UK National Screening Committee

Antenatal Screening for Fragile X Syndrome

External review against programme appraisal criteria for the UK National Screening Committee (UK NSC)

Version: Final

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October 2014

The UK NSC advises Ministers and the NHS in all four UK countries about all aspects of screening policy. Its policies are reviewed on a 3 yearly cycle. Current policies can be found in the policy database at http://www.screening.nhs.uk/policies and the policy review process is described in detail at http://www.screening.nhs.uk/policyreview

Template v1.2, June 2010

Abbreviations List

ACGS	Association for Clinical Genetic Science
ADHD	Attention deficit hyperactivity disorder
ASD	Autism spectrum disorder
CF	Cystic Fibrosis
DD	Developmental disorder
EM	Early menopause
FA	Folic acid
FM	Full mutation
FMR1	Fragile X mental retardation 1 gene
FXS	Fragile X Syndrome
FXPOI	Fragile X associated premature ovarian insufficiency
FXTAS	Fragile X associated tremor/ataxia syndrome
ID	Intellectual disability
IM	Intermediate allele
OR	Odds ratio
PM	Premutation
POI	Primary ovarian insufficiency
POF	Premature ovarian failure

Executive summary

Background

Fragile X Syndrome (FXS) is the most common identifiable hereditary cause of learning difficulty in most western countries, with an estimated UK of around 1 in 5000. It is caused by an expansion of the CGG triplet repeat in the FMR1 (fragile X mental retardation 1) gene, on the X chromosome. Because the condition is associated with the X-chromosome, males are more likely to be symptomatic. Because women have two copies of the X chromosome an FXS expansion on one will not necessarily predict a symptomatic FXS phenotype.

The number of CGG triplet repeats is a continuum from healthy to FXS and the diagnosis of FXS is determined by the number of repeats. A full mutation is characterised by more than 200 repeats, a pre-mutation generally has 55-200 repeats and an immediate allele will have 41 or 45 to 54 repeats, depending on the reference definitions used. Most people will have less than 45 repeats.

The full mutation is associated with a range of outcomes and can result in moderate to severe intellectual disabilities, social impairment and a variety of physical symptoms (notably repetitive movement). Recently a number of outcomes have been linked with pre-mutations in men and women, notable examples include Fragile X associated tremor/ataxia syndrome (FXTAS) and Fragile X associated primary ovarian insufficiency (FXPOI).

Update review

In 2011, the UKNSC published an evidence report on antenatal Fragile X Syndrome and, based on this review, recommended that screening for FXS should not be offered. This update review explores the volume, characteristics, quality and direction of the literature published since the 2011 UKNSC review and focuses on key questions relating to that document's conclusions. The aim of the review is to inform discussion on whether the recent evidence suggests a need to reconsider the current screening recommendation.

The key conclusions from the 2011 UKNSC review and a summary of the evidence published since (addressing each) are listed below:

- While the natural history and prognosis of full mutations in males is well understood, it is not possible to predict whether a female fetus carrying the full mutation will be affected by learning difficulties or to what extent.
    - In the 2014 update, no new evidence (published since 2011) was identified and therefore no further comment can be made on the 2011 evidence review conclusion.

- The clinical impact of carrying an FMR1 pre-mutation (55 to 200 repeats) mutation and the association FXPOI and FXTAS is unclear.
    - In the 2014 update, no prospective cohort studies were identified related to this key question ; this type of study is required to adequately describe the likelihood of developing one or more of the associated FXS conditions in people with a pre-mutation However a number of papers addressing this issue were identified. These suggest that the precise mechanisms by which a pre-mutation status could increase the risk for FXPOI and FXTAS, and the factors that may contribute to this remain unclear.

      Evidence regarding the association between *FMR1* intermediate allele status and Autism Spectrum Disorder (ASD) was inconclusive.

- The current approach to testing, southern blotting, is labour intensive and not a practical use of resource in a universal screening programme requiring a high volume of tests. Alternative screening tests, for example PCR kits, are required.
    - In the 2014 update, no studies were identified that assessed the performance of PCR kits in large, unselected, pregnant populations. However 6 exploratory studies assessing analytical validity were included. Those studies reported test sensitivity ranging from 88.6% to 100%, and specificity from 42.9% to 100%. PCR followed by selective Southern blot remains the only acceptable method for diagnosing FXS; further research is required on the accuracy of PCR tests in the pregnant population.

- There were no curative or preventive treatments for FXS, FXTAS, or FXPOI that could be offered to those identified as having these conditions or of being at risk of the conditions
    - In the 2014 update, no new randomised controlled trials were identified for the two prioritised treatments, folic acid and L-acetylcarnitine. No studies exploring alternative treatments for the effects that decreased levels of the fragile x mental retardation protein has in people with FXS were included. The next update should consider advances made in this area, specifically in infants identified through antenatal screening.

Conclusion

As with the earlier review:

- There is no sufficiently well researched test which could be used for antenatal screening purposes.
- There are no interventions to reduce the risk of developing FXS or the adverse outcomes associated with it.
- The natural history of premutations and intermediate alleles remains insufficiently understood. As such the information from screening and diagnosis would not be adequate to support reproductive decision making.

The 2014 review suggests that the body of evidence identified by the literature search is an insufficient basis on which to change the current screening policy.

Introduction

Fragile X Syndrome

Fragile X syndrome (FXS) is an X-linked condition caused by a mutation in the FMR1 (Fragile X mental retardation 1) gene. The causative mutation is an expansion of a CGG triplet repeat. Individuals are generally classified into four categories based on the number of CGG repeats, although the precise thresholds used to define these categories may vary:

- Full mutations - >200 CGG repeats - once the repeat region reaches a given size hypermethylation occurs, rendering the gene inactive.
- Premutations - generally 55 to 200, draft ACGS guidelines[1] use 59 to 200 - premutations are unstable. A maternal premutation may expand to full mutation in her offspring
- Intermediate alleles – generally between 41 and 54 or 45 and 54 - draft guidelines of the Association for Clinical Genetic Science (ACGS)[1] use 46 to 58 repeats to define this category
- Normal length repeats - generally defined as 11 to 40 repeats, although some thresholds may go up to 45 repeats - the majority of individuals are in this range

Males generally have one copy of the X chromosome, so males with full mutations will develop FXS symptoms, including moderate to severe intellectual disabilities, social impairment and a variety of physical symptoms. As females have two copies of the X chromosome, existence of >200 repeats on one chromosome will not necessarily result in a FXS phenotype.

Recent evidence highlights additional late-onset conditions associated with FXS, notably Fragile X associated tremor/ataxia syndrome (FXTAS) and Fragile X associated primary ovarian insufficiency (FXPOI).

A distinct condition, FRAXE, arises due to large expansions of a GCC repeat in the 5' untranslated region of the *FMR2* gene; however, the clinical phenotype is less severe that that seen in Fragile X Syndrome (also referred to as FRAXA). FRAXE is not covered further in this review.

Basis for current recommendation

The most recent UKNSC external review of antenatal screening for FXS, conducted in 2010, concluded that "the updated evidence published since 2003 does not support a change in national policy regarding antenatal screening programme for Fragile X Syndrome. The full NSC criteria for considering a population screening programme remain unmet."

Several key uncertainties were highlighted by the 2010 evidence review:

- Uncertainty regarding the prevalence of FXS and associated conditions (e.g. FXTAS, FXPOI) in the UK
- Lack of clarity surrounding the natural history of FXS and associated conditions, especially in regards to the prognosis of premutations and full mutations in females
- The availability of a reliable, rapid, high-throughput test amenable for use in population screening programmes
- The availability of treatments for individuals identified by screening as having FXS and associated conditions

Following the 2010 review, the National Screening Committee concluded that screening for FXS during pregnancy is not recommend as "the current available test is labour intensive and unsuitable for high throughput screening purposes. The test would identify carriers of premutations and full mutations. In addition to identifying carriers of a full mutation, the phenotype of which in females is highly variable, the test would also identify premutation carriers for whom the epidemiology, natural history and clinical course is currently inadequately understood in both males and females."[2]

Current update review

The current review considers whether the volume and direction of the evidence produced since the 2010 external review indicates that the previous recommendation should be reconsidered. Three main criteria will be considered, with particular focus given to areas the 2010 review identified as uncertain, or supported by insufficient evidence. The main criteria and key questions reviewed are:

Table 1. Key questions for 2014 FXS update review

Criterion	Key Questions (KQ)	# KQ Studies Included
2 - The epidemiology and natural history of the condition, including development from latent to	Can the prevalence of *FMR1* premutations and intermediate alleles be established from the literature?	9

declared disease, should be adequately understood and there should be a detectable risk factor, disease marker, latent period or early symptomatic stage	Has understanding of the natural history of premutations and intermediate alleles developed since the previous review?	12
	What is the prognosis for women with a FXS full mutation?	0
5 - There should be a simple, safe, precise and validated screening test	Is a high volume/rapid throughput test available which is suitable for whole population screening?	6
10 - There should be an effective treatment or intervention for patients identified through early detection, with evidence of early treatment leading to better outcomes than late treatment	What are the most effective treatments for symptomatic full and premutations of FXS?	1

A systematic literature search of studies published between 2010 and August 2014 yielded 1,295 studies addressing FXS. Of these, 373 were assessed as being potentially relevant to the key questions outlined in Table 1. These studies were further filtered at title and abstract level, and 59 were selected for appraisal at full text. UK based studies were prioritized, although studies from other countries were also considered. Each section below provides additional information on the evidence selection process for the given criterion.

Appraisal against UK NSC Criteria

These criteria are available online at http://www.screening.nhs.uk/criteria.

2. The epidemiology and natural history of the condition, including development from latent to declared disease, should be adequately understood and there should be a detectable risk factor, disease marker, latent period or early symptomatic stage

Description of 2010 UKNSC evidence review conclusion

The most recent evidence review for antenatal screening for FXS concluded that "the natural history of FXS in males is adequately understood, and the presence of the full mutation or premutation is detectable using available methods. In females, it is still not possible to predict which carriers of the full mutation will be affected by FXS or to what extent. The prevalence of the condition in the general population in the UK can be estimated based on studies mainly from other countries, but local information on the prevalence of the premutation would be helpful if a screening programme was to be introduced."

In addition to the known mutation interactions, the previous review highlighted a number of conditions that have been linked with fragile X premutations. In women with premature ovarian insufficiency, recent evidence showed higher than expected numbers of women with a premutation (known as FXPOI). Similarly, there were high numbers of people with premutations in cases of fragile X associated tremor/ataxia syndrome (known as FXTAS) and a variety of neurobehavioral conditions.

The previous evidence review also highlighted that the acceptability of a screening programme is unclear. This issue is complicated further by the potential of screening to identify premutation carriers, and although premutations have been associated with conditions that present later in life, there is currently no way to predict the likelihood of these conditions presenting at the point of screening.

2014 UK NSC key question

Based on these uncertainties, the current evidence review focuses on three main questions regarding the epidemiology and natural history of *FMR1* mutation carriers:

1) Can the prevalence of *FMR1* premutations (50-200 CGG repeats) and intermediate alleles (41 or 45 to 54 CGG repeats) be established from the literature?
2) Has understanding of the natural history of premutations (PM) and intermediate (IM) alleles developed since the previous review?
3) What is the prognosis of women with a FXS full mutation (FM)?

Evidence regarding intermediate alleles, premutations, and FXS associated conditions included FXTAS and FXPOI are assessed separately below.

Furthermore, the scoping of the 2014 update did not identify any studies that would alter the conclusions made about the full mutation in males, which as noted above, is well understood. Therefore this update will not address this population.

Description of the evidence

Overall, 55 studies were identified as potentially relevant during title and abstract sifting and further assessed at full text. Cross-sectional analyses were considered for inclusion for the prevalence question, although studies with population based samples were prioritised. Prospective cohort studies were prioritised for the questions regarding natural history and prognosis, however, case control studies, or studies comparing allele frequency amongst FXTAS and FXPOI populations compared to controls were also included.

Of the 55 studies assessed at full text, 21 were included in the final analysis. The main reasons for exclusion were sample selection (epidemiology in clinically referred samples only) and lack of prospective study design or comparison to a control population (natural history).

Results

Epidemiology

No studies were identified that assessed the epidemiology of *FMR1* intermediate or premutation carriers in the UK population. Eight studies were identified that assessed the prevalence in other countries (four from the USA[3-6] two in Korea[7, 8], one from Japan[9] and one meta-analysis of epidemiology figures from multiple countries[10]). These studies are summarised below and in Table 2.

Overall, no direct evidence was identified regarding the prevalence of *FMR1* intermediate alleles and premutations in the UK population. Variation in prevalence by ethnicity was observed, with studies in Western or Caucasian populations reporting greater frequency of both intermediate and premutation allele carriers than studies in Asian populations. This has implications for the relevance of international studies for use in the UK, and further research establishing the prevalence of these alleles in the UK population (including minority ethnic groups) is needed.

Premutation

Overall, the prevalence of premutations varied according to country, sex, and recruited populations. Prevalence ranged from 0 (Japan) to 1:204 (USA), with frequencies in Asian populations consistently lower than in European or Caucasian populations. Across four studies conducted in the USA,[3-6]

premutation prevalence among males ranged from 1:468 to 1:290, and the prevalence of premutation carriers among females from 1:209 to 1:148. A meta-analysis[10] of studies conducted in various countries suggests a global prevalence of premutation carriers of approximately 1:855 among males and a prevalence of premutation carriers of 1:291 among females. There is evidence of variation in prevalence across ethnicities, with both intermediate alleles and premutation carriers being more frequent in Caucasian and European populations than Asian populations (see Table 2).

Intermediate

Similar to premutation prevalence patterns, Western and Caucasian populations exhibited higher frequency of intermediate alleles than Asian populations. Across three studies in the USA, overall intermediate allele (45 to 54 CGG repeats) carrier prevalence ranged from 1:84 to 1:39, among males prevalence ranged from 1:112 to 1:42, and among females the prevalence of intermediate carriers ranged from 1:66 to 1:33.

Table 2. Prevalence of *FMR1* intermediate (IM) alleles and premutations (PM)

Study	Country	Sample source, (n=)	Overall	Male	Female
Premutation (55 to 200 repeats)					
Seltzer 2012[5]	USA	Population (n=6,747)	1:225*	1:468	1:151
Maenner 2013[4]	USA	Population (n=19,996)	1:204*	1:290	1:148
Hantash 2011[3]	USA	Population [predicted] (n=11,759)		1:400†	1:178†
Tassone 2012[6]	USA	Screening [non-FXS] (n=14,207)	1:284*	1:430	1:209
Hunter 2014[10]	Multiple	Population [predicted] (n approx. 134,000)		1:855∫	1:291∫
Jang 2014[7]	Korea	Clinical (n=10,241)			1:788
Kim 2013[8]	Korea	Clinical (n=5,829)			1:583* overall 1:781 no FH 1:120* FH
Otsuka 2010[9]	Japan	Clinical (n=946)	0:946*		
Intermediate (45 to 54 CGG repeats)					
Maenner 2013[4]	USA	Population (n=19,996)	1:39*	1:62	1:33
Seltzer 2012[5]	USA	Population (n=6,747)	1:38*	1:42	1:35
Tassone 2012[6]	USA	Screening [non-FXS] (n=14,207)	1:84*	1:112	1:66
Jang 2014[7]	Korea	Clinical (n=10,241)			1:137
Kim 2013[8]	Korea	Clinical			1:146*

		(n=5,829)			1:143 no FH 1:179 FH
Expanded intermediate (40 or 41 to 54 CGG repeats)					
Maenner 2013[4]	USA	Population (n=19,996)	1:16*	1:22	1:14
Seltzer 2012[5]	USA	Population (n=6,747)	1:16*	1:21	1:13
Tassone 2012[6]	USA	Screening [non-FXS] (n=14,207)		1:32	1:18
Otsuka 2010[9]	Japan	Clinical (n=946)	1:158*	1:103	1:324
† Predicted prevalence based on CF screening population and Hagerman's equations ∫ Based on meta-analysis of studies from multiple countries * Reviewer calculated FH: Family history of FXS, mental retardation, developmental problems, autism or primary ovarian failure (POF).					

Impact of family history of FXS and related conditions

Limited evidence was identified in the 2014 update search regarding varying intermediate or premutation epidemiology according to family history of FXS or related disorders. One study[8], conducted in Korea among a sample of women clinically referred or requesting *FMR1* testing on their own initiative, suggests that the prevalence of premutation carriers is higher among women with a family history of FXS, mental retardation, developmental problems, autism or premature ovarian failure, than those with no family history.

Another US based study[11] examined the risk of expansion among the offspring of approximately 1,100 female intermediate, premutation and full mutation carriers. Prenatal diagnostic testing found that the intermediate, premutation and full mutation alleles were transmitted approximately 50% of the time, indicating that there is no segregation distortion of the alleles. Some alleles were unstable, and the length of the repeat changed resulting in 1:10 foetuses carrying an intermediate allele, and 1:5 foetuses carrying a premutation and 1:5 a full-mutation. Risk of expansion to the full mutation from a maternal allele with a CGG repeat in the 70-89 CGG range was significantly higher with a history of FXS compared to no family history (70-79 repeats: 54% [family history] vs 11% [no family history], p=0.0081; 80-89 repeats: 88% [family history] vs 33% [no family history], p=0.00085).

Natural History

FXPOI

No prospective cohort studies amongst premutation carriers were identified that assessed the risk of developing FXPOI or the natural history of this fragile X associated condition. One study[3] was identified that estimated the prevalence of FXPOI using prevalence data from a female population screened for cystic fibrosis, however, the estimates varied considerably depending on assumptions made (1:14,240 to 1:890) and it is unclear which FXPOI penetrance estimate is most relevant to the UK due to a lack of UK epidemiological data.

Seven studies assessed either CGG repeat lengths or premutation carrier status amongst infertile women or women with premature ovarian insufficiency compared to controls[1]. These included cross-sectional studies,[12-15] case-control studies[16, 17] and one meta-analysis of case-control studies[17]. The body of evidence provided limited information on the natural history of FXPOI. It should be noted that the comparator groups used in the studies varied from healthy fertile women to women with other forms of infertility.

Intermediate

Six studies (four cross sectional studies[12-15], one nested case-control conducted in the UK[16] and one case-control study[18]) compared the proportion of intermediate allele carriers amongst women with POI vs. controls. Only one study[15] reported significant differences in IM allele prevalence. This study, however, examined occult POI (experiencing menstrual cycles, but with impaired ovarian response), and it didn't follow-up to determine whether participants went on to develop POI.

Premutation

One meta-analysis of case-control studies[17], one UK based nested case-control[16], one case-control [17] and four cross-sectional studies[12-15] assessed premutation carrier prevalence between POI participants and non-POI controls. Three of the four studies found that significantly higher proportions of POI participants carried the *FMR1* premutation (odds ratio [OR] range 5.4 to 6.9). No significant differences were detected in Asian populations.

Table 3. *FMR1* intermediate allele and premutation carrier status, POI patients vs. controls

Study	Country	POI	Controls	p-value	OR (95% CI)	OR p-value
Premutations						
Murray 2014[16]	UK	2.0% (5/254)	0.4% (7/1,915)	p=0.008	OR 5.47 (1.72 to 17.38)	p=0.004
De Geyter 2014[12]	Switzerland	2.1% (1/48)	0.5% (1/199)	p=NS	-	-
Karimov 2011[15]	USA	1.3% (7/535)[a]	0.2% (1/521)[b]	p=0.036	-	-
Tosh 2014[17]	Multiple (SR)	-	-	-	Overall: 5.41 (2.53 to 11.61)	p<0.001
					Asian: 3.91 (0.73 to 20.74)	p=0.11
					European: 6.85 (2.58 to 18.19)	p≤0.001

[1] NB. The studies variously described premature ovarian failure and premature ovarian insufficiency; for the purposes of this review we refer to these conditions as premature ovarian insufficiency [POI]

Ficicioglu 2010[13]	Turkey	0% (0/9)	0% (0/40)[c]	-	-	-
Ishizuka 2011[14]	Japan	1.6% (2/128)	0% (0/98)	-	-	-
Tosh 2014[17]	India	0% (0/289)	0% (0/360)	-	-	-
Intermediate						
Murray 2014[16]	UK	2.8% (7/254)	2.8% (53/1,915)	-	OR 1.01 (0.46 to 2.25)	p=0.98
De Geyter 2014[12]	Switzerland	0% (0/48)	2.0% (4/199)	p=NS	-	-
Voorhuis 2014[18]	Netherlands	2.7% (10/375)	3.7% (123/3,368)	p=NS	-	-
Karimov 2011[15]	USA	3.2% (17/535)[a]	1.3% (7/521)[b]	p=0.046	-	-
Ficicioglu 2010[13]	Turkey	0% (0/9)	0% (0/40)[c]	-	-	-
Ishizuka 2011[14]	Japan	3.9% (5/128)[d]	0% (0/98)	-	-	-
Tosh 2014[17]	India	0% (0/289)	0% (0/360)	-	-	-

[a]Women had 'occult' POI, defined as experiencing menstrual cycles, but with impaired ovarian response
[b]Controls in this study were infertile women or oocyte donors
[c]Controls in this study were infertile but had normal ovarian function
[d]Intermediate alleles had between 41 and 54 repeats in this study

Overall, limited evidence was identified on the association between *FMR1* allele status and FXPOI. No prospective evidence was identified, and most studies were cross-sectional analyses from studies conducted in non-UK populations. One UK based study[16] (a nested case-control study from a large [>100,000] prospective cohort study) reported an OR of 5.47 for premutation carriers in POI vs. controls. The study was not a population based sample, however, and recruited charity organisation volunteers and their friends and families.

Study size was a key limitation across the body of evidence; generally, power calculations were not reported, and it is unclear if the studies were sufficiently powered to detect an association. Furthermore, given the available data on *FMR1* intermediate allele and premutation prevalence (see Table 2), small numbers of participants in each group would not be expected to identify IM and PM carriers. This limitation is particularly relevant for studies that report no cases detected.

Overall, the evidence identified did not substantially alter the understanding of the natural history of females with the *FMR1* premutation, or the development of FXPOI. While the studies largely validated previous findings regarding the association between FMR1 premutation status and POI, they are unable to determine which female premutation carriers will develop FXPOI. Further evidence from large prospective cohort studies amongst female PM carriers is needed in order to

estimate the risk of developing POI in this population and further elucidate the natural history of this condition.

Ethnicity

The body of evidence regarding the epidemiology of *FMR1* intermediate alleles and premutations, as well as that concerning associations between these alleles and POI, suggests that there may be heterogeneity by ethnicity, with lower prevalence seen among Asian populations than that reported for European or primarily Caucasian populations. It is important to note, however, that small sample sizes across the body of evidence on POI may account for some of the lack of *FMR1* CGG expansion carriers seen in these studies (e.g. sample size may have been too small to detect any carriers). Further evidence from large UK studies is needed in order to determine what, if any, impact ethnicity would have on a UK population screening programme.

FXTAS

No prospective cohort studies amongst PM carriers were identified that assessed the risk of developing FXTAS or the natural history of this fragile X associated condition. One US based study[3] was identified that estimated the prevalence of FXTAS as 1:4,848 using prevalence data from a female population screened for cystic fibrosis. This estimate may not be directly applicable to the UK as it relies upon prevalence estimates from a selected population in another country, as well as further assumptions on the ratio of male:female PM prevalence and penetrance of FXTAS in that population.

Two small cross sectional studies were included. One US based study[19] that assessed the phenotypic variance across FXTAS patients, and examined the genotype-phenotype relationship in this group. The other UK based study[20] assessed whether CGG repeat length moderated the relationship between age and executive function amongst male PM carriers who do not exhibit FXTAS symptoms.

These were small cross-sectional studies, and power calculations were generally not reported. As such, it is unclear whether the studies were sufficiently powered to detect associations between CGG repeat lengths and various FXTAS indicators. Larger prospective studies are necessary to further define the natural history of this condition.

Overall, the study amongst FXTAS patients found no association between CGG repeat length and age of tremor/ataxia onset, FXTAS diagnostic category (i.e. definite, probable, possible, indeterminate), disease severity or length of disability in older, male PM carriers with a family history of FXS.

The UK based study suggests that CGG repeat length may moderate the relationship between age and performance on certain cognitive tests amongst PM carriers without FXTAS symptoms (performance declines with increasing age amongst PM carriers with more CGG repeats, but there is no associations at the lower end of the PM scale). However, the study employed a cross-sectional design with no long term follow-up, so it is uncertain whether the participants went on to develop FXTAS, and whether this early moderating effect is part of the natural history of the condition.

Neuropsychological and behavioural

FMR1 expansions have previously been associated with developmental and behavioural disorders, and an understanding of the risk of developing these disorders among carriers of intermediate alleles and premutations was considered as part of the current update review. In particular, associations with Autism Spectrum Disorders (ASD) were assessed, as FXS and ASD are often comorbid conditions.[21, 22] As such, evidence regarding the risk of developing ASD, or the natural history of ASD among *FMR1* intermediate or premutation carriers was assessed.

Three studies[22-24] were included that assessed the neuropsychological or behavioural correlates with premutation carrier status. As with FXPOI and FXTAS, this limited evidence was cross-sectional in nature, and was insufficient in quantity and quality to draw conclusions regarding the natural history of PM carriers in terms of these outcomes.

Evidence from one small cross sectional study[23] suggests that there are no significant differences in cognitive function between female PM carriers and healthy controls. This was the only study identified that assessed this outcome amongst PM females, and given limitations in terms of study design and size, limited conclusions can be drawn regarding cognitive function in this patient population.

Two studies compared the frequency of intermediate alleles between populations with intellectual or developmental disability, autism, other ASDs and ADHD vs. healthy controls. Both studies were cross-sectional analyses, and an overview of results is presented in Table 4.

In the Spanish study which reported significantly lower prevalence amongst participants (all males) with neurobehavioural conditions, male controls were drawn from previously published studies that recruited from clinical settings, who may not be representative of the general population. Furthermore, outcomes and diagnoses were not reported based on age in this study, and a wide range of ages were represented (18 months to 45 years). By comparison, the US based study included participants under the age of 6 years only. It is unclear whether the inconsistency in the significance of these associations is due to variation in participant ages, power to detect a significant effect, or bias due to recruiting methods of the cases vs. controls.

Table 4. IM prevalence (%) across developmental and behavioural conditions

Study	Country	Control	ID/DD	ASD	ADHD
Madrigal 2011[24]	Spain	3.5%* male	1.6%* male	1.33%* male	0.98%* male
Tassone 2013[22]	USA	0.4%** male 2.4%** female	0%** male 2.0%** female	1.3%** male 3.2%** female	NA
* Significant difference vs. control at p<0.05; ** no significant difference vs. control ID intellectual disability; DD developmental disorder; ASD autism spectrum disorder; ADHD attention deficit hyperactivity disorder					

Overall, no conclusions can be drawn regarding intermediate allele prevalence amongst these conditions, due to limited quantity, quality and consistency of the identified evidence. No prospective studies were identified that explored the natural history of these conditions amongst intermediate allele and premutation carriers. This low level evidence was conflicting in terms of both the direction and significance of the identified associations, and was based on cross-sectional analyses.

Further research is required to determine the validity of these studies' findings, and future prospective studies are needed to quantify and qualify the association between *FMR1* expansions and a range of developmental and behavioural disorders, and to determine whether *FMR1* expansions are significant factors in terms of ASD aetiology.

Prognosis amongst full mutation females

The 2010 NSC external evidence review concluded that "the natural history of FXS in males is adequately understood...In females, it is still not possible to predict which carriers of the full mutation will be affected by FXS or to what extent."

No new evidence (published since 2011) was identified in the current update search that addressed the prognosis amongst females with a *FMR1* full mutation.

Summary: Criterion 2 not met. No studies were identified in the update search that determined the prevalence of intermediate alleles or premutations in the UK population. Evidence from the US suggest that the overall prevalence of premutations ranges from 1:284 to 1:204, although the reported prevalence is higher in European and Caucasian populations than Asian populations. No prospective cohort studies were identified that examined the natural history of intermediate and premutation allele carriers, or key conditions previously shown to be associated with premutation, including FXTAS and FXPOI. Understanding of the precise mechanisms by which premutation status increases the risk for these conditions, and the factors that increase the risk of development remains largely unchanged since the previous review. Evidence regarding the association between *FMR1* intermediate alleles status and ASDs was inconclusive, with inconsistent results identified from two small cross-sectional studies. No new studies were identified that assessed the genotype-phenotype relationship or prognosis amongst females with the full *FMR1* mutation.

As more information on the associations between intermediate and premutation carrier status and related conditions emerges, future NSC reviews should consider the impact these associations on acceptability of population wide antenatal screening.

5. There should be a simple, safe, precise and validated screening test

Description of 2010 UKNSC evidence review conclusion

In 2005 the Clinical Molecular Genetics Society's Practice Guidelines for molecular diagnosis of FXS stated that "southern blot analysis should be the method of choice to obtain a conclusive result in prenatal diagnosis."[25] Prenatal southern blot testing, on chorionic villus tissue taken by chronic villus sampling (CVS), is the preferred test because it can detect all sizes of allele expansion. Fluorescent PCR can also be used but its use is limited to only be able to identify normal alleles, furthermore there are uncertainties around its accuracy when it is used with prenatal samples.

Southern blot is, however, labour and time intensive, and not amenable to rapid and high-throughput testing, reducing its suitability to population based screening programmes except as a confirmatory diagnostic test.

The previous 2010 NSC external review on antenatal screening for FXS concluded that "research is ongoing into methods which could eventually reduce the need for Southern blotting and improve throughput. However, these studies are exploratory and do not yet appear to have been applied to screening large unselected populations. These new techniques are likely to need further testing and development before they could be adopted, including showing the sensitivity and specificity of each test, and whether they can be reproducibly and reliably performed in different laboratories."[2]

This conclusion was also made in the 2014 Association for Clinical Genetic Science draft practice guideline for molecular diagnosis of FXS, citing similar concerns that PCR kits were "pending wider validation".[1]

2014 UK NSC key question

The current review focuses on new evidence regarding the development of rapid, high-throughput PCR kits compared to Southern Blot. Prospective studies that were undertaken in a population that is similar to a UK screening cohort were prioritised. In the absence of such evidence, those that

considered testing in a laboratory or research setting were considered. The PCR kit sensitivity and specificity when compared with southern blot testing was the preferred test outcome.

Description of evidence

Overall, 25 studies were identified as potentially relevant when assessed at title and abstract level. None of the identified studies assessed the clinical validity of PCR as part of a population based screening programme. Six observational exploratory studies that assessed the analytical validity of a PCR kit among clinical samples with a confirmed Southern Blot diagnosis were included. One of the included studies[26] reported test performance using prenatal samples (from pregnant women who were all carriers of PM or FM *FMR1* alleles), while the remaining five used whole blood or dried blood spot samples. The study taken

The number of samples included in the studies ranged from 38 to 712, and in three of the studies[27-29], the samples were enriched for a variety of CGG expanded alleles. Three of the studies[27, 28, 30] were conducted in the United States, one[31] in Brazil, one[26] in Belgium, and one[29] in Singapore.

Results

The six exploratory studies assessing the analytical validity of PCR-based *FMR1* kits found that test sensitivity ranged from 88.6% to 100%, and specificity from 42.9% to 100%. The testing strategy and cut-off thresholds assessed varied across the studies (see Appendix Tables and Table 5).

Table 5. Sensitivity and specificity of PCR kits when compared with established southern blot fragile X diagnostic testing

Study	No. samples	Prenatal sample?	Positive test cut-off	Sn	Sp
Testing for full mutations only					
Seneca et al. 2012[26]	67	Y	>200 (FM)	97.4%	100%
Filipovic-Sadic et al. 2010[28]	146	N	>200 (FM)	97.1%*	100%*
Curtis-Cioffi et al. 2012[31]	45	N	>200 (FM)[a]	97.4%	42.9%
Testing for premutations only, or premutations and full mutations					
Curtis-Cioffi et al. 2012[31]	38[b]	N	≥55 to <200 (PM)	88.6%	100%
	75	N	≥55 (PM & FM)	100%	100%
Basehore et al. 2012[27]	712	N	>55 (PM & FM)	100%	100%
Lyon et al. 2010[30]	205	N	≥55 (PM & FM)	100%*	100%*
Teo et al. 2012[29]	44	N	85^{o} C MCA (PM & FM)	100%	100%

* reviewer calculated; Sn sensitivity; Sp specificity; FM full mutation; PM premutation; MCA melting curve analysis

[a]People with PMs were excluded from this calculation

[b]People with FMs were excluded from this calculation

There was variation in test performance depending on the CGG repeat lengths assessed. The four studies that calculated test performance based on ability to distinguish between normal alleles and premutation and full mutation (cut-off of approximately 55 CGG repeats), consistently reported sensitivity and specificity of 100%. Sensitivity was slightly lower among the three studies that assessed the tests ability to identify full mutations (CGG>200), with sensitivity consistently reported as approximately 97% to 98%. These figures suggest that a PCR based testing strategy for full mutations only may be feasible, although further studies that report the detection of premutations, and include an assessment of the clinical validity of such an approach are required.

In terms of study design, six observational studies of analytic validity were identified, representing a very low grade of evidence. These six studies found a high degree of concordance with a gold standard among a small number of samples enriched for expanded alleles While the evidence suggests that some of the previous limitations of PCR kits for FXS diagnosis (e.g. amplification difficulties at higher CGG repeat numbers) have been addressed, the study designs do not allow for assessment of clinical validity of these test, and key performance measures (e.g. PPV, NPV) cannot be established.

Additional uncertainties remain regarding the directness of this body of evidence. Only one study (Seneca et al. 2012[26]) assessed the test's analytic validity in pregnancy, using prenatal samples (chorionic villus tissue and amniotic cells). This sample site, however, would not be appropriate for a screening pregnancy due to its invasive technique and the small risk to the pregnancy that is associated with the tissue removal. The remaining five studies used whole blood or dried blood spot samples. It is unclear whether the timing of the test has an effect on the accuracy of the results.

Overall, this represents a very low level of evidence, and further research efforts that assess the performance of PCR based tests for screening large, unselected prenatal populations are needed in order to establish the clinical validity and utility of this testing strategy.

Summary: Criterion 5 not met. No studies were identified in the update search that assessed the performance of PCR kits in large, unselected populations. Six exploratory studies assessing analytical validity were identified, and found that test sensitivity ranged from 88.6% to 100%, and specificity from 42.9% to 100%. None of these studies, however, were done in pregnant women with samples taken from a suitable site. No major updates to the evidence regarding PCR kits as a rapid and high-throughput screening strategy were identified, and conclusions remain unchanged since the previous 2010 NSC external review for FXS. PCR followed by selective Southern blot remains an accepted method for diagnosing FXS; however, this strategy is labour and time intensive and not amenable to use in a population based screening programme.

10. There should be an effective treatment or intervention for patients identified through early detection, with evidence of early treatment leading to better outcomes than late treatment

Description of 2010 UKNSC evidence review conclusion

The previous 2010 NSC external review on antenatal screening for Fragile X Syndrome concluded that "there is only limited high quality evidence from small RCTs about the effectiveness of the available treatments for the symptoms associated with FXS" and that "the available RCT evidence has not specifically examined whether earlier treatment results in better outcomes."[2]

2014 UK NSC key question

There is no cure for FXS; instead interventions are offered to treat specific symptoms caused by the mutation. While these types of interventions have been shown to have some benefit (often

depending on the symptom severity and presentation), it is unclear if there is a treatment that can be offered before presentation to prevent, or reduce the severity of, symptoms.

For a screening programme to be effective, the identification of fragile X mutations should allow appropriate intervention and management that would have greater benefits than reactive treatment at presentation, in later life.

The current review, therefore, focuses on new evidence regarding the effectiveness of folic acid and L-acetylcarnitine for the treatment of symptomatic full and premutation FXS. Systematic reviews and randomised controlled trials were prioritised, however, in the absence of such evidence, prospective cohort studies would be considered. Where possible, the impact on symptom alleviation is discussed separately, in sub-group analyses according to sex and mutation status (premutation or full mutation). As discussed above, the treatment for specific secondary conditions (for example, FXPOI) caused by FXS and not symptoms directly associated with the mutation are outside of the remit a screening policy and have therefore been excluded.

Description of evidence

Overall, three references were identified as potentially relevant when assessed at title and abstract level; two of which were excluded from further analysis. One systematic review, published in 2011, of randomised controlled trials was included. This review[32] assessed the safety and efficacy of folic acid compared to placebo amongst individuals with FXS. The review included five RCTs, four of which were crossover trials, with a total of 67 participants (all male). The included RCTs were published between 1986 and 1992, four were conducted in the United States and one was carried out in Germany. Four of the five studies were assessed as part of a previous Health Technology Assessment (HTA) review published in 1997[33]; the review did not draw specific conclusions regarding folic acid treatment for FXS.

The protocol for a second systematic review[34] on the effectiveness of L-acetylcarnitine was identified, however, the full review has not yet been published. No new (published post-2011) randomised controlled trials or prospective cohort studies on folic acid or L-acetylcarnitine for the treatment of FXS were identified.

Results

Overall, the systematic review found that folic acid treatment had no significant effect on psychological, intellectual, behavioural or social outcomes among males with FXS. Due to heterogeneity of outcomes and outcome measurements, meta-analysis was not possible (see Table 6).

Table 6. Treatment effects of folic acid (FA) vs. placebo across five randomised controlled trials identified by the systematic review

Outcome	**No. Studies**	**No. Participants (range)**	**Results**
Psychological outcomes and learning capabilities			
IQ	2	n=5 to 25	Non-significant (data NR)*
General Intelligence	1	n=8	MD CPM test: -1.75 (95% CI -17.15 to 13.65); p=0.83
Language Development	2	n=21 to 25	One study reported as non-

			significant (data NR) Mean (SD) PPVT: FA 55.4 (16.2) vs. placebo 59.2 (20.2); p=NS (value NR)
Behaviour or social performance outcomes			
General behaviour	2	n=6 to 21	One study reported as non-significant (data NR) Mean (SD) VABS: FA 51.0 (13.7) vs. placebo 50.9 (15.5); p=NS (value NR)
Hyperactivity	1	n=21	Mean CPTRS: FA 15.55 vs. placebo 13.45; p=NS (value NR) ACTeRS moderate or severe rating: FA 90% vs. placebo 100%; p=NS (value NR)
Autism symptoms	3	n=3‡ to 16†	Studies reported no significant differences (data NR)*
* Narrative study level evidence from one study of effect in subgroup of prepubertal boys (n=8); comparative data not reported ‡Two studies reported outcomes for the 3 males with autism † One study included only a subgroup of participants (16 of25) who completed a battery of Autism outcome assessments. ACTeRS ADD-H Comprehensive Teacher Rating Scales; CPTRS Conners' Parent and Teacher Rating Scale; CPM coloured progressive matrices; FA folic acid; MD mean difference; NR not reported; NS non-significant; PPVT Peabody picture vocabulary test; VABS Vineland adaptive behavioural scale			

The single included systematic review identified low quality evidence regarding the effect of folic acid treatment among males with FXS. While the identified randomised controlled trials suggest that treatment had no significant effect on psychological, behavioural, social and intellectual outcomes, there were substantial limitations in terms of study design and methodology, risk of bias, and directness of the evidence.

Overall, the systematic review identified a small number of trials with a very small number of participants (n=67 total, however, three of the five trials included ten participants or less, and no trial included more than 25 individuals). These studies were underpowered to detect anything other than very large treatment effects.

Furthermore, due to inadequate reporting, the risk of bias among four of the five trials was unclear (all trials published before CONSORT statement); the fifth and largest trial (n=25) was determined to have a low risk of bias. In particular, the review found that insufficient reporting of allocation methods and blinding, and unclear risk of potential carry-over effects among three of the four crossover trials make it difficult to reject the possibility of a high risk of bias across the body of evidence.

All five RCTs included males only, aged 1 to 54, and information regarding the timing of treatment initiation was not reported at the review level, and no trial included younger children only. As such, no conclusions can be drawn regarding the short- and long-term effectiveness of folic acid treatment initiated early in life following identification via antenatal screening. Additionally, the effectiveness

of folic acid treatment among females with either premutation or full mutation *FMR1* alleles cannot be determined based on the identified evidence.

Overall, low quality evidence suggests that folic acid treatment has no significant effect on an array of outcomes among males with FXS. Neither the systematic review nor the update search identified any randomised controlled trials published after 1992 or 2011, respectively.

Summary: Criterion 10 not met. No new randomised controlled trials were identified in the update search that assessed the effectiveness of folic acid or L-acetylcarnitine for the alleviation of symptoms among individuals with premutation or full mutation *FMR1* alleles. A systematic review of five small RCTs identified low quality evidence suggesting that folic acid treatment has no significant effect among males with FXS. Furthermore, none of the studies were in patients identified by antenatal screening. Therefore, based on the identified evidence, it is not possible to determine whether earlier treatment following FXS identification via antenatal screening results in better outcomes. The update search identified a Cochrane review protocol on the efficacy and safety of L-acetylcarnitine treatment, however, the full review has not yet been published.

Conclusions

Implications for policy

This report assesses antenatal screening for Fragile X Syndrome against select UK National Screening Committee (UK NSC) criteria for appraising the viability, effectiveness and appropriateness of a screening programme. This topic was last assessed by an external evidence review in 2010, which concluded that "the updated evidence published since 2003 does not support a change in national policy regarding antenatal screening programme for Fragile X Syndrome. The full NSC criteria for considering a population screening programme remain unmet." In particular, the review identified key uncertainties in regards to the understanding of the epidemiology and natural history of premutations, which would be detected by a screening programme; the prognosis for female carriers of a full *FMR1* mutation; the availability of a rapid and high-throughput screening test; and availability of treatments for individual identified by an FXS screening programme.

The UK NSC subsequently decided to not recommend antennal screening, as "the current available test is labour intensive and unsuitable for high throughput screening purposes. The test would identify carriers of premutations and full mutations. In addition to identifying carriers of a full mutation, the phenotype of which in females in highly variable, the test would also identify premutation carriers for whom the epidemiology, natural history and clinical course is currently inadequately understood in both males and females."

This review assessed key questions to determine if evidence published since the last review resolves any of the identified uncertainties. Limited evidence was identified regarding the availability of a suitable test and treatment. Evidence regarding *FMR1* intermediate and premutation prevalence from other countries was identified, but no UK specific epidemiology research was found. Furthermore, weaknesses in study design and methodology limit the conclusions that can be drawn regarding the natural history of premutations and associated conditions.

The identified body of evidence neither alters the conclusions of the 2010 evidence review nor supports overturning previous UK NSC recommendations regarding a UK antenatal screening programme for Fragile X Syndrome. A summary of key findings for the three assessed criteria is provided below:

- **Epidemiology, natural history and clinical course –**Studies conducted in the US suggest that the prevalence of premutations ranges from 1:284 to 1:204, although the reported prevalence is higher in European and Caucasian populations than Asian populations. Evidence regarding the natural history of premutations - specifically regarding associated

conditions including FXTAS, FXPOI and select neuropsychological, behavioural and developmental disorders – was based on cross-sectional and case control studies only. While these studies generally suggested that individuals with these conditions were more likely to be premutation carriers compared to control groups, no further evidence was available that defined the variation in risk across premutation carriers, nor improved the understanding of which premutation carriers would ultimately develop these conditions. Furthermore, no new evidence was identified regarding the prognosis of females with the *FMR1* full mutation. No UK specific evidence was identified regarding the prevalence of *FMR1* intermediate alleles or premutations.

- **Ethical, safe, simple and robust screening test –** The development of a rapid, high-throughput PCR testing has advanced since the 2010 evidence review, however, research remains in early stages and is able to determine the analytical validity based on testing of clinical samples. No studies were identified that assessed the performance of these tests in large, unselected populations. PCR followed by selective Southern blot remains the accepted method for diagnosing Fragile X Syndrome; however, this strategy is labour and time intensive and not amenable to use in a population based screening programme. The early studies identified in the current update review suggests that new triplet-primed PCR based methods may be suitable for the identification of a large range of repeat lengths including full mutations, potentially overcoming a key limitation of existing PCR based approaches (i.e. amplification failures of larger CGG expansions). This suggests testing strategies for full mutation screening may be possible with further development, although the clinical validity of this approach for antenatal screening remains untested.
- **Effective treatment available –** Two key treatments for the alleviation of symptoms among individuals with premutation or full mutation *FMR1* alleles were included in the 2014 update search: L-acetylcarnitine and folic acid. No new evidence (published between 2010 and August 2014) was identified regarding the former treatment. A single systematic review identified low quality evidence suggesting that folic acid has no significant effect among males with FXS; no evidence was identified regarding females with the full mutation. None of the studies included in the systematic review assessed treatment among FXS patients identified via antenatal screening, and it is not possible to determine, based on the included evidence, whether earlier treatment with folic acid would result in better outcomes.

Implications for research

Given the limited evidence identified for each key question, additional high quality studies in the following areas are needed in order to resolve uncertainties regarding antenatal screening for Fragile X Syndrome:

- Research is required to determine the prevalence of premutations in the UK population, as these alleles can be detected via available testing strategies.
- Current evidence assesses the risk of carrying an intermediate or premutation allele in individuals with Fragile X associated conditions compared to controls, however, prospective cohort studies recruiting individuals with known *FMR1* allele status are needed in order to establish the natural history these conditions and to determine which premutation carriers are likely to go on to develop these diseases.
- Similar prospective cohort studies are needed among women with the full *FMR1* mutation in order to determine whether or not prognosis can be predicted.
- Between 2010 and 2014 several studies were published regarding the analytical validity of PCR based testing strategies for Fragile X Syndrome using clinical samples. Further research

in large, unselected populations is required to assess the clinical validity and utility of these methods, specifically for antenatal screening programmes.

- The research into treatments of Fragile X Syndrome remains active and investigations are on-going into interventions that can offer benefit when used early or after detection through antenatal or newborn screening programmes. Much of the current research is on treating the effects that decreased levels of fragile X mental retardation protein has in people with Fragile X Syndrome, specifically the over and under expression of target genes. While no studies that explored these interventions were found to meet the inclusion criteria for this review, the next update should consider the advances made in this area, in infants identified through antenatal screening.

Methodology

The draft update report was prepared by Bazian Ltd., and then adapted in line with comments from the National Screening Committee.

Search strategy

1. Search approach(es) used

MEDLINE

1 Fragile X Syndrome/ (3374)
2 fragile x syndrome$.ti,ab. (2501)
3 fra X.mp. (440)
4 martin bell syndrome$.mp. (138)
5 x linked mental retard$.mp. (812)
6 (xlmr or fraxa or fraxd or fraxf or fmr1).mp. (1912)
7 or/1-6 (4898)
8 (screen* or test or tests or testing).ti,hw. (1058573)
9 (sensitiv* or diagnos*).ti,hw,ab. (2376079)
10 (prognos* or predict* or course*).ti,hw,ab. (1382843)
11 (incidence or prevalence).ti,hw,ab. (742031)
12 (cost? or costing? or costly or costed).ti,hw,ab. (310541)
13 prevent*.ti,hw,ab. (748260)
14 (systematic* or random* or trial* or retrospective or prospective).ti,hw,ab. (1884927)
15 or/8-14 (6176536)
16 exp Mass Screening/ (82432)
17 (screen* or carrier or (gene* and test*)).tw. (705054)
18 preimplant*.ti,hw. (4444)
19 exp Genetic Testing/ or exp Prenatal Diagnosis/ or exp Genetic Counseling/ (75531)
20 or/16-19 (791228)
21 (case reports or comment or editorial or letter).pt. (2373963)
22 (animal* not human*).sh,hw. (3419622)
23 7 and (15 or 20) (2610)
24 (systematic* or review* or random* or stud* or series or cohort* or retrospective or prospective or meta-analysis or trial* or cost* or economic*).ti,hw,ab,pt. (8716301)
25 (23 and 24) not (21 or 22) (1491)
26 limit 25 to (english language and yr="2003 -Current") (523)
27 from 26 keep 1-523 (523)

Cochrane (in central HTA, NHS EED, DARE)

#1 "fragile x" OR "fra x" OR "martin bell" OR "x linked mental retard" OR xlmr or fraxa or fraxd or fraxf or fmr1:ti,ab,kw, from 2003 to 2010 17

NELH – Screening specialist library
"fragile x" OR "fra x" OR "martin bell" OR "x linked mental retard" OR xlmr or fraxa or fraxd or fraxf or fmr1

Pubmed Guidelines
"fragile x" OR "fra x" OR "martin bell" OR "x linked mental retard" OR xlmr or fraxa or fraxd or fraxf or fmr1

Guideline sites
"fragile x" OR "fra x" OR "martin bell" OR "x linked mental retard" OR xlmr or fraxa or fraxd or fraxf or fmr1

2. Keywords/synonyms included in the search
Fragile X Syndrome/
Fragile X
X linked mental retard*
FXS or FMR1 or FXTAS or FXPOI or FXS
Martin bell syndrome
FX premutation
FX adj3 (mutation* or allele* or repeat*)

Quality

Several factors were assessed to determine the quality of the identified evidence, including study design and methodology, risk of bias, directness and generalisability of the evidence. Factors that were determined to be pertinent to the body of evidence identified for each criteria are outlined in the results section as well as the comment section of the Appendix tables. The overall level of evidence was assessed by considering the quantity, quality and consistency of evidence across the body of studies for each criterion reviewed.

Appendices

Appendix number	1
Relevant criteria	2
Publication details	[20] Cornish KM, Hocking DR, Moss SA, et al. Selective executive markers of at-risk profiles associated with the fragile X premutation. Neurology. 2011;77(7):618-22.
Study details	Cross sectional study, UK
Study objectives	To determine whether CGG repeat length moderates the relationship between age and neuropsychological tests scores in PM carriers asymptomatic for FXTAS.
Inclusions	PM carriers; family history of FXS
Exclusions	FXTAS related symptoms
Population	n=33 PM men with a family history of FXS aged 20 to 68 years (mean 45.33 years), recruited from the UK Clinical Genetics Services and the UK Fragile X Society.

	Mean CGG length was 99.53 (SD 28.56), range 55-161. 16 participants had less than 100 CGG repeats (range 55-97) and 17 had more than 100 repeats (range 101-161). Mean IQ was 104.10 (SD 14.93), range 72-136.
Intervention/test	NA
Comparator	NA
Results/outcomes	<u>Response inhibition</u> *Hayling category B errors* Significant association with age, and interaction between age and CGG repeat length and inhibition tasks. When CGG lengths increased (+1 SD) there was a deterioration in inhibitory control with increasing age. When CGG lengths were low (-1 SD) there was no deterioration with increasing age. There was no interaction with CCG repeat length alone. Repeat length: Standardized B 0.22, p=NS Repeat length x age: Standardized B 0.37, p<0.05 *Stroop Color-Word interference* Significant association with age and CCG repeat length, but no interaction with CGG repeat length x age Repeat length: Standardized B -0.41, p<0.01 Repeat length x age: Standardized B -0.24, p=NS <u>Working memory</u> *Letter-number sequencing (correct response)* Significant interaction effect; relationship between age and score becomes stronger with increasing CGG length. At high CGG, strong inverse relationship between age and sequencing score; a low CGG repeats there was no deterioration with increasing age. Repeat length: Standardized B -0.12, p=NS Repeat length x age: Standardized B 0.44, p<0.01 *PSAT accuracy score* Significant association with age, but no interaction with CGG repeat length or repeat length x age Repeat length: Standardized B -0.30, p=NS Repeat length x age: Standardized B 0.33, p=NS
Comments	Multiple regression analyses used to assess moderating effect of CGG repeat on

	the relationship between age and performance. Findings suggest that individuals in the upper range of *FMR1* PMs may be at risk for age related declines in certain executive function tasks (inhibitory and working memory tasks). Small study size; unclear if sufficiently powered to detect interactive effects. Cross sectional design, so unable to determine if this interaction effect is associated with the later development of FXTAS.

Appendix number	2
Relevant criteria	2
Publication details	[12] De Geyter C, M'Rabet N, De Geyter J, et al. Similar prevalence of expanded CGG repeat lengths in the fragile X mental retardation I gene among infertile women and among women with proven fertility: a prospective study. Genet Med. 2014;16(5):374-8.
Study details	Cross sectional study, Switzerland
Study objectives	To compare CGG repeat lengths between fertile and infertile women.
Inclusions	NR
Exclusions	NR
Population	n=619 women (n=199 fertile women who conceived within three months, n=372 infertile women who experienced ongoing menstrual cycles and n=48 infertile women previously diagnosed with POI (secondary amenorrhea and FSH level >30IU/l before the age of 40).
Intervention/test	NA
Comparator	NA
Results/outcomes	<u>CGG repeat lengths across three groups</u>

CGG repeats	**Fertile controls**	**Infertile women**	**Infertile with POI**	**p-value**
<35 (low normal)	170 (85.4%)	303 (81.5%)	44 (91.7%)	NR
35-44 (high normal)	24 (12.1%)	55 (14.8%)	3 (6.3%)	0.196
45-54 (IM)	4 (2.0%)	9 (2.4%)	0	0.543
55-200 (PM)	1 (0.5%)	5 (1.3%)	1 (2.1%)	0.537
≥35	29 (14.6%)	69 (18.6%)	4 (8.3%)	0.136
≥45	5 (2.5%)	14 (3.8%)	1 (2.1%)	0.643

<table>
<tr><td></td><td>Correlation of four parameters of ovarian reserve with CGG repeat length
<table><tr><th>Parameter</th><th>Correlation coefficient (95% CI)</th><th>p-value</th></tr><tr><td>Age (years)</td><td>0.003 (-0.116 to 0.122)</td><td>0.965</td></tr><tr><td>FSH (U/l)</td><td>0.011 (-0.137 to 0.158)</td><td>0.886</td></tr><tr><td>AMH (pmol/l)</td><td>-0.001 (-0.37 to 0.036)</td><td>0.976</td></tr><tr><td>Inhibin B (pg/ml)</td><td>-0.001 (-0.17 to 0.015)</td><td>0.888</td></tr></table></td></tr>
<tr><td>Comments</td><td>Significant age differences among three groups (mean age fertile women 29.6 years, infertile menstruating women 31.2 years and infertile women with POI 33.5 years).

No women were diagnosed with a FMR1 full mutation.

Unselected cohort of infertile women with no family history of FXS.</td></tr>
</table>

Appendix number	3
Relevant criteria	2
Publication details	[13] Ficicioglu C, Yildirim G, Attar R, et al. The significance of the number of CGG repeats and autoantibodies in premature ovarian failure. Reprod Biomed Online. 2010;20(6):776-82.
Study details	Cross sectional study, Turkey
Study objectives	To determine the threshold of CGG repeat length in premature ovarian ageing and premature ovarian failure.
Inclusions	Infertile group: randomly chosen patients with FSH concentrations mIU/ml Control group: Age under 40, normal FSH concentrations (<12mIU/ml), primary infertility due to tubal ligation or a mild male factor and infertility duration shorter than 2 years.
Exclusions	Infertile group: Infertile patients older than 40y, those who had previously undergone ovarian surgery and those with a history of cytotoxic chemotherapy or pelvic irradiation. Control: Previous IVF treatment, ovarian surgery, pelvic irradiations and cytotoxic chemotherapy, ovarian cysts or endometriosis.
Population	n=39 infertile patients aged 24 to 40 years with premature ovarian ageing (n=30, FSH ≥12 to <50 IU/ml) or premature ovarian failure (n=9, FSH ≥50 IU/ml) and n=40 control patients (age 21 to 39) with normal ovarian function. Conducted between January 2005 and December 2008.

Intervention/test	NA
Comparator	NA
Results/outcomes	Mean (SD) CGG repeats allele 1 POA: 26.8 (3.7) POF: 28.2 (4.6) Control: 26.0 (3.4) NS difference Mean (SD) CGG repeats allele 2 POA: 27.5 (3.7) POF: 27.8 (3.9) Control: 27.2 (3.2) NS difference ≤30 CGG repeats, number (%) POA: 22 (73.3%) POF: 5 (55.6%) Control: 40 (100%) p=0.001 31-40 CGG repeats (high normal), number (%) POA: 8 (26.7%) POF: 4 (44.4%) Control: 0 (0%) NS difference Intermediate, premutation or full mutation, number (%) POA: 0 (0%) POF: 0 (0%) Control: 0 (0%) Outcomes by CCG repeat length, mean (SEM) for non-group outcomes

<table>
<tr><td></td><td>
<table>
<tr><th>Parameter</th><th><30 CGG</th><th>≥30 CGG</th><th>p-value</th></tr>
<tr><td>Control (%)</td><td>40 (100%)</td><td>0 (0%)</td><td>0.001</td></tr>
<tr><td>POA (%)</td><td>22 (73.3%)</td><td>8 (26.7%)</td><td>NR</td></tr>
<tr><td>POF (%)</td><td>5 (55.6%)</td><td>4 (44.4%)</td><td>NR</td></tr>
<tr><td>Age (SD), years</td><td>32.3 (0.5)</td><td>33.7 (1.6)</td><td>NS</td></tr>
<tr><td>FSH (SD), IU/ml</td><td>15.0 (1.8)</td><td>29.8 (6.9)</td><td>0.005</td></tr>
<tr><td>AMH (SD), ng/ml</td><td>1.8 (0.1)</td><td>0.8 (0.2)</td><td>0.05</td></tr>
<tr><td>Inhibin B (SD), pg/ml</td><td>25.8 (3.3)</td><td>18.2 (6.9)</td><td>NS</td></tr>
<tr><td>Adrenal autoantibody</td><td>3</td><td>0</td><td>NS</td></tr>
<tr><td>Thyroid autoantibody</td><td>1</td><td>2</td><td>NS</td></tr>
</table>
</td></tr>
<tr><td>Comments</td><td>No women were found to have intermediate or PM alleles. Small study, unclear whether sufficiently powered to detect differences in CGG repeat length.</td></tr>
</table>

Appendix number	4
Relevant criteria	2
Publication details	[23] Goodrich-Hunsaker NJ, Wong LM, McLennan Y, et al. Adult Female Fragile X Premutation Carriers Exhibit Age- and CGG Repeat Length-Related Impairments on an Attentionally Based Enumeration Task. Front Hum Neurosci. 2011;5:63.
Study details	Cross sectional study, USA
Study objectives	To assess the cognitive function of female *FMR1* PM carriers.
Inclusions	NR
Exclusions	NR
Population	n=50 females (age range 21 to 42) (21 healthy controls and 29 PM carriers).
Intervention/test	NA
Comparator	NA
Results/outcomes	<u>Average enumeration task error rates, range</u> PM: 1.11% (SD 0.03%) to 13.70% (SD 0.18%) Control: 0.00% (SD 0.00%) to 9.50% (SD 0.09%) p>0.25 <u>Enumeration task reaction time</u> PM vs Control p>0.27

Comments	Cross sectional study with small number of participants.

Appendix number	5
Relevant criteria	2
Publication details	[3] Hantash FM, Goos DM, Crossley B, et al. FMR1 premutation carrier frequency in patients undergoing routine population-based carrier screening: insights into the prevalence of fragile X syndrome, fragile X-associated tremor/ataxia syndrome, and fragile X-associated primary ovarian insufficiency in the United States. Genet Med. 2011;13(1):39-45.
Study details	Retrospective cohort, USA
Study objectives	To determine the frequency of fragile X PM and FM in nonselected, unbiased populations undergoing routine carrier screening for other conditions.
Inclusions	NA
Exclusions	NA
Population	Residual anonymized DNA from n=11,759 consecutive cystic fibrosis (CF) carrier screening samples and n=2,011 samples submitted for screening for genetic disorders prevalent among the Ashkenazi Jewish population.
Intervention/test	Triplet primed PCR to detect PM and FM
Comparator	NA
Results/outcomes	<u>PM prevalence in screening sample, number (frequency)</u> CF screened: 48 (1:215) Ashkenazi Jewish: 15 (1:134) Total: 63 (1:219) <u>Estimated PM prevalence</u> Female 1:178 Male 1:400 <u>Estimated prevalence of FXTAS</u> FXTAS 1:4,848 males <u>Estimated prevalence of FXPOI</u> FXPOI: 1:890 to 1:14,240 females (variation due to alternative penetrance estimates)
Comments	CF screened samples unlikely to be representative of the US population, as CF carrier screening recommendations are mainly for whites and Ashkenazi Jews. >99% of samples were from females. Majority of prevalence estimates are predicted based on female PM prevalence

	estimates; not reflective of observed prevalence. Predicted population frequency estimated by stratifying self-reported ethnicity specific frequency to the overall US population. Hagerman's equations used to calculate predicted frequencies: predicted FM frequency=PM frequency in females x rate of expansion of PM into FM [calculated to be 0.107] x 0.5 predicted PM in males=PM frequency in females x (1- rate of expansion of PM into PM) x 0.5 predicted prevalence of FXTAS= PM frequency in males x penetrance of large PM alleles [estimated as 33%] x frequency of alleles ≥70 repeats predicted prevalence of FXPOI= PM frequency in females x penetrance of PM alleles in FXPOI [estimated as 12-28%] x frequency of alleles ≥70 repeats

Appendix number	6
Relevant criteria	2
Publication details	[10] Hunter J, Rivero-Arias O, Angelov A, et al. Epidemiology of fragile X syndrome: a systematic review and meta-analysis. Am J Med Genet A. 2014;164(7):1648-58.
Study details	Systematic review and meta-analysis, various countries (19 USA/ Canada/ Australia, 16 Europe, 9 Asia, 10 other countries)
Study objectives	To determine the prevalence of FXS
Inclusions	Studies estimating the frequency of PM or FM *FXMR1* alleles in any population (no language or date limit); prospective or retrospective primary studies; studies assessing any of three populations (total population without any selection bias [generally pregnant women and newborns]; normal population studies without individuals with intellectual disabilities (ID); populations with intellectual disability).
Exclusions	Reviews and editorials
Population	**Studies:** n=54 overall, 25 were published prior to 2000 and 29 after the year 2000. n=31 population based screening among over 153,500 individuals (78,000 males and 75,500 females) without bias against individuals with intellectual disability n=15 assessed prevalence among 7,475 individuals with intellectual disability **Population (for PM):** Primary analysis: approximately 134,000 (45,000 males, 89,000 females)
Intervention/test	PM or FM status assessed by PCR or Southern Blot
Comparator	NA
Results/outcomes	<u>Predicted mean PM frequency in total population (random effects model):</u> Male: 11.7 per 10,000 (95% CI 6.0 to 18.7) or 1:855 Female: 34.4 per 10,000 (95% CI 6.3 to 83.3) or 1:291

	Predicted mean FM frequency in total population (random effects model): Male: 1.4 per 10,000 (95% CI 0.1 to 3.1) or 1:7,143 Female: 0.9 per 10,000 (95% CI 0.0 to 2.9) or 1:11,111 FM prevalence in ID population, number (frequency) Male only: 148 (1:46)* Female only: 28 (1:18)* Male and female (combined in primary studies): 9 (1:35)* * Reviewer calculated
Comments	Review estimated overall mean PM and FM prevalence by pooling estimates from several different countries/populations, and there was high heterogeneity across all logistic regressions; direct applicability of mean frequency figures to UK population is not known. Some of the included studies were small, and identified few individuals with either PM or FM alleles.

Appendix number	7
Relevant criteria	2
Publication details	[14] Ishizuka B, Okamoto N, Hamada N, et al. Number of CGG repeats in the FMR1 gene of Japanese patients with primary ovarian insufficiency. Fertil Steril. 2011;96(5):1170-4.
Study details	Cross sectional study, Japan
Study objectives	To compare the number of CGG repeats between women with POI and controls
Inclusions	Patients with normo-karyotypic, sporadic, and nonsyndromic POI (≥3 months amenorrhea, <40 years of age, FSH levels ≥40 IU/L). No family history of early menopause or mental retardation.
Exclusions	NR
Population	n=128 patients with POI and n=98 controls with proven fertility and normal menstruation or who had undergone normal menopause.
Intervention/test	NA
Comparator	NA
Results/outcomes	Age, mean (SE) [range] POI: 37.82 (0.50) [20-54]

<table>
<tr><td></td><td>Control: 39.12 (1.09) [21-59]

<u>CGG repeats short allele, mean (SE) [range]</u>
POI: 28.88 (0.23) [12-43]
Control: 28.90 (0.23) [20-36]

<u>CGG repeats long allele, mean (SE) [range]</u>
POI: 32.91 (0.51) [26-68]
Control: 31.90 (0.33) [23-40]

<u>Onset of amenorrhea in POI patients, age (SD)</u>
>38 CGG repeats: 25.1 (2.50)
≤38 CGG repeats: 29.9 (0.62)
p<0.05
<u>Distribution of repeat length in women with POI and controls</u>
<table>
<tr><td>Repeat length</td><td>POI</td><td>Control</td></tr>
<tr><td>≤30</td><td>71 (55.5%)</td><td>54 (55.1%)</td></tr>
<tr><td>31-36</td><td>40 (31.3%)</td><td>38 (38.8%)</td></tr>
<tr><td>37-40</td><td>10 (7.8%)</td><td>6 (6.1%)</td></tr>
<tr><td>41-54</td><td>5 (3.9%)</td><td>0 (0%)</td></tr>
<tr><td>55-200</td><td>2 (1.6%)</td><td>0 (0%)</td></tr>
</table>

Prevalence of >36 repeats reported as significantly higher among POI patients compared to controls; p-value NR.</td></tr>
<tr><td>Comments</td><td>Statistical comparison poorly reported</td></tr>
</table>

Appendix number	8
Relevant criteria	2
Publication details	[7] Jang JH, Lee K, Cho EH, et al. Frequency of FMR1 premutation carriers and rate of expansion to full mutation in a retrospective diagnostic FMR1 Korean sample. Clin Genet. 2014;85(5):441-5.
Study details	Retrospective cohort study, South Korea
Study objectives	To estimate female PM carrier frequency and risk of expansion to FM
Inclusions	Samples from previous *FMR1* mutation analysis at Green Cross Laboratories

	between December 2011 and December 2012.
Exclusions	
Population	n=10,241 clinical samples for *FMR1* gene testing from pre-conceptional or pregnant women. 95 of the samples were from patients with a positive family history.
Intervention/test	Commercialised CCG repeat primed PCR.
Comparator	NA
Results/outcomes	<u>IM allele prevalence, number (frequency)</u> 75 (1:137, 95% CI 1:172 to 1:110) <u>PM allele prevalence, number (frequency)</u> 13 (1:788, 95% CI 1:1,250 to 1:455)
Comments	PM prevalence is reported as lower than Western populations, reducing direct applicability to UK screening population. All samples were from women who were tested on their own initiative, or on the advice of their physician, on a self-pay basis. Repeat length thresholds used to classify IM and PMs was not reported.

Appendix number	9
Relevant criteria	2
Publication details	[19] Juncos JL, Lazarus JT, Graves-Allen E, et al. New clinical findings in the fragile X-associated tremor ataxia syndrome (FXTAS). Neurogenetics. 2011;12(2):123-35.
Study details	Cross sectional study, USA
Study objectives	To assess the phenotypic variance in patients with FXTAS and to examine genotype-phenotype relationship for the condition.
Inclusions	*FMR1* PM carriers over the age of 50 from known FXTAS pedigrees. Motor symptoms or abnormal results (>1SD) on tremor/ataxia detection instruments.
Exclusions	NR
Population	n=50 males, mean age 65 (SD 7) years. 21 participants with definite FXTAS, 10 with probable, 9 with possible, and 10 indeterminate.
Intervention/test	NA
Comparator	NA
Results/outcomes	<u>Disease presentation</u> Mean age of onset of motor symptoms: 59 (SD 11) years. 31 of 50 (62%) participants reported motor symptoms at the study recruitment stage

7 of the 15 subjects reporting no motor symptoms at recruitment exhibited some tremor or ataxia at testing (5 tremor only, 2 ataxia and parkinsonism)

Following testing 46 of 50 (92%) participants recognized tremor ataxia symtoms, and recalled the chronology of the symptoms.

28 of 46 participants with symptoms presented with intention and/or postural tremor, 9 presented with ataxia, 6 with tremor plus ataxia, and 3 with parkinsonism.

Major presentation patterns

16/50 (32%) presented with tremor only; of these 6/16 (38%) had cognitive impairment at study screening.

21/50 (42%) presented with tremor plus ataxia; of these 15/21 (69%) had evidence of cognitive impairment at study screening. Motor symptoms presented 5 to 7 years later than tremor only presentation.

Duration and severity

In 8/20 subjects presenting with tremor who later developed other motor symptoms, tremor preceded new symptoms by 10 years or more. This may be confounded by participants having essential tremor before FXTAS onset.

Motor symptom duration by diagnostic category

Definite: 7.9 (SD 5)

Probable: 2.6 (SD 2)

Possible: 4.7 (SD 5)

$p<0.01$

Average duration of symptoms (all participants)

Mean: 5.1 (SD 5.2)

Range: 0 to 18 years

Longer duration associated with more severe symptoms on Rankin scale ($r=0.42$, $p<0.01$)

Outcome		Cognitive Impairment		p-value
		No	Yes	
Diagnosis	Definite FXTAS	7 (33%)	14 (67%)	p=0.01
	Not definite	20 (69%)	9 (31%)	
Presenting	Tremor only presentation	10 (62.5%)	6 (37.5%)	p=0.039
	Ataxia or	6 (29%)	15 (71%)	

<table>
<tr><td></td><td>
<table>
<tr><td></td><td>ataxia/tremor</td><td></td><td></td><td></td></tr>
<tr><td rowspan="2">Duration (motor symptoms)</td><td>Tremor</td><td>3.3y (SD 4)</td><td>31.67y (SD 1.86)</td><td>p=0.32</td></tr>
<tr><td>Ataxia</td><td>1.5y (SD 2)</td><td>4.1y (SD 5)</td><td>p=0.03</td></tr>
</table>
<u>CGG repeat correlations</u>
Age of onset: r=-0.08, p=0.61
Definite vs. probable, possible and intermediate FXTAS: 95.4 (SD 15) vs. 104.6 (36), 84.7 (25) and 85.0 (32) respectively. ANOVA p=0.25
Disease severity:
Clinical Rating Scale for Tremor: r=0.12, p=0.40
International Cooperative Ataxia Rating Scale: r=0.03, p=0.81
United Parkinson's Disease Rating Scale: r=0.01, p=0.97
Level of disability (Rankin score): r=0.07, p=0.6</td></tr>
<tr><td>Comments</td><td>Small cross sectional study; unclear if sufficiently powered to detect associations between CGG repeat lengths and various FXTAS indicators.
Disease progression data based on self-report.
Overall, found no association between repeat length and age of onset, FXTAS diagnostic category, disease severity or length of disability in older, male PM carriers with a family history of FXS.</td></tr>
</table>

Appendix number	10
Relevant criteria	2
Publication details	[15] Karimov CB, Moragianni VA, Cronister A, et al. Increased frequency of occult fragile X-associated primary ovarian insufficiency in infertile women with evidence of impaired ovarian function. Hum Reprod. 2011;26(8):2077-83.
Study details	Cross sectional study, USA
Study objectives	To compare IM and PM prevalence among women with occult POI and no family history to controls
Inclusions	Menstruating women aged <42 years with occult POI defined as experiencing menstrual cycles, but with impaired ovarian response. Control subjects had infertility due to other reasons or were oocyte donors.
Exclusions	Amenorrhea greater than three months duration (overt POI), previous surgical removal of an ovary, chemotherapy and radiation therapy. Family history of unexplained mental retardation, autism or FXS.

<table>
<tr><td>Population</td><td>n=1,056 women (535 occult POI, 521 control) presenting for infertility treatment or as oocyte donors between January 2006 and December 2010.</td></tr>
<tr><td>Intervention/test</td><td>NA</td></tr>
<tr><td>Comparator</td><td>NA</td></tr>
<tr><td>Results/outcomes</td><td>
<u>PM (55-200 repeats) prevalence, number (%)</u>
Occult POI: 7 (1.3%)
Control: 1 (0.2%)
p=0.036

<u>IM (45-54 repeats) prevalence, number (%)</u>
Occult POI: 17 (3.2%)
Control: 7 (1.3%)
p=0.046

<u>Repeats on longest allele, mean (SD)</u>
Occult POI: 32.7 (7.1)
Control: 31.6 (4.3)
p<0.01

<u>Repeats on shortest allele, mean (SD)</u>
Occult POI: 27.1 (4.5)
Control: 27.0 (4.4)
p=0.4

<u>ROC analysis</u>
AUC: 0.56 (SD 0.2), p<0.01
<table>
<tr><td>Repeat cutoff</td><td>Sn</td><td>Sp</td></tr>
<tr><td>31.5</td><td>72%</td><td>36%</td></tr>
<tr><td>35</td><td>17%</td><td>88%</td></tr>
<tr><td>45</td><td>5%</td><td>98%</td></tr>
<tr><td>55</td><td>1.6%</td><td>99.8%</td></tr>
</table>
</td></tr>
</table>

Comments	Four year study included all women presenting with infertility due to occult POI at three clinics. Controls were also infertility patients or oocyte donors and may not be representative of women of reproductive age, and it is not known whether control subjects would eventually develop occult POI.

Appendix number	11
Relevant criteria	2
Publication details	[8] Kim MJ, Kim do J, Kim SY, et al. Fragile X carrier screening in Korean women of reproductive age. J Med Screen. 2013;20(1):15-20.
Study details	Retrospective cohort study, Korea
Study objectives	To estimate the prevalence of PM and FM *FMR1* alleles among women of reproductive age.
Inclusions	NR
Exclusions	NR
Population	n=5,829 women of reproductive age (n=5,470 low risk, n=359 high risk) between September 2003 and December 2011. Risk status based on family history of FXS, mental retardation, developmental problems, autism or premature ovarian failure.
Intervention/test	PCR and Southern Blot
Comparator	NA
Results/outcomes	<u>Overall prevalence, number (frequency)</u> IM: 40 (1:146*) PM: 10 (1:583*) FM: 1 (1:5,829*) <u>Prevalence among low risk women, number (frequency)</u> IM: 38 (1:143) PM: 7 (1:781) FM: 0 (NA) <u>Prevalence among high risk women, number (frequency)</u> IM: 2 (1:179) PM: 3 (1:120*) FM: 1 (1:359*) * Reviewer calculated
Comments	Prevalence reported as lower than Western studies, and is not directly applicable

	to UK screening population. All samples were from women who were tested on their own initiative, or on the advice of their physician.

Appendix number	12
Relevant criteria	2
Publication details	[24] Madrigal I, Xuncla M, Tejada MI, et al. Intermediate FMR1 alleles and cognitive and/or behavioural phenotypes. Eur J Hum Genet. 2011;19(8):921-3.
Study details	Cross sectional, Spain
Study objectives	To determine the frequencies of IM alleles (45 to 54 repeats) among males with ID, ADHD or ASD.
Inclusions	NR
Exclusions	NR
Population	n=9,730 males (aged 18m to 45y) referred for fragile X testing (9,015 ID, 415 ADHD, 300 ASD). n=6,525 controls from general population recruited from multiple hospitals as part of previous studies (5,775 males and 750 females).
Intervention/test	NA
Comparator	NA
Results/outcomes	<u>Prevalence of IM among behavioural/cognitive phenotypes, number (%)</u> ID: 142 (1.6%)* ADHD: 4 (0.96%)* ASD: 4 (1.33%)* Control (male): 204 (3.5%) * significantly different from control population at $p<0.05$ When prevalence was compared to controls within the same region, differences were non-significant (unclear if regional samples were powered to detect differences).
Comments	Large cross sectional study suggests that intermediate alleles are lower in the ID, ADHD and ASD male populations than the general population. All participants were referred for FXS testing, and may not be representative of ID/ADHD/ASD population. All controls were drawn from previous studies and recruited from clinical settings; may not be representative of general population. Wide age range represented, no subgroup analysis based on age. Unclear ID/ADHD/ASD distribution across participant ages.

Appendix number	13
Relevant criteria	2
Publication details	[4] Maenner MJ, Baker MW, Broman KW, et al. FMR1 CGG expansions: prevalence and sex ratios. Am J Med Genet B Neuropsychiatr Genet. 2013;162B(5):466-73.
Study details	Retrospective cohort study, USA
Study objectives	To estimate the prevalence of *FMR1* PM (55 to 200 repeats) and grey zone (i.e. IM, 45-54 repeats) CGG repeat expansions in a population based sample.
Inclusions	Samples from the Marshfield Clinic Personalized Medicine Research Project (PMRP), an ongoing population cohort study (since 2002) with stored DNA, plasma, and serum, as well as linkages to electronic health records.
Exclusions	NR
Population	n=19,996 samples from 8,469 male and 11,527 female adults in Wisconsin; 98.4% White Caucasian, year of birth ranging between pre-1922 and 1991.
Intervention/test	PCR
Comparator	NA
Results/outcomes	<u>PM (55 to 200 repeats) prevalence, number (frequency)</u> Female: 72 (1:148, 95% CI 1:207 to 1:113) Male: 26 (1:290, 95% CI 1:530 to 1:194) Overall: 98 (1:204*) <u>IM (45 to 54 repeats) prevalence, number (frequency)</u> Female: NR (1:33, 95% CI 1:39 to 1:29) Male: NR (1:62, 95% CI 1:78 to 1:50) Overall: 512 (1:39*) <u>Expanded IM (41 to 54 repeats) prevalence, number (frequency)</u> Female: NR (1:14, 95% NR) Male: NR (1:22, 95% NR) Overall: 1,217 (1:16*) *reviewer calculated
Comments	

Appendix number	14
Relevant criteria	2
Publication details	[16] Murray A, Schoemaker MJ, Bennett CE, et al. Population-based estimates of the prevalence of FMR1 expansion mutations in women with early menopause and

<table>
<tr><td></td><td>primary ovarian insufficiency. Genet Med. 2014;16(1):19-24.</td></tr>
<tr><td>Study details</td><td>Nested case control study, UK</td></tr>
<tr><td>Study objectives</td><td>To estimate the population based IM and PM prevalence among women with POI and early menopause (EM)</td></tr>
<tr><td>Inclusions</td><td>Women with natural menopause prior to age 40 (POI cases) or between 40 and 45 (EM cases). Controls were matched on age, date of study enrolment, and source of recruitment.</td></tr>
<tr><td>Exclusions</td><td>History of breast cancer</td></tr>
<tr><td>Population</td><td>n=4,045 women with primary ovarian insufficiency (254), early menopause (1,881) or age matched controls (1,915)</td></tr>
<tr><td>Intervention/test</td><td>NA</td></tr>
<tr><td>Comparator</td><td>NA</td></tr>
<tr><td>Results/outcomes</td><td><u>Normal, IM and PM carrier prevalence</u>
<table>
<tr><td></td><td>Control</td><td>POI</td><td>EM</td><td>POI and EM</td></tr>
<tr><td>PM</td><td>7 (0.4%)</td><td>5 (2.0%)</td><td>14 (0.7%)</td><td>19 (0.9%)</td></tr>
<tr><td>IM</td><td>53 (2.8%)</td><td>7 (2.8%)</td><td>56 (3.0%)</td><td>63 (3.0%)</td></tr>
<tr><td>CGG <45</td><td>1,855 (96.9%)</td><td>242 (95.3%)</td><td>1,811 (96.3%)</td><td>2,053 (96.2%)</td></tr>
</table>
<u>Mutation OR (95% CI)</u>
<table>
<tr><td></td><td>PM carrier</td><td>IM carrier</td></tr>
<tr><td>POI vs control</td><td>5.47 (1.72 to 17.38)
p=0.004</td><td>1.01 (0.46 to 2.25)
p=0.98</td></tr>
<tr><td>EM vs control</td><td>2.04 (0.82 to 5.08)
p=0.12</td><td>1.08 (0.74 to 1.58)
p=0.68</td></tr>
<tr><td>POI + EM vs control</td><td>2.45 (1.03 to 5.83)
p=0.04</td><td>1.07 (0.74 to 1.56)
p=0.71</td></tr>
</table></td></tr>
<tr><td>Comments</td><td>Population based on large (n=110,000) UK based prospective cohort study of women over the age of 16. Risk of selection bias as recruitment is based on Breakthrough Breast Cancer volunteers responding to publicity, their family, friends and other contacts. Participants reported to “include substantial members from all sections of society and geographical areas of the UK”

Assessment of age at menopause based on self-report/recall, which could introduce bias into the classifications of POI, EM and controls.

This population based study reports lower ORs for PM than studies based on clinical referrals for POI.

Family history of FXS not reported.</td></tr>
</table>

<table>
<tr><td>Appendix number</td><td>15</td></tr>
<tr><td>Relevant criteria</td><td>2</td></tr>
<tr><td>Publication details</td><td>[11] Nolin SL, Glicksman A, Ding X, et al. Fragile X analysis of 1112 prenatal samples from 1991 to 2010. Prenat Diagn. 2011;31(10):925-31.</td></tr>
<tr><td>Study details</td><td>Retrospective cohort study, USA</td></tr>
<tr><td>Study objectives</td><td>To determine the risk of expansion for normal, IM and PM FMR1 CGG repeats.</td></tr>
<tr><td>Inclusions</td><td>All mothers carried the IM, PM or FM allele.</td></tr>
<tr><td>Exclusions</td><td>NR</td></tr>
<tr><td>Population</td><td>n=1,112 prenatal samples collected from 1991 to 2010 as part of clinical diagnostic analyses.</td></tr>
<tr><td>Intervention/test</td><td>PCR of chorionic villus samples (n=927) and amniotic fluid samples (n=193) or both (n=8); diagnostic confirmation via Southern Blot</td></tr>
<tr><td>Comparator</td><td>NA</td></tr>
<tr><td>Results/outcomes</td><td>
<u>Allele transmission (present in foetuses), number (frequency)</u>
Normal: 558 (1:2*)
IM: 106 (1:10*)
PM: 216 (1:5*)
FM: 232 (1:5*)

<u>Transmission of IM, PM and FM alleles by maternal repeat size</u>
<table>
<tr><th rowspan="2">Maternal repeat size</th><th colspan="5">Foetal outcome</th></tr>
<tr><th># IM</th><th># PM</th><th># FM</th><th>FM</th><th>Total</th></tr>
<tr><td>45-49</td><td>55</td><td>0</td><td>0</td><td>0%</td><td>55</td></tr>
<tr><td>50-54</td><td>45</td><td>5</td><td>0</td><td>0%</td><td>51[a]</td></tr>
<tr><td>55-59</td><td>0</td><td>86</td><td>0</td><td>0%</td><td>86</td></tr>
<tr><td>60-69</td><td>2</td><td>77</td><td>2</td><td>2%</td><td>81</td></tr>
<tr><td>70-79</td><td>2</td><td>30</td><td>15</td><td>32%</td><td>47</td></tr>
<tr><td>80-89</td><td>1</td><td>15</td><td>45</td><td>74%</td><td>61</td></tr>
<tr><td>90-99</td><td>0</td><td>2</td><td>31</td><td>94%</td><td>33</td></tr>
<tr><td>≥100-200</td><td>1</td><td>1</td><td>93</td><td>98%</td><td>95</td></tr>
<tr><td>>200</td><td>0</td><td>0</td><td>46</td><td>100%</td><td>46</td></tr>
<tr><td>Total</td><td>106</td><td>216</td><td>232</td><td>42%</td><td>555</td></tr>
</table>
[a] One allele contracted to normal size
<u>Unstable transmissions (excluding full mutation expansions)</u>
<table>
<tr><td></td><td colspan="3">Foetal outcome</td></tr>
<tr><td>Maternal repeat</td><td>Unstable/total</td><td>Average increase</td><td>Average decrease</td></tr>
</table>
</td></tr>
</table>

	size	transmissions (%)	in repeat size (n)	in repeat size (n)
	45-49	5/55 (9)	1.0 (5)	0 (0)
	50-54	13/51 (26)	5.1 (10)	8.7 (3)
	55-59	37/86 (43)	5.9 (36)	2 (1)
	60-69	60/79 (76)[b]	13.2 (57)	12.3 (3)
	70-79	32/33 (97)[b]	22.4 (30)	20.5 (2)
	80-89	16/16 (100)[b]	29.3 (11)	18.2 (5)
	90-99	0/1 (0)[b]	0	0 (0)
	≥100-200	2/2 (100)[b]	-	68.0 (2)

[b] Excluding full mutation expansions

Foetal allele by maternal repeat size and family history of fragile X

Maternal repeat size	Foetal allele: family history FXS			Foetal allele: no family history		
	Stable PM	Unstable PM	FM (%)	Stable PM	Unstable PM	FM
55-59	0	2	0	35	31	0
60-69	2	2	1	14	48	1
70-79	**0**	**11**	**13**	**1**	**15**	**2**
80-89	**0**	**5**	**35**	**0**	**8**	**4**
90-99	1	2	29	0	0	0
≥100	0	3	86	0	0	2
Total	3	29	164	50	108	9

Comparison of expansion to FM in the 70-79 maternal CGG repeat range:

FXS family history: 13/24 (54%)

No FXS family history: 2/18 (11%)

p=0.0081

Comparison of expansion to FM in the 80-89 maternal CGG repeat range:

FXS family history: 35/40 (88%)

No FXS family history: 4/12 (33%)

p=0.00085

* Reviewer calculated

Comments	All women had IM, PM or FM. Distribution of these alleles unclear based on provided data (information provided only for 555 IM, PM or FM transmissions). Women with a family history tended to carry longer alleles. There was overlap in

	the maternal repeat range of 70-89 CGG repeats, which formed the basis of the expansion comparison.

Appendix number	16
Relevant criteria	2
Publication details	[9] Otsuka S, Sakamoto Y, Siomi H, et al. Fragile X carrier screening and FMR1 allele distribution in the Japanese population. Brain Dev. 2010;32(2):110-4.
Study details	Retrospective study, Japan
Study objectives	To estimate the prevalence of various *FMR1* alleles in a Japanese population.
Inclusions	NR
Exclusions	NR
Population	n=946 control samples (576 male, 370 female) and n=109 samples from autistic individuals (102 male, 7 female)
Intervention/test	PCR hybridization methods
Comparator	NA
Results/outcomes	<u>IM (40 to 54 repeats) prevalence control population, number (frequency)</u> Total: 6 (1:158*) Male: 5 (1:103) Female: 1 (1:324) <u>PM or FM prevalence control population</u> No carriers of PM or FM were detected. <u>Alleles prevalence, autistic population</u> No IM, PM or FM alleles detected
Comments	Control samples recruited via response to public internet invitation to collect healthy control population samples; reported as “self-declaring Japanese control population in Tokyo area with the absence of major illness confirmed by the physician”. Methodology could not amplify repeat regions of 155 alleles; may not completely capture premutation prevalence.

Appendix number	17
Relevant criteria	2
Publication details	[5] Seltzer MM, Baker MW, Hong J, et al. Prevalence of CGG expansions of the FMR1 gene in a US population-based sample. Am J Med Genet B Neuropsychiatr Genet. 2012;159B(5):589-97
Study details	Retrospective cohort study, USA

Study objectives	To estimate the prevalence of IM and PM of the *FMR1* gene in a population based sample of older adults in Wisconsin.
Inclusions	Surviving participants from the Wisconsin Longitudinal Study (WLS, random sample of 10,317 individuals who graduated from Wisconsin high schools in 1957) and their siblings who returned saliva samples in 2006 and 2007 (56% of WLS survivors included).
Exclusions	NR
Population	n=6,747 saliva samples yielding sufficient DNA (3,273 male, 3,474 female) from older adults
Intervention/test	PCR
Comparator	NA
Results/outcomes	<u>Prevalence of IM (45-54 CGG repeats), number (frequency)</u> Males: 78 (1:42, 95% CI 1:54 to 1:34) Females: 98 (1:35, 95% CI 1:44 to 1:29) Overall: 176 (1:38*) <u>Prevalence of IM (41-54 CGG repeats), number (frequency)</u> Males: 157 (1:21, 95% CI 1:25 to 1:18) Females: 272 (1:13, 95% CI 1:15 to 1:11) Overall: 429 (1:16*) <u>Prevalence of PM, number (frequency)</u> Males: 7 (1:468, 95% CI 1:1,628 to 1:252) Females: 23 (1:151, 95% CI 1:249 to 1:105) Overall: 30 (1:225*) <u>Phenotypic characteristics of female PM (n=20) vs normal controls (n=1,893)</u> Age (years) at last menstruation: PM 48.1 vs. Control 50.8, p<0.05 <u>Phenotypic characteristics of male & female PM (n=30) vs normal controls (n=2,800)</u> % reporting experiencing symptoms weekly or more: Aching muscles: PM 43% vs. Control 43%, p=NS Dizziness/faintness: PM 17.9% vs. Control 3.9%; p<0.001 Numbness: PM 28.6% vs. Control 13.3%, p<0.05 Has child with disability: PM 23.3% vs. Control 11.9%, p=0.07

	* Reviewer calculated
Comments	2,632 of the 6,747 samples had a sibling included in the analysis, and siblings' CGG repeat numbers were correlated (*r*=0.45); to account for this dependence CIs for prevalence estimates were calculated using bootstrapped sampling approach. Of the seven PM carriers with a child with a disability, 2 children with ID, one with learning disability, four with mental health disorders (1 major depression, 2 bipolar, 1 substance abuse) Sample consisted mainly of Whites of European descent, may increase applicability to UK population. Prevalence estimates ascertained from a population based sample, and may be lower than estimates from studies assessing clinically referred samples. Phenotypic characteristics ascertained via self report; authors suggest that their population approach reduces likelihood of reporting bias compared to clinically referred participants as individuals are unlikely to be aware of PM status or risk. Due to small sample size, statistical power of phenotype comparison may be limited, and the analyses could not be stratified by sex. All participants were high school graduates; may not be representative of source population.

Appendix number	18
Relevant criteria	2
Publication details	[6] Tassone F, Iong KP, Tong TH, et al. FMR1 CGG allele size and prevalence ascertained through newborn screening in the United States. Genome Med. 2012;4(12):100.
Study details	Cohort study, USA
Study objectives	To determine the prevalence rate of IM alleles and PM of the *FMR1* gene in a population based newborn screening programme
Inclusions	Parental consent for additional FXS screening (beyond state mandated screening)
Exclusions	NR
Population	n=14,207 (7,312 male, 6,895 female) newborns screened in three birthing hospitals, between in November 2008 and May 2012
Intervention/test	PCR based DBS screening with Southern Blot diagnostic confirmation
Comparator	NA
Results/outcomes	<u>IM (45 to 54 repeats) Prevalence, number (frequency)</u> Male: 65 (1:112, 95% CI 1:145 to 1:88) Female: 105 (1:66, 95% CI 1:80 to 1:54) Overall: 170 (1:84*) <u>Expanded IM (40 to 54 repeats) Prevalence, number (frequency)</u>

	Male: NR (1:32, 95% CI 1:37 to 1:28) Female: NR (1:18, 95% CI 1:20 to 1:16) <u>PM Prevalence, number (frequency)</u> Male: 17 (1:430, 95% CI 1:736 to 1:268) Female: 33 (1:209, 95% CI 1:303 to 1:149) Overall: 50 (1:284*) * Reviewer calculated
Comments	

Appendix number	19
Relevant criteria	2
Publication details	[22] Tassone F, Choudhary NS, Tassone F, et al. Identification of expanded alleles of the FMR1 Gene in the CHildhood Autism Risks from Genes and Environment (CHARGE) study. J Autism Dev Disord. 2013;43(3):530-9.
Study details	Cross-sectional, USA
Study objectives	To assess the frequency of IM *FMR1* alleles in a population with developmental or behavioural conditions.
Inclusions	Participation in the CHARGE case control study
Exclusions	Serious disabilities that would impeded the ability to obtain valid scores on developmental tests (e.g. blindness, deafness, serious motor disability)
Population	n=945 children mean age 42-46 months across groups (range 15-72 months); 346 typical development (TD), 313 autism, 144 ASD, 152 developmental delay (DD). TD controls matched on age, sex and broad geographic area.
Intervention/test	NA
Comparator	NA
Results/outcomes	<u>Prevalence of FM, n (%)</u> <u>AUT: 2</u> <u>DD: 2</u> <u>TD, ASD: none detected</u> <u>No statistical comparison</u> <u>Prevalence of PM, n (%)</u> TD, AUT, ASD: none detected

<table>
<tr><td></td><td>DD: 2 (1.4%)

No statistical comparison

<u>Prevalence of IM, % (95% CI)</u>

No significant differences observed between AUT/ASD vs TD or DD vs TD in males or females.

<table>
<tr><td></td><td colspan="2">IM</td></tr>
<tr><td></td><td>Males</td><td>Females</td></tr>
<tr><td>TD</td><td>0.4% (0.0 to 2.1%)</td><td>2.4% (0.7 to 8.3%)</td></tr>
<tr><td>AUT/ASD</td><td>1.3% (0.5 to 2.9%)</td><td>3.2% (0.9 to 11.0%)</td></tr>
<tr><td>DD</td><td>0% (0.0 to 3.8%)</td><td>2.0% (0.1 to 10.3%)</td></tr>
</table>

<u>CGG repeat size, mean (SD)</u>

TD: 30.0 (4.3)

Autism: 30.1 (4.9)

ASD: 29.6 (4.8)

AUT/ASD: 29.9 (4.8)

DD: 29.8 (4.6)

(NB FM and PM alleles excluded from analysis)

<u>Association between IQ and CGG repeat among AUT, AST and DD participants (b=change in test score for each additional repeat)</u>

Vineland score: b=0.01 (95% CI -0.31 to 0.34)

Mullen score: b=0.11 (95% CI -0.27 to 0.50)</td></tr>
<tr><td>Comments</td><td>No differences in prevalence of IM between AUT, ASD and DD patients and controls; no significant differences in CGG repeat length or correlation between repeat length and IQ scores.</td></tr>
</table>

Appendix number	20
Relevant criteria	2
Publication details	[17] Tosh D, Rao KL, Rani HS, et al. Association between fragile X premutation and premature ovarian failure: a case-control study and meta-analysis. Arch Gynecol Obstet. 2014;289(6):1255-62.
Study details	Case control study plus SR and meta-analysis of case controls, India (present study) and various countries (SR)

Study objectives	To assess the association between *FMR1* PM and POF
Inclusions	Present study: NR SR: case-control studies including women diagnosed with POF
Exclusions	Present study: NR SR: studies with randomly selected control samples with no information on reproductive life/fertility; uncontrolled studies; abstracts only, comments, reviews, editorials or letters.
Population	n=289 POF patients attending the Infertility Institute and Research Centre in Hyderabad. POF defined as at least six months amenorrhea before the age of 40, FSH ≥40IU/l. n=360 healthy females with regular menstrual history and successful pregnancies (population based controls) SR: 11 case-control studies (including current study) including 1,313 POF cases and 3,132 controls
Intervention/test	NA
Comparator	NA
Results/outcomes	*FMR1* IM and PM prevalence (present study) 0 IM or PM in cases and controls. OR 1.26 (0.03 to 63.61) *FMR1* PM prevalence (meta-analysis of n=11 studies, 1,313 POF cases and 3,132 controls) OR 5.41 (95% CI 2.53 to 11.61), p<0.001 I^2=0%, p=0.8 Subgroup meta-analysis Asian populations: OR 3.91 (0.73 to 20.74), p=0.11 European descent: OR 6.85 (2.58 to 18.19), p≤0.001 Other ethnicities: OR 3.59 (0.61 to 21.34), p=0.15
Comments	In present case control study, no PM events reported in either case or control group, however, non-significant OR reported. No significant publication bias detected by Begg's and Egger's tests. Murray et al. 2013 included in meta-analysis and current NSC update review.

Appendix number	21

Relevant criteria	2
Publication details	[18] Voorhuis M, Onland-Moret NC, Janse F, et al. The significance of fragile X mental retardation gene 1 CGG repeat sizes in the normal and intermediate range in women with primary ovarian insufficiency. Hum Reprod. 2014;29(7):1585-93.
Study details	Case control study, The Netherlands
Study objectives	To determine whether *FMR1* CGG repeats in the normal and intermediate range is associated with primary ovarian insufficiency (POI)
Inclusions	PI was defined as spontaneous cessation of menses for at least 4 months in women younger than 40 years of age with FSH concentrations exceeding 40IU/l
Exclusions	NR
Population	N=375 phenotyped women with POI and n=3,368 controls with natural menopause at age 40 or later. Cases recruited from two cohort studies, controls from the Prospect EPIC study.
Intervention/test	NA
Comparator	NA
Results/outcomes	<u>IM (45 to 54 repeats) prevalence, number (%)</u> POI: 10 (2.7%) Control: 123 (3.7%) <u>Normal *FMR1* (<45 repeats) prevalence, number (%)</u> POI: 365 (97.3%) Controls 3,245 (96.3%) OR 0.72 (0.38 to 1.39), p=0.38 <u>Regression analysis, age at POI diagnosis and CGG repeat size</u> Beta=-0.018, p=0.74
Comments	

Appendix number	22
Relevant criteria	5
Publication details	[27] Basehore MJ, Marlowe NM, Jones JR, et al. Validation of a screening tool for the rapid and reliable detection of CGG trinucleotide repeat expansions in FMR1. Genet Test Mol Biomarkers. 2012;16(6):465-70.
Country	United States

Population	Samples from patients previously tested for FXS. Cohort 1: n=88 male and female samples, a high percentage of which had expanded alleles. Cohort 2: n=624 female only samples.
Test	Triplet repeat-primed *FMR1* PCR assay and capillary electrophoresis. 55 CGG repeat was set as the threshold for normal vs. expanded *FMR1* alleles.
Comparator/gold standard	Previous diagnostic results from Southern Blot and fluorescent PCR.
Study results / outcomes	*Criterion 5:* **Analytic validity** Sn=100% (95% CI 99.58% to 100%) Sp=100% (95% CI 99.39% to 100%)
Comments	Methods initially described in Lyon et al. 2010 (see Appendix Table 25). Analysis was repeated in different laboratory, with 100% concordance of results. Sampling methods and timing not specified, however, methods suggest that PCR cycles selected to increase robustness for testing from whole blood and dried blood spots (DBS).

Appendix number	23
Relevant criteria	5
Publication details	[31] Curtis-Cioffi KM, Rodrigueiro DA, Rodrigues VC, et al. Comparison between the polymerase chain reaction-based screening and the Southern blot methods for identification of fragile X syndrome. Genet Test Mol Biomarkers. 2012;16(11):1303-8.
Country	Brazil
Population	n=78 DBS samples from 40 males and 38 females, some suspected of having FXS.
Test	PCR-based screening (not further defined)
Comparator/gold standard	Southern Blot.
Study results / outcomes	*Criterion 5:* Conclusive results from 75 of 78 samples (96.2%) **Analytic validity (normal vs. premutation plus full mutation)** Sn=100% Sp=100% Accuracy=100%

	Analytic validity (normal vs. premutation) Sn=88.6%% Sp=100% Accuracy=89.5% **Analytic validity (normal vs. full mutation)** Sn=97.4% Sp=42.9% Accuracy=88.9%
Comments	Reported validity results exclude the three inconclusive PCR results. All three PCR inconclusive results were identified as full mutations in SB analysis. If these three inconclusives are considered negative PCR results then **overall validity figures**: Sn=96.0% Sp=100% Accuracy=96.2% **For normal vs. full mutation:** Sn=90.2 Sp=42.9% Accuracy=83.3% Study reportedly reproduced results of a previous validation study (Tassone F, Pan R, Amiri K, et al. (2008) A rapid polymerase chain reaction-based screening method for identification of all expanded alleles of the fragile X (FMR1) gene in newborn and high-risk populations. J Mol Diagn 10:43–49).

Appendix number	24
Relevant criteria	5
Publication details	[28] Filipovic-Sadic S, Sah S, Chen L, et al. A novel FMR1 PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome. Clin Chem. 2010;56(3):399-408.
Country	United States
Population	n=146 blinded whole blood samples from individuals evaluated at the MIND Institute Clinic
Test	*FMR1* specific PCR and capillary electrophoresis
Comparator/gold standard	Southern Blot.
Study results /	*Criterion 5:*

outcomes	**Analytic validity (full mutation)*** Sn=97.1% Sp=100%
Comments	Samples were enriched for FM *FMR1* alleles. Authors suggest large number of repeats (including >1,000 CGG repeats) were successfully amplified and detected. *Sn and Sp reviewer calculated

Appendix number	25
Relevant criteria	5
Study details	[30] Lyon E, Laver T, Yu P, et al. A simple, high-throughput assay for Fragile X expanded alleles using triple repeat primed PCR and capillary electrophoresis. J Mol Diagn. 2010;12(4):505-11.
Country	United States
Population	n=205 previously characterised whole blood samples.
Test	Triplet repeat primed PCR and capillary electrophoresis, positive results ≥55 CGG repeats.
Comparator/gold standard	Sizing PCR test and Southern blot.
Study results / outcomes	*Criterion 5:* **Analytic validity (premutation plus full mutation)*** Sn=100% Sp=100%
Comments	Test classified normal or intermediate genotype as normal, and premutation, full mutation or mosaic as expanded. Authors' proposed screening strategy uses whole blood or DBS. *Sn and Sp reviewer calculated

Appendix number	26
Relevant criteria	5
Study details	[26] Seneca S, Lissens W, Endels K, et al. Reliable and sensitive detection of fragile X (expanded) alleles in clinical prenatal DNA samples with a fast turnaround time. J Mol Diagn. 2012;14(6):560-8.
Country	Belgium
Population	n=67 blinded clinical prenatal samples (34 male/33 female foetuses), obtained from chorionic villi (n=56) or cultured amniotic cells (n=11). All women were carrying a PM or FM *FMR1* allele.
Test	CGG triplet repeat primed PCR and capillary electrophoresis, with 200 CGG

	repeats as full mutation threshold.
Comparator/gold standard	Southern blot.
Study results / outcomes	*Criterion 5:* **Analytic validity (full mutation)** Sn=97.4% (95% CI 84.9% to 99.9%) Sp=100% (85.0% to 100%)
Comments	CGG repeats ranged from 17 to 1,100. All samples were from women who were carried a *FMR1* PM or FM allele.

Appendix number	27
Relevant criteria	5
Study details	[29] Teo CR, Law HY, Lee CG, et al. Screening for CGG repeat expansion in the FMR1 gene by melting curve analysis of combined 5' and 3' direct triplet-primed PCRs. Clin Chem. 2012;58(3):568-79.
Country	Singapore
Population	n=44 blinded clinical whole blood samples enriched for PM and FM alleles (21 male/23 female).
Test	5'- and 3'-weighted direct triplet repeat primed PCR and capillary electrophoresis plus melting curve analysis (MCA); 85^{o}C cutoff.
Comparator/gold standard	PCR and/or Southern blot.
Study results / outcomes	*Criterion 5:* **Analytic validity, 5' dTP-PCR with 85^{o}C MCA cutoff** ROC AUC=1.000 (p=0.000) Sn=100% Sp=100% **Analytic validity, 3' dTP-PCR with 90^{o}C MCA cutoff** ROC AUC=0.964 (p=0.000) Sn='near 100%' Sp=100%
Comments	Samples enriched for PM and FM alleles.

Appendix number	28
Relevant criteria	10
Publication details	[32] Rueda JR, Ballesteros J, Guillen V, et al. Folic acid for fragile X syndrome.

	Cochrane Database Syst Rev. 2011(5):CD008476.
Study details	Systematic review of randomised controlled trials of folic acid at any dose and by any administration, compared with placebo for any person diagnosed with FXS, healthcare setting not reported
Study objectives	To review the efficacy and safety of folic acid in the treatment of people with FXS.
Inclusions	RCTs that assessed the effect of folic acid treatment (any dose or route of administration) among individuals diagnosed with FXS on psychological and learning capabilities, behaviour or social performance, and adverse effects.
Exclusions	Studies with unreported allocation or randomisation procedures, or treatment periods too short to sufficiently assess relevant outcomes. (Threshold not reported; a study among two inpatients with 8 day treatment period was excluded, although randomisation procedure was also unclear in this study). An additional publication was excluded because it did not provide information on results and on treatment allocation procedures.
Population (Studies)	**Studies** 5 RCTs, published between 1986 and 1992. 4 performed in the USA and 1 in Germany; 4 utilised a cross-over design, and 1 a parallel group design. **Participants** n=67 total, range 5-25. All participants were male aged 1 to 54. Intellectual disability among participants ranged from borderline to severe. Two studies included participants with additional diagnoses of autism or autistic behaviour.
Intervention	All identified studies utilised an oral administration route. Doses included from 10 mg/day (three studies), 15 mg/day (one study) and 250 mg/day (one study). Treatment period ranged from 2 to 8 months. Overall study duration ranged from 2 to 12 months.
Comparator	Placebo (4 studies), control preparation of folic acid at a dose of <0.0015mg/day (1 study).
Review results / outcomes	**Included studies** 5 RCTs (n=67 total, range 5-25) published between 1986 and 1992. 4 performed in the USA and 1 in Germany; 4 utilised a cross-over design, and 1 a parallel group design. Meta-analysis not conducted due to heterogeneity of outcome measures. **Psychological outcomes and learning capabilities – no significant effect** <u>IQ</u> Two studies (n=5 to 25). Assessment tool varied (outcome assessed via Wechsler test [n=2], Leiter test [n=3] and Stanford Binet and Yale Revised Developmental Schedules [n=25]). Suggestive narrative evidence from a small subgroup (n=8) in the larger study that there may have been a significant improvements of IQ among prepubertal males (data NR). <u>General Intelligence</u> One study (n=10), assessed via Coloured Progressive Matrices, MD -1.75 (95% CI -

	17.15 to 13.65, p=0.83). Language Development Two studies (n=21 to 25), outcome assessed via Peabody Picture Vocabulary Test (n=21) (mean score folic acid 55.4 [SD 16.2] vs. placebo 59.2 [SD 20.2]) and Test of Language Development and an apraxia battery (NS difference, data NR). **Behaviour or social performance – no significant effect** General behaviour or social outcomes Two studies (n=6 to 21), assessed via the Vineland Adaptive Behaviour Scale; results presented graphically in one study, numerically in the other (mean score folic acid 51.0 [SD 13.7] vs. placebo 50.9 [SD 15.5]). Hyperactivity One study (n=21), assessed via Conners' Parent and Teaching Rating Scales (mean scores: folic acid 15.55 vs. placebo 13.45, p=NR) and ADD-H Comprehensive Teacher's Rating Scales (severe or moderate hyperactivity rating: folic acid 90% vs. placebo 100%, p=NR). Autism metrics Subgroup of one study (n=16 [of 25 randomised]), assessed via Autism Behaviour Checklist and Childhood Autism Rating Scale, no significant difference across all patients; some evidence that significant effects may be seen in prepubertal boys (n=8). No effect in two studies (n=3 each) with participants with additional diagnosis of autism, assessed via Autistic Descriptors Checklist and Alpern-Ball test for communication skills; no statistical analyses conducted. **Adverse effects (AEs)** No severe AEs reported across studies. One study (n=21)reported minor transient effects during treatment period (e.g. diarrhoea, sleep delays, mood swings)
Comments	**Review authors assessment of study quality and risk of bias:** Insufficient reporting of allocation and blinding make judgements regarding risk of bias (or to reject the possibility of a high risk of bias) difficult. One study (n=25) was determined to have a low risk of bias. Areas in which the risk of bias was determined to be low included attrition bias/incomplete outcome data (all five studies assessed as low risk), and performance bias or detection bias/blinding (3 of five studies at low risk, 2 at unclear risk; all reported as double blinded). Four of five studies were cross-over trials, with potential risk for a carry-over effect. Three studies were determined to have a high risk of bias due to potential carry-over effect. Study duration was too short to detect potentially relevant changes in psychological outcomes and learning capabilities. Poor methodological reporting makes it difficult to assess risk of bias; authors

	note that all trials were published before CONSORT statement, however. Overall, low quality and quantity of evidence (five studies published over 20 years ago, with 67 total patients and insufficient power to detect anything other than very large treatment effects).

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