Health Technology Assessment 1997; Vol. 1: No. 4

Review

Screening for fragile X syndrome

Jenni Murray
Howard Cuckle
Graham Taylor
Jenny Hewison

Health Technology Assessment
NHS R&D HTA Programme

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Screening for fragile X syndrome

Jenni Murray [1]
Howard Cuckle [1]
Graham Taylor [2]
Jenny Hewison [3]

[1] Reproductive Epidemiology, Centre for Reproduction, Growth & Development, Research School of Medicine, University of Leeds, 26 Clarendon Road, Leeds LS2 9NZ

[2] Regional Clinical Molecular Genetics Laboratory, St. James's University Hospital, Beckett Street, Leeds LS9 7TF

[3] Centre for Reproduction, Growth & Development, Research School of Medicine, University of Leeds, 34 Hyde Terrace, Leeds LS2 9LN

Final manuscript received December 1996

This report should be referenced as follows:

Murray J, Cuckle H, Taylor G, Hewison J. Screening for fragile X syndrome. *Health Technol Assessment* 1997; **1**(4).

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This report is one of a series covering acute care, diagnostics and imaging, methodology, pharmaceuticals, population screening, and primary and community care. It was identified as a priority by the Population Screening Panel (see inside back cover).

The views expressed in this publication are those of the authors and not necessarily those of the Standing Group, the Commissioning Board, the Panel members or the Department of Health.

Series Editors: Andrew Stevens, Ruairidh Milne and Ken Stein
Assistant Editor: Jane Robertson

The editors have tried to ensure the accuracy of this report but cannot accept responsibility for any errors or omissions. They would like to thank the referees for their constructive comments on the draft document.

ISSN 1366-5278

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Published by Core Research, Alton, on behalf of the NCCHTA.
Printed on acid-free paper in the UK by The Basingstoke Press, Basingstoke.

Copies of this report can be obtained from:

The National Coordinating Centre for Health Technology Assessment,
Mailpoint 728, Boldrewood,
University of Southampton,
Southampton, SO16 7PX, UK.
Fax: +44 (0) 1703 595 639 Email: hta@soton.ac.uk
http://www.soton.ac.uk/~hta

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List of abbreviations and glossary

Technical terms and abbreviations are used throughout this report. The meaning is usually clear from the context but a glossary is provided for the non-specialist reader. In some cases usage differs in the literature but the term has a constant meaning throughout this review.

AGG (adenine–guanine–guanine) Trinucleotide usually interspersed in the CGG repeat sequence in the FMR-1 gene.

Carrier Individual with an FM or PM.

Cascade screening Testing members of a proband's family.

Case-finding Actively trying to diagnose probands for cascade screening.

CGG (cytosine–guanine–guanine) Trinucleotide repeated in the FMR-1 gene.

CVS (chorionic villus sampling) Invasive procedure to obtain placental tissue for prenatal diagnosis.

Detection rate Proportion of affected individuals with positive results.

Expansion Increase in the repeat sequence between generations.

False-positive rate Proportion of unaffected individuals with positive results.

FM (full mutation) Array of repeat size over 200.

FMR (fragile X mental retardation) Fragile X syndrome.

FMR-1 Gene which is mutated in fragile X syndrome.

FMRP Protein product normally transcribed by FMR-1.

FRAXA Fragile site associated with fragile X syndrome.

FRAXE Fragile site on the X chromosome which is associated with mild mental handicap.

FRAXD & F Fragile sites close to FRAXA & E.

Haplotype Combination of linked genetic markers.

LCR Ligase chain reaction.

Mosaicism Individual with more than one cell line of different genetic composition.

nCGG A sequence of n repeats.

Negative predictive value Probability that an individual with a negative result is unaffected.

NTM (normal transmitting male) Unaffected male with a PM.

Obligate carrier Person who from pedigree analysis must have passed on an affected gene.

PCR (polymerase chain reaction) Method of amplifying small amounts of DNA.

PM (pre-mutation) Array of repeat size 53 or 55–200.

Positive predictive value Probability that an individual with a positive result is affected.

Proband Affected individual through whom attention is drawn to a pedigree.

PUBS (peripheral umbilical cord blood sampling) Invasive procedure to obtain foetal blood for prenatal diagnosis.

Pure repeat size The largest contiguous number of CGG repeats in an array without intervening AGGs.

RED Repeat expansion detection.

Repeat sequence Section of DNA containing the CGG repeats.

Repeat size Total number of repeats in an array, both CGG and AGG.

Triplet Trinucleotide.

Xq27.2 Locus of FRAXD.

Xq27.3 Locus of FRAXA.

Xq28 Locus of FRAXE & F.

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Executive summary

Background and aim of review

In 1991, the gene responsible for fragile X syndrome, a common cause of learning disability, was discovered. As a result, diagnosis of the disorder has improved and its molecular genetics are now understood. This report aims to provide the information needed to decide whether to use DNA testing to screen for the disorder.

How the research was conducted

A literature search of electronic reference databases of published and 'grey' literature was undertaken together with hand searching of the most recent publications.

Research findings

Natural history

Physical characteristics of fragile X syndrome include facial atypia, joint laxity and, in boys, macro-orchidism. Most affected males have moderate-to-severe learning disabilities with IQs under 50 whereas most females have borderline IQs of 70–85. Behavioural problems are similar to those seen with autism and attention-deficit disorders.

Although fragile X syndrome is not curable there are a number of medical, educational, psychological and social interventions that can improve the symptoms.

About 6% of those with learning disabilities tested in institutions have fragile X syndrome. Population prevalence figures are 1 in 4000 in males and 1 in 8000 in females.

Genetics

The disorder is caused by a mutation in a gene on the X chromosome which includes a trinucleotide repeat sequence. The mutation is characterised by hyper-expansion of the repeat sequence leading to down-regulation of the gene. In males an allele with repeat size in excess of 200, termed a full mutation (FM), is always associated with the affected phenotype, whereas in females only half are affected. Individuals with alleles having repeat size in the range 55–199 are unaffected but in females the sequence is heritably unstable so that it is at high risk of expansion to an FM in her offspring. This allele is known as a pre-mutation (PM) to contrast it with the FM found in the affected individual. No spontaneous expansions directly from a normal allele to an FM have been observed.

Screening strategies

The principal aim of screening for fragile X syndrome is to reduce the birth prevalence of the disorder, by prenatal diagnosis and selective termination of pregnancy, or by reducing the number of pregnancies in women who have the FM or PM alleles.

Possible screening strategies are: routine antenatal testing of apparently low risk pregnancies, pre-conceptual testing of young women, and systematic testing in affected families ('cascade' screening).

A secondary aim is to bring forward the diagnosis of affected individuals so that they might benefit from early treatment. Active paediatric screening and neonatal screening could achieve this but there is no direct evidence of any great benefit from early diagnosis.

Screening tests

Cytogenetic methods are unsuitable for screening purposes. Southern blotting of genomic DNA can be used but is inaccurate in measuring the size of small PMs, there is a long laboratory turn-round time, and it is relatively expensive. The best protocol is to amplify the DNA using polymerase chain reaction on all samples and, when there is a possible failure to amplify, a Southern blot.

Practical experience

There is little published information on the practical consequences of offering antenatal or pre-conceptual screening.

In one study, antenatal tests were offered to women about to have prenatal diagnosis for other conditions. They had to pay for themselves to be tested and uptake was only 21%. In another study, testing was offered to those with a family history of mental retardation but the uptake rate was not reported.

Pre-conceptual screening has only been reported among potential egg donors for *in vitro* fertilisation.

Four programmes of active cascade screening have been reported. In the largest study (conducted in Australia) in women with an FM or PM detected by screening and counselled, there was an estimated 26% reduction in births. In those who had further children, similar acceptance rates for invasive prenatal diagnosis were reported in Australia (77%), New York, USA (50%) and Kuopio, Finland (100%).

Pregnancy is generally terminated when an affected male foetus is found and, from all the reported cases in the literature combined, 64% of female foetuses with an FM are also terminated.

In the UK and elsewhere, it is established practice for children with learning difficulties or developmental delay to be tested to exclude fragile X syndrome. However, only one active testing programme has been examined. In Colorado, USA, educators were trained to select school children believed to be at high risk for testing and 1% were found to have an FM.

Neonatal screening has not been tried in practice.

Modelling allele dynamics

A model of allele inheritance was constructed. The critical parameters are the FM frequency (1 in 4000 for both sexes), PM frequency (1 in 273 for females, 1 in 800 for males), the risk of a PM allele expanding to FM (60–78% in families, 10% in the general population), and the reproductive fitness of individuals with an FM (50% for females, 0% for males).

Assessment of screening

Antenatal screening can be expected to have a detection rate and a negative predictive value approaching 100%. The false-positive rate would be 0.4% and the positive predictive value 1 in 20.

It is known that invasive prenatal diagnosis has a high acceptability among carriers and that the termination rate for affected pregnancies is high, even for female foetuses. However, information on likely screening uptake is lacking so it is not possible to completely predict effectiveness.

Pre-conceptual screening is completely unevaluated but is unlikely to be a realistic option.

Within the affected families known to the cascade screening programme, there has been a dramatic reduction in affected births through avoidance of future pregnancies and prenatal diagnosis. However, there is no reliable information on the impact of this screening on the total population birth prevalence of fragile X syndrome.

Paediatric screening is widely practised but its effectiveness is unproven and neonatal screening is untried.

Human and financial costs

Screening may result in psychological harm and, if invasive prenatal diagnosis is involved, there is also an approximately 1% foetal loss rate.

Care is needed to explain that the prognosis for a female with an FM cannot be predicted. Also, some apparently unaffected female carriers of mutations may have subtle cognitive problems and have difficulty understanding some of the complex information.

The average cost of preventing an affected birth was estimated as $14,200 (Australia, 1986) and $12,740 (USA, 1992). This is a small fraction of the estimated lifetime cost of care for an affected individual, which is a minimum of $1 million (USA).

Using the model, routine antenatal screening will cost between £90,000 and £143,000 depending on uptake. Although this is more than the cost of screening for Down's syndrome (£30,000) or cystic fibrosis (£40,000–104,000), technical developments may eventually lead to a reduction in cost.

Main recommendations

Limited paediatric screening for fragile X syndrome and some cascade screening in affected families is currently being carried out at many UK centres. This is of clinical value and should continue. However, more research will be needed before any active screening programmes should be considered for implementation in the NHS.

- Studies should be carried out to assess the current practice of paediatric screening when there is developmental delay.
- There should be a national audit of the current practice of cascade screening in affected families.
- Research should be commissioned into the psychosocial implications of being identified as having a PM.
- The feasibility of routine antenatal screening should be assessed.
- A central register for all diagnoses should be established, based mainly on reports from DNA laboratories.

Chapter 1

Background

Fragile X syndrome is a common cause of learning disability. Among known causes of learning disability it is the most frequent **inherited** cause and the second most frequent cause in general after Down's syndrome. Precise estimates are lacking but, in the UK, there may be between 100 and 200 affected births each year compared with 1000 for Down's syndrome. Most affected males have learning disability in the moderate to profound range but females are often less severely affected. Although the disease has long been known to be hereditary there is a non-Mendelian pattern of inheritance with several unusual features. Recently, the molecular genetics of the disease have been elucidated and this has explained many of these features. As well as clarifying the aetiology, the new genetic knowledge has important clinical consequences. In particular, diagnosis can now be improved and the possibility is opened for routine screening. These developments have been rapid and there is an urgent need for health planners to have information on which to base screening policy for the fragile X syndrome.

Molecular genetics

As the name suggests the disorder is associated with 'fragility' on the X chromosome. Most affected individuals manifest fragility at a specific locus on the chromosome and the DNA at this site is now known to contain a sequence which is heritably unstable in affected families. The sequence comprises multiple copies of a specific section of DNA. In the normal X chromosome the sequence contains a variable but limited number of copies, whereas in affected families the sequence becomes expanded in successive generations. When the expanded sequence reaches a critical size it is liable to hyper-expansion within one generation, thus causing fragile X syndrome.

Improved diagnosis

In the past, confirmation of a clinical suspicion of fragile X syndrome relied on cytogenetic techniques that were not completely reliable or valid. This also limited the ability of geneticists to predict with confidence which members of affected families were carrying the affected gene. Molecular genetic techniques are now beginning to replace cytogenetics, bringing an increase in reliability and validity, and also a reduction in cost.

Routine screening

The technical improvements and better understanding of the genetics raise the possibility of testing apparently unaffected individuals for the affected gene, either as the full mutation (FM) or as the pre-mutation (PM). There would be two distinct purposes for screening; namely, to reduce birth prevalence and to bring forward a clinical diagnosis. Each requires different strategies: for the former, antenatal testing of apparently low risk pregnancies, pre-conceptual testing of young females, and systematic testing within the families of affected individuals; for the latter, routine testing of neonates and an active search for paediatric cases among high risk children.

Screening policy

In this document, structured reviews of the literature are used to obtain the information needed by planners to make policy decisions. Detailed information is presented and synthesised concerning the natural history, genetics, prevalence, laboratory techniques and screening strategies. Statistical modelling techniques are used to explore the likely consequences of each strategy. In addition to screening efficiency, we consider the human and financial costs including the provision of counselling services. Finally, those areas where information is lacking are highlighted and suggestions made for future research.

Chapter 2

Systematic review

In conducting this review, we followed the guidelines produced by the NHS Centre for Reviews and Dissemination at the University of York.

A literature search was undertaken and this was kept updated during the review. All references collected before August 1996 were included in the review. The majority of publications were identified using two electronic reference databases: Medline and the Science Citation Index (SCI) accessed via Bath Information and Data Services. In general, SCI is more up to date than Medline. The search was also extended to include the Citation Index for Nursing and Allied Health and Sociofile (or CINAHL) for related articles. Key text words or phrases such as fragile X, fragile X mental retardation (FMR), fragile X sites (FRAX), cytosine–guanine–guanine (CGG), PM and FM were used to ensure a high recall rate of relevant references. The terms triplet and trinucleotide repeat also produced review material on genetically similar disorders.

Over one thousand publications were considered. For each the abstract was read electronically (where available) and papers were excluded if they were outside the scope of the review or the results were biased by poor study design or based on anecdotal information. Occasionally studies were also excluded if the DNA status of the participants was unclear. Case reports were excluded unless they provided vital information on the molecular genetics of the fragile X mutation. A full photocopy was obtained of all the remaining references. Further relevant publications were found by examination of the references listed in papers and also by searching SCI for 'descendants' of important papers. In addition to electronic methods, hand searches were undertaken in the most recent issues of those journals in which articles on the subject are most often found; viz: *Journal of Medical Genetics, American Journal of Medical Genetics, American Journal of Human Genetics, Human Molecular Genetics, Human Genetics, Nature Genetics* and *The Lancet.*

After careful examination, half of the papers identified were considered to be relevant to this review. A full listing of these, in alphabetical order, is given in the Bibliography. A computer database of publications was maintained using Idealist software. In addition to the standard reference information (authors, title, journal, year, volume, pages), the database contains the source, category (e.g. prevalence estimates, genetic counselling, prenatal diagnosis), and current status. This was kept up to date to avoid duplication of references. A manual filing system for hard copies was established using the same categories as Idealist.

The so called 'grey' literature is an important source of review material and this was accessed from several sources. The electronic database, System for Information on Grey Literature in Europe (or SIGLE) accessed via the British Library Automated Information Service (BLAISE-LINE), was searched using the same key words and phrases as for the published literature. Unpublished relevant conference proceedings were also obtained and reviewed for inclusion. The principal scientific forum for the subject is the biennial *International Workshop on Fragile X and X-linked Mental Retardation*. In recent years, the Workshop reports, abstracts and papers have been published. As members of the Fragile X Society in the UK and the Fragile X Foundation in the USA, we received bi-annual and monthly newsletters from these bodies. These provided useful information on dates of impending meetings and unpublished study results. Authors of important papers were contacted for the most up-to-date unpublished information. We also maintained contact with the group led by Professor Pembrey at the Institute of Child Health, who were also carrying out a similar structured review to our own for the NHS Research & Development Programme on Health Technology Assessment.

Where appropriate, studies on the same subject were entered into a meta-analysis. The results of each study are tabulated together with an overall value that is the best estimate of the combined data.

Chapter 3

Natural history

The fragile X syndrome phenotype is characterised by a complex mixture of physical, cognitive and behavioural features. Although males and females display similar features, most of the published studies on the phenotype refer to males.

Physical characteristics

There are several physical features that distinguish affected individuals. These include facial atypia, orthopaedic abnormalities, skin manifestations, unusual growth patterns, cardiac anomalies, and endocrine dysfunction. Despite so many systems being affected, there is no evidence for a substantial impairment of physical health. In one study, life expectancy was reduced by 12 years on average but the authors concluded that this was probably due to biased ascertainment of cases (Partington *et al,* 1992).

Facial features can include prominent ears (increased length and breadth), reduced bizygomatic diameter, prognathism, high arched palate, dental overcrowding and increased head circumference (Simko *et al,* 1989; Hagerman, 1991; Partington, 1984; Sutherland & Hecht, 1985; Butler *et al,* 1991a; Meryash *et al,* 1984). Ocular abnormalities such as strabismus are associated with a variety of chromosomal abnormalities (Elston, 1989; Catalono, 1990) and, in fragile X syndrome, they are present in about one-third to a half of cases (Schinzel & Largo, 1985; Storm *et al,* 1987; Maino *et al,* 1991; King *et al,* 1995). Macro-orchidism is present in over three-quarters of adult males, although it is not specific to fragile X syndrome and is a common secondary feature to other forms of X-linked mental retardation (Brondum Nielson *et al,* 1981; 1982; Sutherland & Hecht, 1985). Common musculo-skeletal findings include pes planus, scoliosis and excessive joint laxity (Davids *et al,* 1990). In one study, about three-quarters of boys under the age of 10 years displayed hyper-extension of the metacarpophalangeal joints (Davids *et al,* 1990). Although recurrent ear infection is a common complaint in clinically normal children, it is found more frequently in boys with fragile X syndrome – about two-thirds of cases in one study (Hagerman, 1987). There may be characteristic markings on the skin, usually including either simian or Sydney palmar creases (Simpson, 1986), and calluses are often present on the hand as a result of hand-biting. General overgrowth (de Vries *et al,* 1995a) as well as abnormal growth patterns have also been reported in both males and females, with a premature growth spurt resulting in a higher than average height in childhood but a reduced final height in adulthood (Loesch *et al,* 1995; Loesch *et al,* 1988; Meryash *et al,* 1984).

Mitral valve prolapse is present in about one-quarter to one-half of affected individuals and, although this finding is generally benign, it can predispose to cardiac arrhythmias (Loehr *et al,* 1986; Sreeram *et al,* 1989). Epilepsy has also been linked with fragile X syndrome (Brøndum Nielson *et al,* 1983; Partington, 1984; Musumeci *et al,* 1988; Wisniewski *et al,* 1991), although it is not clear whether the association is primary to the syndrome or whether it is non-specific or familial (Vieregge & Froster-Iskenius, 1989).

Cognitive profile

Approximately 80% of affected males are moderately to profoundly mentally-impaired, with an IQ of less than 50 (Maes *et al,* 1994). Females with fragile X syndrome usually display a milder phenotype, the majority having a borderline low IQ of between 70 and 85 (Rousseau *et al,* 1994; Taylor *et al,* 1994). A substantial decline in IQ with increasing age has been observed in a proportion of affected individuals (Fisch *et al,* 1991a; Dykens *et al,* 1989; Hagerman *et al,* 1989; Lachiewicz *et al,* 1987) and is suggested as being a consequence of progressive neurological dysfunction (Fisch *et al,* 1991a; Sutherland & Hecht, 1985). However, IQ studies must be interpreted with caution as they are complicated by the use of different measures of intelligence and the presence of cognitive and behavioural problems (Hay, 1994).

Some of the cognitive deficits observed in affected males and females are not specific to fragile X syndrome and are consistent with other forms of mental retardation (Fisch, 1993; Einfield & Hall, 1992). However, published studies also suggest that specific cognitive impairments are present. Most affected individuals experience difficulty in numeracy and visual–spacial tasks, although they perform relatively better in language skills, particularly reading (Freund & Reiss, 1991; Miezejeski *et al,* 1986). Speech is often delayed and is generally described as echolalic, perseverative (i.e. repetition of words, phrases or topics) and cluttered (Sudhalter *et al,* 1990; Sudhalter *et al,* 1992). The ability to process information sequentially, such as following a set of instructions given together, creates particular challenges (Kemper *et al,* 1988).

Behavioural features

Unlike many of the physical features, such as the long face or macro-orchidism, which only become apparent around the onset of puberty, the principal behavioural characteristics are often already observable in early childhood. However, the heterogeneous nature of fragile X syndrome means that not every child will display characteristic behavioural features. Social impairment may present as anxiety, sensory defensiveness, ritualistic behaviour, self-injury (mainly hand-biting) and other stereotyped behaviour such as hand-flapping (Hagerman *et al,* 1986). Poor eye contact, to the point of turning the head and torso away from the line of gaze, is also not uncommon (Hagerman *et al,* 1986; Wolff *et al,* 1989).

Autism

At one time the behavioural features were believed to be associated with autism (Brown *et al,* 1982; Meryash *et al,* 1982; Partington, 1984; Levitas *et al,* 1983), but the relationship between the two disorders was not confirmed by subsequent studies (Einfeld *et al,* 1989; Fisch, 1992). It is now generally accepted that although autism does occur in males with the fragile X syndrome, the incidence is no greater than in other groups of children with learning disabilities (Einfeld *et al,* 1989; Einfeld *et al,* 1994). Detailed neuropsychological studies comparing the two disorders have shown that males with fragile X syndrome more commonly display a range of autistic-like features that differ subtly from true autism (Schapiro *et al,* 1995; Freund & Reiss, 1991; Cohen *et al,* 1989). For example, some studies have found that although problems of language and communication are common to both disorders, males with fragile X syndrome appear to have a greater understanding of conversation than autistic males (Sudhulter *et al,* 1990; 1991).

Attention-deficit disorders

Several studies have reported on the presence of attention deficits and hyperactivity in young boys with fragile X syndrome (Largo & Schinzel, 1985; Fryns *et al,* 1984c; Finnelli *et al,* 1985; Hagerman, 1987). This behaviour becomes apparent in the second year of life and may, to some extent, improve after puberty (Largo & Schinzel, 1985). Controversy exists over whether attention-deficit, hyperactivity disorders are specific to fragile X syndrome. It has been suggested that the behaviour simply relates to the severity of the learning disability in general (Einfield *et al,* 1991). However, recent research indicates that, when compared to those with similar learning disabilities, boys with fragile X syndrome are more restless and fidgety, and have poorer concentration (Turk, 1995b). Also, in children with fragile X syndrome who have mild learning disabilities, the presenting features may be significant hyperactive and attentional problems (Hagerman *et al,* 1985).

Treatment

Although fragile X syndrome is not curable, there are a number of medical, educational, psychological and social interventions that can improve the symptoms.

Medical treatment is available for common problems such as recurrent ear infections, strabismus and joint laxity. Treatment of ear infections is particularly important, since existing or potential language deficits may be further complicated by fluctuating hearing loss.

Folic acid medication is sometimes used in an attempt to control the behavioural problems. Interest in the therapeutic potential of folic acid arose from the observation that fragile site expression *in vitro* could be reduced by the its addition to the culture medium. Lejeune (1982) first reported anecdotally on the beneficial effects of folic acid for improving ‘psychotic-like’ behaviour in affected males. Seven out of eight patients studied showed dramatic improvement, although the method used to measure improvement was not specified. There have since been other such reports, including a survey carried out by the Fragile X Society (unpublished) in which 10 out of 16 children treated were said to have improved. However, seven double-blind, placebo-controlled crossover studies on a total of 65 affected males have failed to demonstrate a marked improvement when hyperactive behaviour and attentional problems were measured by standard objective instruments (see *Table 1*). The patients only showed an improvement when the assessment was judged by the impressions of parents, doctors and teachers. It is possible that the

TABLE 1 Folic acid treatment: results of seven double-blind, placebo-controlled, crossover studies of males

Study	**Number of patients**	**Age range (years)**	**Folic acid per day**		**Improvement in symptoms (%)**†	
			mg	**mg/kg**	**Objective**	**Subjective**
USA, NY I Brown *et al,* 1984	2	10–18	–	1.6	1 (50)	2 (100)
USA, NY II Brown *et al,* 1986	5	8–26	250	–	0 (0)	0 (0)
USA, Colorado Hagerman *et al,* 1986	25	1–31	10	–	2 (8)	13 (52)
Sweden Gillberg *et al,* 1986	4	6–14	0.5	–	3 (75)	3 (75)
USA, NY III Fisch *et al,* 1988a	6	3–15	10	–	0 (0)	0 (0)
USA, Chicago Strom *et al,* 1992	21	2–22	15	–	2 (10)	10 (48)
UK Turk, 1992a	2	5–8	NS	NS	NS	2 (100)
All	65	1–31	–	–	8 (12)	30 (46)

† *Hyperactive behaviour and attentional deficits, judged using objective instruments and by the subjective impression of parents, doctors and teachers; NS = not specified.*

subjective view is correct but that the instruments were insufficiently sensitive or that the studies were not large enough. Alternatively, the results might indeed be poor due to inappropriate dosage of medication, inclusion of post-pubertal males or perhaps deliberate misbehaviour by patients.

Stimulants including methylphenidate (Ritalin®, Ciba Laboratories) and dextroamphetamine (Hagerman *et al,* 1988b) and other medications such as clonidine (Hagerman *et al,* 1995) have been shown to have a beneficial effect on hyperactivity and attentional problems. It has been suggested that reducing these symptoms might enable affected children to improve social relationships and maximise their cognitive potential (Hagerman, 1987). However, unlike folic acid, side-effects have been observed with stimulant use including increased irritability, nausea, headaches and nocturia (Hagerman *et al,* 1988b; 1995). Moreover, the overall effect on learning and long-term cognitive development is unknown.

In addition to medication for behavioural problems, specialised educational programmes have been shown to be effective in the overall management of children with fragile X syndrome. Occupational therapy with sensory integration techniques is helpful where joint instability, tactile defensiveness and behavioural problems occur (Levitas *et al,* 1983). Specific calming techniques, such as deep pressure and deep breathing, and calming music and sounds are thought to be helpful when the individual becomes overwhelmed and overanxious with their surroundings (Hagerman, 1987).

Speech therapy will further improve quality of life on a daily basis by enabling better communication as a result of decreased verbal perseverance and dyspraxia. Finally, it is possible that many of the educational needs of children with fragile X syndrome may to be best met in special classrooms where the social environment can be tailored to minimise the behavioural problems.

Chapter 4

Genetics

The genetics of fragile X syndrome was first investigated in the 1940s when Martin and Bell (1943) reported on a family whose pedigree showed a specific form of X-linked mental retardation. Hence the eponymous description of the disorder as the Martin–Bell syndrome. However, it was a further 25 years before genetic linkage to a fragile site on the long arm of chromosome X was established (Lubs, 1969). Subsequently, the discovery of the cytogenetic media conditions needed to demonstrate expression of the fragile site reproducibly *in vitro* (Sutherland, 1977) enabled segregation studies to be performed. However, these early techniques were crude and, as a result, only limited information regarding the nature of inheritance could be gained. With the recent cloning of the affected gene itself (Verkerk *et al,* 1991) and the development of DNA-testing techniques, many of the previously inexplicable features of the syndrome can now be understood.

Population genetics

Formally, fragile X syndrome is an X-linked dominantly-inherited disorder with reduced penetrance but it does not have a simple Mendelian pattern of inheritance. Females as well as males can be affected, albeit to a lesser extent. In addition, both males and females can be unaffected carriers. Although the children of unaffected female carriers are at increased risk of the disorder, those of unaffected male carriers are not. These individuals, known as normal transmitting males (NTMs), have sons who are neither affected nor carriers and daughters all of whom are unaffected carriers but whose children are at increased risk of fragile X syndrome. This is the 'Sherman paradox', a particular case of the general genetic phenomenon of 'anticipation' (Sherman *et al,* 1984). Thus, in affected families the number of individuals with fragile X syndrome increases with each generation.

Cytogenetics

Fragile sites on human chromosomes are characterised cytologically as specific regions that exhibit constrictions, gaps or breaks when cells are cultured and karyotyped (Sutherland & Hecht, 1985). They are areas of late replication (Webb, 1992; Hansen *et al,* 1993) and their expression can be induced *in vitro* by blocking the normal replication pattern of DNA. This is generally achieved by altering the media conditions of the cultured cells. Over 100 fragile sites have been found on the human genome (Sutherland & Ledbetter, 1989). Some are common but most are rare, and only two are of clinical significance. Designated FRAXA and FRAXE, they are located on the long arm of the X chromosome, at Xq27.3 and Xq28, respectively (Sutherland & Baker, 1992), and both occur in families affected by mental retardation. However, whilst FRAXA expression is specific to fragile X syndrome, FRAXE is inconsistently associated with non-specific, mild mental retardation (Sutherland & Baker, 1992; Knight *et al,* 1993; 1994; Hamel *et al,* 1994; Mulley *et al,* 1995; Bullock *et al,* 1995; Murray *et al,* 1996). Two other fragile sites situated close by on the X chromosome, the common FRAXD at Xq27.2 (Sutherland & Baker, 1990) and the rare FRAXF at Xq28 (Hirst *et al,* 1993b; Parrish *et al,* 1994), are not related to mental retardation.

Until recently the diagnosis of fragile X syndrome was based on the cytogenetic expression of FRAXA in a proportion of cultured cells. However, there were a number of technical problems with the method. First, cytogenetics cannot reliably distinguish FRAXA from the other three neighbouring fragile sites, requiring fluorescence *in situ* hybridisation with DNA probes to separate them (Sutherland & Baker, 1992; Hirst *et al,* 1993b). Second, although it was initially thought that the frequency of cytogenetic expression was mainly controlled by genetic factors (Soudek *et al,* 1984; Hecht *et al,* 1986), between-laboratory variation has been shown to contribute more to the variance (Fisch *et al,* 1991b). There are differences in the proportion of affected cells regarded as diagnostic; although guidelines have been published recommending 4% as the lower limit (Jacky *et al,* 1991), some laboratories use a cut-off as low as 2%. There is also variability because of differences in the tissue culture medium (Sutherland, 1977), levels of folic acid and thymidylate synthetase activity (Glover, 1981; Cantu *et al,* 1985; Sutherland *et al,* 1985), and cell density (Cantu *et al,* 1985; Krawczun *et al,* 1986). (The 1991 guidelines recommended standardisation of the number and type of induction systems used and the number of cells counted.) Third, the assay is affected by factors other than the presence of fragile X syndrome. Oral intake of folic acid in the diet might decrease the frequency of FRAXA expression (Brown *et al,* 1984; Gustavson *et al,* 1985). Also, an inverse relationship between age and cytogenetic expression has been observed in females (Rousseau *et al,* 1991b), although this relationship has not been demonstrated in males (Brøndum Nielsen & Tommerup, 1984).

Molecular genetics

Classically, genetic diseases (e.g. cystic fibrosis, Tay–Sachs disease, sickle cell anaemia) are either inherited in a recessive or dominant Mendelian form or the result of a new mutation. Fragile X syndrome does not behave like this, and is now known to be an example of a different type of genetic disease caused by a 'dynamic' mutation which is heritably unstable (Richard & Sutherland, 1992). Here, an initial change in the DNA sequence increases the tendency to further mutation within subsequent generations. Fragile X syndrome is the result of a dynamic mutation in a gene at the FRAXA locus that is referred to as fragile X mental retardation-1 (FMR-1) (Kremer *et al,* 1991; Verkerk *et al,* 1991; Fu *et al,* 1991). Dynamic mutations are now known to be also responsible for spinobulbar muscular atrophy (Kennedy's disease) (La Spada *et al,* 1991), myotonic dystrophy (Brook *et al,* 1992; Mahadevan *et al,* 1992; Fu *et al,* 1992), Huntington's disease (Huntington's Disease Collaborative Research Group, 1993), spinocerebellar ataxia type 1 (Orr *et al,* 1993), dentatorubral pallidoluysian atrophy (Koide *et al,* 1994; Nagafuchi *et al,* 1994) and the mental retardation associated with FRAXE (Knight *et al,* 1993).

FMR-1 gene

This gene spans 39 kb containing 17 exons (Eichler *et al,* 1993). The FRAXA site, located in the untranslated region of the first exon (Verkerk *et al,* 1991; Yu *et al,* 1992; Caskey *et al,* 1992; Ashley *et al,* 1993b), is characterised by the presence of an array comprising a repeat sequence of the trinucleotide CGG interspersed with single adenine–guanine–guanine (AGG) repeats along its length (Verkerk *et al,* 1991; Fu *et al,* 1991; Kremer *et al,* 1991). A CpG island, thought to be the gene promoter, is located approximately 250 bp distal of the CGG repeat (Verkerk *et al,* 1991; Bell *et al,* 1991; Oberlé *et al,* 1991). FMR-1 normally transcribes a cytoplasmic protein product (Verheij *et al,* 1993), FMRP, which is ubiquitously expressed at low levels and at a higher levels in the testes and brain (Devys *et al,* 1993; Bachner *et al,* 1993; Hinds *et al,* 1993; Verheij *et al,* 1995). Although the exact function of the gene product is not known, protein characterisation has shown that it contains sequence motifs characteristic of ribosomal RNA-binding proteins (Siomi *et al,* 1993; Ashley *et al,* 1993a; Feng *et al,* 1995a; Khandjian *et al,* 1996). The absence of this product is believed to be responsible for the clinical phenotype of fragile X syndrome (Gedeon *et al,* 1992; Wöhrle *et al,* 1992a; Verheij *et al,* 1993; Meijer *et al,* 1994).

The array is polymorphic in respect of the number of CGG repeats it includes, as well as the number and position of the interspersed AGGs (Fu *et al,* 1991; Snow *et al,* 1993; Eichler *et al,* 1994; Hirst *et al,* 1994; Kunst & Warren, 1994; Snow *et al,* 1994). The different alleles are usually referred to by the 'repeat size' of the array, that is, the total number of both CGG and AGG repeats. Size is the principal determinant of whether an allele is regarded as normal or mutated.

Normal alleles

Distribution of repeat sizes

In the unaffected population the most common repeat size is 30. The lowest reported size is 5 and the upper limit of normal is generally taken to be 54 (Fu *et al,* 1991) although some studies use 52 as the upper limit. These alleles are inherited stably, although small changes in size can occur between generations. The frequency distribution of repeat sizes in the unaffected population, compiled from five studies, is shown in *Figure 1* (Snow *et al,* 1993; Dawson *et al,* 1995; Eichler *et al,* 1995a; Brown *et al,* 1996; Kunst *et al,* 1996), and includes a total of 6052 normal X chromosomes.

Female alleles

In a proportion of females with normal alleles there is a difference in the size of the repeat sequences on the two X chromosomes; such females are referred to as heterozygous normal. The remaining females with equal repeat sizes are referred to as homozygous normal. *Table 2,* compiled from five studies, shows that in a total of 1518 normal females, 29% were homozygous.

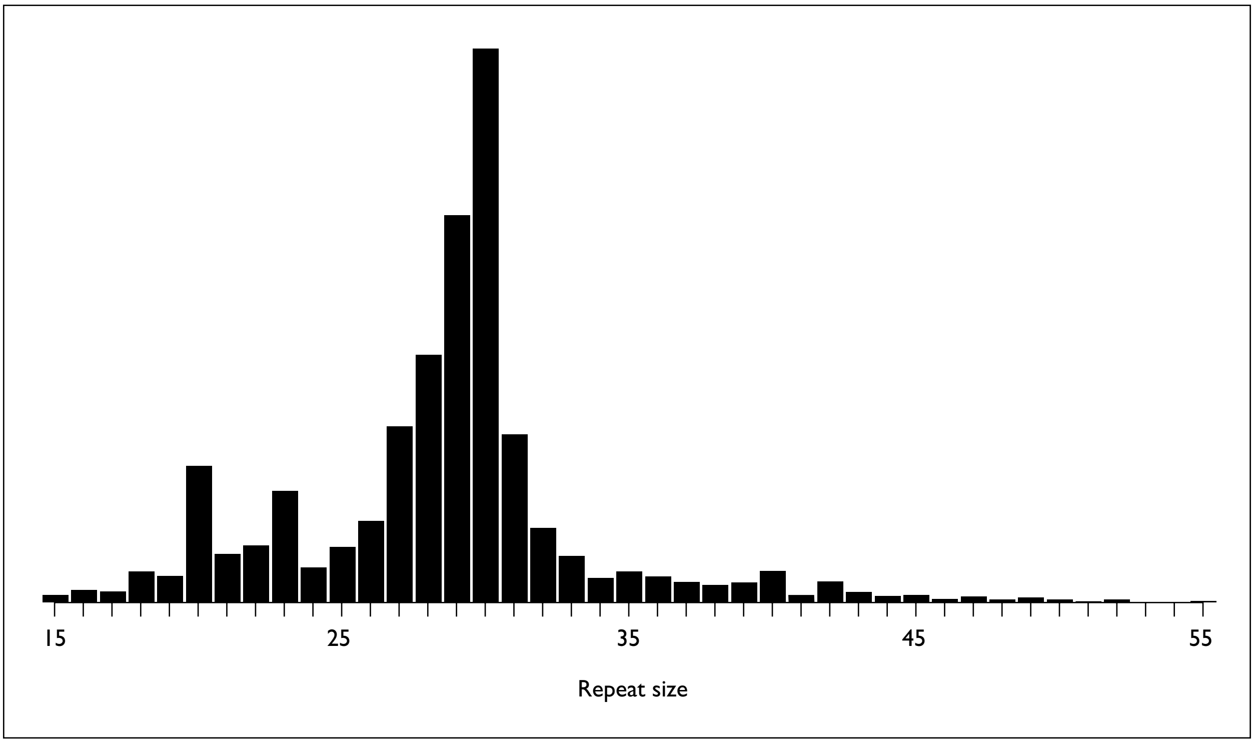

FIGURE 1 *Population distribution of the normal allele size*

TABLE 2 Proportion of normal females who are homozygous for the repeat size: results from five studies

Study	Number of individuals	Homozygous (%)
USA, NY Brown *et al*, 1993	206	44 (21)
Japan Arinami *et al*, 1993	227	66 (29)
USA, Rochester Snow *et al*, 1993	197	35 (18)
Canada Dawson *et al*, 1995	735	242 (33)
UK, Leeds (Unpublished data)	153	51 (33)
All	1518	438 (29)

Mutated alleles

In affected families there are mutations in the FMR-1 gene which lead to hereditary instability and which, ultimately, cause the disorder (Bell *et al,* 1991; Kremer *et al,* 1991; Oberlé *et al,* 1991; Verkerk *et al,* 1991; Yu *et al,* 1991). The mutations are characterised by a substantial increase (or 'expansion') in repeat size compared to normal; two principal classes of mutation have been defined, the PM and the FM, according to the size. FMs are associated with clinical fragile X syndrome; PMs are not but carry a high risk of expansion between mother and offspring (see *Figure 2*).

Full mutation

If the repeat size exceeds 200 there is said to be an FM. This generally coincides with abnormal methylation of the nearby CpG island (Verkerk *et al,* 1991; Bell *et al,* 1991) and is thought to be partly responsible for down-regulation of the FMR-1 gene (Pieretti *et al,* 1991; Sutcliffe *et al,* 1992); in individuals with an FM and methylation, the FMR-1 mRNA cannot be detected.

Pre-mutation

The PM repeat size ranges from approximately 55 to 199, although there is a grey zone between normal and PM alleles (see page 13). In cells with a PM, the FRAXA site is rarely cytogenetically expressed and there is no methylation of the FMR-1 gene; several studies have observed both FMR-1 mRNA and FMRP in these cells (Pieretti *et al,* 1991; Devys *et al,* 1993; Siomi *et al,* 1993; Feng *et al,* 1995a).

Mosaics

There are various types of mosaicism commonly found in individuals with an FM genotype. First, there is 'size' mosaicism, in which those with an FM also have PM cell lines. The results from seven studies which included a total of 604 males and

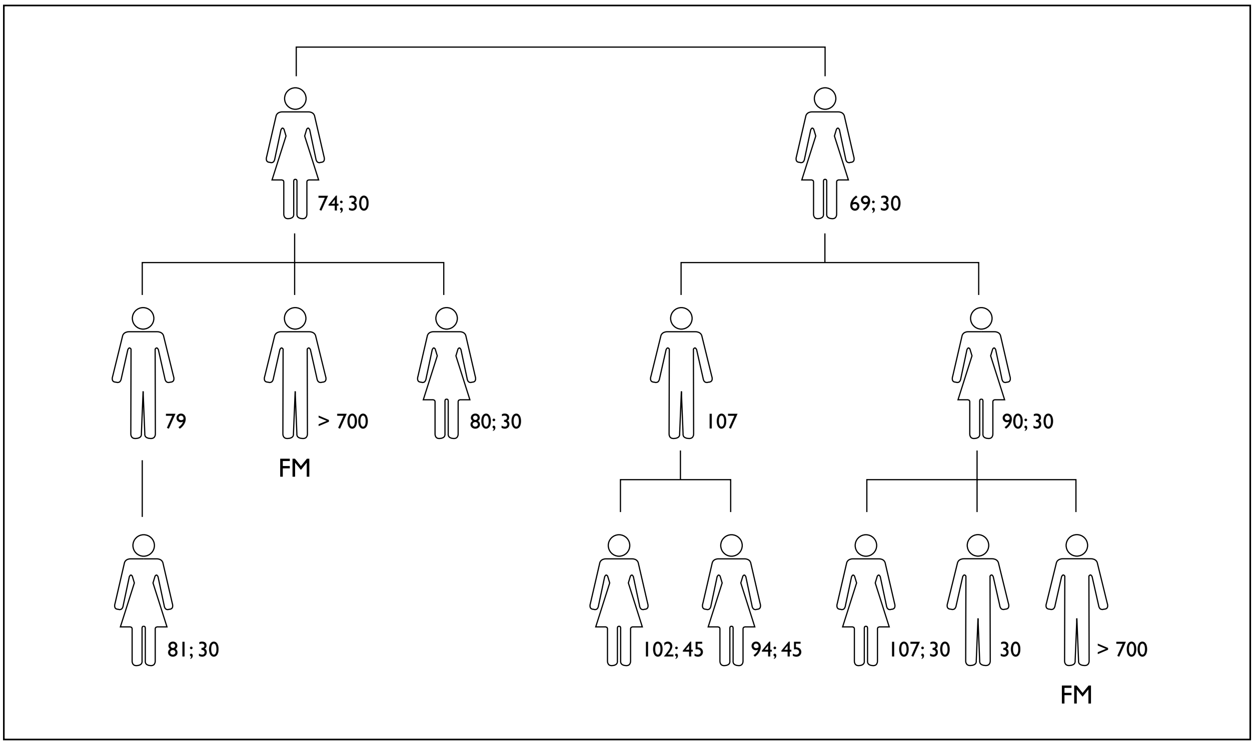

FIGURE 2 *Pedigree of a family with fragile X syndrome (after Warren & Nelson, 1994; numbers are repeat sizes)*

298 females with an FM genotype are shown in *Table 3*. About one-quarter of the males tested had size mosaicism but this proportion was considerably lower for females. One study has also reported an affected male with an FM who has some cell lines with normal alleles (van den Ouweland *et al,* 1994a). Second, there is 'methylation' mosaicism where a proportion of those with an FM in every cell have cell lines in which the FMR-1 gene is either partially or completely unmethylated (Loesch *et al,* 1993c; McConkie-Rosell *et al,* 1993; Hagerman *et al,* 1994a; Pieretti *et al,* 1991). Methylation mosaicism is less common than size mosaicism. In males with either size or methylation mosaicism, FMR-1 mRNA can be detected, albeit at considerably reduced levels (Pieretti *et al,* 1991; Hagerman *et al,* 1994a; Feng *et al,* 1995b). Sperm from all males tested so far with the FM, also have a PM (Reyniers *et al,* 1993; de Graaf *et al,* 1995b). Also, the size of the FM can vary between different cell lines within an individual, resulting in a smear rather than a sharp band on DNA electrophoresis. Some of these mosaic forms are likely to be due to a post-zygotic expansion from PM to FM and somatic instability in the FM lines in early embryonic life (see page 14).

Other mutations

Fragile X syndrome is sometimes caused by defects other than the FM in the FMR-1 gene. These include deletions (Mila *et al,* 1996; van

TABLE 3 *Proportion of those individuals with an FM who have size mosaicism: results from seven studies*

Study	Number of individuals	Mosaic (%)
Males		
France I Rousseau *et al,* 1991a	109	19 (17)
USA, Rochester Snow *et al,* 1993	91	20 (22)
The Netherlands de Vries *et al,* 1993	52	14 (27)
USA, Colorado Hagerman *et al,* 1994a	133	21 (16)
Finland Väisänen *et al,* 1994	71	11 (15)
USA, New York Nolin *et al,* 1993	148	61 (41)†
All males	604	146 (24)
Females		
France I Rousseau *et al,* 1991a	62	6 (10)
USA, Rochester Snow *et al,* 1993	66	6 (9)
France II Rousseau *et al,* 1994	170	9 (5)
All females	298	21 (7)

† *In 20% the mosaicism was slight.*

den Ouweland *et al,* 1994b; Gedeon *et al,* 1992; Wöhrle *et al,* 1992b; Quan *et al,* 1995; Hart *et al,* 1995; Meijer *et al,* 1994; Trottier *et al,* 1994; de Graaff *et al,* 1995a) and point mutations (de Boulle *et al,* 1993); although the exact frequency is unknown, they are thought to be rare.

Grey zone

Although in most centres the division between normal and PM is taken to be a repeat size of 55, this is, to some extent, arbitrary. So far no-one has reported a smaller allele expanding to an FM in one generation but they have demonstrated hereditary instability, that is, a substantial increase in size between generations. In one family with fragile X syndrome, a repeat size as low as 52 demonstrated an increase in size to 73 repeats (Fu *et al,* 1991). In another family, an increase from 46 to 52 repeats was observed (Reiss *et al,* 1994).

Another way of characterising alleles, especially those in the 'grey zone' around 55, which may help to distinguish stable from unstable alleles, is to consider the repeat structure of the array. The 'pure' repeat size is defined as the largest contiguous number of CGG repeats without intervening AGGs. It has been suggested that the loss of AGGs is responsible for increased hereditary instability. In one unaffected family, an allele of repeat size 66 but pure size 46 (array structure of 9 CGGs, AGG, 9 CGGs, AGG and 46 CGGs) was transmitted stably, although unstable pure sequences of 34 repeats have been observed (Eichler *et al,* 1994). A pure repeat size of 56 has also been reported to have resulted in an affected offspring, the total repeat size being 66 (Eichler *et al,* 1994).

Phenotype–genotype relationship

Full mutation

The majority of clinically-affected individuals with fragile X syndrome have an FM with complete methylation (Smits *et al,* 1994). Males with an FM nearly always have a typical fragile X phenotype, and there does not appear to be any correlation between the degree of mental retardation and the repeat size (de Vries *et al,* 1993). It has been suggested that the existence of high functioning males with the fragile X phenotype relates to the level of FMRP produced (Hagerman *et al,* 1994a). However, attempts to correlate IQ with the degree of mosaicism (both size and methylation) have yielded conflicting results (de Vries *et al,* 1993; McConkie-Rosell *et al,* 1993; Hagerman *et al,* 1994a; Rousseau *et al,* 1994).

Only about half of the females with the FM have a fragile X phenotype with obvious mental retardation (Steinbach *et al,* 1993; Väisänen *et al,* 1994; Taylor *et al,* 1994), with 20% having a moderate to severe phenotype (Rousseau *et al,* 1994). In those without a reduced IQ, specific neuro-cognitive deficits have been observed (Mazzocco *et al,* 1992; 1993). The milder phenotype in females and the variable expression are due to X-chromosome inactivation.

X-chromosome inactivation

During early development, undifferentiated female embryos undergo a process whereby in each somatic cell one of the two X chromosomes becomes inactivated (Puck *et al,* 1992; Fialkow, 1973). This is believed to be a random process so that maternally or paternally inherited X chromosomes have an equal chance of being active or inactive in each cell. Although it is becoming apparent that not all genes on the X chromosome are inactivated, recent evidence indicates that the FMR-1 gene is (Kirchgessner *et al,* 1995). Studies of females with FM have found skewed X-chromosome activation ratios (Watkiss & Webb, 1995; Rousseau *et al,* 1991b), and this may help predict the severity of the disease phenotype. For example, a skewed activation ratio in favour of the abnormal allele might suggest a more severe phenotype. Although there is evidence to support this (Reiss *et al,* 1995), tissue differences in X activation ratios have been observed suggesting that any correlation based on peripheral blood cells should be interpreted with caution (Azofeifa *et al,* 1996).

Pre-mutation

Female obligate carriers of fragile X syndrome who do not have an FM always have a PM; no spontaneous expansion directly from a normal allele to an FM has been observed. NTMs have a PM which is relatively stable so that when the X chromosome is passed on to a daughter only small changes in repeat size occur (Fisch *et al,* 1995). Thus, the daughters are also unaffected and generally have a PM.

Although individuals with a PM are phenotypically normal, there is a substantial increase in obstetric and gynaecological problems. Specifically, there is a reported increase in the twinning rate compared with women who have normal X chromosomes or an FM (Tizzano & Baiget, 1992; Turner *et al,* 1994b). Two studies have demonstrated that in those with a PM the rate of premature ovarian failure is 4–5 times higher than controls but similar to those with an FM (see *Table 4*). This effect may be even greater when the ovarian failure is familial rather than sporadic (Conway *et al,* 1995).

Although neither males nor females with a PM have been shown to have a reduced level of IQ (Reiss *et al,* 1993; Taylor *et al,* 1994; Thompson *et al,* 1994; Sobesky *et al,* 1994b), subtle emotional and neuro-cognitive effects may be present (Loesch *et al,* 1993c; Sobesky *et al,* 1994b). Although Reiss and colleagues (1993) found no significant difference in any cognitive or neuro-psychological measure between women with a PM and a control group of mothers of developmentally-delayed children, Steyaert and colleagues (1994a) found, in a small study, that women with a PM showed faster visual information processing in a divided attention task.

TABLE 4 *Rate of premature ovarian failure: comparison of women with normal, PM and FM alleles: results from two studies*

Study †	Number of individuals	Rate (%)
Normal allele		
USA–Canada	135	8 (6)
USA, Colorado	74	3 (4)
All normal	209	11 (5)
PM allele		
USA–Canada	140	34 (24)
USA, Colorado	33	6 (18)
All PM	173	40 (23)
FM allele		
USA–Canada	44	8 (18)
USA, Colorado	32	6 (19)
All FM	76	14 (18)

† *USA–Canada, Schwartz* et al, *1994; USA, Colorado, Hull & Hagerman, 1993; the former study defined premature ovarian failure as permanent cessation of menses prior to the age of 40 years, whereas the latter did not make a firm definition.*

Cytogenetic–molecular genetic comparison

Several studies have compared the two methods for the same individuals. The cytogenetic results from five studies of individuals with different types of FMR-1 allele are shown in *Table 5*. A total of 534 males with an FM were tested, and 99% had positive cytogenetic results; in females, the proportion detected cytogenetically was lower, with only 81% of the 161 tested having positive results. As indicated in the table, a small proportion of those with PM alleles and even normal alleles have false-positive cytogenetic results.

Aetiology of the expansion

Origin

Genealogical studies have inferred the silent passage of mutations in affected families through several generations prior to a clinically significant event (Holmgren *et al,* 1988; Smits *et al,* 1993). However, these studies cannot distinguish if the affected gene persisted as a PM over the generations or in a more stable form until recent expansion.

Analysis of microsatellite markers located close to the FMR-1 repeat sequence suggest that several mutational pathways may be operating. Various combinations of these markers (i.e. haplotypes) have been studied and linkage disequilibrium has been observed whereby specific haplotypes are enriched in affected families. The results of haplotype analysis in 13 studies in different populations are shown in *Table 6*. The extent of disequilibrium is more obvious in some populations than others. For example, in Finland about three-quarters of affected chromosomes have one specific haplotype which is found in only 3% of controls (Oudet *et al,* 1993a). It is thought that in Finland the majority of affected chromosomes originate from a single mutational event: a ‘founder effect’. In other countries, where the linkage disequilibrium is not so pronounced, it is speculated that more than one mutational event may have occurred at different times.

Mechanism

An initial event pre-disposing an allele to instability may be intrinsic to the repeat sequence, such as the loss of an interrupting AGG. Support for this theory comes from several studies of repeat array structure (Eichler *et al,* 1994; Hirst *et al,* 1994; Kunst & Warren, 1994; Snow *et al,*

TABLE 5 *Proportions of positive cytogenetic results* † *in males and females with normal, PM and FM alleles: results from five studies* *

Study	**Males**		**Females**	
	Number	**Positive**	**Number**	**Positive**
Normal allele				
USA Hull & Hagerman, 1993	–	–	74	0
Canada Rousseau *et al*, 1994	221	0	252	3
All	221	0 (0%)	326	3 (1%)
PM allele				
USA Hull & Hagerman, 1993	–	–	37	4
Finland von Kuskull *et al*, 1994	10	0	62	14
Canada Rousseau *et al*, 1994	39	1	239	12
UK Macpherson *et al*, 1992a	10	0	42	0
Brazil Mingroni-Netto *et al*, 1994	–	–	29	3
All	59	1 (2%)	409	33 (8%)
FM allele				
USA Hull & Hagerman, 1993	–	–	28	17
Finland von Kuskull *et al*, 1994	50	50	28	25
USA, Rochester Snow *et al*, 1992	61	60	34	34
Canada Rousseau *et al*, 1994	392	386	19	169
UK Macpherson *et al*, 1992a	31	31	22	18
Brazil Mingroni-Netto *et al*, 1994	–	–	30	21
All	534	527 (99%)	161	131 (81%)

† *The fraction of cells expressing FRAXA required for a positive results varies for each study.*

* *A further large study of 525 subjects (Wang* et al, *1993) was not included as it did not distinguish males and females, FM and PM.*

1994; Zhong *et al,* 1995b; Zhong *et al,* 1996). In *Table 7* the results from five such studies are summarised. Overall, in a total of 722 normal chromosomes the majority had repeat sequences interrupted by two AGGs, whereas in those with a PM over half had pure uninterrupted sequences. Also, in X chromosomes with a PM having intervening AGGs, the longest pure run of CGGs is found at the 3' end suggesting that expansion may be the result of a replication defect (Kunst & Warren, 1994).

Although the actual mechanism of expansion is unproven, one plausible theory is that the loss of an AGG which resulted in a longer pure sequence may lead to slippage during DNA replication (Richards & Sutherland, 1994). The function of the AGGs would be to anchor otherwise pure repeat sequences, thus preventing the formation of large slippage structures. Once a pure sequence approaches the length of an Okazaki fragment (about 150–200 bp) (Thommes & Hubscher, 1990), the chances of expansion to an FM would be greatly increased (Eichler *et al,* 1994). Further

***TABLE 6** Haplotypes occurring more frequently in families affected by fragile X syndrome: results from 13 studies in different populations*

Population (Study)	**Marker †**	**Haplotype**	**Controls**		**Affected families**	
			Tested	**Present (%)**	**Tested**	**Present (%)**
Australian Richards & Sutherland, 1992	1–2	AF	202	12 (6)	134	42 (31)
Finnish I Oudet *et al*, 1993a	2–3	153-196	34	1 (3)	26	19 (73)
French Oudet *et al*, 1993b	2–3	155-204	153	2 (1)	102	14 (14)
Dutch-Belgian Buyle *et al*, 1993	3	204	134	1 (1)	68	25 (37)
Swedish Malmgren *et al*, 1994	2–3	147-194	28	1 (4)	28	8 (29)
Czech Malmgren *et al*, 1994	2–3	149-204	20	1 (5)	15	4 (27)
British I Macpherson *et al*, 1994	3–1–2	6-4-4	188	8 (4)	44	7 (14)
Japanese Richards *et al*, 1994b	1–2	DB	142	18 (13)	40	8 (20)
Finnish II Haataja *et al*, 1994	3–2	196-153	135	11 (8)	60	48 (80)
Caucasian I Kunst & Warren, 1994	1–4–5	D-A-A	172	10 (6)	97	45 (46)
Caucasian II Snow *et al*, 1994	3	196	50	8 (16)	64	22 (34)
British II Hirst *et al*, 1994	1–3	D6	102	2 (2)	70	17 (24)
Italian Chiurazzi *et al*, 1996	1–3	C-3	235	0	137	12 (9)
British III Eichler *et al*, 1996	3–2–1	7-1-3	154	0	44	3 (7)

† Microsatellite markers used include, 1 = FRAXAC1, 2 = FRAXAC2, 3 = DSX548, 4 = FMRa, and 5 = FMRb.

to this, Chen and colleagues (1995) suggest that the repair mechanism by which slippage structures are normally excised may be impaired.

Timing

The expansion in repeat size might take place either during oogenesis or in early embryonic development, but three types of study suggest that it is likely to be a post-zygotic event. First, observations on methylation status during early development show that although the foetal tissue may be methylated the chorionic villi are hypomethylated, indicating that methylation is acquired after fertilisation. Expansion is a separate process that appears to precede methylation, as evidenced by the observation of fully expanded, hypomethylated chorionic villi in an affected foetus. Second, if expansion took place before cell differentiation in the foetus there would be somatic homogeneity, whereas individuals with an FM display mosaicism among and between tissues. Moreover, *in vitro* studies have shown that cells carrying the FM exhibit clonal stability (Wöhrle *et al*, 1993), suggesting that somatic variation is restricted to a period during early foetal development. Lastly, expansion may occur after the twinning event since monozygotic twins discordant for repeat size and methylation status have been observed (Malmgren *et al*, 1992; Devys *et al*, 1993; Kruyer *et al*, 1994).

TABLE 7 *Proportion of FMR-1 genes (%) with different number of interspersed AGGs in those with normal and PM alleles; results from five studies*

Study	Number	Repeat sizes	Number of interspersed AGGs			
			> 2	**2**	**1**	**0**
Normal allele						
USA, Georgia Kunst *et al*, 1996	81[†]	14–49	7	51	19	4
UK Hirst *et al*, 1994	102	16–48	2	65	29	6
USA, New York Zhong *et al*, 1994	132	20–52	4	94	32	2
USA, Texas I Eichler *et al*, 1994	406	13–49	11	299	87	9
All	722	13–52	24 (3%)	509 (71%)	167 (23%)	21 (3%)
PM allele						
USA, Georgia Kunst *et al*, 1996	2	90–105	0	0	2	0
UK Hirst *et al*, 1994	2	70–85	0	0	0	2
USA, New York Zhong *et al*, 1994	54	56–180	20	0	0	34
USA, Texas II Eichler *et al*, 1995a	13	> 55	0	3	9	1
All	71	55–180	20 (28%)	3 (4%)	11 (15%)	37 (52%)

[†] *One allele excluded as CGG repeat included TGG interruption.*

Chapter 5

Definitions

Interpreting the literature on fragile X syndrome is complicated by variable and changing usage of the terms **affected, carrier** and **screening.** For the purposes of this review, explicit definitions are used in an attempt to avoid ambiguity in the conclusions and recommendations.

Affected

Studies vary in the extent to which individuals with a normal phenotype but a genetic lesion are regarded as affected. Males do not constitute a problem as the clinical, cytogenetic and DNA diagnosis is likely to coincide but this is not the case for females (see page 13). In this review, we have restricted the term affected to individuals with the fragile X syndrome phenotype.

Carrier

Before the introduction of DNA testing that was capable of distinguishing females with an FM or PM, the term carrier was used ambiguously. Women who were phenotypically normal mothers of affected individuals or who themselves had fragile X syndrome were classified together as obligate carriers. Other family members may only have been classified as carriers if this could be demonstrated cytogenetically. In this review we have tried to avoid the term carrier; however, where its use was deemed necessary it is prefixed by PM or FM, and in FM carriers we distinguish between those with and without clinical fragile X syndrome by referring to affected and unaffected carriers, respectively.

Screening for fragile X syndrome

In common usage, screening includes any kind of testing carried out to detect a disorder. This is too vague a concept for public health purposes, where four aspects of the screening process need emphasis. These are that

- it is routine and systematic
- it is applied to apparently healthy individuals
- it aims to select those who are at high risk of a well defined disease
- those in the high risk group are offered proven preventive action which would be too expensive or hazardous for general use.

Sometimes fragile X testing is carried out on individuals with learning disabilities in order to improve or confirm a clinical diagnosis. This is not screening. However, if such a population is systematically tested it might be regarded as screening, depending on the intention. If the aim is simply case-finding, for example, in order to provide an estimate of prevalence, this would not be considered screening. If, however, the endpoint of the case-finding is preventive, say as the starting point of a systematic attempt to identify affected families for counselling or testing, then it would be considered as part of a screening programme.

Chapter 6

Prevalence

Studies of prevalence fall into two distinct groups. In one group of studies, the investigators studied the cases of fragile X syndrome found in institutions. In the absence of information on the proportion of affected individuals who are institutionalised, these studies cannot be used to estimate a population prevalence. Nonetheless, they do allow an estimate of the frequency of the disorder among individuals with mental handicap, which is useful for case-finding (see page 27). In a second group of studies either multiple sources or a systematic method of ascertainment was used to obtain a more complete yield of cases in the whole of a defined population.

Bias

There is likely to be marked between-study variability in the estimated prevalence of fragile X syndrome owing to important differences in study design. Those studies which are based on a cytogenetic diagnosis of fragile X syndrome will tend to yield higher estimates of prevalence than those using DNA methods. Studies that include females will tend to underestimate prevalence if diagnostic testing is restricted to institutions and special educational units. The relatively large number of mild cases may escape detection. Studies that include a disproportionate number of children will also tend to yield relatively low estimates. Diagnosis will be delayed in individuals in whom the clinical features of fragile X syndrome are not apparent until puberty; indeed, in some individuals the intellectual deficit may not be noticed until they are of secondary school age.

Frequency in the mentally handicapped

The frequency of fragile X syndrome in institutionalised males was examined in ten studies (*Table 8*). Of the 2019 males tested in all the studies combined, 6% were found to be affected. However, the frequency varied greatly between studies and the heterogeneity may mean that the overall frequency is not a reliable estimate of the true rate. The variability was due to a number of differences in study design; viz:

- admission patterns for the institution
- completeness of ascertainment due to patient and parental non-compliance with diagnostic testing
- selection criteria for testing (e.g. in some studies only those with typical features of fragile X syndrome were tested, while in others everyone was tested)
- definition of the denominator population.

***TABLE 8** Frequency of fragile X syndrome among males in institutions: results of ten studies*

Study	**Selection criteria***	**Number**	**Fragile X (%)**
Finland Kähkönen *et al*, 1983	UA	150	6 (4)
Germany Froster-Iskenius *et al*, 1983	All	242	15 (6)
USA, Boston Paika *et al*, 1984	UA	44	6 (14)
Belgium Fryns *et al*, 1984c	UA	354	57 (16)
Japan Arinami *et al*, 1986 Hofstee *et al*, 1994	All	305	11 (4)†
UK Primrose *et al*, 1986	UA	100	7 (7)
USA, Colorado Hagerman *et al*, 1988a	UA	267	7 (3)
Sicily Neri *et al*, 1988	All	155	12 (8)
USA, Tennessee Butler & Singh, 1993	UA	201	4 (2)
Poland Mazurczak *et al*, 1996	All	201	6 (3)†
All		2019	131 (6)

† *All males were tested except in studies selecting those with unknown mental impairment of aetiology (UA).*

* *Confirmed by DNA analysis.*

General population prevalence

In seven studies, attempts have been made to estimate the population prevalence of fragile X syndrome in males (see *Table 9*). The usual approach was to carry out diagnostic tests on young people with special educational needs. The number of individuals found to be affected was then related to the size of the population of the same age from which they were drawn. Since testing was not acceptable to all subjects, the figures were generally adjusted upwards proportionally on the assumption that there was no bias in the those accepting testing. This may not be a valid assumption as, in one study (Tranebjaerg *et al,* 1994), the rate of acceptance was higher in those individuals with severe learning disabilities than in those with milder symptoms. One of the studies was based on a cytogenetic register. Of the studies using school populations, one relied solely on special schools but, for the age range studied, it is unlikely that many boys with the disorder would be in mainstream education.

The results from each of the seven studies are shown in *Table 9*. The study based on a register yielded a particularly low prevalence but it is unclear how complete the register was. The studies of young people using a cytogenetic technique for diagnosis yielded much higher prevalence estimates than the remainder. This is likely to reflect the known tendency for cytogenetics to produce false-positive results. Of the four studies using DNA methods of diagnosis, two had originally been based on cytogenetics but were subsequently updated. The cytogenetically-based figures for these studies have been widely quoted but, when those with positive results were DNA tested, it became clear that these were gross overestimates. In the Australian study, four of those originally believed to have fragile X syndrome could be excluded, thus reducing the observed prevalence from 3.8 (Turner *et al,* 1986) to 2.3 per 10,000 males (Turner *et al,* 1996). In the UK – Coventry study, DNA analysis has enabled the reclassification of ten patients diagnosed with fragile X syndrome in the original cytogenetic study. Thus, the original prevalence of 10.5 per 10,000 boys (Webb *et al,* 1986b) has been reduced to 2.4 per 10,000 (Morton *et al,* 1997). Only one study was completely based on DNA techniques (Jacobs *et al,* 1993) and this yielded a prevalence of 2.6 per 10,000 in males.

Taking all the DNA studies together, the combined prevalence is about 2.5 per 10,000 males or 1 in 4000. From what information is available, this must be regarded as the best estimate of population prevalence. However, it should be regarded as a minimum estimate since, in general, boys were only tested if they were at state schools. In the

TABLE 9 *Population prevalence of fragile X syndrome in males: results from seven studies*

Study	Diagnostic technique*	Age range (years)	Source of samples †	Number tested	Fragile X syndrome	
					Number	Rate per 10,000
Sweden Gustavson *et al,* 1986	CG	< 18	PD	89	12	5.9
Finland Kähkönen *et al,* 1987	CG	8–9	MS	61	4	8.3
Denmark Tranebjaerg *et al,* 1994	CG	All	CR	31	31	0.4
UK, Wessex I Jacobs *et al,* 1993	DNA	5–18	MS	180	4	2.8
UK, Wessex II Murray *et al,* 1996	DNA	5–18	MS	1013	5	2.0
UK, Coventry Morton *et al,* 1997	CG/DNA	11–16	SS	219	6	2.4
Australia Turner *et al,* 1996	CG/DNA	5–18	SS & MS	472	10	3.0

* *CG, cytogenetic method; DNA, DNA method; CG/DNA, cytogenetic method originally, but positives later re-analysed using DNA method.*

† *PD, individuals identified from paediatric records; MS, those with mental handicap in mainstream schools; SS, from special schools; CR, from cytogenetics register.*

Australian study it was thought that a large number of children may have been excluded because they were at private schools (Turner *et al,* 1992). Four of the studies also considered the prevalence of fragile X syndrome in females. In the UK – Wessex studies, an estimate was not possible as no cases were found in the 74 girls tested (Jacobs *et al,* 1993), but in the other three studies the prevalence was about half of that found in males (Kähkönen *et al,* 1987; Webb *et al,* 1986b; Turner *et al,* 1986).

Chapter 7

Screening and diagnosis

Aims of screening

The ultimate public health purpose of genetic screening is prevention. With fragile X syndrome there are two possibilities, namely, primary or secondary prevention aimed at reducing the birth prevalence of the disorder, and tertiary prevention aimed at improving prognosis by appropriate management when the diagnosis is brought forward through screening. Closely allied to this is the provision of information for its own sake which, in general, also appears to be of value (Mooney & Lange, 1993). The two aims are not mutually exclusive in that, for example, the early diagnosis of one affected child in a family may lead to the avoidance of further affected children. Similarly, as a consequence of an affected pregnancy being detected and terminated, family studies may be initiated which bring forward the diagnosis of some affected relative.

Reducing affected births

The ability to reduce the number of affected births is contingent on the identification of young women with a PM or an FM, and therefore at high risk of an affected pregnancy. Once these have been identified, there are several preventive options. The first is prenatal diagnosis and selective abortion of foetuses with an FM. Other options are to avoid pregnancy, have *in-vitro* fertilisation of a donated ova and, in the near future, pre-implantation diagnosis and selected implantation of an unaffected zygote.

Earlier diagnosis

Although there is a general awareness of fragile X syndrome amongst health professionals, the clinical features which separate this disorder from other non-specific forms of learning disability are not well known. This is compounded by the lack of obvious clinical features which accompany developmental delay early in life. Thus, as the prevalence studies show, many cases go unrecognised or there is a considerable delay in diagnosis.

Improving prognosis

Although fragile X syndrome is not curable there are a number of interventions that can improve the prognosis (see page 6). Nonetheless, there is no specific evidence that intervening at an early stage will achieve a better long-term outcome than doing so at the usual time of presentation, but quality of life for the affected individual and family may be improved by early intervention. A more palpable advantage of early diagnosis is the provision of an explanation to the family for the child's behavioural and intellectual problems. Subsequent genetic counselling may also remove some of the burden of responsibility and guilt associated with bearing affected children (Roy *et al,* 1995). On the negative side, a specific diagnosis may lead to stigmatisation of the affected individual and even the family.

When all these factors are taken into consideration, many would agree that preventing affected births is a more achievable aim of screening than attempting to improve prognosis.

Screening strategies

There are three possible strategies aimed at identifying females at high risk of an affected pregnancy. These are:

- antenatal testing of apparently low risk pregnancies
- pre-conceptual testing of young women
- systematic testing within the families of affected individuals (cascade screening).

Two strategies aimed at improving prognosis are:

- an active search for paediatric cases among high risk children
- routine testing of neonates.

Antenatal testing

The testing of pregnant women for a PM or FM could be incorporated into existing screening programmes for neural tube defects, Down's syndrome and ultrasound detectable structural abnormalities (Palomaki, 1994). Those who are found to have a mutation would then be offered prenatal diagnosis. Antenatal screening could be offered either routinely to all women or selectively to those at relatively higher prior risk. The latter group would be principally those with a family history of fragile X syndrome, but could be extended to include those with a family history of mental retardation in general, possibly including non-specific or mild disability.

Pre-conceptual testing

Low risk females of reproductive age would be encouraged to undertake testing for a PM or FM. In a properly constituted screening programme of this kind, a target population would be identified and systematically offered the test. Possible target populations would include school leavers, women of reproductive age on general practitioners' lists, and women attending family planning clinics. Experience of pre-conceptual screening for another genetic disorder, cystic fibrosis, is that the uptake rate for testing is lower than for antenatal screening, even though it offers more reproductive choice (Brock, 1994).

Cascade screening

Following the clinical diagnosis of an affected individual, the genetic services normally offer counselling and DNA testing to family members. Close relatives are tested first and, depending on the results, more distant relatives might be contacted. This is a well-established approach in clinical genetics and some would not regard it as screening *per se*. With cystic fibrosis screening, a distinction has been made between this practice and a more active type of cascade testing whereby a systematic approach is made to identify all affected families. Active cascade screening in its most complete form begins with a concerted attempt at case-finding. This may involve systematic testing at institutions and special schools in the area (see page 27).

Paediatric testing

It is established practice for paediatricians to request cytogenetic tests on children referred to them because of developmental delay. Blood samples from children with this indication are usually tested for Down's syndrome and fragile X syndrome. Since the new molecular genetic technology has become available, fragile X syndrome testing is starting to be performed in DNA rather than cytogenetic laboratories. Many diagnoses are made in this manner but they tend to be the more severe cases.

In the UK, a system exists in schools whereby a formal statement is made by the local education authority when a child is considered to have a special educational need. Paediatricians may contribute to the assessment on which the statement is based but this is not routine practice. The 1981 Education Act requires that needs identified in such a statement are met. It is not known what proportion of children with fragile X syndrome will have such a statement made. Also, there is often a considerable delay before the formal statement is made. In order to produce a substantial increase in the number of early diagnoses, a more systematic and active approach is needed. One possibility would be to alert teachers to specific cognitive and behavioural traits that indicate a high risk of the disorder.

Neonatal testing

In the UK, all newborn infants are subject to routine testing for phenylketonuria and hypothyroidism using a heal prick blood sample absorbed on to a Guthrie card. Increasingly, some centres are using spare blood spots on the card to test for cystic fibrosis and it would be technically feasible to further extend such testing to include fragile X syndrome.

Prenatal diagnosis

Females discovered to have a PM or FM through antenatal screening or as a result of any of the other screening strategies described above will have the option of prenatal diagnosis. Foetal DNA can be obtained from any one of three different invasive procedures, namely amniocentesis, chorionic villus sampling (CVS) and peripheral umbilical cord blood sampling (PUBS). Each procedure has its own technical limitations and hazards, and these are discussed later.

The DNA test on the foetal material obtained at prenatal diagnosis also has its limitations. The test can only determine whether or not the foetus has an FM. If it is a male foetus this is equivalent to diagnosing fragile X syndrome. However, only half the female foetuses with an FM will have the disorder. Those accepting an offer of prenatal testing, need to give considerable thought to how they will proceed if a female FM carrier is detected. In addition to the options to terminate or continue with the pregnancy, which also apply to male foetuses, there is a third possibility. They could agree in advance only to be informed that there is an abnormality if the foetus is male. In view of this problem and other considerations (discussed in chapter 12), genetic counselling prior to prenatal diagnosis in women with a PM or FM is particularly difficult.

Pre-implantation diagnosis

The detection of inherited diseases in very early embryos allows the selection and transfer of only one healthy zygote to the uterus. After pre-implantation diagnosis, couples with a high prior risk can embark on the pregnancy with the certainty that it is free from the specific condition in the family.

This service is currently available for cystic fibrosis and X-linked diseases. For fragile X syndrome such testing has been done in research laboratories and is likely to become more generally available in the future. If the mother has an FM, any zygotes which also have an FM need not be transferred. If she has a PM there are two possibilities; either to avoid using any mutated zygotes or to reject only those with an FM. If there are not enough good quality zygotes, the latter option might have to be adopted. However, expansion may not have taken place in the blastomere (see page 14) and, if there is a later expansion to FM, the foetus may have fragile X syndrome.

Case-finding

Active cascade screening is dependent on finding index cases. The efficiency of this step can be improved by carrying out a prior screening of the target population to identify those at increased risk of fragile X syndrome and to focus testing on this subgroup. Medical records and direct examination of the individuals can be used.

Several physical and behavioural checklists have been developed for this purpose (Laing *et al,* 1991; Hagerman *et al,* 1991; Butler *et al,* 1991b; Nolin *et al,* 1991; Gabarron *et al,* 1992). Discriminant analysis on the items listed shows that most affected males can be identified by using relatively few of them. For example, Butler and colleagues (1991b) found that five physical criteria, together with a family history of learning disabilities, would correctly classify over 90% of affected males. These include the presence of plantar crease, simian crease, macro-orchidism (mainly post-pubertal), large or prominent ears and hyper-extensibility.

Chapter 8

Screening and diagnostic tests

Many of the assay methods used to detect a PM or FM produce different results in males and females. This is because of the presence of two copies of the X chromosome in females and to X-chromosome inactivation. Thus, in some assays a mutation in one X chromosome may be masked by a normal response in the other. In other assays the inactivated X chromosome will react differently to the other.

Cytogenetic tests

This method is only suitable for detecting an FM. The technique is to culture cells under specific conditions that lead to the expression of FRAXA. Folic acid or thymidine deprivation is the principal condition, although other methods can be used (Sutherland, 1977). False-positives and false-negatives occur for both intrinsic and technical reasons (see *Table 5* and page 9). The test requires a high degree of operator skill, together with long culture times (Jacky *et al,* 1991). Consequently, it is time-consuming and expensive (about £75–150 per sample). The usual laboratory turn-round time is 2–3 weeks.

Southern blotting of genomic DNA

Both the PM and FM can be detected in males and females by directly assessing the size of restriction enzyme generated DNA fragments encompassing the repeat sequence. Deletions in the FMR-1 gene can also be identified (Gedeon *et al,* 1992; Wörhle *et al,* 1992a; Trottier *et al,* 1994; de Graaff *et al,* 1995a; Meijer *et al,* 1994; Mila *et al,* 1996). The detection of point mutations will require sequencing (de Boulle *et al,* 1993) but they are rare.

DNA is extracted from whole blood samples, digested with the restriction enzymes, blotted and detected on the hybridising gel with a radioactive probe (Hirst *et al,* 1991a; Nakahori *et al,* 1991; Oberlé *et al,* 1991; Mulley *et al,* 1992; Rousseau *et al,* 1992) or a chemiluminescent (El-Aleem *et al,* 1995) probe. Various combinations of restriction enzymes and probes may be used. Common restriction enzymes are *Eco*RI, *Pst*I, *Bgl*II, *Hind*III, *Bcl*I, *Sac*II, *BssH*II, *Eag*I and *BstZ*I; the last four are methylation-sensitive. Recommended probes include Ox1.9, StB12.3 and StB12XX (Snow *et al,* 1992; Macpherson *et al,* 1992a; Rousseau *et al,* 1991a; Rousseau *et al,* 1991b). Choice of restriction enzyme will depend on the type of mutation being tested. For example, the detection of large PMs using *Pst*I or small PMs using *Bcl*I will allow improved size resolution (Kremer *et al,* 1991; Fu *et al,* 1991; Rousseau *et al,* 1992), whereas *Eco*RI or *Bgl*II are more appropriate for the detection of an FM mutation (Rousseau *et al,* 1992). The locations of some of the restriction enzymes and probes in relation to the FMR-1 gene are shown in *Figure 3*.

The heterogeneous nature of large expansions (see page 11) means that a smear of repeat sizes across the hybridising gel is often seen rather than sharp bands. In order to increase the signal-to-noise ratio

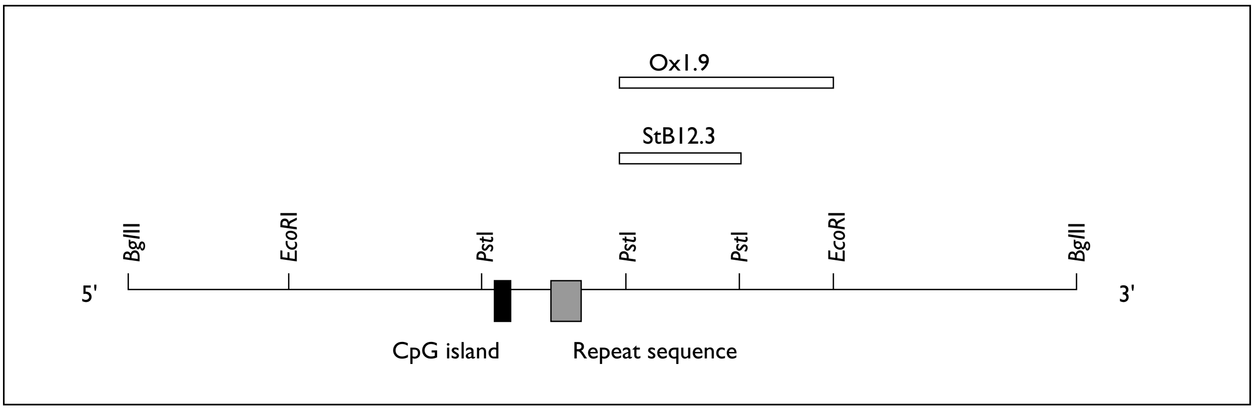

FIGURE 3 *Location of restriction sites and probes close to FMR-1*

and, hence, optimise detection, a radioactive probe which is compatible with restriction enzyme choice is recommended (Rousseau *et al,* 1992).

A simplified profile of the DNA banding pattern produced from a double digest is shown in *Figure 4.* Normal males have a single band whereas, in females, a second band is seen representing the methylated, inactivated X chromosome. A four-banded pattern is produced by a female with a PM, since she will have the mutated and normal X chromosome in both the inactive and active state. In males, a methylated band is seen only for an FM.

Southern blotting has several disadvantages in the screening context. First, it cannot be used to determine accurately the repeat size, which is important for small PMs. Second, there is a long laboratory turn-round time of up to 10 days, largely because of the radioactive detection of fragments; using a phospho-imaging detection system (such as that produced by Molecular Synthetics Ltd) could reduce turn-round time to 1 day. Third, the test is expensive at about £50–75 per test.

DNA amplification by polymerase chain reaction

A rapid and relatively cheap method of assessing the repeat size is to amplify part of the DNA. The most common technique is the polymerase chain reaction (PCR), in which the enzyme DNA polymerase is used to process and copy a specified sequence. The PCR product can be detected by radioactive (Fu *et al,* 1991; Pergolizzi *et al,* 1992; Erster *et al,* 1992) or other means (Brown *et al,* 1993; El-Aleem *et al,* 1995; Nanba *et al,* 1995; Haddad *et al,* 1996; Wang *et al,* 1995a). Thus, the method works on smaller quantities of less purified starting material, either blood or mouthwash (Hagerman *et al,* 1994a), than Southern blotting. The turn-round time for PCR testing is approximately 1 week and, for a high throughput, the cost may be reduced to £10 per sample.

PCR is most suitable for detecting PMs and large normal alleles as it enables improved size resolution of small repeat sequences (Heitz *et al,* 1992; Macpherson *et al,* 1992a). Its use is limited for the detection of large FMs, and it is unable to determine methylation status. There is a tendency for large FMs to fail to amplify. This is less of a problem in males since absence of an expanded fragment can be taken to indicate that an FM may be present and demonstration of a small repeat size is sufficient to exclude an FM. In females, however, the absence of one of the two bands expected after PCR analysis is ambiguous. A small single band is consistent with

(a) preferential amplification of the smaller of two alleles, one of which might be mutated (Brown *et al,* 1993; Erster *et al,* 1992; Pergolizzi *et al,* 1992)
(b) normal alleles homozygous for size
(c) normal alleles differing by only one CGG repeat, which are practically indistinguishable.

Brown and colleagues (1993) have devised a methodology which avoids selective amplification of the smaller allele in heterozygotes enabling them to be differentiated from homozygotes. However, a single allele on PCR is still not fully informative.

PCR and selective Southern blotting

Sequential testing is a reasonable compromise between Southern blotting and PCR. The protocol

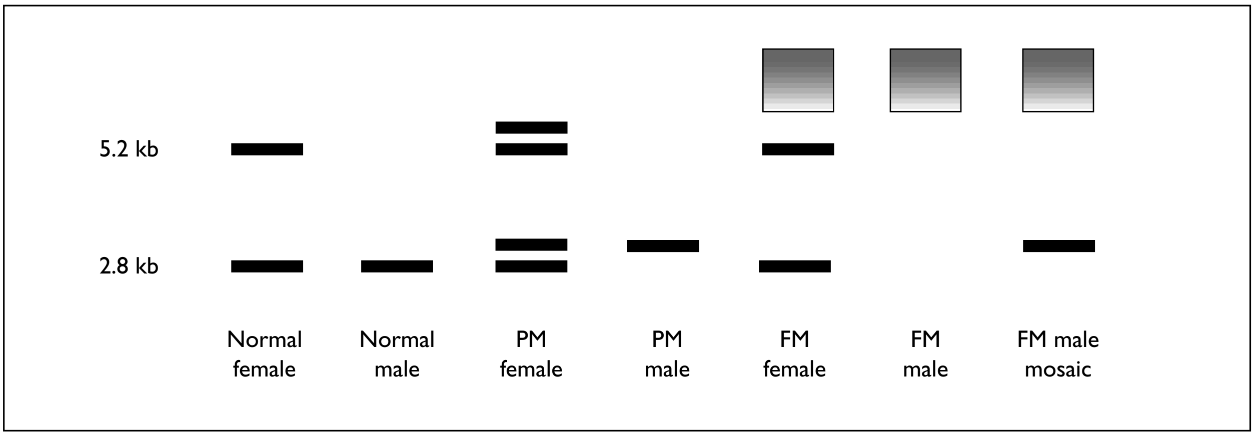

FIGURE 4 *Example of test results using a double digest*

is to perform PCR on all samples but when there is a failure to amplify or there is a single band in a female to perform a Southern blot. Over one-quarter of females are homozygous for repeat size (see *Table 2*) and, in our experience, a further 8% have alleles differing by one repeat. Thus, this approach requires a fair amount of Southern blotting, adding an extra week to the turn-round time and, thus, additional costs.

Blotting PCR products

Although FMs are amplified inefficiently, their large expansions become ideal hybridisation targets for CGG containing oligonucleotides. Blotting methods for these PCR products have been described for fragile X syndrome. Because the initial reaction uses DNA amplification, only small amounts of starting material are required. Non-radioactive blotting of amplified products is feasible (Pergolizzi *et al,* 1992).

PCR-based methylation assay

This method relies on the fact that the unmethylated FMR-1 gene can be digested by certain enzymes, whereas the methylated gene is resistant to digestion. Thus, in males, where methylation occurs with an FM but not a PM or normal allele, digestion with a methylation-sensitive enzyme followed by amplification should only yield products if there is an FM. Females with an FM are not detected by this method since they always have one of their X chromosomes methylated (Wang *et al,* 1995a). There is also the risk of incomplete digestion.

Measurement of FMRP

An antibody test has been described to measure FMRP (Willemsen *et al,* 1995). This could be used to detect an FM in males since those affected should produce no protein. The test will not be useful in females since protein is produced from the normal active X chromosome even when the other chromosome has an FM. Also, even for males the test is limited, because mosaics produce FMRP to a degree. PM alleles yield normal quantities of FMRP.

Alternatives to standard PCR

Several alternative methods to standard PCR have been described, although none are in routine use.

Repeat expansion detection

First described by Schalling and colleagues (1992), repeat expansion detection (RED) uses a thermostable DNA ligase in the usual cycling reaction. Complementary oligonucleotides are used, which anneal along the entire length of one strand of the repeat sequence. Adjacent oligonucleotides are then joined by adding ligase. The ligated products from one cycle continue to ligate to products generated from the original DNA in all subsequent cycles, until the whole expansion has been replicated. However, sensitivity is rather poor in comparison with PCR because the technique involves no amplification.

Ligase chain reaction

The ligase chain reaction (LCR) does allow amplification but to date has generally been restricted to the mapping of point mutations involving the generation of short stretches of DNA. LCR is less suited to the amplification of longer stretches of DNA because of its reliance on complementary self-annealing primers. It has an advantage over PCR in that it is a non-processive system and should not show undue bias against the detection of large expansions.

Hybrids

A method combining LCR and PCR has been described by both Abbott Laboratories Ltd and Roche Diagnostics Ltd for the detection of micro-organisms, and this may adapt well to detecting CGG amplifications although it has not as yet been tried. A modified RED with the hybrid PCR–LCR detection system is another possibility.

Testing protocols

Given the technical, practical and financial constraints of the various laboratory techniques available, it is possible to devise a reasonable testing protocol for the main applications.

Screening

For antenatal, pre-conceptual, cascade and neonatal screening, the first choice would be PCR followed by selective Southern blotting of ambiguous alleles. Since antenatal and pre-conceptual screening concentrate on females, one-third of samples may also need Southern blotting, thus increasing the average cost of testing to about £30 per sample. Nonetheless, this would offer a cheaper, faster and more accurate alternative to Southern blotting of all samples. A more sophisticated protocol would involve the sequencing of grey zone alleles for the number of interrupting AGGs. In case-finding for cascade screening or in paediatric screening, genomic Southern blotting would be used to detect the FM allele.

Prenatal diagnosis

The first prenatal diagnosis of an affected foetus was made using cytogenetics in amniocytes (Jenkins *et al,* 1981; Shapiro *et al,* 1982). Since then, over 100 cytogenetic prenatal diagnoses have been made in amniocytes, chorionic villi and foetal blood with, as expected, large numbers of false-negative and false-positive results (Jenkins *et al,* 1995b).

There have been no reported diagnostic errors using DNA technology in amniocytes (Jenkins *et al,* 1995b) but care is needed when chorionic villi are analysed. CVS has the advantage over amniocentesis of an earlier and speedier diagnosis but there are technical problems. Several studies have reported abnormal methylation patterns in chorionic villi whereby FMs genes are hypomethylated relative to foetal tissue (Sutherland *et al,* 1991a; Iida *et al,* 1994; Castellvi-Bel *et al,* 1995; Yamauchi *et al,* 1993; Jenkins *et al,* 1994b; Sutherland *et al,* 1991b). Consequently, lyonisation of the inactive X is not observed and foetal sex determination requires additional PCR amplification of X- and Y-specific DNA (Devys *et al,* 1992). Also, it is difficult to interpret a band in the PM range which could be the result of incomplete methylation of a FM size mosaic (Strain *et al,* 1994). In such cases, an amniotic fluid or foetal blood sample would be needed to determine the full range of the mutation and the methylation status of the foetus. The latter could only be confirmed by performing a double digest using a methylation sensitive restriction enzyme. Maternal contamination has also been reported (Maddalena *et al,* 1994).

Pre-implantation diagnosis

Dreesen and colleagues (1995) suggest that pre-implantation diagnosis may be performed by genotyping the polymorphic RS46(DXS548) locus, which is closely linked with the FMR-1 gene. However, there are two limitations with this method: a diagnosis cannot be made when the paternal and maternal alleles are the same at this locus or when the female is homozygous at the locus.

Chapter 9

Practical experience of screening and diagnosis

Antenatal screening

There have not yet been any large-scale, population-based screening programmes in the general population. However, there is some information from two studies in which attempts to screen selected populations have been made.

Fairfax, USA

A pilot study was initiated at the end of 1993. All women referred to a genetics institute for prenatal diagnosis were offered screening on a self-pay basis (Howard-Peebles *et al,* 1995; Spence *et al,* 1996). The principal reason for referral was advanced reproductive age. A total of 3345 pregnant women were offered the test, of whom 688 (21%) accepted. A large proportion (31%) of those accepting the offer had a family history of mental retardation, learning disability, autism or attention-deficit disorders, which may have influenced their decision. Of the three women who were found to have a PM, all had an unremarkable family history.

New York, USA

A small antenatal screening programme has been in place since 1992 for women with a family history of learning disability of unknown aetiology (Brown *et al,* 1996). Some 344 women have been screened, but no information is available on the number being offered the test. Six women were found to have FMR-1 mutations – two had an FM and four a PM.

Pre-conceptual screening

There are no reported studies of screening low risk populations prior to pregnancy. However, one centre has reported on testing 271 potential egg donors in an *in-vitro* fertilisation programme (Spence *et al,* 1996). One woman was found to have a PM and another produced a result in the grey zone, with a repeat size of 52. Both women were counselled and offered prenatal diagnosis in a subsequent pregnancy.

Active cascade screening

Three fully active screening programmes have been reported so far; a fourth did not start by case-finding among the mentally-handicapped but used cytogenetic records as its basis, together with an educational campaign among health care providers.

New South Wales, Australia

A state-wide screening programme has grown out of the studies aimed at case-finding which were started more than 10 years ago in New South Wales, Australia (Turner *et al,* 1986). Over the years the project has become a full-scale cascade screening programme, whose stated aim is to inform extended families about the reproductive risks before childbearing.

By 1990, the clinical history of over 14,000 intellectually-handicapped individuals, adults and schoolchildren, had been reviewed and just over half were selected for testing (Turner *et al,* 1992). Permission to test was received from 79%, which resulted in 253 putative cases being identified, 30% of whom were not already known to have fragile X syndrome. (Subsequent DNA testing showed that some of these were false-positive diagnoses).

A case-control study has been carried out to examine the influence of genetic counselling on subsequent reproduction among female FM and PM carriers identified in the early stages of the scheme. In 303 case-control pairs, those given counselling had 26% fewer pregnancies (77 versus 104). This reproductive decline was most marked in the 85 pairs of women who already had an affected child; there were 70% fewer pregnancies (six versus 20). There were 77 pregnancies in the women who had been counselled, and although prenatal diagnosis was offered, only 61% accepted. Since the introduction of DNA testing methods, 44 female carriers identified by the scheme have subsequently became pregnant (Robinson *et al,* 1996). All were offered prenatal diagnosis and 34 (77%) accepted.

New York State, USA

In 1987, a programme of cascade screening was started in seven regions of New York State (Nolin *et al,* 1991; 1992). Mentally-impaired, post-pubertal males from institutions and community residences were considered for diagnostic testing. Physical examinations were carried out to select those who might have fragile X syndrome and, as a result, 42% were tested. By 1991, 43 cases had been identified in 38 families. Of the 33 families followed-up so far, in order to offer genetic counselling, appropriate relatives could not be found for nine and, of the remainder, 12 (50%) took up the offer.

Murcia, Spain

In 1986, a programme of cascade screening was begun in the southern Spanish region of Murcia. Index cases were found in special schools and sheltered workshops. The medical records were examined in an attempt to preselect those for diagnostic testing. In the event, the records were not good enough to enable such a selection to be made, so most males were selected. A total of 22 cases were found, out of 223 males tested, and the cascade screening used these together with a further 31 males referred directly to the genetic services. Overall figures on the uptake rate of genetic counselling are not available but, in the 18 families identified from cases in special schools, 11 (61%) accepted referral; those that did not had no appropriate relatives or had been sterilised.

Kuopio, Finland

An attempt has been made to systematically test the families of index cases diagnosed since 1991 in Kuopio, Finland (Ryyänen *et al,* 1995). From cytogenetic records, 28 probands were identified and, following a campaign to raise awareness among relevant health care providers, a further 31 were found. In each family a contact was approached, usually a parent or guardian, and asked to make arrangements for other family members to be contacted. On the basis of the pedigree, a total of 1017 relatives were identified who had at least a one in eight chance of having fragile X syndrome. Of the 48% who agreed to be tested, 72 (14%) were found to have an FM and 163 (32%) a PM. Of the unaffected females with an FM or PM who were identified as a result, 21 subsequently became pregnant. All accepted prenatal diagnosis and the nine foetuses with an FM were terminated. Twelve of these women had no previous knowledge that they were at high risk of having an affected pregnancy.

Prenatal diagnosis and termination of pregnancy

It is to be expected that most of those undergoing invasive prenatal diagnosis for fragile X syndrome

TABLE 10 *Prenatal diagnosis*[†] *of a female foetus with an FM – outcome of pregnancy. Results from seven studies*

Study	Number of pregnancies	Outcome		
		Birth	Termination	Unknown
France Devys *et al,* 1992	3	1	1	1
USA, NY I Brown *et al,* 1993	4	1	3	0
Finland von Koskull *et al,* 1994	2	2	0	0
USA, Fairfax Maddalena *et al,* 1994	6	2	0	4
Finland, Kuopio Ryynänen *et al,* 1995	6	0	6	0
USA, NY II Brown *et al,* 1996, plus personal communication	20	7	13	0
Italy Grasso *et al,* 1996	6	2	4	0
Total	47	15 (32%)	27 (57%)	5 (11%)

[†] *Using DNA technology.*

will opt to terminate the pregnancy if the foetus has an FM and is male. The reverse might be expected in female foetuses with an FM, but this is not born out by the published studies. In *Table 10*, the combined results from seven studies for the outcome of pregnancy are shown for a total of 47 such foetuses. Over half are known to have opted for termination and, excluding those of unknown outcome, the proportion increases to 64%.

Paediatric screening

Referral

A working group from the American College of Medical Genetics (1994) has published guidelines to aid clinicians in making referrals for fragile X testing. The group recommends that individuals of either sex with mental retardation, developmental delay, or autism be referred, especially if features of the disorder are present or there is a family history of fragile X syndrome or any other mental abnormality. Thus, in New Mexico (Kaplan *et al,* 1994), of 271 individuals tested at one laboratory, 61% were referred because of mental retardation, 27% for developmental delay and 4% for attention-deficit disorders. Only 5% had a family history of fragile X syndrome. An FM was found in 11 individuals, of whom seven were from the 11 referrals with a family history of fragile X syndrome.

In the UK, children with learning difficulties or developmental delay who are referred to genetics centres usually have tests to exclude fragile X syndrome. Since DNA testing became possible, samples are generally being sent to regional DNA laboratories for testing. There are no published data on the numbers of samples tested for the country as a whole but some regional figures are available. In Leeds, we have tested samples from about 1500 boys, five of whom were found to have an FM. Of 153 girls referred for testing, none had an FM. Similar results were obtained in Edinburgh where, over a 5-year period, about 1000 boys have been referred and 18 FMs detected (Professor DJH Brock, personal communication). More details of referral patterns have come from the South East Thames Regional Cytogenetics Laboratory (Barnicoat *et al,* 1993). Of 680 referrals, half were for developmental delay, 79% were male, 17% were related and 3% had pedigrees already known to be affected. As a result, 17 individuals, all male, were diagnosed as having fragile X syndrome.

It is not known to what extent this diagnostic testing leads to cascade screening, active or otherwise. In Strasbourg, France, Mandel and colleagues (1994) have used DNA methods since 1991 to test individuals referred because of mental retardation. Over the first 2 years, about 5% of those tested had an FM. Consequently, 38 female carriers were identified in 28 families of the probands. It is unclear why there is a higher rate of FM in this study compared with the UK studies. However, the referring doctors included clinical geneticists, and there was a requirement to complete a clinical evaluation form for each referral, which may have resulted in a degree of pre-selection.

Active

Hagerman and colleagues (1994b) carried out a pilot project to determine the feasibility of using special education personnel to select and screen schoolchildren at high risk of fragile X syndrome. The project was carried out in five Colorado, USA, school districts. The first stage was to train teachers, and other professionals who have contact with pupils needing special education, to use a physical and behavioural checklist. Those determined to be at high risk were, with parental consent, screened using a mouthwash sample. Of the 439 pupils tested so far, one-third of whom are girls, 51% had a learning disability and 35% had an attention-deficit disorder. Only four of those tested had an FM, a mosaic male and three females, and all except one of the females were already known to local paediatric services.

Chapter 10

Modelling allele dynamics

The published studies describing practical experience with screening do not provide sufficient information in themselves to assess the potential of the different screening strategies for fragile X syndrome. Instead, we found it necessary to rely on theoretical calculations using the best estimates available from the literature. Part of this required the construction of a statistical model which described the population dynamics of the PM and FM alleles between generations. Although several such models have been constructed they do not meet the current needs. The earliest models (Winter, 1987; Sved & Laird, 1990) did not have the benefit of knowledge of the molecular basis of the defect. Later models (Morton & Macpherson, 1992; Kolehmainen, 1994; Ashley & Sherman, 1995) were increasingly detailed, taking account of the latest DNA studies, but are unnecessarily complicated for the purposes of screening.

A simple model

A simple and understandable way of modelling the screening process is to begin with a population of 1 million couples and consider the various possible outcomes for their children, in terms of allelic inheritance and the presence of fragile X syndrome. These possibilities are shown in *Figure 5*, using what is known about the molecular biology of the PM and FM alleles. Some of the combinations of couples have been left out (e.g. both parents have a PM), as they would add complexity but make little difference to the results. Both males and females with an FM are included, although the reproductive fitness of males is thought to be effectively zero and affected females tend not to reproduce (Sherman *et al,* 1984). The critical components of the model are the frequency of the alleles and the risk of expansion from PM to FM in one female generation. It is likely that different values will need to be assigned to these components according to whether the couples are members of families affected by fragile X syndrome or of the general population.

PM frequency

The results of nine studies in which the repeat size has been determined among unrelated individuals with no family history of fragile X syndrome are

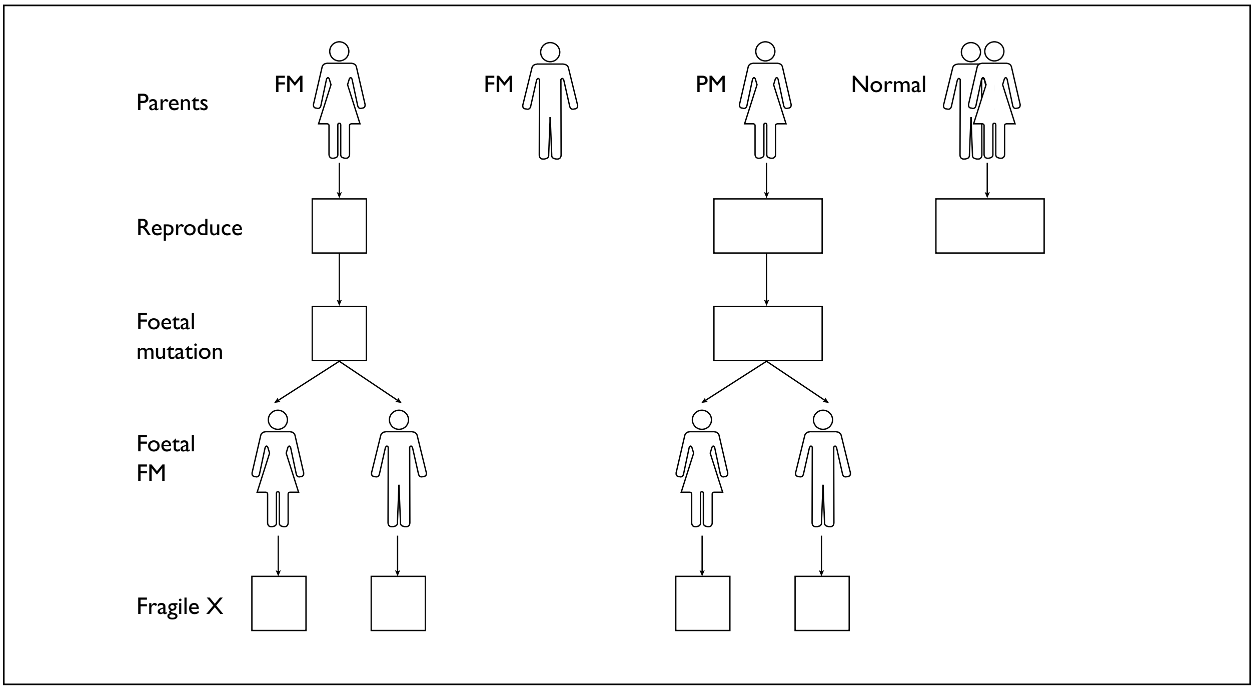

FIGURE 5 *Possible outcomes of pregnancy*

shown in *Table 11*. The cut-off size used to define a PM was the same across the studies (54) so that they can be readily combined. In 26,178 females examined, the PM frequency was 1 in 273, and in 13,592 males the PM frequency was much lower at 1 in 800. Althought most of the combined data comes from one large Canadian study, the smaller studies yielded similar results. The study from Fairfax, USA (Spence *et al*, 1996) may have been slightly biased since testing was offered on a self-payment basis and there was a low uptake (see page 33). It is possible that those accepting testing were a biased subgroup. Nonetheless, the frequency was comparable with the other studies. One of the studies shown in *Table 11* looked at the array structure of PM alleles (Eichler *et al*, 1994). Using a pure repeat size of 34 as the cut-off, the PM prevalence was 1 in 203.

Between the studies shown in *Table 11* there was sufficient information on the repeat size for each PM to produce a crude frequency distribution. In total there were 48 PMs in females and the frequency distribution of sizes is shown in *Figure 6*.

TABLE 11 *Frequency of PM in the general population: results from nine studies*

Study	Source of samples	Number of X chromosomes	Lowest PM †	Number of PMs	Frequency (1 in)
Females					
USA, New York Snow *et al*, 1993	BD	394	57	1	197
Japan Arinami *et al*, 1993	FH	454	–	0	> 227
USA, Texas I Reiss *et al*, 1994	FH	1122	75	1	561
Canada, Quebec I Rousseau *et al*, 1995	OP	21,248	55	41	259
Canada, Manitoba Dawson *et al*, 1995	GC	1470	55	2	368
USA, Fairfax Spence *et al*, 1996	FH/ED	1490	60	3	248
All females	–	26,178	55	48	273
Males					
USA, New York Snow *et al*, 1993	BD	50	–	0	> 50
Japan Arinami *et al*, 1993	FH	370	–	0	> 370
USA, Texas I Reiss *et al*, 1994	FH	416	–	0	> 416
USA, Texas II Eichler *et al*, 1994	BD	406	–	0	> 406
Canada, Manitoba Dawson *et al*, 1995	GC	778	57	3	259
Canada, Ontario Holden *et al*, 1995c	GC	1000	61	1	1000
Canada, Quebec II Rousseau *et al*, 1996	NS	10,572	NS	14	755
All males		13,592	57	17	800

NB: *A further study (Fu* et al, *1991) found 1 female PM among 492 X chromosomes but did not differentiate between males and females.*

† Most used a cut-off repeat size of 54.

BD, blood donors; FH, members of families having DNA tests unrelated to mental impairment; OP, general out-patients; CG, Guthrie cards; ED, egg donors; NS, not specified.

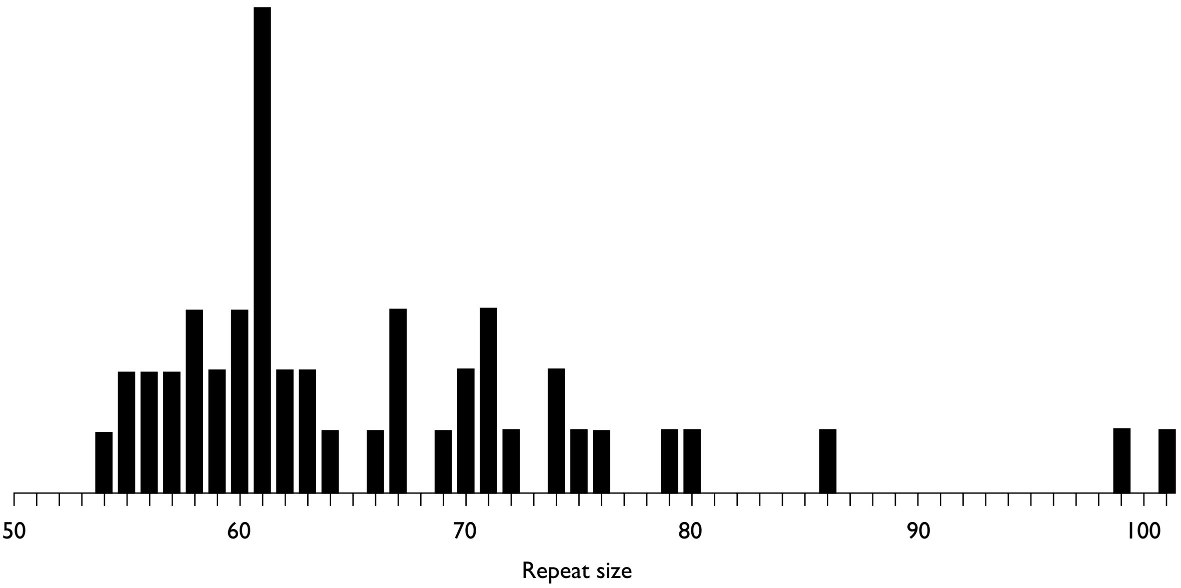

FIGURE 6 *Population distribution of PM size*

FM frequency

From the fragile X syndrome prevalence studies, 2.5 per 10,000 males in the general population, or 1 in 4000, are estimated to have an FM (see page 22). Since the X chromosome with an allele that has expanded to an FM is equally likely to be passed on to a female foetus as to a male, the prevalence of FMs in females will also be 1 in 4000.

Risk of expansion from PM to FM in families

Bias in family studies

The retrospective nature of pedigree analysis within affected families can lead to a biased estimate of the expansion risk. This is evidenced by the observed segregation ratio of normal to mutated alleles in the offspring of women with a PM or FM. There should be an equal number of each but, in pedigree studies, there are more mutations than normals. This occurs because families with fragile X syndrome are ascertained by the presence of an affected proband, leading to an excess of FMs. Removing either one proband from each family or by removing the family from the study altogether will improve the segregation ratio but it may be an over-correction for the bias. A related bias leads to a deficit of non-expanding PMs in parts of the pedigree more distant from the proband. If a female with a PM did not have affected offspring she may not have been tested. Other untested distant family members might include apparently normal individuals such as NTMs or unaffected females with an FM. Finally, there is a bias whereby families with more than one affected member are more likely than less affected families to be ascertained again biasing towards an excess of FMs.

Estimated risk

Four studies have reported on the risk of expansion using direct pedigree analysis within affected families. Thus, the offspring of women with a PM were studied and the proportion with an expansion to an FM in those inheriting the relevant X chromosome is shown in *Table 12*. From three of the studies, the combined risk among 360 offspring was 78% when the proband was included. Two of three studies gave results after exclusion of probands and the risk is seen to be reduced. When the results of these two studies are combined with those from a fourth unbiased study, the risk of expansion in a total of 447 offspring is 60%. A large prospective multicentre collaborative study is now underway in an attempt to overcome the biases of family studies and, to date, 24 pregnancies have occurred in which the X chromosome with a PM was passed to the offspring (Sherman *et al*, 1994). The observed expansion risk so far is 87% but this high risk may be due to chance since the majority of the mothers happened to have a particularly large PM.

TABLE 12 *Risk of expansion from PM to FM in affected families: results from four studies*

Study	**Mothers with PM**		**Offspring with her mutation**		**Excluding probands**	
	No.	**Repeat size**	**No.**	**No. with FM (%)**	**No.**	**No. with FM (%)**
USA, Texas Fu *et al,* 1991	32	50–113	63	44 (70)	–	–
France Heitz *et al,* 1992	102	55–205	175	145 (83)	131	101 (77)
Finland Väisänen *et al,* 1994	66	60–130+	122	92 (75)	79	49 (62)
USA, NY Fisch *et al,* 1995	110	50–130+	–	–	184	119 (65)
All	310	50–205	360	281 (78)	447	269 (60)

Risk of expansion according to PM size

The risk of expansion to the FM is correlated with the size of the PM allele of the carrier mother. The larger the repeat size of PM, the greater the risk of expansion to an FM. There also appears to be a threshold PM size at which expansion to FM always occurs. This is shown in *Table 13,* based on the four studies of expansion risks in affected families shown in *Table 12.*

TABLE 13 *Risk of expansion to FM according to PM size in affected families: results from four studies*

PM size in mother	**Study**			
	USA, Texas Fu *et al,* 1991	**France Heitz *et al,* 1992**	**Finland Väisänen *et al,* 1994**	**USA, New York Fisch *et al,* 1995**
50–55	0%			20%
55–60		10%		
60–70	17%		46%	17%
70–80	77%	56%	48%	39%
80–90			92%	76%
90–100	100%	90%	100%	89%
100–105				91%
105–110				100%
110–115		100%		
115–120				
120–130				
> 130				

The studies report risks for groups of mothers and, since the groupings do not coincide between the studies, it is difficult to combine the data. However, we have performed a meta-analysis and logistic regression analysis of risk on size using the combined results, assuming that the risk for the group applies at its mid-point. In addition, we were able to compare the effect of ascertainment bias by analysing data according to whether the proband was included or excluded. The values predicted from the regression equations are shown in *Table 14* and appear to be close to the observed risks. The equations are given in a footnote to the table. The effect of excluding the proband from the analysis can be seen in *Figure 7.*

Risk of expansion in the general population

The risk of expansion from PM to FM is likely to be lower in the general population compared with that observed in families with fragile X syndrome. There are two reasons for expecting this. First, in affected families the distribution of PMs must, of necessity, be shifted towards greater repeat sizes: which is why the proband presented. Second, for a given size it is possible that in affected families the risk of expansion from PM to FM is greater than in others due to some unknown factor. It is believed that, in the general population, silent PMs may take several generations to expand to the FM (Richards *et al,* 1992; Chakravarti, 1992; Morton & Macpherson, 1992; Oudet *et al,* 1993a), and a PM that took three generations to expand has actually been observed (Brown *et al,* 1993). There are no published studies reporting the expansion risk in the general population but indirect estimates can be made.

From PM size distribution

One indirect approach is to estimate the expected expansion risk for the known PM size distribution in the general population. Applying the regression curves from *Figure 7* to the PM size frequency distribution in *Figure 6* yields an average expansion risk of between 27% and 37%, less than half the 60–78% risk seen in affected families (see *Table 12*). However, even this lower rate is too great to be consistent with the PM and FM population frequency. Thus, if one in 273 women has a PM allele (see *Table 11*) which is passed on to half her children, and in 27–37% of cases it expands, the FM frequency would be between 1 in 2000 and 1 in 1500 (27% and 37% multiplied by half of 1 in 273), even discounting the FM children born to women who have an FM allele. This is at variance with the observed frequency of 1 in 4000. Thus, either one of the frequency estimates is wrong, or the curve in *Figure 7* only applies to affected families, or there is a tendency for the normal X chromosome to be transmitted to the conceptus rather than the mutated one. There is some evidence to support the latter from a segregation ratio of 30:51 observed in a prospective study, although this could be a chance finding in a relatively small series (Sherman *et al*, 1994).

Working backwards

Another indirect way of estimating the general population risk is to work backwards from the FM

TABLE 14 *Logistic regression analysis* to predict the expansion risk from the PM size*

PM size in mother	Risk (%)		
	Number	**Observed**	**Predicted (confidence interval)**
With proband			
60	22	18	23 (6–57)
65	32	41	34 (11–70)
75	41	56	62 (27–88)
80	22	68	74 (40–93)
85	42	88	84 (54–96)
95	21	100	95 (79–99)
100	69	93	97 (87–100)
105	23	100	99 (92–100)
Excluding proband			
55	5	20	9 (5–16)
60	20	10	15 (9–25)
65	45	27	23 (14–36)
75	60	38	46 (31–61)
80	16	56	59 (43–73)
85	51	78	71 (56–82)
95	40	90	87 (79–93)
100	50	90	92 (86–96)
105	20	95	95 (91–97)

* *Based on studies in* Table 13, *the regression formulae were risk =* $100\%/(1 + 2963 \times 0.8933^{size})$ *for studies with the proband, and risk =* $100\% / (1 + 3080 \times 0.9004^{size})$ *for those where the proband was excluded.*

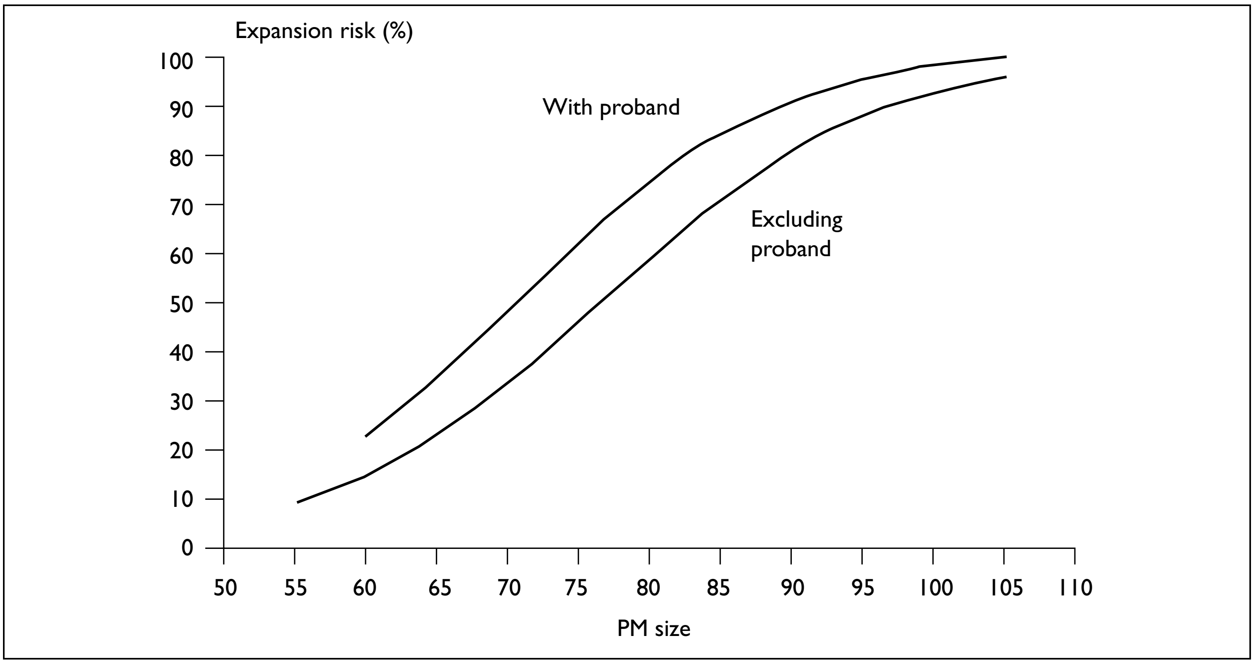

FIGURE 7 *Risk of expansion from PM to FM in one female generation*

frequency. This requires the assumption that the FM allele is in a steady state in the population insofar as the frequency does not change between generations. The allele only arises in the next generation from a mutated X chromosome in a woman, either an FM or an expanding PM (see *Figure 5*). Taking the reproductive fitness of FM carrier women to be 50%, 1 in 16,000 foetuses in the next generation (50% of half of 1 in 4000) will receive an FM from an FM-carrier mother. For the remaining 1 in 5300 (1 in 4000 minus 1 in 16,000), the FM will come from an expanded PM allele. Since 1 in 273 women is a PM carrier, the expansion risk must be 10% (1 in 5300 divided by half of 1 in 273). This calculation relies on the assumption that a steady state exists, but this may not be so, particularly if there have been intervention studies aimed at changing reproductive practice in PM and FM carriers. An evens segregation ratio of mutated to normal alleles is also assumed, and this too may be incorrect (Sherman *et al,* 1994). Nonetheless, the estimated values of 1 in 16,000 and 1 in 5300 do fit with the observed ratio of 1:3.5 for FM:PM carriers in mothers of children with an FM in the large New South Wales study (Professor G Turner, personal communication).

General population model

From the above analysis it is possible to complete a model for the general population screening situation. One caveat is that a range of values need to be considered because of the uncertainty of the precise rate of expansion from PM to FM. However, of the two sets of values, those generated from the risk calculated in the previous paragraph are the more plausible. Moreover, this second set of values are conservative with regard to the potential of screening. In the following chapter we use the latter, as shown in *Figure 8*.

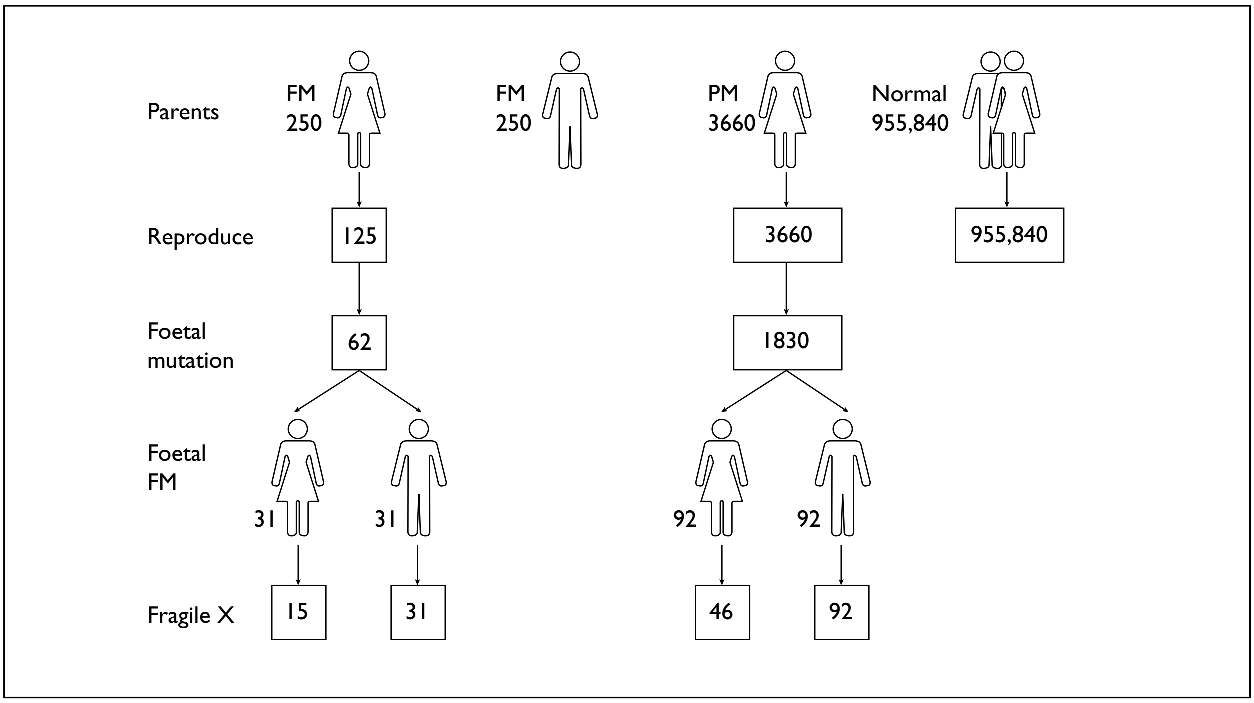

FIGURE 8 *General population model*

Chapter 11

Assessment of screening potential

Ultimately the decision whether or not to introduce any of the screening strategies for fragile X syndrome in the UK will depend on a variety of different factors. Nonetheless, the starting point of this decision-making process must be an assessment of the potential performance of the screening tests involved (Cuckle & Wald, 1984; Wald & Cuckle, 1989).

Measures of screening performance

The most important measures of the performance of a screening test quantify the ability to distinguish affected from unaffected individuals. The usual measures are the sensitivity or detection rate (proportion of affected individuals with positive results) and the false-positive rate (proportion of unaffected individuals with positive results). An alternative way of expressing the latter is the specificity, which is 100% minus the false-positive rate.

The purpose of screening is to identify the high risk group for further action and to reassure the remainder that their risk is low. The predictive value of the test quantifies these risks. The positive predictive value is the probability that an individual with a positive result is indeed affected and the negative predictive value is the chance of being unaffected given that the result is negative. These parameters are a function of the prevalence of the disorder in the population being tested as well as of the sensitivity and specificity of the test itself.

To be effective, any screening strategy needs to make an impact on one or more outcome measures. In addition to the sensitivity of the test, this will depend on the uptake rate of the screening test, the acceptability of the diagnostic and other options offered to those with positive results, and the effect of these on the outcome being measured. The impact of screening can be assessed both in the population being targeted and overall.

Potential of screening for fragile X syndrome

All the screening strategies we have considered that aim at preventing affected births, test for a PM or FM in women. If the test indicates that the woman has such a mutation, it is a positive result. If the woman has a pregnancy affected with fragile X syndrome it is a true-positive result; otherwise, it is a false-positive. The strategies that are aimed at improving prognosis, test for the FM in males or females. If the individual tested has fragile X syndrome, the test is a true-positive result; otherwise it is a false-positive result. Although screening for fragile X syndrome may have other benefits, effectiveness will be judged by the extent to which it reduces the birth prevalence of the disorder or improves prognosis.

Antenatal screening

Nearly all the affected foetuses can be expected to have a mother who has a PM or FM. Therefore, testing mothers for these mutations can be regarded as having a detection rate and negative predictive value close to 100%. False-negatives may occur because of point mutations, deletions and technical errors but all of these will be rare.

There are three ways in which an antenatal screening test will yield a false-positive result. A mother with an FM or PM may pass her normal allele to the foetus, a mother with a PM may pass it to the foetus but it does not expand to an FM, or a female foetus of such a mother may have an FM but is phenotypically normal. The first two will be resolved by prenatal diagnosis but, with current technology, the last will not.

From *Figure 8*, antenatal screening in a general population of 1 million couples will yield 184 true-positives and 3601 (3785 minus 184) false-positives. This is a false-positive rate of 0.4% and a positive predictive value of 1 in 20. Such results are considerably better than those currently achieved by antenatal screening for Down's syndrome, which has a 5% false-positive rate and a 1 in 50 positive predictive value (Cuckle, 1996).

There are no results from studies of general population antenatal screening for fragile X syndrome from which to assess the likely uptake of screening in the UK. The only published data are from tests that were not free at the point of entry. The acceptability of invasive prenatal diagnosis in women found to be carriers, and the consequent termination of pregnancy, would probably be similar to that found in cascade screening and other studies of prenatal diagnosis. Hence, about three-quarters of these women would take up prenatal diagnosis (see chapter 9), and nearly all the affected male and half the affected female foetuses would be terminated (see *Table 10*). Thus, if antenatal screening for fragile X syndrome were shown to be acceptable to pregnant women, its effectiveness in reducing birth prevalence might be comparable with screening for Down's syndrome.

Pre-conceptual screening

Insofar as the next step to pre-conceptual screening is prenatal diagnosis, the same detection rate, false-positive and predictive values will apply. Some women may avoid further pregnancies as a result of screening. Strictly, those who would not have had an affected foetus if they had conceived constitute additional false-positives but it would be impractical to take account of this.

Effectiveness cannot be estimated at present, although the effect of a positive result on future reproduction is likely to be similar to that seen with cascade screening. In the absence of pilot studies, it is difficult to judge how feasible and acceptable pre-conceptual screening for fragile X syndrome would be. If the experience with cystic fibrosis (Brock, 1994) is true for all genetic diseases then antenatal screening is likely to be much more effective than pre-conceptual screening.

Cascade screening

The detection rate and negative predictive value of cascade screening will be similar to pre-conceptual screening but the other parameters will differ because the target population are selected because of high prior risk. Therefore the positive rate is high and, although a large proportion of the PMs will expand, the false-positive rate must be greater than in the general population. The positive predictive value will be higher too, primarily because of the greater prior risk.

The feasibility of cascade screening for fragile X syndrome rests on the practical experience of the four studies in which it has been attempted (see page 33). The most informative study in terms of effectiveness is that from New South Wales. Within the affected families known to the screening programme, there has been a dramatic reduction in affected births both through avoidance of future pregnancies and through prenatal diagnosis (Robinson *et al,* 1996). However, there is no reliable information on the impact of this screening on the total population birth prevalence of fragile X syndrome.

Paediatric screening

With paediatric screening, the child is the affected individual and a test for the FM will also have an approximately 100% detection rate and negative predictive value. The test will only yield a false-positive result if a female has an FM but does not have fragile X syndrome. Thus, false-positives will be rare and, assuming that an equal number of males and females are tested, the positive predictive value will be 3 in 4.

There is practical experience of paediatric screening but its effect on prognosis is unknown. There are medical, educational, psychological and social interventions which are believed to improve symptoms (see page 6). However, there are no clinical trials or other comparable data that can be used to confirm that **early** treatment leads to improved long-term benefit compared with treatment applied at the usual time of presentation. Common sense dictates that some benefits will accrue but they need to be quantified.

Neonatal screening

The screening performance parameters will be similar to paediatric screening. There is no practical experience of this kind of screening for fragile X syndrome and, as with paediatric screening, there is the same problem of proving effectiveness.

Chapter 12

Human and financial costs of screening

Hazards of prenatal diagnosis

Amniocentesis

The principal hazard of amniocentesis is miscarriage but the excess risk associated with the procedure is difficult to quantify precisely. Some 3–4% of mid-trimester pregnancies will miscarry without amniocentesis and, in a particular case of foetal loss following the procedure, it is only rarely possible to directly attribute the adverse outcome to the procedure. Cases of amnionitis or chronic amniotic fluid leakage would be attributable but these are relatively rare consequences. Studies of women having amniocentesis and matched controls are biased. When amniocentesis was a new procedure, it was more available to women of higher social class with a lower miscarriage rate, so early studies were biased towards the safety of the procedure. Later, when the main indications were advanced age and abnormal biochemistry or ultrasound, factors associated with increased risk of miscarriage, the bias went the other way.

There has been only one randomised trial of amniocentesis (Tabor *et al,* 1986). The foetal loss rate in more than 2000 women randomised to the procedure was 0.8% higher than in the control group. While this is necessarily limited to the skills and experience of a single obstetric unit, the results provide the only unbiased estimate of hazard. Thus the excess miscarriage rate is usually quoted as between 0.5% and 1%.

Chorionic villus sampling

Five major comparative studies have shown that, when performed at 9–12 weeks by a skilled operator, CVS has a comparable foetal loss rate to amniocentesis (Canadian Collaborative CVS–Amniocentesis Clinical Trial Group, 1989; Rhoads *et al,* 1989; MRC Working Party on the Evaluation of Chorionic Villus Sampling, 1991; Smidt-Jensen *et al,* 1992; Ammala *et al,* 1993). The possibility of another important consequence of the procedure has been raised, namely the causation of limb reduction defects in the foetus. An international registry of CVS organised by the World Health Organization has been monitoring the procedure, so that any iatrogenic effects will not go unnoticed. The latest reported results based on 138,000 infants found no excess of limb reduction defects compared with data on the background prevalence of these conditions (Froster & Jackson, 1996).

Peripheral umbilical cord blood sampling

The sampling of blood from the umbilical cord would appear to be more hazardous than both amniocentesis or CVS. However, there is no evidence that it results in more foetal losses than the other procedures. There have been no randomised trials but a meta-analysis has been performed on six series, each including more than 100 cases (Ghidini *et al,* 1993). Patients with foetal pathological conditions were excluded, because a compromised foetus is often the indication for carrying out PUBS. The miscarriage rate in the remainder was only 1.4%, which is reassuringly low. There are other complications but they are not major.

Psychological burden

Screening for fragile X syndrome in common with other screening programmes will generate anxiety. First, for many the offer of screening itself will raise the possibility of a congenital abnormality not previously considered. Second, there is likely to be extreme anxiety in those found to have an FMR-1 mutation. In some cases there will be,in addition, the negative psychological effects of terminating a previously wanted pregnancy, or the possibility of stigma in those born despite screening. Although many of these problems are a necessary consequence of screening, they can be ameliorated by high quality information being given at all stages of the screening process, and by genetic counselling when appropriate.

Information giving

A particular problem with genetic screening is that complex information needs to be given to those offered the test. This concerns clinical effects, patterns of inheritance, laboratory tests, and calculations of risk. With fragile X syndrome there is the particular problem of explaining that the prognosis of an individual female with an FM cannot be predicted from the DNA test. Those offered antenatal screening will need to understand in advance the possible dilemma over termination of pregnancy that might arise if the mother is found to be an FM or PM carrier, and prenatal diagnosis demonstrates a female foetus with an FM. To date there are no published studies on the psychological aspects of prenatal diagnosis in fragile X syndrome.

Genetic counselling

In carrier females with a normal IQ, any emotional and cognitive problems may affect their ability to understand some of the information given during genetic counselling sessions. This may be more apparent in FM females; in PM females the problems may be very slight or even absent. Specific advice has been published on how to undertake genetic counselling for fragile X syndrome (McConkie-Rosell *et al,* 1995b). Also, the NHS Research & Development Programme on Mother and Child Health has now commissioned this research group to carry out an empirical study on counselling and patient variables in genetic diseases including fragile X syndrome. This study will examine, among other things, the ways in which the cause of the condition is discussed by counsellor and patient, and the ways in which the counsellor checks that information has been understood.

Genetic counselling should encourage individuals to discuss fragile X syndrome with other family members. Those at risk of having affected offspring could then request testing. It is possible that feelings of guilt and stigmatisation may hinder the transmission of information between family members; however, the Fragile X Society have reported that none of their members have experienced stigmatisation (Nuffield Council on Bioethics, 1993).

Costs

Measures of cost

The cost-effectiveness of a screening programme is usually expressed as the average cost of detecting one affected individual. This can be readily estimated from the separate unit costs for each component of the screening process. For example, with antenatal screening there is information giving, DNA testing, genetic counselling and prenatal diagnosis (some would also include the cost of therapeutic abortion). The average cost is computed from the estimated detection and false-positive rates, prevalence and uptake rates. Sensitivity analysis can then be used to vary one or more of the component costs and determine what aspect of the programme is most price sensitive.

A more complex approach is to carry out a cost–benefit analysis in which the benefits are also measured and valued. In the example of antenatal screening, the avoidance of treatment costs incurred by an affected individual may be seen as a large benefit. The welfare or utility experienced by a person with the disorder and their family, in not having to care for the affected person, or that gained by an early diagnosis, even when it is decided to continue the pregnancy, are more difficult to quantify and are usually ignored. Another possibility is to value the benefit to those being screened by performing a willingness-to-pay analysis, that is, by asking people how much they would be prepared to pay for the service.

Estimated financial costs and benefits

The costs of two of the reported cascade screening programmes have been estimated. In New South Wales, it was estimated that screening costs were $14,200 (Australian, at 1986 prices) to prevent one affected birth through prenatal diagnosis (Turner *et al,* 1986). In Murcia, Spain, the estimated cost was $12,740 (US, at 1992 prices) per affected birth prevented (Gabarron *et al,* 1992). At that time cytogenetic testing was used so that, even allowing for inflation, a modern screening protocol (see page 31) would be more cost-effective. Neither of the studies estimated or took into account the savings that might result from births averted by means other than prenatal diagnosis. If these were included, the average cost of preventing an affected birth would be even lower. The corresponding lifetime costs of care for an affected individual have been estimated to be in the region of $1–2 million (Lauria *et al,* 1992) and may be as high as $4 million (Nolin *et al,* 1991).

There are no published costs for strategies other than cascade screening. Antenatal screening can be simply costed using *Figure 8* and the unit costs of testing given in chapter 8; the unit costs of information giving, genetic counselling and prenatal diagnostic procedures are taken from Cuckle and colleagues (1996). These are shown in *Table 15.* First, we make a baseline assumption that no-one refuses the offer of screening, or prenatal diagnosis and termination of pregnancy, as appropriate, and that everyone has two pregnancies. Then the average cost of preventing each affected birth is £93,000. Reduced uptake does not

TABLE 15 *Cost per case detected under different assumptions*

Assumptions	**Number at each stage (unit cost)**				**Cases detected**	**Cost/case detected (£000)**
	Information giving (£2)	**DNA testing (£30)**	**Genetic counselling (£25)**	**Diagnosis † (£275)**		
Baseline*	996,000	996,000	7570	7570	368	93
75% uptake	996,000	747,000	5678	5678	276	95
75% PND	996,000	996,000	7570	5678	276	122
75% uptake & 75% PND	996,000	747,000	5678	4259	207	124

† *Diagnostic procedure (£200) and Southern blotting (£75).*

* *100% uptake of screening, 100% uptake of prenatal diagnosis (PND) in those with a PM or FM and two pregnancies per couple.*

alter this markedly, since screening and diagnostic tests comprise most of the cost. In contrast, the cost increases in direct proportion to the number of PM and FM carriers who do not want prenatal diagnosis or termination of pregnancy. If, in addition, we assume that half the affected female foetuses are not terminated, the average cost of preventing an affected pregnancy will increase by 20%.

Therefore, unless there are future technical developments which obviate the need for Southern blotting in a third of pregnancies, screening for fragile X syndrome will be more expensive than other antenatal screening tests. Maternal serum screening for Down's syndrome, which is well-established in the UK, costs about £30,000 per affected pregnancy detected (Sheldon & Simpson, 1991; Shackley *et al,* 1993; Piggott *et al,* 1994). Antenatal screening for cystic fibrosis is the only form of genetic screening where the costs of antenatal testing have been fully evaluated. This is estimated to cost £40,000–104,000 per affected pregnancy depending on the population carrier frequency, uptake and methodology (Cuckle *et al,* 1996). However, unlike screening programmes for Down's syndrome and cystic fibrosis, the discovery of a proband will inevitably lead to some cascade testing in the affected family. The effect of this on costs is not known.

Pre-conceptual screening for fragile X syndrome would be expected to have a comparable cost to antenatal screening if the denominator were affected births prevented by prenatal diagnosis. If all affected pregnancies avoided were considered the average cost may be lower, although should pre-implantation diagnosis become widespread the cost would be higher. For the screening strategies aimed at improving prognosis, paediatric and neonatal testing, the relevant unit is the diagnosis of an affected individual. Since there are fewer steps in the screening process, it is reasonable to assume that neonatal screening will be cheaper than paediatric screening which, in turn, will be cheaper than cascade screening.

Estimating utilities and disutilities

To fully explore the balance of human costs and benefits of screening, utilities and disutilities need to be assigned in a decision analysis (Thornton & Lilford, 1995). Antenatal screening involves a small disutility for many through the raising of anxiety, and a larger disutility for a few (those with positive results) against a putative even larger gain for a few of avoiding an affected birth. However, there is at present no published information on which to base such an analysis for fragile X syndrome.

Ethics

Screening tests differ from other tests performed in normal medical practice in that they are carried out pro-actively rather than in response to symptoms or concerns raised by patients. While the efficacy of normal medical tests may not be quantifiable they can be justified by the patient's needs. This is not the case for screening tests; it is only ethically justifiable to offer screening if the full consequences can be predicted.

Genetic screening raises additional questions. The Nuffield Council for Bioethics (1993) has produced a report on the ethical issues that arise for the individual and for society as result of genetic screening. The NHS Central R&D Committee (1995) has endorsed this report and raised its own concerns. The Committee expressed the view that genetics differs from other biomedical areas in that it involves not only the individual being tested but also other family members. The Department of Trade and Industry (1996), in response to a report of the House of Commons Select Committee on Science and Technology, has advised the Government to establish an Advisory Committee on Genetic Testing. This committee has now been established (chaired by Professor Polkinghorne) and it is likely that the Department of Health will seek its ethical advice should screening for fragile X syndrome be seriously considered.

Chapter 13

Recommendations

This review brings together a vast body of literature on fragile X syndrome. Much is now known about the natural history, genetics and prevalence of the disorder. The various meta-analyses we have performed on published studies in these areas form a sound basis for health planners to judge whether or not the syndrome should be considered for screening. However, when it comes to the potential screening strategies the position is less clear.

There is considerable practical experience with active cascade screening, particularly in New South Wales. Cascade screening has been shown to be both feasible and effective in reducing affected births within affected families; no comparable data are available on the impact on birth prevalence for the whole population. Other options have not been studied sufficiently for general statements to be made about potential screening performance from practical experience. Nonetheless, reasonable estimates of efficacy can be arrived at for antenatal screening. A simple model of the screening process suggests that performance could be high, and certainly comparable with antenatal screening for Down's syndrome and cystic fibrosis. It is known that invasive prenatal diagnosis has a high acceptability among carriers and that the termination rate for affected pregnancies is high even for female foetuses. However, information on likely uptake is lacking so it is not possible to completely predict effectiveness. Pre-conceptual screening is completely unevaluated but is unlikely to be a realistic option. Paediatric screening is widely practised but its effectiveness is unproven, and neonatal screening is untried.

On the basis of our structured review, we make five recommendations for further research.

1. Studies should be carried out to assess the current practice of paediatric screening when there is developmental delay. Large numbers of samples are being sent to DNA laboratories, mainly by paediatricians, for fragile X diagnosis. The percentage yield of cases is not very high, and it might be more efficient to preselect samples so that only those with the highest risk of the disorder are tested. A survey that includes a large number of laboratories is needed to determine the variability of practice throughout the country and whether it could be improved.
2. There should be a national audit of current practice in cascade screening of affected families. Cascade screening must be regarded as of proven benefit and, in this country, it is carried out to some extent as part of normal genetic practice. However, there is no information on how actively this is undertaken or how successful the practice is. Before the funding of new active schemes of cascade screening for fragile X syndrome is considered, current practice needs to be evaluated.
3. Research should be commissioned into the psychosocial implications of being identified as having a PM. As we have emphasised, screening for fragile X syndrome has potential human benefits but, for some individuals, it may carry a high psychological price. It is important for this to be quantified, and a need for research into methods of ameliorating it. This will be an important part of any pilot studies of antenatal screening and any other screening programmes that may be proposed.
4. Pilot studies should be carried out to assess the feasibility of routine antenatal screening. In the UK over the last 20 years, a number of antenatal screening services have been introduced into the NHS. Most women are now routinely offered maternal serum and ultrasound screening for neural tube defects, Down's syndrome and a number of gross structural abnormalities. Pilot studies of screening for cystic fibrosis have been successfully undertaken and this service is beginning to be introduced. Now that fragile X screening is technically feasible, some centres may want to add this to their routine practice. At present, cost will be a deterrent but, eventually, this is likely to be reduced. Well-designed pilot studies aimed at determining the practicality and acceptability of such testing would be of value to planners in the future.

5. A central registry should be established for all diagnoses, based mainly on reports from DNA laboratories. There are no official statistics for this country of the number of individuals born with fragile X syndrome. It is therefore not possible to monitor the effect of screening or other intervention on the prevalence of this common serious disorder. The present voluntary system of birth defect notification to the Office of National Statistics is inadequate for this purpose. One solution, as has happened with Down's syndrome, would be to develop a national register, starting with those cases ascertained by DNA laboratories equipped to perform diagnostic testing.

Acknowledgements

The authors wish to thank Lyn Cookman for help with the manuscript and keeping track of the references, and Carol Wilson for organising the electronic reference management system. We are grateful to Professor Ted Brown, Professor Marcus Pembrey, Professor Gillian Turner, Professor David Brock, Dr Wayne Miller and Dr Christine Spence for providing us with unpublished information. Finally, we wish to thank Dr Jeremy Turk for his helpful comments.

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There are more than 500 papers on fragile X syndrome which we have consulted in detail while carrying out this systematic review. Not all of them are referred to directly in the report but they all are listed here in alphabetical order. Other papers are referenced (e.g. on the risks of amniocentesis) that do not relate to fragile X syndrome and these form a separate alphabetical list at the end of this bibliography.

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The National Coordinating Centre for Health Technology Assessment,
Mailpoint 728, Boldrewood,
University of Southampton,
Southampton, SO16 7PX, UK.
Fax: +44 (0) 1703 595 639 Email: hta@soton.ac.uk
http://www.soton.ac.uk/~hta

ISSN 1366-5278