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Clinical implication of *FMR1* intermediate alleles in a Spanish population

Running title: *FMR1* intermediate alleles in Spain

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Conflict of interest: The authors declare no conflicts of interest.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/cge.13257

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ACKNOWLEDGMENTS

This study was supported by the Instituto de Salud Carlos III (ISCIII) [PI17/01067], co-financed by Fondo Europeo de Desarrollo Regional (FEDER) "una manera de hacer Europa" and Agència de Gestió d'Ajuts Universitaris i de Recerca from the Autonomous Catalan Government [2014 SGR603 and 2017 SGR1134] from the Generalitat de Catalunya. The 'CIBER de Enfermedades Raras' is an initiative of the ISCIII.

AUTHOR CONTRIBUTIONS

MIAM, MM, IM, LRR contributed to the conception and design of the study.

MIAM, PSV, FM, MIT, SIA, AC, OV, BRS, LAPJ, MM contributed to the acquisition and analysis of data.

MIAM, MM, IM, LRR contributed to drafting the text and preparing the figures.

All coauthors reviewed and commented on the manuscript and agree on publication.

ABSTRACT

FMR1 premutation carriers (55-200 CGGs) are at risk of developing Fragile X-associated primary ovarian insufficiency as well as Fragile X-associated tremor/ataxia syndrome. *FMR1* premutation alleles are also associated with a variety of disorders, including psychiatric, developmental, and neurological problems. However, there is major concern regarding clinical implications of smaller CGG expansions known as intermediate alleles (IA) or gray zone alleles (45-54 CGG). Although several studies have hypothesized that IA may be involved in the etiology of *FMR1* premutation associated phenotypes, this association still remains unclear. The aim of this study was to provide new data on the clinical implications of IA. We reviewed a total of 17,011 individuals: 1,142 with primary ovarian insufficiency, 478 with movement disorders, 14,006 with neurodevelopmental disorders and 1,385 controls. Similar IA frequencies were detected in all the cases and controls (cases 1.20% vs. controls 1.39%, p=0.427). When comparing the allelic frequencies of IA e50CGGs, a greater, albeit not statistically significant, number of alleles were detected in all the cohorts of patients. Therefore, IA below 50 CGGs should not be considered as risk factors for *FMR1* premutation associated phenotypes, at least in our population. However, the clinical implication of IA e 50CGGs remains to be further elucidated.

Keywords: *FMR1* intermediate alleles; grey zone; primary ovarian insufficiency; movement disorders; neurodevelopmental disorders; genetic counseling

INTRODUCTION

The *FMR1* gene is associated with significant morbidity. Since the description of the mutation responsible for fragile X syndrome (FXS) in 1991, we have made great progress in the knowledge of this gene and its broad spectrum of clinical implications.

The CGG expansion in the *FMR1* gene was the first disease described caused by a dynamic mutation. In the general population the repeat is between 6-44 CGG, while in FXS patients it is of more than 200 CGG which is considered as a full mutation (FM). This mutation generally leads to methylation of the repeat itself and the promoter region accompanied by silencing of the *FMR1* gene. There are two more classes of alleles. The alleles between 55-200 CGG correspond to premutation alleles (PM) and are present in individuals who may develop different pathologies, the most relevant being Fragile X-associated primary ovarian insufficiency (FXPOI) in females and a progressive neurodegenerative disorder called fragile X-associated tremor/ataxia syndrome (FXTAS) in both males and females (over 50-60 years). Lastly, there is another category of alleles called intermediate alleles (IA) or gray zone alleles. IA are between the normal and premutation ranges and are defined by the American College of Medical Genetics as having between 45 and 54 CGG repeats [1]. Despite population differences, according to a newborn screening in the United States the prevalence of IA is relatively high (1/66 females and 1/112 males) [2]. However, the clinical relevance of these IA alleles has been poorly studied.

Although most IA are stable, some can occasionally expand to a PM in offspring and to a FM after two or three subsequent generations [3]. IA are more prone to expansion

when transmitted through males compared to females [4], and similar to PM the risk of expansion is affected by the AGG interruptions [5]. On the other hand, using highly sensitive quantification, Kenneson and collaborators [6] demonstrated significantly diminished FMRP levels, and a 1.5-fold increase of *FMR1* mRNA which negatively correlated with the repeat number.

While it is clear that the FM involves intellectual disability and autism and the PM involves a wide range of clinical manifestations with reduced penetrance, the clinical implications of IA remain unclear. Several studies have been carried out to determine whether these alleles have clinical repercussions or not. In relation to the frequency of IA in different disorders such as FXPOI, FXTAS, Parkinsonism, cognitive or behavioral disorders, there is controversy regarding whether the frequency is higher compared to the general population [7]. Therefore, further research is needed to fully elucidate the clinical significance of IA. The main objective of this study was to shed light on this issue in order to improve genetic counseling in these individuals.

MATERIAL AND METHODS

Patients

A retrospective review was performed of 15,626 cases recruited from four different genetic departments from around Spain (Table 1). All patients were referred for *FMR1* testing corresponding to unrelated individuals presenting a *FMR1*-associated disorder: 1,142 females with primary ovarian insufficiency (POI), 478 (272 males and 206 females) with movement disorders (MD) and 14,006 (12,319 males and 1,687 females) with neurodevelopmental disorders (NDD). Part of the NDD cohort was previously published in Madrigal and collaborators [8] (Table 1). All POI cases were unrelated cases referred by gynecological units for ovarian failure, infertility and/or early menopause. All the patients with MD were unrelated individuals referred by neurological units for unspecific spinocerebellar ataxia (SCA). Finally, patients with NDDs were referred by pediatricians, psychiatrists and neuropediatricians and included unrelated children with a broad spectrum of cognitive and behavioral phenotypes such as global developmental delay, attention deficit and hyperactivity, intellectual disability and autism spectrum disorders.

All the patients or their parents provided written informed consent for testing and for the use of their phenotypic/clinical and genetic data. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona and was carried out in accordance with the Declaration of Helsinki (2013).

Control population

The population based frequency of *FMR1* alleles was obtained by screening of 1,385 individuals (460 males and 925 females) recruited from the general population (Table 1). Among these, we selected a subset cohort of 232 individuals above 40 years (135 males and 97 females) in order to compare IA frequency from late-onset disorders.

Molecular analysis

DNA extraction from patients and controls was performed from peripheral blood using the Gentra Puregene blood kit (Qiagen) or a similar standard method. Molecular analysis of the *FMR1* CGG repeat locus was performed in all laboratories of the participating centers following the same methods of PCR amplification using fluorescently labeled primers (upon request) and the GC-Rich PCR System (Sigma-Aldrich) or the AmplideX PCR/CE FMR1 Kit (Asuragen). The results of the repeat number from all the laboratories were validated.

Statistical analysis

Statistical analyses were performed using commercially available software (SPSS-PC, version 23.0; SPSS Inc.). The chi-square test of frequencies and Fisher's exact test were applied to the contingency tables in order to compare IA frequencies between control and patient populations. Significance was accepted for exact asymptotic bilateral P-values < 0.05. Bonferroni correction was performed for multiple comparisons.

RESULTS

The allelic and genotypic frequencies of *FMR1* IA alleles are summarized in Table 2.

A total of 223 patients (29 with POI, 7 with SCA and 187 with NDD) and 32 controls from the general population were identified with IAs. Only one female with POI was found to be a carrier of two IA (50 and 53 CGGs).

Similar IA frequencies were detected in all the cases and controls (cases 1.20% *vs* controls 1.39%, p=0.427). In order to adequately compare populations, the IA frequencies of the cohorts with POI and MD were compared with the entire control population and with a subset of the control population over 40 years of age. Regarding the NDD cohort, IA frequencies were compared to those detected in the whole control population. Statistical analysis did not show an excess of IA among any of the populations screened (Table 2). In fact, the frequency of IA carriers was significantly lower on comparing females with NDD and women in the control population (p=0.02), although this difference did not reach significance on applying the Bonferroni correction for multiple comparisons.

On the other hand, a similar distribution was observed between IA harbored by cases and controls, being the most common allele (~25%) 45 CGGs in both groups (Figure 1A). Moreover, using regression analysis we found a negative correlation between the CGG repeat size and the number of individuals carrying a specific allele (Figure 1B and 1C). However, the coefficient of correlation was stronger in controls (R2 = 0.90) than in cases (R2 = 0.63).

Based on this observation, when comparing the allelic frequencies of IAe50 CGGs between cases and controls, we detected higher allelic frequencies in all the cohorts of patients (Table 3). However, statistical analysis did not identify significant differences neither in allelic or genotypic frequencies when comparing cases and controls.

DISCUSSION

Taking into account the high frequency of IA in the general population, it is important to determine their possible effects on different disorders. The description of increased *FMR1* mRNA levels in IA opened the debate regarding a possible clinical effect [9]. We performed a comparative analysis of the *FMR1* CGG repeat locus in a large case-control study of several groups of individuals with *FMR1* premutation associated pathologies. The frequency of IAs in the present study did not significantly differ from that obtained in the control population, suggesting the presence of an IA is not associated with the risk of POI, MD or NDD.

In the last years, clinical and neuropsychological involvement has been associated with individuals carrying IA [9]. To date, 5 cases of FXTAS have been described in carriers of these alleles [10, 11]. In addition, an increased prevalence of IA has been reported in children with NDD [12-14] as well as in adults with POI or Parkinson's disease (PD) [15-19]. Nevertheless, these findings do not agree with those of other authors [e.g. 8, 20, 21]. However, it should be taken into account that the prevalence rates are difficult to compare across studies given the variable definitions of the IA CGG repeat range.

In this study, 15,626 *FMR1* alleles were screened among Caucasian patients diagnosed with MD, POI and NDD, being one of the largest reported up to now. Our results suggest that IA might not confer an increased risk of developing these disorders at least in Spanish populations. These results are consistent with a previous study by our group which included a subset of NDD patients [8].

In the present study the age of the control population may be a limitation. In order to overcome this, we selected controls that were over 40 years of age to compare IA frequencies in late-onset disorders (POI and MD). Overall, we detected 2.31% of IA carriers among our Spanish control population including all males and females. This frequency did not significantly differ from that previously reported by Seltzer and collaborators [22] in 6,747 controls among males and females between 67-78 years in the United States. These authors reported a prevalence rate of 2.6% IA with the defined range of 45–54 repeats [22]. Therefore, our results could be compared with this latter study.

In contrast with our results, some studies have reported an association between POI and IA [23]. However, they are not comparable with our data since the CGG repeat range used was different [15, 16]. In addition, other authors have described an increased frequency of IA in females with occult POI using the same 45-54 CGG repeat size as in our study [24]. Nevertheless, the association between IA and POI has not been replicated by several authors [20, 25, 26].

On the other hand, it has been reported an increased frequency of IA in patients with PD from different populations [17-19]. However, in agreement with our results a previous study by a Spanish group reported similar IA frequencies in 206 PD patients and 227 control subjects [27].

In the last years, there have been more than 15 papers trying to elucidate the implication of IA in clinical disorders. Most support the fact that these alleles do not confer risk at least for the disorders associated to the *FMR1* premutation, but we can not ignore the

reported cases such as patients with FXTAS or POI carrying IA and the molecular characteristics of IA which have a slight elevation of *FMR1* mRNA and reduced FMRP levels, resembling what occurs in PM. If we accept a continuous transition from the normal to PM or FM status and taking into account the reduced penetrance in the *FMR1* premutation associated disorders, a model similar to the polygenic multifactorial inheritance could be considered. The IA could contribute to the development of the disease with less impact than PM alleles. In this model very few patients with IA would develop a disorder. This is in consonance with the results observed in the regression analysis of IA distribution. The present study showed that in the group of patients there were more larger expanded alleles compared to the control population (Figure 1). In fact, when considering the range between 50 to 54 CGGs, we found higher IA frequencies among females with MD and in males with ID compared to sex-matched controls (Table 3). However, these differences were not statistically significant, although a higher number of controls with alleles within this range would be necessary to draw conclusions regarding this subgroup of carriers. Finally, other predisposing factors likely play an additional role in the development of the disease.

In summary, our study provides one of the largest case control studies. Based on the screening of *FMR1* CGG repeats in 15,626 patients, the results of this study do not support the implication of the IA in the appearance of MD, POI or NDD considering the current range of 45-55CGGs. According to these observations, IA below 50 CGGs should not be considered as risk factors for *FMR1* premutation associated phenotypes, at least in our population. However, the clinical implication of IA e 50CGGs remains to

be further elucidated.Genetic counseling should undoubtedly be performed in all IA carriers in order to assess the risk of expansion in the next generations.

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Table 1. Individuals recruited for this study.

	POI	MD		NDD		Controls	
		B	@	B	@	B	@
Hospital Clínic[†] (Barcelona)	508	165	139	5,259	212	460	108
Hospital Cruces‡ (Barakaldo-Bizkaia)	337	77	15	2,496	492	-	-
Hospital La Fe[§] (Valencia)	239	26	50	4,053	744	-	-
Hospital Miguel Servet (Zaragoza)	58	4	2	511	239	-	-
qGenomics Laboratory (Barcelona)	-	-	-	-	-	-	817
TOTAL	**1,142**	**272**	**206**	**12,319**	**1,687**	**460**	**925**

POI: primary ovarian insufficiency; MD: movement disorders; NDD: neurodevelopmental disorders; B: male individuals; @: female individuals

[†]This cohort includes 4,753 male patients with intellectual disabilities and 275 male controls previously published [8].

[‡]This cohort includes 2,142 patients with intellectual disabilities (1,800 males and 342 females) previously published [8].

[§]This cohort includes 1,550 male patients with intellectual disabilities previously published [8].

Table 2. Allelic and genotypic frequencies of *FMR1* intermediate alleles (45-54 CGGs) in 1,142 females with primary ovarian insufficiency (POI), 478 patients with movement disorders (MD), 14,006 patients with neurodevelopmental disorders (NDD) and 1,385 controls from the general population. Exact asymptotic bilateral P-values are shown.

			Patients			**Control population**	
			POI	**MD**	**NDD**	**Whole cohort**	**>40 years[†] (n=232)**
Allelic frequencies		IA (%)	**1.31%** (30/2,284)	**1.02%** (7/684)	**1.19%** (187/15,693)	**1.39%** (32/2,310)	**2.13%** (7/329)
		p-value (rl whole cohort)	P=0.90	P=0.57	P=0.48	-	-
		p-value (rl older cohort)	P=0.22	P=0.16	-	-	-
Genotypic frequencies	Females	IA (%)	**2.54%** (29/1,142)	**2.91%** (6/206)	**1.42%** (24/1,687)	**2.70%** (25/925)	**5.15%** (5/97)
		p-value (rl whole cohort)	P=0.89	P=0.82	P=0.02		
		p-value (rl older cohort)	P=0.18	P=0.34	-		
	Males	IA (%)	NA	**0.37%** (1/272)	**1.32%** (163/12,319)	**1.52%** (7/460)	**1.48%** (2/135)
		p-value (rl whole cohort)	-	P=0.27	P=0.68		

		p-value (rl older cohort)	-	P=0.26	-		

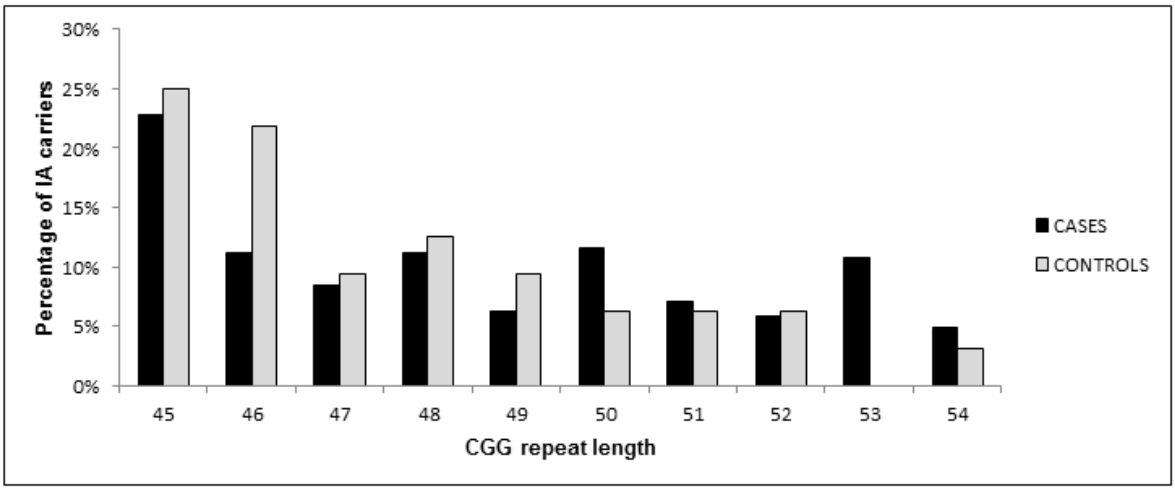
† This cohort is a subset of the global control population recruited. rl: related to

Table 3. Allelic and genotypic frequencies of *FMR1* intermediate alleles e50 CGGs in 1,142 females with primary ovarian insufficiency (POI), 478 patients with movement disorders (MD), 14,006 patients with neurodevelopmental disorders (NDD) and 1,385 controls individuals from the general population. Exact asymptotic bilateral P-values are shown

			Patients			**Control population**
			POI	**MD**	**NDD**	
Allelic frequencies		IA (%)	**0.35%** (8/2,284)	**0.44%** (3/684)	**0.50%** (79/15,693)	**0.30%** (7/2,310)
		p-value	P=0.8	P=0.7	P=0.3	-
Genotypic frequencies	Females	IA (%)	**0.61%** (7/1,142)	**1.46%** (3/206)	**0.30%** (5/1,687)	**0.54%** (5/925)
		p-value	P=0.8	P=0.7	P=1	
	Males	IA (%)	NA	**0%** (0/272)	**0.56%** (69/12,319)	**0.43%** (2/460)
		p-value	-	P=0.5	P=1	

FIGURE LEGENDS

Fig. 1 Distribution of *FMR1* intermediate alleles (45-54 CGGs). Black bars represent the cohort of patients (n=15,626) and grey bars represent the control population (n=1,385). (a) Percentage of cases and controls carriers of intermediate alleles; Regression analysis between the CGG repeat length (X-axis) and the number of cases (b) and controls (c) carriers of intermediate alleles (Y-axis)



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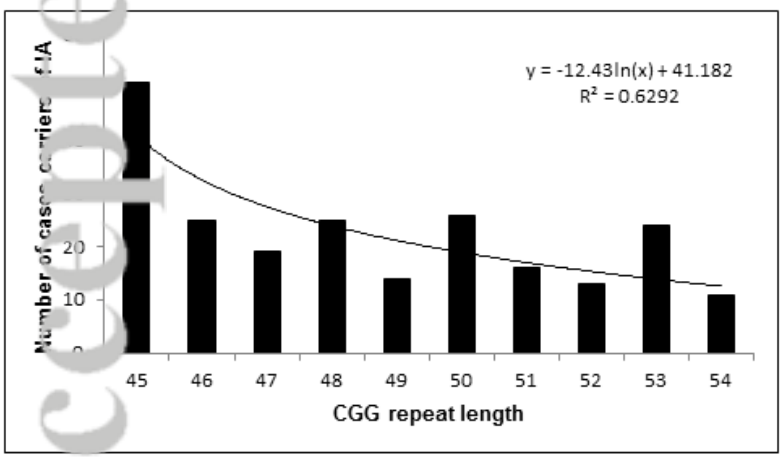

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