2007; Raymond et al.,2007). In addition, it could be present in exons that have not been annotated yet or because the mutation affects transcript levels or results in defective splicing. In the meantime more studies are needed on larger populations to reveal the contribution of UPF3B gene to this syndrome.

It is likely that within a few years we will be able to (routinely) sequence the entire human genome for a reasonable cost. The interpretation and the assignment of the relevance of the nucleotide changes (including copy number variation) identified will pose a major challenge for the entire human genetics field. The availability of detailed clinical data and extended family material will be extremely valuable and will greatly contribute to the functional annotation of individual changes in patient's DNA. Our project, among other, more immediate goals (e.g. linkage mapping for indirect diagnosis by linkage), also aimed to

achieve this level of preparedness for such future technologies. We ought to look into the future with great optimism, that one day we will be able to identify the majority, if not all genetic lesions leading to heritable forms of intellectual disability and use that information for the benefits of the patients and their families.

Chapter Five: Summary and Conclusions

Chapter Five: Summary and Conclusion

5.1 Summary

This study was conducted to ascertain the South Australian families with XLMR and evaluate them in order to identify the cause of their intellectual disability in the affected males. A total of 40 families were identified and characterized. Among those 6 families were already mapped or diagnosed and 7 were excluded. The rest of the 27 families were than classified, based on the type of XLMR, into syndromic and non-syndromic. The history and the clinical manifestations of all families were documented and a database was created which will be a reference for clinical geneticists to assist them in the follow up in the future. In addition, it will be used by the GOLD SA project to follow up these families and will be a permanent record of the families. 6 of these families were large enough to perform the linkage mapping and thus provide them with indirect test for their condition. Unfortunately only two of these agreed to participate in the study. The linkage mapping for the first family revealed the carrier status of the females within the family. The clinical manifestations in the affected individuals did not meet any of the manifestations reported for any of the 9 known XLMR gene mutations within the linkage interval. According to the facilities and time available the proband was tested for mutations in the coding regions of two of them PHF6 and GRIA3 which were the best candidates but no pathological mutation was found. Therefore, the carrier status was revealed but the exact gene causing the abnormality was not identified. However, the family members can now make their reproductive decision more confidently.

Altogether 109 DNA samples were collected. All families had fragile X molecular test, standard karyotyping, ST MLPA which gave normal results. In addition, some of them had array CGH performed, which also did not show any abnormality. The GOLD SA project will be following up those families. Families with XLMR for which no diagnosis is established

can reduce the risk of having an affected male child by sex selection, either by prenatal diagnosis or PGD.

5.2 Conclusion

Mental retardation is a neuropsychiatric condition that results in emotional, psychological, and financial costs to affected individuals, their families and society. Having an individual with MR within the family is difficult for the family members and this was obvious in the interviews with the families and made approaching these families not an easy task. Therefore, it is important to know its cause in order to provide a reliable molecular diagnosis for the families to help them have normal children. Great advances have been made in identifying the molecular basis of MR.

X-linked mental retardation is one of the important causes of MR and has been the focus of many research studies around the world over many years. To date, researchers have mapped many MRX and MRXS genes but many are yet to be identified. The exact number of XLMR genes remains unknown.

In South Australia there were 27 families with XLMR the cause of which was not identified. The aim of this study was the ascertainment, diagnostic evaluation and gene mapping of these families. Some of these families were seen for 20 years and until now we were unable to provide them with an answer on what is the cause of MR in their family members. The families were ascertained and evaluated and several tests were carried out but the cause is still unknown. However, There are 109 DNA samples available from these families which can be used for further studies to reveal the cause of the MR. It is just a matter of time. Nevertheless, we were able to provide the mapped family with information about the carrier status of the females to help them in making decisions about future reproductive plans. The only practical way of reducing the couple's risk is now by sex selection either by prenatal diagnosis or PGD.

5.3 Future directions

This research opens the door for other researches to be carried on these families in order to provide them with a molecular diagnosis. New technologies are being discovered. It is now possible to do the entire X chromosome sequencing which is a great advance in this field and in the near future it can be offered to the public. Providing these families with a molecular diagnosis will help in delivering them with proper genetic counseling and a more accurate estimate of the recurrence risk within the family. In addition, it might raise the possibility of prenatal diagnosis which is considered by many families. On the other hand, identifying these genes might improve our understanding of the molecular basis underlying cognitive function and might lead to the discovery of new therapeutic approaches. It might give invaluable information about the brain development which might increase our understanding of other brain development disorders which are caused by non-genetic factors.

Research into the genetic basis of XLMR is being conducted in laboratories around the world aiming at discovering the unidentified genes. Once gene identification is complete, the challenge will be to provide cheap, reliable and effective methods of sequence analysis to families with MR.