



# **FRAGILE X SYNDROME** IN RICAURTE, COLOMBIA

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Flora Tassone - Randi J. Hagerman



Programa ditorial



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Colección Salud  
Research

**Universidad del Valle**  
**Programa Editorial**

Title: *Fragile X syndrome in ricaurte, colombia*  
Author-editor: Wilmar Saldarriaga Gil  
ISBN PDF: 978-958-765-918-4  
Collection: Health - Research

**First english edition**

Traslation: Wilmar Saldarriaga Gil, Claudia Valencia Peña, Randi J. Hagerman.

Rector of the Universidad del Valle: Édgar Varela Barrios  
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Copy editor: Luz Stella Grisales H.  
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Cali, Colombia, november 2018.

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To the inhabitants of Ricaurte, who opened the doors of their homes  
and their hearts with this research work.





## REFLECTION

I can't stop saying that as a child and teenager I heard my father Adolfo Saldarriaga talking with relatives and friends in social gatherings full of hypothesis colloquially supported in the depth of simple observation and popular beliefs of people that lived or visited the Divino Ecce Homo in Ricaurte, and the ones which tried to answer the question: Why are there so many fools in Ricaurte? As a medicine student at the Universidad del Valle, at the end of the 90s I brought that question to the academic field and managed to spread the enthusiasm for the answer to my classmate Cesar Payán (today also my friend), to Carolina Isaza, our genetics professor (today also my mentor), and Alberto Alzate, epidemiologist; and with the technique that we had within reach, the karyotype, we found that what causes the intellectual disability in Ricaurte, was a rare disease with a genetic origin, fragile X syndrome.

Now as a genetics professor and PhD student of the Universidad del Valle, with a battalion of countless students and professors (also the coauthors of this book, of course) and the most important group of investigation in the subject, the MIND institute of the University of California, Davis, we have accomplished not just demonstrate that Ricaurte is a genetic cluster of FXS with highest prevalence in the world and analyze the social issue, but perform an intervention that will surely decrease the number of new FXS cases and will stop the perpetuation of the cluster. Maybe that impact could be quantified just up to three generations and nobody will remember us by then.



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## PROLOGUE

This is an extraordinary book that has grown out of a wonderful collaboration between scientists, clinicians, and advocates at two institutions committed to improving the lives of families affected by neurodevelopmental disorders: the UC Davis MIND Institute in the United States and the Universidad Del Valle in Colombia. The collaboration has been led by Drs. Randi J. Hagerman and Wilmar Saldarriaga Gil, but has involved many dozens of people over a period of three years.

This book presents information about the cutting-edge scientific findings and technologies used to study fragile X syndrome (FXS), the leading cause of inherited intellectual disability and the most common single-gene cause of autism spectrum disorder. What makes the book unique, however, is the application of this scientific knowledge to the team's work in Ricaurte, Colombia. Ricaurte is a small rural town in the Valle del Cauca region, Southwest of Bogota.

As the reader will see, Ricaurte has an unusually high concentration of people affected with FXS, owing no doubt to its geographic isolation and poverty, which have kept its residents in the town for many decades and discouraged new residents from settling there. Thus, there is a strong "founder effect." This high prevalence of FXS is also, unfortunately, accompanied by a lack of appropriate health care and social services to help affected individuals and their families, leaving them to struggle and all but ensuring less than optimal developmental outcomes.

Ricaurte, therefore, possess both a scientific opportunity and a humanitarian challenge. In this book, the authors detail how their collaboration is advancing its scientific understanding and beginning to improve the circumstances of the people of Ricaurte. The book also provides insight into how collaborations can be formed despite barriers of language and distance, when the parties are committed to using science for the betterment of

all citizens. I commend the authors and their teams for all they have done. This book, however, is only a road map of the collaboration thus far. The collaboration will continue as there is still so much work to be done, and I know Drs. Hagerman and Saldarriaga Gill will stay on the path of commitment to Ricaurte.

Leonard Abbeduto, PhD  
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## FRAGILE X SYNDROME

*Wilmar Saldarriaga Gil, Jose Vicente Forero Forero,  
Laura Yuriiko Gonzalez Teshima, Carlos A Fandino Losada,  
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Wilmar Saldarriaga Gil, Jose Vicente Forero Forero, Laura Yuriiko González Teshima, Carlos A. Fandino Losada, Carolina Isaza, Randi Hagerman.

Fragile X syndrome (FXS) is a genetic disease inherited through the X chromosome. It is classified as a trinucleotide repeat disorder and it has a special non-mendelian inheritance pattern. It is generated by a dynamic and abnormal expansion of the Cytosine-Guanine-Guanine (CGG) triplet within the promoter of the FMR1 gene. Xq27.3, which leads to the silencing of the gene when the CGG repeat is over 200 (1). FXS was described by Martin and Bell in 1943 (2) and now it is considered the most common inherited cause of intellectual disability (ID) and it is second most prevalent genetic cause after Down syndrome. It is also considered the most common cause of the autism spectrum disorder (ASD).

The classic phenotype of the affected males includes anxiety, aggression, hyperactivity, large or prominent ears, joint hypermobility and macroorchidism; the phenotype in females is nonspecific, they may have mild intellectual disability, nevertheless between 30% and 50% of them may have normal IQ. Their facial characteristics are similar but less noticeable than in males (1,3,4). Females with FXS are less affected than males because they have two X chromosomes and the activation ratio of the X chromosome (the percentage of cells with the normal X chromosomes as the active X) correlates positively with IQ and negatively with the severity of the phenotype (3).

## GENOMICS

FXS is caused by functional changes in the FMR1 (Fragile X Mental Retardation 1) gene, with a locus at Xq27.3, genomic coordinates; X:147,911,950-147,951,126; it has 17 exons spanning 38 Kb, OMIM \*309550 (5). This gene is characterized by variable numbers of CGG repeats in the 5' untranslated region (UTR5'). Depending on the number of repetitions, four types of alleles are defined with different clinical manifestations: Normal alleles with up to 44 CGG repeats; intermediate or "gray zone" alleles between 45 and 54 repeats, premutation (PM) alleles, between 55 and 200 and full mutation alleles (FM) with more than 200 repeats. Intermediate or gray zone alleles are precursors for PM alleles and these are precursors of FM alleles (6–8).

It has been proposed that the expansion of CGG repeats in FMR1 is associated with an anomalous function of DNA polymerase II enzyme in feminine meiosis, in which this enzyme would continue adhering the CGG triplet to the new DNA chain/strand although the repetitive sequence had already been completed in the template chain (1 this is not the correct reference for this statement). FM expanded alleles typically have epigenetic modifications which lead to the silencing of this gene (9). In male patients with the FM allele all the cytosines in CpG islands in the FMR1 gene promoter go through a process of methylation, acquiring a methyl (-CH<sub>3</sub>) group in carbon 5 of the pyrimidine ring of the cytosine, while there is a complete absence of methylation in healthy males who have a normal CGG repeat number (24,25,26). Likewise, individuals with FXS lose the function of another element within the FMR1 gene, named boundary sequence (methylation limit), found 650 to 800 nucleotides upstream from the repeated region that has methylation. This boundary sequence is responsible for limiting the hypermethylated region of the genome, because of its structural conformation in chromatin, protecting the FMR1 gene promoter from possible methylation (10,11,13). So, the alteration of the number of CGG triplets and its relation with the structural conformation of chromatin in the boundary sequence favors the epigenetic changes that finally lead to silencing the FMR1 gene and preventing the production of the FMR1 protein (FMRP) (14).

Cases of FXS caused by alterations in exons of FMR1 gene, like point mutations, reading frame shifts and deletions have also been described. These point mutations, deletions or duplications in the gene produce changes in FMRP leading to a functional deficit of the protein and the consequent phenotype, and account for less than 1% of FXS cases (5).



FMRP is a RNA binding protein that regulates the stability, subcellular transport and translation of neural mRNAs that codify for proteins involved in the development of synapsis, neural plasticity and brain development (15–17). This protein binds preferably to RNA homopolymers (18) and selectively to subgroups of brain transcripts (19). The lack of this protein alters a wide range of neural mRNAs, increases the synthesis of some neural proteins including matrix metalloproteinase 9 (MMP-9) producing spine dysmorphogenesis joined by an increase of mGluR5 pathway activity and a decrease in the pathway regulated by GABA. These changes produce a clear excitation/inhibition imbalance (Glutamate/GABA), and phenomena present in patients with FXS including ID (intellectual disability), anxiety, hyperactivity, and aggressiveness among others (20, 21). This spine dysmorphogenesis plays an important role in the clinical manifestations of patients affected with FXS, due to the alteration in synaptic connections caused by this imbalance of neurotransmitters (7).

### INHERITANCE PATTERN OF THE FXS

The FXS is inherited through the X chromosome and does not have a classic Mendelian inheritance pattern. It depends on the number of trinucleotide repeats upstream from the promoter of the FMR1 gene (4). Expansion phenomena during the transmission of the maternal X chromosome with the PM producing a rising number of CGG repeats, leading to the inheritance of an allele with the FM (4). The probability of the occurrence of the expansion depends on the number of repetitions of the CGG repeats within the allele with the PM from the progenitor (see Table 1); Therefore, the alleles with 55 up to 59 repeats have a 3.7% probability of expanding to FM and the alleles with 99 or more repeats will expand to FM with a 100% probability (22-24). It is worth saying that this expansion phenomena do not occur within the transmission of the X chromosome with PM when it is inherited from a male to his daughters and it stays as a PM in the daughters. It has been proposed that the spermatic selection is why the PM remains in the offspring (25). Alleles in gray zone with 45 up to 54 repeats are precursors of alleles with PM, this expansion occurs due to both paternal or maternal meiotic instability phenomena and the occurrence probability of the expansion or contractions not clear (22).

Chances of an expansion in the number of CGG triplets decrease if there are AGG anchors for every 9 or 10 CGG repeats; these triplets are named 'AGG interruptions' and are considered protective. See 1.1. Also, males with

FM perform a process of contraction of the number of CGG repeats in spermatogenesis; therefore, all his daughters will be PM carriers (26-28).

The probabilities of males and females with PM or FM of having children with PM or FM will be developed in this chapter under the subtitle “Genetic counseling in FXS”

**Table 1.1. Risk comparison of expansion from PM to FM among carriers considering CGG repetitions and considering AGG interruptions and maternal age**

Range of CGG triplets at the maternal PM	Risk of expansion to FM depending on the CGG repeats only (%)	Risk of expansion to FM depending on CGG repeats and AGG interruptions (%)*		
		0 interruptions AGG	1 interruption AGG	2-3 interruptions AGG
55-59	3,7	0,4-6,5	0,1-2,3	0-0,1
60-69	5,3	1,5-56,6	0,5-30,2	0-2,1
70-79	31,1	22,7-96,1	8,9-89,1	0,5-28,9
80-89	57,8	84,7-99,8	64,8-99,4	8,4-88,4
90-99	80,1	99-100	97,2-100	63,3-99,3
>99	100	99,9-100	99,8-100	97-100

\* The percentage risk increases within the range as the maternal age and the CGG repeats increase.

Table taken from: Saldarriaga W, Tassone F, González-Teshima LY, Forero-Forero JV, Ayala-Zapata S, Hagerman R. Síndrome de X Frágil. Colomb Med. 2014;45(4):190-8.

## PREVALENCE

Prevalence of FXS in the general population is 1 in 5000 males and 1 in 2500 to 8000 females (29-31). The estimate of FXS prevalence has varied depending on the populations, on factors like the studied population and the diagnostic tests used. Prevalence studies have been conducted in groups of children or young people with ID, extrapolating the frequency to the general population, individuals with or without family background of FXS, pregnant females, couples in a preconception appointment, general population or screening in newborns. The estimate of prevalence is also affected by the diagnostic test used in the studies, karyotype with folate deficit, PCR (Polymerase Chain Reaction) with one, two or three primers, Q-MSP (Quantitative Methylation-specific PCR), southern blot or combinations of them. In addition, as these tests were developed and the counting of CGG repeats in FMR1 was established, the identification of its four allelic variants, normal, GZ, PM and FM were achieved; allowing the documentation of their prevalence (32). As studied populations become comparable and the sensitivity

and specificity of diagnostic tests improve the estimated prevalence gets closer to the real prevalence of FXS.

Other factors that influence the estimation of the FXS prevalence are the poor identification of the phenotype, especially in females, by health personnel and the limited accessibility to molecular test for the diagnosis, especially in developing countries. In Colombia FXS prevalence has not been established, and neither has that of the allelic variants of FMR1 although genetic clusters have been identified (Saldarriaga et al 2018 J med Genetics).

Hunter et al 2014, conducted a meta-analysis of the prevalence of FXS where they approached the issue through an analysis of fixed and random effects in which they group the studies according to the characteristics of the included population samples. From 5582 identified studies, they selected 54 articles (none of them made in Latin America), which approached FXS epidemiology using PCR and/or Southern Blot for the diagnosis.

They analyze prevalence data of two groups of studies, one named total population in which they included individuals with and without intellectual disability (mainly screenings in newborns and pregnant females) and another group named normal population in which the studies included individuals without intellectual disability (mainly screenings in females without intellectual disability and studies that sought to establish PM prevalence).

In the first group, in the reviewed studies they found 78,104 evaluated males and 14 with FM, 75,539 females evaluated and 9 with FM; they obtained a prevalence of 1.4 in 10,000 males (95% CI: 0.1-3.1) and 0.9 in 10,000 females (95% CI: 0.0-2.9), 1: 7,143 and 1: 11,111 respectively, for FM. Likewise, in this group they found 45,253 evaluated males and 63 with PM, 88,673 evaluated females and 539 with PM; they reported a prevalence of the PM of 11.7 in 10,000 males (95% CI: 6.0-18.7) and 34.4 in 10,000 females (95% CI: 6.3-83.3), 1:855 and 1:291 respectively. For the second group they found 42647 evaluated females and 237 with the PM, the prevalence calculation of the PM was of 34.4 in 10,000 females (95% CI: 8.9-60.3), which is equivalent to 1:291, result equal to the estimated in the total population group (33).

Prevalence of GZ, determined by the number of CGG repeats between 45 and 54, and using variants of PCR as diagnostic test, has been established in several studies with differences in the results.

Tassone and collaborators in 2012 reported a screening in 14,207 newborns in 3 hospitals of the United States of America USA, where 1 in 112 males and 1 in 66 females were in GZ. In 2005 Cronister and collaborators

reported 29,103 pregnant females finding that 1 in 143 were in GZ (28). In Australia, Metcalfe and collaborators studied 21,411 not pregnant females and found that 1 in 86 were in GZ (34). In Spain variable results have been found among studies conducted in newborns in that country. Fernández and collaborators in 2008 reported that out of 5267 males studied 1 in 26 had GZ (35); while Rife and collaborators in 5000 males reported that 1 in 4449? (This is too high) had GZ (36). In the mentioned studies we find that observed prevalence for GZ oscillates in females between 1 in 66 to 143; and in males between 1 in 26 to 449. Variations in the prevalence of GZ found by different studies could be explained by the different ethnic groups studied and variants of the molecular test used by every research group (37).

### FENOTIPO DEL SXF

The phenotype of FXS patients is of complete penetrance in males and incomplete and of variable expressivity in females. This will depend on the amount of FMRP being produced, according to the number of triplets and the methylation degree that FMR1 presents in each one of the patients (3). The phenotypic spectrum will then be wide, varying from a mild emotional affectation and normal IQ in females, when FMRP levels are not low; to a severe cognitive impairment and pronounced physical characteristics usually in males and less frequently in females, as the production of this protein decreases or completely disappears (4).

Among the most frequent clinical findings are mild to moderate intellectual disability (ID), autistic spectrum disorder, long face, prognathism, large and prominent ears, hyper-extensible finger joints, macroorchidism, language deficit, hyperactivity, anxiety and aggression, See table 1.2;

Macroorchidism allows distinguishing patients with FXS after adolescence from patients with ID not associated with FXS (4,5).

Females with FM and FXS present a phenotypic range much wider than that of males, according to the activation ratio. females can present from ID and typical physical characteristics of FXS (see Table 1.2) to the absence of these, joined by a mild learning issue. 70% of females with a FM have a borderline or lower IQ. The remaining 30% present a normal IQ; nevertheless, they will generally have emotional or learning difficulties (4).

**Table 1.2. Clinical characteristics of patients with FM and Fragile X Syndrome**

Characteristics	Male	Female*	Figures
Facial	Large and prominent ears (75-78%)	Large and prominent ears (75-78%)	1.1
	Long face	Long face	1.2 A
	Mandibular prognathism (80% adult males)	Mandibular prognathism	1.2 B1, 1.2 B2
	Cleft palate	Prominent forehead	
	Macrocephaly		1.2 C, 1.2 D
Ophthalmologic	Strabismus (8%)		1.2 E
	Refractive errors		
Inner ear	Conductive hearing loss due to the high recurrence of ear infection		
Neurologic	Seizures (16%)		1.3
	Hypotonia (children)		
	Clonus (adults)		
	Positive palmomentary reflex		
Psychiatric	Poor eye contact		1.3
	Attention deficit hyperactivity disorder in 90%	ADHD in 30%	
	Anxiety	Shyness and anxiety	
	Repetitive motor behavior		
	Autistic characteristics (ASD) in 60%	ASD in 30%	
	Aggression and distress crisis		
Development	ID in 80% - ID in 30%		
	Cognitive and language deficit (last one repetitive)		
Orthopedic	Flat feet		
	Hyper-extensibility in the metacarpophalangeal joint		
	Scoliosis in 20%		
Thorax	Double jointed thumbs		
	Pectus excavatum		
Genitourinary	Macroorchidism (95% of adult males)		1.4
Cardiovascular	Cardiac abnormalities (mitral valve prolapse)		
Other	Obesity, cramped teeth, tall or short stature		1.5A, 1.5B, 1.5C, 1.6
* The majority of the phenotypic characteristics have been described in males with FXS, females typically have similar features although often less severe.			



Figure 1.1 Large and prominent ears typical in FXS



(A) Long face. (B1, B2) Mandibular prognathism. (C, D) Macrocephaly. (E) Strabismus.

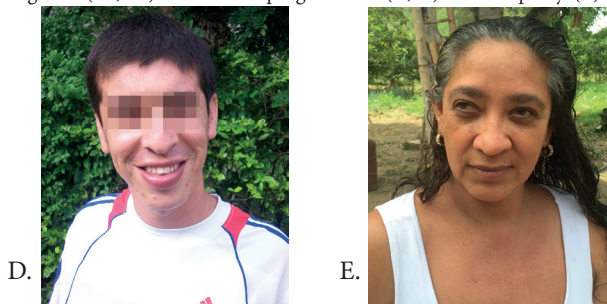


Figure 1.2. Typical facial characteristics in FXS





*Figure 1.3. Scars secondary to acts of self-injury in a patient with FXS*



*Figure 1.4. Macroorchidism. Large testicles in male affected by FXS*



*Figure 1.5 Obesity in patients with FXS*



Figure 1.6. Tall stature of a patient with FXS

### Differential diagnosis

Differential diagnosis of FXS includes Sotos, Prader-Willi (38), Klinefelter and FRAXE syndromes, phenotypic characteristics and molecular tests of these syndromes compared with FXS are seen in the table 1.3.

There have also been described individuals with ID and chromosomal fragility in two other fragile sites (FRAXD and FRAXF).

Tabla 1.3 Diagnósticos diferenciales del síndrome de X frágil (21,38,39)

Syndromes	Mental retardation	Stature*	Cephalic perimeter*	Testicular size*	Weight*	Diagnostic tests
Fragile X syndrome	Mild to moderate	+++	++	+++	++/+++	PCR, Southern Blot
Sotos syndrome	Mild to moderate learning problems	+++	+++	++	++	Sequencing and/or FISH gene NSD1
Prader-Willi syndrome	Mild to moderate	+	++	+	+++	FISH 15q11.2-q13; methylation Studies or microarray
Klinefelter syndrome	Absent or mild	+++	++	+	++/+++	Karyotype

\* The following conventions are used:

+ Smaller size than general population

++ Similar size to general population

+++ Bigger size than general population

Table taken from: Saldarriaga et al. (8).



## DIAGNOSIS

Before the identification of the FMR1 gene in 1991 (25), the diagnosis of FXS was made through cytogenetic techniques such as karyotype using cell cultures with a folate-deficient medium. In light microscopy a narrowing of the distal end of the long arm of chromosome X was visualized in the band 27.3 (Xq27.3-23.8), sometimes the distal constriction is so subtle that it is not observed in the karyotype and only fragments of independent DNA are found, which are called satellites and are observed as two points distal to the long arm of the X chromosome, given that these findings are known as fragile points, the name of Fragile X Syndrome was generated (4, 37).

However, in patients with FM the observation of fragile points was only found in a percentage of the cells, reaching in males at least 10% of the metaphases, but in females the fragile points could not be seen and in some cases fragile sites were observed in several chromosomes including the X chromosome without a correlation with a specific phenotype; thus, the karyotype has limited sensitivity and specificity to diagnose cases of FXS especially in females (41). This technique also fails to diagnose carriers of the PM (37).

At present, molecular tests are more sensitive and specific than the karyotype for the identification of the different allelic variants and methylation status of FMR1. They are based on PCR and Southern blot; these are usually used to confirm the clinical suspicion of those affected by FXS and carriers of the PM (35,37). Also, there are molecular tests that allow quantifying messenger RNA (mRNA) and the amount of the protein (FMRP) that the affected gene encodes, which are conducted for a better understanding of the pathophysiology of the disease when correlating them with the phenotype of patients with the FM and the PM. These studies, mRNA and FMRP are not easily accessible because they are usually expensive and are carried out in research laboratories (1, 37, 42, and 43).

### **Polymerase chain reaction (PCR)**

Since the relationship between the CGG repeats and its methylation status with the FXS phenotype was found, different PCR approaches have been used for the quantification of CGG repeats and the diagnosis of the intermediate alleles or GZ (45-54), PM (55-200) and FM (> 200) in FMR1 (37) (43).

At first, with the use of PCR that uses a specific primer for FMR1, allowing the amplification of the sequence downstream (5' → 3') of the promoter that contains the region with the expansion of the triplets, and the use of agarose or polyacrylamide gel, alleles with more than 200 CGGs would make the diagnosis of FM. However, it did not establish differences between

normal, intermediate or PM alleles, nor did it quantify the number of triplets (4, 37, 44, 45).

In the evolution of PCR in the diagnosis of FXS, two primers flanking FMR1 were used downstream (5' → 3') and upstream (3' → 5'); DNA amplified and analyzed through capillary electrophoresis produce a peak visualization depending on the number of base pairs that the patient under study has. This technique would have low sensitivity to identify FM cases with over 500 repeats especially in females. Therefore, through this test it is possible to quantify the repeats, but some cases of FM could remain without a diagnosis (31, 37, 44).

Currently, the method called TP-PCR (triplet repeat-primed PCR) is recommended for the differentiation of each one of the alleles of FMR1, because its low cost, and the possibility of being used in DNA extracted of blood drops on filter paper, and therefore recommended for screening in different population groups but mainly in newborns; This test uses three primers, the first two mentioned earlier and a third chimeric primer of the region of the CGG repeats producing a series of peaks which lead to specific patterns in images produced by capillary electrophoresis (CE) and that allows the differential diagnosis of the allelic variants of FMR1 (45, 46). Nevertheless, this technique does not tell us if the DNA is methylated or not, and in some cases, it is hard to distinguish between alleles in range of PM and FM, and establish the number of triplets; Therefore, it has been suggested that when expanded alleles have been identify through TR-PCR, then another technique called Southern Blot should be used (37, 44).

### **Southern blot**

The Southern Blot technique is the gold standard test for the FXS diagnosis. In this technique, two restriction enzymes are used over the extracted DNA of the patient for the analysis of the allelic variants of FMR1. Among the most frequently used enzymes are ECOR1 and Nru1, which produce cuts in specific locations of the X chromosome, the first one in the methylated DNA and the second one in the non-methylated DNA. The fragmented DNA migrates -stimulated by electrophoresis- in agarose gel and it is transferred to a nylon membrane to which it gets attached stimulated by ultraviolet light. Then the membrane incubates with a radioactive probe that is specific for FMR1 and that hybridizes with the previously digested DNA and added to the membrane DNA of the patient. The most commonly used probe is called "tB12.13", and then the excess of non-hybridized probes of the nylon membrane is removed. Then, the positions of the hybridized probes that persist on the nylon membrane are observed through autoradiography

with an X-Ray film. Thus, specific patterns will be observed in the autoradiography according to the genre and the allelic variant found. (47, 48).

The Southern Blot technique used on the diagnosis of FXS can quantify the triplet repeats, differentiate all the allelic variants of FMR1, and quantify the triplets in cases of extensive repeats. Moreover, its main advantage is that it can be used for detecting the status of the methylation of the CpG islands. Nevertheless, in comparison with the RT-PCR, the process takes longer, and it needs more DNA so that the sample for the DNA extraction must be obtained from -at least- 3cm<sup>3</sup> of blood, and it is more expensive. For these reasons, the Southern Blot is considered as a confirmatory test of the results of RT-PCR in PM range and FM. The combination of both tests reaches a sensitivity and specificity of 99% and 100% respectively. That combination is the one used currently by most of the reference laboratories and it has been suggested for screening programs (37).

#### **MANAGEMENT OF PATIENTS AFFECTED WITH FRAGILE X SYNDROME**

The management of patients affected with FXS is based on an integral intervention that includes schools specialized in the education of children with particular learning needs and in adults consists of vocational training. Specific therapies that are helpful for children and adults includes occupational therapy which will handle the sensory and occupational needs of the patient;; physical therapy -focusing on fine and gross motor skills- and language therapy for both receptive and receptive language deficits. Medication is oriented to improve specific symptoms. For instance, in patients with convulsions the first option will be carbamazepine and the second will be valproic acid; Phenytoin and phenobarbital must be avoided. In those who have sleep disorders the use of melatonin and clonidine has shown beneficial effects. To control ADHD or hyperactivity the use of guanfacine can be helpful and efficacy has been demonstrated in the use of stimulants, specifically methylphenidate. To treat anxiety the use of selective serotonin reuptake inhibitors (SSRIs) such as sertraline or fluoxetine has been helpful. Low dose sertraline (2.5 to 5.0 mg) has also demonstrated efficacy in improving multiple aspects of development in children with FXS as young as 2 years of age (Greiss-Hess et al 2016 JDBP) (50). The use of targeted treatments that may help to reverse the neurobiological changes in the brain due to the lack of FMRP have included minocycline which can improve synaptic connections and behavior but it can also improve loose connective tissue such as joint dislocations (49). Metformin is a new targeted treatment that can reduce obesity which is often a problem in adult patients with FXS

but it appears to be helpful in development and language and behavior in children and adults with FXS (Dy et al 2017 Clinical Genetics). Cocktails of antioxidant and vitamins C, D and E have been used with no definitive conclusions (49).

### **DISORDERS ASSOCIATED WITH THE FMR1 PM**

FMR1 PM carriers with 55 up to 200 CGG repeats can develop a variable phenotype with incomplete penetrance which is noticeably different from the phenotype of the ones affected by the FM. The pathologies found in carriers have been named the fragile X-associated PM disorders. The best known ones are fragile X-associated tremor/ataxia syndrome (FXTAS) and the fragile X-associated primary ovarian insufficiency (FXPOI) (51). Also, adults with and without FXTAS and FXPOI are more at risk than the general population of developing affective disorders, depression, anxiety, obsessive-compulsive disorder (OCD), sleep disorders, sleep apnea, neuropathies, psychiatric disorders, hypertension, migraine, fibromyalgia, and thyroid disorders (52-58). Likewise, in early childhood anxiety, attention deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD) and seizures commonly occur (53, 59).

#### **Fragile X associated tremor/ataxia syndrome**

FXTAS is a progressive neurodegenerative disorder characterized by neurologic deficits that include progressive intention tremor, cerebellar ataxia, Parkinsonism, neuropathy, autonomic dysfunction and cognitive deficit. These neurologic problems affect 40% of the PM carrier males by the age of 60, 75% of the carrier males by the age of 80 and 20% of the females. In the general population the estimated prevalence of FXTAS is 1 in 4000 for males after the age of 55 and 1 in 7800 in females. (53, 60).

In FXTAS the alteration within FMR1 does not silence the gene expression and on the contrary, it produces an overexpression causing an increase in between 2 and 8 times higher than the usual quantity of FMR1-mRNA. (51, 53, 61). The excess of mRNA is thought to be responsible for the neurotoxicity induced by the increase of CGG repeats within the mRNA which also leads to late-onset neurodegenerative disorders (61, 62).

Different models of toxicity by the excess of mRNA have been proposed, the main one takes the myotonic dystrophy model as reference, in which the mRNA that contains the excess of the CGG triplet sequesters multiple binding proteins in the cytoplasm of neurons and astrocytes, among them is the protein complex Droscha-DGCR8 which is a mediator for micro-RNA

(miRNA) production., Therefore several miRNAs are decreased in those with FXTAS and this may lead to immune dysfunction seen in carriers and also may participate in the neurotoxicity of FXTAS. (53, 63). Another model proposes that the mRNA with CGG excess would be translated including the region with CGG triplets and this would produce the FMRpolyG peptide which is toxic to neurons. In those affected with FXTAS inclusions are formed that are ubiquitin positive and tau negative and these inclusions can be found in neurons throughout the CNS and also in the peripheral nervous system (PNS) in addition to heart muscle, the heart conduction system, pericardial ganglia, adrenals, kidneys, thyroid gland and islets of Langerhans. Inclusions have also been found in the ovarian stroma cells of a PM carrier with primary ovarian insufficiency (POI) (53, 61, 63).

A model apart from the one that points at the excess mRNA as the responsible pathology has been proposed as well. This model states that there is a secondary damage -aside from the hairpin structure formation- that takes place at the transcription within the DNA at the region of the CGG repeats. Thus, DNA repair mechanisms are activated but when they are produced incorrectly they cause cell death. A phosphorylated variant of the H2AX histone that takes part in the DNA damage repair has been observed in inclusions of patients with FXTAS (64, 65). Nevertheless, this model is the one with the least evidence (53).

Those affected by FXTAS have neurologic deficits that include progressive intention tremor, and cerebellar ataxia, which are the two major clinical criteria for its diagnosis (53, 61, 66-69). Other signs within the patients and that appear before the beginnings of the major clinical criteria are neuropathy, vestibular dysfunction which generates dizziness, vertigo, tinnitus, and auditory deficits (70-72) and eventually cognitive decline. Signs shared with Parkinson disease (PD) are tremor and ataxia plus bradykinesia, resting tremor, anxiety, mood alterations, and cognitive disorders. In some patients who are PM carriers the differentiation of the diagnosis is achieved through the findings in the MRI or PET scanning for PD. However, approximately 50% of those with FXTAS can have parkinsonian symptoms and both FXTAS and PD can coexist.

Additional features of FXTAS include: short-term memory problems of moderate-severe intensity, decreased volume of hippocampus which has been reported in both males and females (51); executive function deficits, anxiety, depression, irritability, poor social skills, psychiatric disorders such as dementia (56, 74) and parkinsonism (61, 75). Nevertheless, it is important to highlight that the FXTAS is not associated with ID as in those with FXS. Patients have a normal IQ and, in most cases, they preserve verbal

comprehension and language despite the motor symptoms. The dementia and the prominent executive dysfunction which leads to a working memory deficits and learning disabilities, as well as troubles with understanding the time sequence of events, and the slow information processing speed alters the quality of life and independence of those affected with FXTAS (69).

The beginning of the tremor and ataxia usually occurs by the age of 62, afterwards there is a motor and neurologic deterioration. Ataxia usually follows the initial symptoms of tremor by 2 years followed 6 years later by an increase in the fall frequency and 15 years later mobility aids are needed (Leehey et al 2007). Finally, 21 years after the beginning of the symptoms it is expected that the patient dies in absolute disability / total permanent disability (TPD), prostrate in bed, dysarthric, dysphagic, with total absence of sphincter control, and severe Parkinsonism. So, life expectancy among people who develop this syndrome is 5-25 years, depending on the conditions of each case (76).

FXTAS diagnosis also requires the evidence of major and minor radiologic findings in the MRI of at least 2 Teslas (T2). The major radiologic criterion is hyperintensity of the middle cerebellar peduncle (MCP) observed by MRI. This finding is characteristic but not pathognomonic (68) of FXTAS and it is usually an indicator of a prognosis of something serious as, for example, greater cognitive deficit. It is present on 60% of males and 13% of females with FXTAS; Minor radiologic criteria are; White matter hyperintensity in different zones of the CNS including the pons, insula, splenium of the corpus callosum and the periventricular regions.

Diagnosis of FXTAS is done with the summation of major and minor clinical and radiologic criteria. For a definitive diagnosis it is necessary to have one major clinical criterion and one major radiologic criterion, nevertheless, the presence of typical FXTAS intranuclear inclusions in neurons and astrocytes is criterion recently included for neuropathological diagnosis. (61, 66). The presence of major radiological criterion accompanied by a minor clinic criterion or two major clinic criteria makes up a probable FXTAS diagnosis. Finally, a possible FXTAS diagnosis is built from the presence of one minor radiologic criterion and one major clinical criterion (61, 69). Therefore, in patients with a PM, the diagnosis of FXTAS requires a detailed neurological workup including an MRI, although such a diagnosis is likely if an older carrier has both tremor and ataxia.

Exposure to environmental toxins as general anesthesia, chemotherapy, agent orange, chronic opioid intake, and alcohol abuse, have been reported in patients with appearance of FXTAS symptoms at an earlier age or with deterioration of the clinic condition (78). Opioids and alcohol abuse have



been associated with cerebral atrophy and white matter disease and faster progression of FXTAS (79).

The management of patients with FXTAS is based on two parameters; A lifestyle change to prevent and control symptoms, and an individualized pharmaceutical management of the symptoms of each patient. General recommendations include the elimination of toxins such as drugs of abuse and alcohol, perform aerobic exercises regularly, improve the nutritional status including adding antioxidants to the diet, ingest zinc, and avoid vitamin D or other vitamin deficiency. Treatment of sleep apnea to avoid hypoxia, treatment of hypertension, depression, stress, anxiety and hypothyroidism is important. A variety of medications can help the tremor such as primidone or a beta blocker as detailed elsewhere (Tassone and Hall editors 2016 of FXTAS book).

In the treatment of FXTAS, different intervention strategies, and constant and detailed monitoring of the progression of the clinical neurologic and psychiatric manifestations should be done. It is essential to supply an interdisciplinary medical care with different specialists that allow the patient to be functional and independent (67) and provide genetic counseling to the patient and his family to make the comprehension of this process easier for them and so making the family members aware of their risk of suffering this type of syndromes (67, 68).

### **Fragile X associated primary ovarian insufficiency (FXPOI)**

Fragile X-associated primary ovarian insufficiency (FXPOI) is one of the most important disorders observed in PM females (51) (85). About the 20% of the females who are PM carriers have a disappearance of the menstrual bleeding for one year before the age of 40, which is an indicator for the diagnosis of FXPOI. This frequency is 20 times higher in comparison with the general population, in which POI is present in the 1% of females. (26, 86). In addition, it has been found that the frequency of POI in PM carriers of all ages with which studies have been done is above normal. These results were; 1.4% at 18 years old, and 3% at 29 years old (the frequency in the general population of this age is 1 in 1000). Also, PM carriers have menopause earlier than normal after the age of 40 (87). Other signs of ovarian alterations in reproductive age as menstrual cycle disorders, oligomenorrheas, mild but persistent metrorrhagias, and decrease of the reproduction have been described as well. The consultation frequency of females who are PM carriers is higher than normal; 3% in PM carriers and between 1 in 150 to 1 in 250 in general population of females (88).

It has been demonstrated that up to 7% of the POI cases have a genetic cause and among the genes involved are the FMR1, FSHR, LHR, ATM, AIRE, GDF9, FSH  $\beta$ , PF1 B, and others. The alterations are related to the regulation of the hypothalamus-hypophyseal-ovarian axis, the number and response of FSH and LH receptors within the granulosa cells, the regulation of the oogenesis, and the coordination of the evolution of the germ cell to posterior stages, and systemic endocrine functions (88, 90). The most frequent cause of genetic POI in females, in whom Turner syndrome was dismissed, was the PM, whereby molecular diagnostics for detecting the PM are indicated in the protocols for POI etiologic diagnosis.

The ovarian failure in carriers of the PM is joined by the alteration of hormone production with high amounts of FSH ( $> 40$  UI/L) and low estradiol levels ( $< 50$  pg/ml). FSH/Estradiol correlation greater than 20 and diminished levels of anti-Müllerian hormone are the main markers for suspecting and/or confirming the ovarian failure (88, 91, and 92). The ovarian dysfunction will lead to consequences as the reduction of the probability of a successful pregnancy and the effects of the decreased levels of estrogen, just as in females without the PM. Early onset osteoporosis, increase in fractures, depression, anxiety, psychologic problems, among others, have been reported as well (87).

Management of FXPOI must be individualized and integrated following usual protocols for females with early menopause. Changes in lifestyle must be done; quitting smoking, exercising more, improving nutritional habits including vitamin D and calcium on the diet, among others. Hormone replacement therapy (HRT), medication for depression disorders, as well as for anxiety and osteoporosis treatment, must be individualized. In case of fertility problems, fertility treatments -with previous genetic counseling- have shown good results. Ovulation induction, assisted reproductive technology with pre and post implantation genetic diagnosis (PGD) have been successfully done (87, 88).

Despite of all the advances in the knowledge of FXPOI, no pathophysiological explanation has been found for the occurrence of FXPOI in PM carriers (87, 93). The hypothesis based on the FXTAS models in which increased levels of mRNA could alter the hypothalamus-hypophysis-gonads axis or the ovary directly is one possibility and the granulosa cells may be the most vulnerable to RNA toxicity. (94). However recent studies have found the FMRpolyG peptide in the ovary and this can also play an important role in toxicity. (95).



## SUMMARY

Both the PM and the FM affect many family members with a variety of symptoms in a family once a proband is diagnosed. Therefore, it is important for the physician to consider the whole family tree in the workup of individuals affected by these mutations and cascade testing for FMR1 mutations through the generations is essential for those at risk to be affected by the PM or the FM. Because of the myriad of problems that can be manifested by multiple patients, the primary health care provider needs to involve many specialists for a wide range of ages of affected individuals.

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## TREATMENT IN THE FRAGILE X SYNDROME

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Young children who are diagnosed with fragile X syndrome (FXS) require multi-modality interventions, including speech and language therapy, occupational therapy, special education support to enhance academic learning and the utilization of medications to improve behavior and cognition (1), see table 2.1. The National Fragile X Foundation website ([www.fragileX.org](http://www.fragileX.org)) has a variety of educational materials in both English and Spanish.

The medical intervention for those with fragile X syndrome include treatment of recurrent otitis media infections which are common in young children in the first three years of life. Antibiotics and typically the placement of pressure equalizing (PE), tubes can be beneficial to normalize hearing and decrease infections. Usually these recurrent ear infections continue for the first 3-4 years of life and then they are less common or disappear. Treatment of gastrointestinal (GI) reflux which is sometimes associated with recurrent vomiting early in childhood often will require an anti-reflux medication or the thickening of foods. Obstructive sleep apnea is an occasional problem in young children with fragile X syndrome and it is associated with snoring and obstruction at night. Typically, treatment with an adenoidectomy will resolve this problem (2).

Strabismus can be seen in up to 25% of children with FXS. This requires an ophthalmological evaluation and then treatment either with lenses or patching or perhaps surgery to release extra ocular muscles. Sleep disturbances are very common in the first 2-3 years of life and they include frequent awakenings or even getting up in the middle of the night and this can lead to sleep disorders in the parents. It is best treated with melatonin (1 to 3 mg at bedtime) which has been shown to improve sleep in those with FXS or

autism (3). Seizures occur in 10-20% of young children with fragile X syndrome and they usually emerge in the first five years of life. They can include staring spells, partial motor seizures or grand mal seizures (4). The seizures usually respond well to an anticonvulsant such as valproic acid, carbamazepine or lamotrigine; and these can also be helpful in stabilizing mood and improving aggression. Some occasions may need a second anticonvulsant to control seizures. It is important to treat seizures early because recurrent seizures are known to worsen the symptoms of autism (2).

### **PSYCHOTROPIC MEDICATION**

A variety of behavior problems occur in individuals with fragile X syndrome including hyperactivity along with a short attention span and impulsivity in addition to anxiety. Autism Spectrum Disorder (ASD) occurs in approximately 60% of young boys with FM and approximately 20% of girls with FM (4,5).

Anxiety can often be associated with autism, such that the greater the anxiety the greater the severity of ASD (6). Significant anxiety and ASD necessitates the use of medications such as selective serotonin reuptake inhibitors (SSRIs), specifically, sertraline. Sertraline has less activation compared with fluoxetine, and a controlled trial of sertraline demonstrated improvement in children ages 2-6 with FXS (7). This trial, which involved more than 50 patients, included a dose of 2.5 mg to 5 mg given for a 6 month period. The controlled trial demonstrated that visual reception, fine motor coordination and average cognitive T scores were significantly improved on the Mullen in those that were treated on sertraline compared to placebo. For those with fragile X syndrome and autism, there was a significant improvement in expressive language on the Mullen Scales of Early Learning. In general, sertraline can also improve anxiety in many with fragile X syndrome and since anxiety occurs in >80%, it is a worthwhile medication to consider in those with fragile X syndrome of any age (7,8). Approximately 20% of those treated with sertraline can become overactivated and if this occurs the dose can be lowered or discontinued.

ADHD symptoms have been shown to improve with stimulant medication including methylphenidate and dextroamphetamine compared to placebo (9). However, when stimulants are given to children under 5, they often lead to irritability, whereas when given after 5 years of age, they are usually efficacious. An overall positive response is seen in approximately 60% of those with FXS (9). A long-acting stimulant will give a steady blood level during most of the day and stimulants can be combined with other medi-

cations such as an SSRI. Guanfacine is an alpha2A agonist which can also calm down hyperactivity and has been shown to be beneficial in young children with FXS (9). A similar drug to guanfacine is clonidine which causes more sedation than guanfacine. Although clonidine can be utilized during the day, it typically will cause significant sedation particularly in the first week of beginning the medication so low doses should be used. Clonidine has an overall calming effect and the sedation side effect is quite helpful for individuals who have difficulty in falling asleep (2). Typically, melatonin in a dose of 1-3mg at bedtime can be tried at first, but if this is not efficacious then clonidine can be utilized (10). A summary of these medications can be seen in table 2.1.

**Tabla 2.1 Medicamentos utilizados en síntomas particulares de afectados por SXF**

Medication	Dose	Indication	Response
Melatonin Neuro hormone that acts on the MT1 and MT2 receptors Decreases oxidative stress.	Children: 1 to 3 mg at bedtime	Circadian rhythm disorder	Increase time of intervals of night sleep. AE*: hypotension, tachycardia.
Valproic acid Blocks constant and repetitive high frequency firing of the neurons.	10-15 mg/kg/day administered in two or three doses increasing 5-10 mg/kg/day weekly until regulation. It is recommended 30-60 mg/kg/day for dose maintenance.	Seizures. Aggression, mood instability.	Seizure control stabilizes mood, decreases aggression. AE*: Nausea, vomiting and gastrointestinal discomfort, abdominal pain and heartburn.
Sertraline Increases BDNF r (Brain Derived Neuro-trophic Factor). Selective serotonin reuptake inhibitor.	2.5 mg per day in children between 2 and 3 years old. 5mg per day in children between 4 and 7 years old, > 7 years old 10 to 20 mg per day. Adults 25 to 200 mg per day.	Anxiety, depression, autism, language deficits, irritability.	Decreases anxiety and irritability, improves language and psychomotor development including fine motor coordination. AE*: aggression and hyperactivity or hyperarousal.
Clonidine Selective $\alpha$ -2 agonist	Not for children under the age of 3, 0.1 mg tab, half tab at night. After 6 years 1 tab..	Sleep disorders, attention deficit hyperactivity disorder (ADHD)..	Increase time of intervals of night sleep. Manages hyperactivity. AE*.

Continues

Medication	Dose	Indication	Response
Methylphenidate stimulant. Blocks noradrenaline and dopamine reuptake in the presynaptic neuron I	Children over 5 years old: 18 to 54 mg per day at morning (long acting) 5 to 40 mg per day twice a day (short acting). Usually not used in adults.	ADHD	Improves attention, impulsivity, and decreases hyperactivity. AE*: Sleeping and decreased appetite. Elevation of blood pressure
Minocycline Decreases MMP-9.	25 mg each day in children under the age of 3. 50 mg between 4 and 12 years old. 100 mg between 12 and 18 years old. 200mg in adults.	Language deficits and behavior issues.	Improves behavior and language. AE*: Rash, symptoms similar to lupus, if anti-nuclear antibodies (ANA) increase or when there is joint edema, discontinue.

\*AE: Adverse effects.

## TARGETED TREATMENTS FOR FRAGILE X SYNDROME

Advances in understanding the molecular underpinnings of fragile X syndrome with the development of animal models have led to a new field of targeted treatments in those with fragile X syndrome. The first targeted treatment utilized in individuals with fragile X syndrome was Fenobam which is an mGluR5 antagonist. Fenobam studies were carried out in two centers and demonstrated efficacy in behavior and in a quantitative measure of inhibition (9). Subsequent mGluR5 antagonists including Malvoglutant and Basimglutant were tried in adolescents and adults but were not found efficacious in behavioral measures (11). However, cognitive measures were not utilized in these studies and it is likely that Malvoglutant would be helpful in young children with fragile X syndrome.

Therefore, a new clinical trial ([www.clinicaltrials.gov/nct02920892](http://www.clinicaltrials.gov/nct02920892)) has been initiated in multiple centers in the U.S., funded by the NeuroNext network of the National Institutes of Health. This is a placebo controlled treatment with Malvoglutant (AFQ056) combined with intensive parent implemented language intervention through Skype (PILI) to examine whether targeted learning in 3-6 year old children with fragile X can be facilitated by this mGluR5 antagonist compared to placebo. PILI has been shown to be efficacious in improving language development in young children with fragile X syndrome (12,13). A study is currently taking place at the MIND institute in children between the age of 10 and 18 with lovastatin a medica-

tion that downregulates the mGluR5 pathway that is high in patients with FXS in absence of FMRP.

Another targeted treatment is minocycline, which lowers the level of matrix metalloproteinase 9 (MMP-9) which is elevated in fragile X syndrome. The absence of FMRP leads to upregulation of many proteins in the central nervous system including the MMP-9 protein. High levels of MMP-9 can interfere with synaptic connections and these levels are brought down to normal with minocycline treatment. A controlled trial of minocycline demonstrated significant benefits in children with fragile X syndrome (16). The use of event related potentials (ERPs) have demonstrated improvement in a habituation paradigm, meaning improvement in the inhibitory system to habituate normally to sensory stimuli, when comparing minocycline to placebo (17). Because Minocycline is clinically available through a pharmacy, it can be prescribed in children with fragile X syndrome. However, side-effects can occasionally occur, including darkening of the skin, gums, fingernails and even teeth; although this seems to occur only rarely. The antinuclear antibody (ANA) titer should be followed for children treated with minocycline at least once a year, although if a rash or swollen joints develop then minocycline should be discontinued (16).

Although the mGluR5 pathway is up-regulated in fragile X syndrome, the GABA pathway which is an inhibitory pathway is down-regulated in fragile X syndrome. Arbaclofen, which is a GABAA agonist has been studied in a controlled trial in fragile X syndrome and this initially demonstrated efficacy but only for those who had significant social deficits or autism in addition to their fragile X syndrome (18). A subsequent large study of Arbaclofen in children and adults with fragile X syndrome did not demonstrate efficacy in adults but did demonstrate benefits in some outcome measures in children, suggesting that the earlier it is started the better the effect. Other GABAA agonists have been studied or will soon be studied, specifically Ganaxolone (19) and Gaboxadol, which will be studied in the near future.

Animal studies demonstrated that metformin, a type II diabetes medication that regulates insulin signaling, can be beneficial in the *Drosophila* model (20) and in the mouse model of fragile X syndrome (21). Metformin lowers the activation of the mTOR pathway, which helps to re-regulate the DGKkappa system in fragile X syndrome (21). A preliminary review of the clinical use of metformin in children and adults with fragile X syndrome, including those with obesity, those with the Prader-Willi phenotype of fragile X syndrome, and even in a four year old child with fragile X syndrome, demonstrated benefits particularly in behavior and language abilities (22). A controlled trial of metformin is currently underway for children between



the ages of 6-25 years of age with a focus on improvements in language and behavior at both the MIND Institute and at two sites in Canada.

Trofinetide, which is an analog of the terminal tripeptide of IGF-1 (insulin-like growth factor 1) can decrease abnormal ERK and Akt activity and normalize the phenotype in the fragile X mouse (23). A preliminary treatment study in adolescents and adults with fragile X syndrome was carried out at multiple centers and the preliminary results are positive (24). Future controlled trials of Trofinetide will be carried out.

Additional targeted treatments such as Metadoxine developed by Alcobra also demonstrated some preliminary positive results on the Vineland Adaptive Behavior Scale. Acamprosate, a medication available by prescription at the current time as a treatment for alcoholism, also demonstrated benefits in an open-label study in individuals with fragile X syndrome (25) and is currently undergoing a controlled trial.

Aripiprazole (Abilify) and risperidone are the only medications that are FDA approved for treatment of children and adults with autism. Aripiprazole has been utilized frequently in the treatment of children with fragile X syndrome, particularly for aggression, hyperactivity and anxiety where it shows significant benefits (9). However, only an open-label study has been carried out (26) and a controlled trial is currently taking place. This medication can be prescribed clinically and is available in most countries in the world.

Although many targeted treatments are not yet available for clinical use, there are several which are available including SSRIs such as sertraline, atypical antipsychotics such as risperadone and aripiprazole, a variety of stimulant medications which also show efficacy in a controlled trial of fragile X syndrome, and acamprosate. Lithium is another targeted treatment which lowers the mGluR5 pathway and has been studied in an open-label assessment in those with fragile X syndrome for treatment of aggression (27) and is also available clinically. However, renal side-effects which may be more likely with a long-term use of lithium have led to less frequent use of this medication. Many of the currently available medications can make a significant difference for improvement in behavior in those with fragile X syndrome and should be utilized clinically.

Newer medications targeting language improvement and behavior in individuals with FXS will be studied in the near future but will require evaluation with regulated trials to demonstrate the efficacy, see table 2.2



**Tabla 2.2 Medicamentos dirigidos a normalizar las vías neuronales alteradas en afectados por SXF, en estudios**

Medication	Mechanism of action	Results	Studies
Fenobam NOT available	mGluR5 Antagonist	Behavior and self-control improvement	Preclinical, phase I and phase II.
Mavoglurant and Basimglurant NOT available	mGluR5 Antagonists	Not effective in behavior disorders in teenagers. In trials with children, await language improvement.	Preclinical and phase I.
Lovastatin	mGluR5 Antagonist	May improve behavior, still in studies. Not below 10 years old or in those with low cholesterol.	Preclinical, phase I and phase II.
Arbaclofen Not available	GABA Agonist	Improvement in social deficits in children with FXS and autism	Preclinical, phase I, phase II and phase III.
Trofinetide Not available	Decreases ERK and Akt activity	Behavior improvement	Preclinical and phase I.
Metadoxine	GABA agonist	Results improvement in Vineland	Preclinical, phase I and phase II.
Acamprosate	GABA Agonist	Improvement in the interaction of children and adults with autism, decreasing aggression, hyperactivity and anxiety	Preclinical, phase I and phase II.
Lithium	mGluR5 Antagonist	Improves behavior and decreases aggression.	Preclinical and phase I.

As our understanding of the mechanisms underlying FXS continues to improve, more refined targets will be revealed, leading to more specific therapeutic agents. One such example is the recent discovery (Kashima et al., 2016) that the noncanonical BMP type II receptor (BMPR2) mRNA is a target of FMRP, and that in the absence of FMRP in FXS, BMPR2 is elevated. Elevated BMPR2 leads in turn to increased LIM domain kinase 1 (LIMK1), resulting in altered dendritic and synaptic morphology and function. Targeting LIMK1 led to reductions in the morphological abnormalities, suggesting a possible therapeutic role of LIMK1 inhibitors in FXS.

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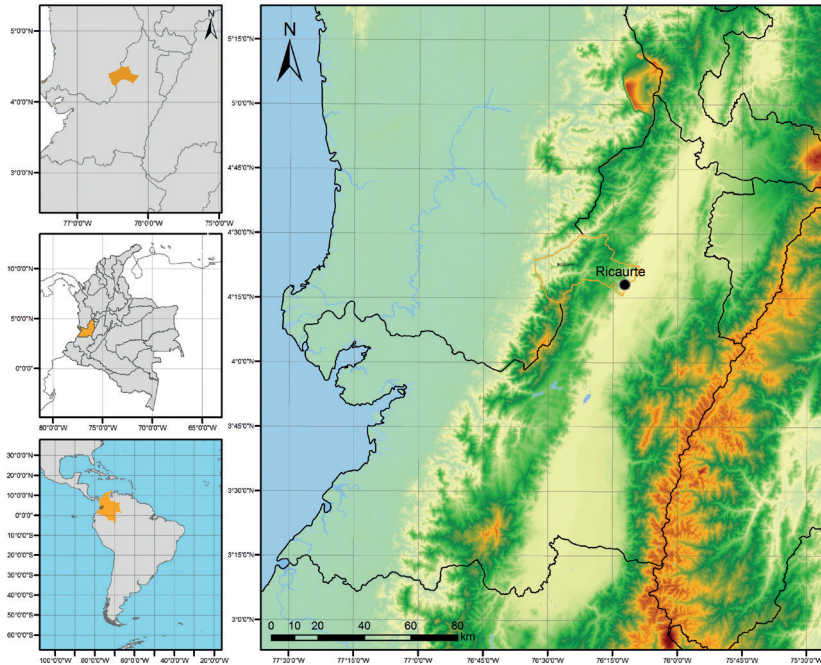
## RICAURTE

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Ricaurte is one of the villages of the municipality of Bolivar, located in the valley in between the western range and the Cauca River, left of the Panorama road in the north of Valle del Cauca Department in Colombia, Figure 1.1. With an altitude above sea level of 930m, it covers 1338m<sup>2</sup>, latitude: 4° 20' 26" north, longitude: 76° 10' 17" west, Greenwich Meridian (1). Ricaurte has a population of 1350 inhabitants according to the census done by local health authorities in 2013.

Ricaurte has a social and economically depressed population, whose main sources of employment are the agriculture and cattle raising areas. Figure 3.2. The remuneration for these activities may be in kind or financial, either with food or generally an amount of money much less than the minimum monthly salary. On the other hand, generally the workers do not have social security services such as: health, pension or insurance against professional risks.

There are important limitations to accessibility of health services, at this time a general practitioner does twice a month external consultation. Such consultations include meeting with specialists, occupational, physical or language therapy, laboratory tests or second level complexity imaging for the inhabitants of this region. There the people of Ricaurte are unlikely, given that its municipality lead, Bolivar does not have these experts available. The people of Ricaurte often do not have transportation to go to Bolivar even if the experts were available.



**Figure 3.1 Ricaurte's location**

The village of Ricaurte is located in northern South America, specifically in Colombia's southwest region, in the inter-Andean valley of the Cauca River, with an altitude of 950m above the sea level.

Given the social conditions many young people migrate to other Colombian cities or other countries for finding better life conditions.



**Figure 3.2 (A) Patient with a FM for FXS developing his daily activity as a farmer. (B) Patient with a FM for FXS hauling firewood from alongside the river.**



## ESTABLISHMENT

There is not a precise date for the founding of Ricaurte. Nevertheless, there are documents that sustain historic facts which suggest that the settlement of families in the area of Ricaurte is around the beginning of the XIX century (1). The first name that zone received was Yegüerizo, to be an area designated for the breeding of mules, mares and horses, besides sheep and goats (2).

There is a 1834 parochial file in Bolivar's archive church which shows the naming of the Yegüerizo's patron to "Nuestra Señora de las Mercedes." (2). This suggests that the parish construction took place years before this document, surrounding the final part of the XVIII century or beginning of the XIX century. Other important documents would suggest an approximate date for Ricaurte's founding is the public instrument number one, from January 31st 1852 in the Canton of Roldanillo, in which Vicente Torres De La Cruz tests his children. This document at the same time empowers José Cornelio Torres Aldana to sell Yegüerizo's chaplaincy where now it is documented that 11 people live in the area that is now Ricaurte. This sale is finalized in 1858 to the 11 inhabitants including Eugenio DelaCruz, Manuel Vicente DelaCruz, Rumaldo DelaCruz, Laureano Gordillo, Manuel Quintero García, Segundo García, Dionisio Valderrama, Manuel Triviño, Luis José Gómez, Diógenes Quintana and Leandro Palacios. A public deed number 42 in Roldanillo from 1858 documents this sale (2).

About the families that bought the grounds, the document "Levantamiento Histórico del Corregimiento de Ricaurte del Municipio de Bolivar," affirms that the families came from different parts of Colombia: Rodriguez and Palacios from Tolima, Sánchez, Gordillo, González and Molina from Buga, Domínguez and DelaCruz from Bugalagrande; and one family from Spain surnamed Rengifo (1). Nonetheless, the historian Hector Herney Arias affirms that the families' background that bought the area is this way: The Rengifos and Triviño from Buga, Quintero de Anserma viejo and Sánchez from Spain (Asturias) (1,2). All the mentioned surnames in both documents still exist in Ricaurte now and some are related to individuals with Fragile X syndrome

## REGIONAL AND NATIONAL VISIBILITY IN RICAURTE

Ricaurte is well-known in the Department for having a religious image known as the “Divino Ecce Homo,” who has awarded miracles. Ricaurte has become a pilgrimage center, especially on Sundays’ mass and in August when the festivities “Fiestas patronales,” are celebrated. Additionally, Ricaurte is known in Valle del Cauca and in Colombia, by the writer Gustavo Álvarez Gardeazabal’s work titled “El Divino” (3) published in 1986, that was highlighted on national television in 1988. In this work the author makes reference to “dumbs,” people with intellectual disability.

In this work there are descriptions of families and individuals with intellectual disability including the phenotype that is typical of Fragile X syndrome. In specific phrases the writer allows us to glimpse various Fragile X syndrome characteristics in Ricaurte. It describes 39 males with intellectual disability in a population of 1124 inhabitants (3), who make part of the families which traditionally have had members with intellectual disability and it describes a pattern of X linked inheritance. This work also affirms that the families have been there for centuries consistent with the founding of Ricaurte over 200 years ago. This work also describes the typical speech pattern including commonalities in phrases, chopped phrases and bursts of words, all these described and within the array of language deficits characteristic of FXS. The author documents that these individuals have intellectual disability so they do not understand the chants at church, but they can herd cattle, take care of sheep or clean up vineyards, therefore the intellectual deficits are mild or moderate. The author also describes their facial phenotype including a long face with prognathism, drooling and the tendency to keep their mouth open. All these characteristics described by the author are part of the phenotype of FXS.

In addition, throughout the book “El Divino”, the author describes the FXS phenotype and makes demographic references that show a high prevalence of intellectual disability in Ricaurte since the village’s founding. So, the author in this work without knowing describes in a literature language the genetic cluster of FXS that existed in Ricaurte.



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## EXPLANATIONS OF RICAURTE'S HABITANTS TO THE INTELLECTUAL DISABILITY'S ORIGIN

Ricaurte is a Colombian village, in which traditionally the population itself have identified large number of people with intellectual disability (ID), and colloquially the question "Why are so many dumbs in Ricaurte?" was repeated, so the population suggested answers with various hypotheses. Some suggested the ID was caused by high magnesium concentrations that would reach the inhabitants of Ricaurte from a nearby mountain through various tracks. One track would be through the water, given that mountain is where the river is born, from which formally the water was taken for the village's aqueduct; another would be the air, given the wind's direction would go from mountain towards the village and the people would breathe it and would get impregnated from it; the third track would be the ground, given that the magnesium in the air would accumulate in the ground.

Another hypothesis that would explain the intellectual disability in Ricaurte, argued that in the village grew a wild form of a vegetable called "Chamico," (Jimson Weed). Some females performed witchcraft using Chamico to achieve marriage, and when given to the males it caused ID. Others said the vegetable was ingested by goats which then were consumed by the inhabitants and the eating of excessive goat meat would produce ID.

A third hypothesis suggested the ID in Ricaurte originated from consanguineous unions, which the population believed was frequent in Ricaurte, and the hypothesis was reinforced because one affected boy was the son of his grandfather. The last hypothesis affirmed that people with ID came from specific families and their members when procreating with other families' individuals led to cases in the families which did not have a record of individuals with ID.

There were no scientific approaches to these hypotheses that confirmed or dismissed them until the Payan's study – Saldarriaga in 1999. This study documented the presence of FXS in Ricaurte and this confirmed the last hypothesis. (4).

**FIRST SCIENTIFIC MEDICAL APPROACH TO DETERMINE THE CAUSES OF MENTAL RETARDATION IN RICAURTE: “ENDEMIC FOCUS STUDY OF MENTAL RETARDATION IN RICAURTE, VALLE”**

En el estudio, realizado por investigadores de la Universidad del Valle a finales de los años noventa, se encontró que la causa de la DI en Ricaurte es una enfermedad genética heredable a través del cromosoma X, denominada síndrome de X frágil (SXF). En ese estudio se lograron diagnosticar 19 pacientes con el síndrome a través de citogenética con cariotipos con bandas G en medio de cultivo con déficit de folatos inducido por metotrexate (4). Además, se hizo el diagnóstico clínico en 16 más a quienes no se les logró realizar la prueba, encontrando en total 35 pacientes afectados. Los casos fueron encontrados en tres familias y se postuló un probable ancestro común dadas las características de la fundación del pueblo y patrones migratorios. Para el año 1999 se determinó una prevalencia de SXF de 1 en 38 hombres y de 1 en 100 mujeres, lo que excedió 100 veces lo reportado en la literatura para el SXF, no encontrándose en la literatura ninguna población con esa prevalencia (4).

**The Endemic Focus of Intellectual Disability and FXS in Ricaurte**

The observations of the writer Álvarez-Gardeazábal published in 1986 in the book “El Divino,” showed there were 1124 in Ricaurte with 39 “Dumbs” (3); and the study of Payan-Saldarriaga in 1999 (4), found 35 affected by FXS in 1128 habitants, a prevalence similar to the numbers reported by Álvarez-Gardeazábal reported 13 years previous. In the study of Universidad Del Valle there were 8 individuals with FXS under 14 years old that could not have been observed by Álvares- Gardeazábal in 1986. By 2014 according to health authorities information of Ricaurte there were 1334 habitants and 45 people with intellectual disability, among them 4 children under 10 years old, without knowing clearly how many of them have Fragile X syndrome. These observations suggested that the endemic focus of ID and FXS, are perpetuated without any intervention from the health authorities.

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## POPULATION MEDICAL GENETICS AND GENETIC CLUSTERS

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Population medical genetic fieldwork studies focus on populations with a high prevalence of genetic diseases and / or congenital anomalies (1). Individuals affected by a disease are treated traditionally as a general practitioner or specialist would treat their patients, but also the needs of the whole community are addressed so population medical genetics becomes a public health tool. From now forward we will address both of these perspectives regarding fragile X syndrome (FXS) and PM disorders and Ricaurte.

### GENETIC GEOGRAPHIC CLUSTERS

Geographic cluster is defined by a higher than the expected prevalence (globally accepted prevalence) for a disease, within a population that lives in a defined geographical area, over a long period of time. Nevertheless, for genetic diseases, this definition was reformulated, since in general these diseases are rare or the prevalence of these diseases are unknown; There are asymptomatic individuals such as heterozygotes of recessive diseases, carriers of genes of dominant conditions but of incomplete penetrance, or carriers of the PM in diseases caused by trinucleotide repeat disorders. Therefore what must be considered is the gene frequency and genotypes instead of phenotypes (1).

In Latin America, the country with the highest number of probable genetic clusters reported is Brazil. The National Institute of Population Me-

dical Genetics (INAGEMP) is in charge of reporting and classifying these populations. In Brazil of 191 alleged clusters that have been described and proposed, 86 clusters are registered mainly in the northeast region of the country with a prevalence of 1.017 cases per million inhabitants. Of these 86 clusters, 58 correspond to autosomal recessive diseases, 17 autosomal dominant, 5 are X- linked, three to multifactorial diseases and one case of environmental exposure disorder by thalidomide (2). This distribution is predictable because of the high inbreeding rate that normally occurs in genetic clusters. In the majority of cases these clusters are genetically isolated populations due to geographic or cultural reasons. In the majority of clusters there is an autosomal recessive disease (1).

One example of a probable genetic cluster in Brazil is the community of Cândido Godói district of Linha São Pedro, where there is a high prevalence of mono and dizygotic twins that occurs in 2%, whereas in the rest of the country the rate of twinning is 1%, a statistically significant difference. (3). This high prevalence is secondary to a founder effect of German ancestors in this community of 6700 inhabitants. This high prevalence is due to a decrease in apoptosis due to a reduction of P53 levels present in this region caused by the 172 allele of the TP53 gene, the main risk factor for having twins (3,4). Also seven regions in South America had been described, with a high prevalence of palatal and labial (do you mean labial?) fissures and four regions with palatal fissures that are related to an Amerindian inheritance and low social stratum (5). In Colombia a population with high prevalence of juvenile Alzheimer has been reported in Antioquia where individuals with the same mutation in the presenilin-1 gene were identified (6).

However, in none of the populations in which a high prevalence of genetic disease has been identified have molecular studies been carried out to identify possible asymptomatic carriers of the alleles involved.

Therefore, none of the previously mentioned clusters would meet with the definition of genetic geographic cluster. Fragile X syndrome is a rare disease that does not fulfill Mendelian inheritance patterns. It is a trinucleotide repeat disorder inherited through the X chromosome where PM carriers can pass on to their children the same number of triplets (being equally carriers) or expand the number of triplets and these present with phenotypes with different degree of expression. There is significant phenotypic variability in females with FM, including mild or nonexistent intellectual disability. In FXS it is not necessary that the two parents are carriers of a mutated gene, therefore it does not occur with greater frequency by consanguineous unions (7). FXS has genomic and phenotype

characteristics that would make it prone to be endemic in a population of the sociodemographic conditions of Ricaurte, a small village, economically depressed, with poor access to health services, and migratory patterns in which the most skilled people, usually migrate to look for a better quality of life and individuals with intellectual disabilities stay in the village. In addition, Ricaurte is a geographic place with very few immigrants.

### **RICAUORTE AS A GENETIC GEOGRAPHIC CLUSTER**

Ricaurte has a genetic cluster of FXS since it fulfills several of the characteristics of the definition. 1. Ricaurte is a geographic place defined and recognized by the competent authorities as a village of the municipality of Bolívar. 2. The prevalence of people with FXS is high; in Ricaurte 1 in 38 males and 1 in 100 females were diagnosed with FXS in 1999 whereas in the rest of the world the overall prevalence of FXS is 1 in 7143 males and 1 in 11,111 females (8–10). 3. The prevalence of affected people has been maintained over time. Álvarez Gardeazábal in 1986, in his book *El Divino*, described 1124 inhabitants with 39 "bobos", individuals with intellectual disability and suggestive physical characteristics of FXS (11); In the study carried out by the Universidad del Valle in 1999, a population of 1128 inhabitants and 35 people with intellectual disabilities were reported, 19 of them were confirmed for FXS due to cytogenetics and 16 for phenotype (10); very similar numbers as the ones described by Álvarez-Gardeazábal. Nevertheless, in the Universidad del Valle study, 8 individuals under the age of 14 were included as affected by FXS, who could not have been seen by Álvarez Gardeazábal in 1986 (10). Álvarez-Gardeazábal suggests that the high frequency of inhabitants with intellectual disability in Ricaurte has been observed since its foundation. According to data from 2013 and endorsed by the health promoter of Ricaurte there are 1334 inhabitants and 45 people with intellectual disabilities.

Thus, between 1986 and 2013, the prevalence of individuals with intellectual disabilities and probably with FXS in Ricaurte has remained high. 4. Given that the prevalence of people with FXS is high, it can be hypothesized that the prevalence of the PM of the FMR1 gene should also be high. To prove that Ricaurte is a genetic cluster of FXS, we must establish the prevalence of the PM and FM of FMR1 with molecular testing and determine if they are appreciably elevated compared to the world-wide prevalence stated above.

## GENETIC COUNSELING IN THE CONTEXT OF PUBLIC HEALTH

Public Health has extensive fieldwork that seeks the modification of factors that undermine the welfare of the population. In the first half of the twentieth century, several authors were focused on achieving improved public health. Among them was the Swiss physician Henry Sigerist, who was for the time the leading theorist of socialized medicine in the Western Hemisphere. He developed a system of medical care where he gave fundamental importance to primary care and health promotion activities, being the first to set up this concept (12).

Afterwards, with the development of preventive medicine, Leavell and Clark relied on the work of Sigerist to distinguish the four categories of medicine developed by him: health promotion, disease prevention, cure or recovery of the sick and rehabilitation, as stages of the health-disease process. In this way, health promotion was a synonym of health education and the concepts of primary, secondary and tertiary prevention arose.(13).

In the second half of the twentieth century, possibly influenced by the postwar period, public health actions were reevaluated and new approaches, proposals and revisions of health and disease concepts emerged. Thus, the proposal of the World Health Organization (WHO) of Primary Health Care (PHC) and Health for All in the Year 2000 (SPT 2000) states the imperative need to bring health, understood as a personal state of well-being that allows a person to develop a socially and economically productive life; eliminating factors that make access impossible, such as financial and coverage reasons (14). Achieving this goal involves community participation and appropriate technology, turning the individual, the family and the community into the base of the health system and the primary care agent into the central health worker.

Despite the apparent acceptance of the program and the political will of those involved, the results obtained in the twentieth century were not the expected since the inequity and health deficiency especially in developing countries, continued despite efforts. For this reason, in the 80s, in an International Conference about Health Promotion organized by the WHO, the Canadian Public Health Association and the Ministry of Health and Social Welfare of Canada, set the Ottawa Charter that established a new paradigm in Public Health.



### **Promotion and Prevention in Public Health**

According to the Ottawa charter, health promotion consists of providing to people the necessary resources to improve their health and having greater control over it, thereby becoming a concept that involves all of those involved in the achievement of a welfare state for all individuals (15). Public policy must be aimed at identifying and eliminating obstacles that prevent the adoption of measures that favor health through living and working conditions. Health promotion affects the social determinants of disease through socio-political models (13).

Health promotion must impact the life of the individual in a positive way in its different spheres, providing security and the necessary tools so that throughout life one is empowered to achieve strength within the community. (15).

On the other hand, the concept of prevention requires acting early, based on knowledge of the natural history of a disease in order to reduce or make unlikely its negative consequences, reducing its incidence and prevalence in populations. Prevention projects use the dissemination of scientific information and specific policy recommendations about habit changes to neutralize a specific disease, unlike promotional actions aimed at increasing health and well-being in general (16).

Prevention has been divided into levels according to its impact and timing of intervention in the health of an individual. Primary prevention refers to measures aimed at preventing the appearance of a disease or health problem by controlling the causal, predisposing or conditional factors; its objective is to reduce the incidence of the disease. Secondary prevention includes actions to achieve an early diagnosis, well timed catchment and adequate treatment through periodic medical examination and the search of cases through screening tests in apparently healthy patients. The objective is to avoid the appearance and progress of the sequelae of the disease and thus reduce the prevalence. Tertiary prevention consists in providing the conditions for recovery through correct diagnosis, treatment and physical, psychological and social rehabilitation, thereby seeking to reduce the sequelae, minimize suffering and facilitate the rehabilitation of the previously ill individual (17).

### **Molecular diagnosis and genetic counseling in public health**

In this context, all the actions concerning the physical, mental and social well-being of an individual or a community are involved in the concepts of promotion of health and prevention of disease. With the development of molecular biology in the second half of the 20th century, from the discovery of the DNA structure in 1953 up to the achievements obtained

with the Human Genome Project in 1997, the new knowledge was used among others to develop molecular tests, such as Polymerase Chain Reaction (PCR), Fluorescence in situ hybridization (FISH), Multiplex Ligation-dependent Probe Amplification (MLPA), targeted gene sequencing, molecular panels, Microarray-based Comparative Genomic Hybridization (aCGH), whole exome Sequencing (WES) and whole genome sequencing (WGS).

The new molecular technologies allow accurate diagnosis of genetic diseases, detect asymptomatic carriers and determine which patients have the possibility of developing disease or transmitting it to their offspring. These technologies are used to promote health and prevent disease, becoming another tool of public health to achieve welfare in the population through the spread of information and preventive actions aimed at decreasing the frequency of genetic disease, reduce complications of those affected and provide adequate treatment and specific rehabilitation processes. (18,19).

Genetic counseling is established as a communicative process of primary prevention, between an individual or a family and trained personnel who address the problems associated with the occurrence or risk of recurrence of a genetic disease. This implies that the ones involved understand the natural history of the disease, the diagnosis, inheritance, health issues and the available treatment. Therefore, with appropriate counseling the family will be able to make decisions in order to achieve better living conditions for the affected individual and their environment, based in adequate information and they will also help guide reproductive choices in the patient and the rest of the family. (20,21)

### **Genetic Counseling in Fragile X Syndrome**

FXS is caused by an expansion in the number of CGG repeats in the promoter of the FMR1 gene to a FM, affecting male individuals to a greater extent than females. During the transmission of the maternal chromosome X that contains the PM, the phenomenon of expansion of the CGG repeats can occur, resulting in a mutant allele (>200 CGG repeats); this expansion to a FM does not happen during the transmission of the PM paternal X chromosome to a female offspring because only the PM is passed on to the daughters from their carrier father. The possibility of the expansion occurring from maternal transmission is determined by factors such as the number of repetitions of the CGG sequence, the number of AGG interruptions and the gender of the carrier.

Genetic counseling plays an important role in the management of the fragile X spectrum of disorders, allowing a differential approach according to the clinical suspicion or the diagnosis, the possibility for treatment and the reproductive expectation of the individual and the members of their family. Fragile X DNA testing should be done in patients with intellectual disability or autism and suggestive characteristics of FXS, family members suspected of being carriers in the family history, children from carrier patients or family members who present with ataxia, tremors, neurological symptoms or premature ovarian failure (22).

This way, the patient can be guided from the moment of clinical suspicion, to obtaining access to the information without any type of coercion and avoiding a distorted understanding of the implications from the possible diagnosis. The patient should be allowed to have a process in which he/she can reflect the impact on his/her life, appreciate the role of inheritance of the disease and understand the options to manage the risk of recurrence, choose the most appropriate courses of action according to the information obtained and adapt to his/her medical condition. The ultimate goal of genetic counseling should be to provide the patient with tools to make an autonomous decision after having undergone a molecular test (19).

The genetic counseling in the FXS is based on the gender of the individual to whom it is done and on the identification of the allelic variants of the FMR1 gene, which is also classified according to the number of CGG triplets. An FMR1 gene with less than 44 CGG repeats is considered normal, between 45 and 54 is called gray zone, within 55 to 200 repeats is considered a PM and more than 200 repeats is considered a FM (23–25). According to the number of triplets there will be a chance of expansion and having children with PM, with FM and/or affected (Table 1.1 chapter 1).

The probability of being affected having the FM changes according to the gender of the individual. Males with FM have a penetrance close to a 100%, they will have ID and the other characteristics of the phenotype; while females that inherit the FM have a 25% chance of having ID and a 30% chance of having a normal IQ, but 60% have learning problems and emotional difficulties (7,26,27). Genetic counseling in people with ID must be carried out with the legal representative of the patient since the issues discussed are complex.

Male patients with FM will have a 100% probability of conceiving daughters with PM, given the phenomenon of spermatid contraction, and will not have affected male children (7,28). However, it must be taken into account that less than 5% of males with FM reproduce and have offspring (7). Given the situation of ID in this group, the counseling is given to the res-

possible relative. Male carriers of the PM have a 100% chance of passing on the PM to their daughters, although the PM repeat number may go up or down but it will stay in the PM range.(7).

Counseling is of great importance in PM carrier females and in females with FM, the latter case may or may not have ID and do not have reproductive problems. The probabilities of expansion of the CGG triplet in females carriers of PM increase as the number of repetitions is higher (Table 1.1); the calculations of having affected children can be visualized from this table.

For a female with repeats between 56 and 59 the risk of expansion to the FM is 3.7%, considering that the risk of transmitting the affected chromosome is 50%, the probability of having children with FM is of 1.8%. If the number of repetitions is between 90 and 99, the risk of expansion increases to 80% and having children with FM to 40%. In females with 100 or more repetitions, the risk of expansion is of 100% and having children with FM is 50%. Females with FM have a risk of children inheriting the FM is 50% (22).

Individuals with gray zone alleles can expand the number of repeats to the PM, so their children should have the FMR1 DNA test to see what the CGG repeat number has expanded to. (29).

In genetic counseling for carriers of the FMR1 gene mutation, it should be explained to them that although they do not have the classic phenotype, they can develop pathology associated with the PM such as depression, anxiety, hypertension, FXTAS and FXPOI. The signs and symptoms of these diseases should be explained and basic medical tests should be suggested to be taken before symptoms start to appear or schedule a medical appointment if they already show the symptoms. 20% of female carriers and 40% of male develop FXTAS typically in their 60s, with intention tremor and/or cerebellar ataxia, neuropathy, and cognitive decline. 20% of females develop FXPOI before age 40 and/or hormonal and reproductive disorders.

Taking into account the sociodemographic conditions of Ricaurte, where accessibility to health services is precarious, genetic counseling is an essential tool for individuals and families affected by the alterations of the FMR1 to prevent the perpetuation of the FXS genetic cluster in Ricaurte (30).

From now on the following study will be described.

## POPULATION MEDICAL GENETICS IN A COLOMBIAN DISTRICT WITH A HIGH PREVALENCE OF FRAGILE X SYNDROME\*

Given that Ricaurte is the village with the highest prevalence of people affected by FXS reported in literature, the FXS heritability characteristics in which carriers of the PM and females with FM do not have a clearly recognizable phenotype but they can have offspring with the syndrome; plus, the definition of geographic cluster for genetic diseases includes prevalence not only of those affected by the disease, but all the allelic variants involved, questions must be answer: Are the gray zone, PM and FM of the FMR1 gene elevated in Ricaurte? Is Ricaurte a FXS cluster?

In order to answer these questions, it was necessary to develop a study that will address all the inhabitants of Ricaurte by performing molecular tests such as PCR (screening) and Southern Blot (confirmatory), which would allow the identification of all the allelic variants of the FMR1 gene. This would establish the prevalence while allowing comparison with the data reported in literature to demonstrate the hypothesis that Ricaurte is an FXS cluster. In addition, performing genetic counseling after identifying individuals with allelic variants at risk of having children affected by the syndrome and/or suffering from FXTAS and FXPOI. With this last intervention it would be expected that over the years the number of new cases of FXS would be reduced, preventing the perpetuation of the cluster.

### Global methodology

The general objective of this study was to demonstrate that Ricaurte is a genetic FXS cluster.

The specific objectives of this study were:

- Establishing the prevalence of the gray zone, PM and FM of the FMR1 gene in the inhabitants of Ricaurte and comparing them to the prevalence reported in literature.
- Determine interfamilial relations of the cases with PM and FM allelic variants.
- Carry out genetic counseling in inhabitants of Ricaurte with PM and FM of the FMR1 gene.

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\* Project co-financed between Universidad del Valle (Cali, Colombia) and the University of California (Davis, CA, United States), carried out between February 2015 and May 2017. Researchers: Wilmar Saldarriaga-Gil, Jose Vicente Forero-Forero, Laura Yuriko Gonzalez Teshima, Julian Ramirez Cheyne, Carolina Isaza, Carlos Andres Fandiño Losada, Flora Tassone, Jose Rafael Tovar, Marisol Silva, Sergio Aguilar-Gaxiola, Randi Hagerman.

In order to reach these objectives, a study of prevalence was designed based on population medical genetics, which included all the inhabitants of Ricaurte (1334) who signed the informed consent of the study.

This study was approved by the institutional ethics committee of the Faculty of health of the Universidad Del Valle (Cali, Colombia).

### **Steps prior to the start of the study**

Before starting the study, various preparations were made, including meetings with the members of the community affected directly with disorders associated to the FXS and their families, community leaders, government officials, the local priest and health services officials. Community-based participatory research principles such as confidence, respect, clear communication about roles and shared mission were explained. We also made educational presentations about the genetics of the FXS and the phenotype of the disorders associated with the PM and the FM, objectives and development of the study methodology, and emphasizing the importance of comprehending the meaning of being a carrier of a PM or having a FM in a family member. We also guaranteed inhabitants with the privacy of the results, individualized delivery and personalized genetic counseling that would be provided accordingly.

The following chapters will break down each of the specific objectives of this study in order to describe in a timely manner the methodology, results, discussion and conclusions.

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**PREVALENCE OF GRAY ZONE, PREMUTATION AND FULL MUTATION ALLELES OF THE FMR1 GENE IN THE POPULATION OF RICAURTE\***

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**INTRODUCTION**

Fragile X syndrome (FXS) is a genetic disease inherited through the X chromosome, it is the most common genetic disorder associated with intellectual disability (ID) and autistic spectrum disorders (1). It is caused by expansion greater than 200 CGG repeats in the FMR1 gene, which leads to hypermethylation of the promoter region preventing transcription, and resulting in the absence or deficit of FMRP expression levels in the brain and connective tissue (2–4). The patients affected by FXTAS have a spectrum of clinic involvement that include ID, autism, macroorchidism, long face, prominent ears hyperextensible fingerjoints and poor eye contact (1,5).

Four FMR1 allele categories have been defined according to the CGG repeat number ; 5 to 44 repeats is a normal allele; between 45-55 is a gray zone (GZ); between 55-200 is a PM; and FM alleles with more than 200 CGG repeats (FM), which cause FXS with the typical clinical features (4,5). Several

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\* Una versión menos extensa de los resultados presentados en este capítulo fue publicada en línea en el artículo “Genetic cluster of fragile X syndrome in a Colombian district” en el *Journal of Human Genetics*, el mes de enero del 2018 [doi:10.1038/s10038-017-0407-6].

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molecular screening studies using such as PCR and Southern blot analysis have been carried out to identify expanded FMR1 alleles in newborns, in pregnant females, in females prior to conception or with ovarian insufficiency and in adults with or without ID (6–11). However, FXS screening has never been carried out in the inhabitants of a specific locality.

Hunter and collaborators in 2014 carried out a meta-analysis in which they found the prevalence of the FM being 1 in 7,143 males and 1 in 11,111 females and the PM being 1 in 855 males and 1 in 291 females (12). Tassone and collaborators in 2012 carried out a study in newborns using a screening methodology, which is the same used in this study and they found an intermediate (gray zone) prevalence of 1 in 112 males and 1 in 66 females and a PM prevalence of in males and of in females (7).

In 1999, Saldarriaga and Payan performing diagnosis by karyotype with folate deficient tissue culture media described a FXS endemic focus, in Ricaurte a Colombian village located in the Valle del Cauca (13). They found a prevalence of FXS of 1 in 38 males and 1 in 100 females. However, in that study carriers were not diagnosed nor was the GZ, because the karyotype did not identify those allelic variants (13)(14).

The goal of this study, which results are reported in this Chapter, was to establish the prevalence of the different allele categories of the FMR1 gene: gray zone, PM and FM in Ricaurte and, to determine if the prevalence is elevated compared with the prevalence values reported in literature.

## METHODOLOGY

### ***Tipo de estudio***

Transversal de prevalencia.

### ***Type of Study***

1334 are the inhabitants of Ricaurte a Village of Bolivar municipality, in Valle del Cauca, according to available information before the beginning of the study.

### ***Criteria of Inclusion***

The inhabitants of Ricaurte from who signed, by themselves and/or their legal guardian, an informed consent to participate to the study, comprising 7-year-old minors, adults or under-age (between 7 and 18 years old), with or without intellectual disability were included in this study.

### ***Census construction and sample collection***

Based on the village's map from the health authorities where each inhabitant's house was drawn, a walker a walk through the locality led to rebuilding this map (see figure 5.1), and a numeric code was assigned to each

house. A project's health personal went door to door to each house in the village and completed a form with sociodemographic information: name, two surnames, birthday, age, scholarship, civil status, occupation, monthly income, if they were native from Ricaurte, if they belonged to one of the families in which there were cases of Fragile X Syndrome (FXS) and if the person had intellectual disability of another nature; socioeconomic data and pathological personal records. In addition, informed consent was taken for the sample collection.

When the informed consent was obtained to participate in the study, a code was assigned to each individual that included the house code and a second individual code per person. This code was used to label the blood samples guaranteeing the anonymity of the participants and their results.

To determine if the participants were natives from Ricaurte the following question was included in the survey: "When were you born? Are your father and/or mother living in Ricaurte?" If the answer was positive, the participant was defined as native from Ricaurte. If the answer was negative, the subject was considered non-native from Ricaurte.

### **INFORMED CONSENT**

Informed consent was signed by each person before taking blood drops for the sample on filter paper. There were different informed consents for people with disability and under-7-year-old, which were signed by one of their parents or legal guardians. Additional informed consents were used for the adult population and for minors between 7 and 18 years old, signed by the minor and one of their parents or legal guardians. Furthermore, the informed consents were signed by the staff, which explained the consent and, by a witness. When the investigators collected a larger blood sample, (10 ml) and additional consent was also signed.



**Figure 5.1** Map of the houses in Ricaurte

A code was assigned to each house and a secondary code to each individual per house.

## **SAMPLING, STORAGE AND TRANSPORTATION PROCEDURE**

For the CGG sizing using a PCR-based approach, at least 2 blood drops were taken on filter paper by a digit-puncture. Before the procedure, the filter paper was properly marked with the unique code of the individual.

Southern Blot was performed to confirm positive cases (presence of an expanded allele) identified by the screening PCR, and in special cases where the clinic characteristics were not compatible with the PCR result. For example, in a male with a PM allele but with a phenotype compatible with an FXS FM.

To carry out the Southern Blot, a new informed consent was signed, and 10ml of peripheral blood sample was collected through a venous puncture in the upper limbs, stored in EDTA containing tube previously marked with the unique code of the individual. All the procedures were performed under current biosafety regulations.

Blood spot cards and peripheral blood samples collected in EDTA containing tubes were stored and shipped at a temperature of 4 °C (39.2 °F) by mail to the MIND Institute laboratory in California, USA according to protocols approved by the UC Davis Institutional Review Board (IRB).

## **LABORATORY PROCEDURES**

### **DNA extraction of blood samples in filter paper**

The DNA isolation was executed using the DNA extraction kit QIAxtractor Pure Advantage<sup>®</sup> (Qiagen) and the instructions of the manufacturer were followed. The blood sample in filter paper (903 Whatman) 2 mm diameter discs were removed by puncture and each one was placed in a well of a S-block plate; from this plate a map was built on paper, registering each sample code. Each sample was lysed with 300 µl of lysis buffer Tissue Digest (BTD) with 20 µl of digest enzyme, followed by 60 minutes incubation at 56 °C. The supernatant was removed and placed in a new plate of S-block following the same location of each sample as previously (7,15). Elution of the DNA in a DNA stabilization solution was obtained as manufacture instructions. At the end of the process a new plate is obtained in which its wells are found Isolated DNA was stored at -20 °C (-4 °C) or perform PCR immediately.

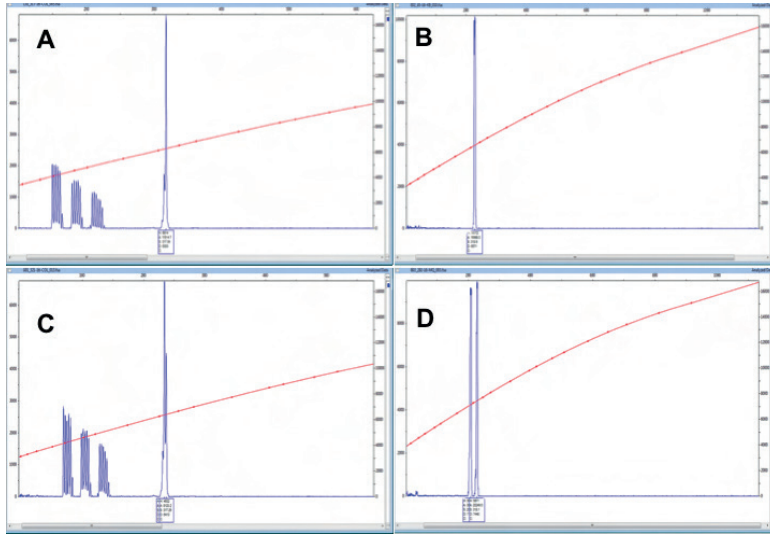
### **Polymerase chain reaction**

Asuragen AmplideX<sup>®</sup> was used for CGG sizing. Master mixture was added in an Eppendorf tube and included 11.46 µl AMP buffer rich in Guanine

and Cytosine; 0.50  $\mu\text{L}$  of primers FMR1 F (TCA GGC GCT CAG CTC CGT TTC GGT TTC A) and R (FAM-AAG CGC CAT TGG AGC CCC GCA CTT CC), marked with 6-carboxyfluorescein (FAM) and a 0.2  $\mu\text{L}$  of the FMR1 CGG forward (CGG linker); 0.05  $\mu\text{L}$  of polymerase mix and a rich content of the nucleotides guanine and cytosine; besides thinner solution 0.5  $\mu\text{L}$ . The master mix was vortex by a vertical agitator before being applied into a microtiter plate (96 wells, Phenix Research Products) or depending on the number of samples to process to PCR tubes; to each plate's well or each PCR tube 14  $\mu\text{L}$  of the master mix and 2  $\mu\text{L}$  (20 ng / $\mu\text{L}$ ) of DNA were applied (7,15). The sealed plate (ABGene Aluminum, Phenix Research Products) or the PCR tubes were put in a vertical agitator, and then they were centrifuged for 20 seconds and transferred to a thermocycler (ABI 9700; Applied Biosystems). The samples were amplified with a stage of initial thermal denaturation at 98 °C for 5 minutes, 25 cycles at 97 °C for 35 seconds, 62 °C for 35 seconds and 72 °C for 4 minutes; and a final extension at 72 °C for 10 minutes. After the PCR, the samples were light-protected stored under 20 °C before the capillary electrophoresis analysis (16). Two positive controls with known CGG repeat size were used in each PCR. The PCR products were visualized by capillary electrophoresis (CE) ABI 3730 (AppliedBiosystems, Foster City, CA, USA). For this process an optic plate was prepared (Phenix, MPC-3420) by adding to each plate's well 13  $\mu\text{L}$  of capillary electrophoresis mixture (11  $\mu\text{L}$  Hi-Di formamide + 2  $\mu\text{L}$  of Rox 1000) and 2  $\mu\text{L}$  of the amplified DNA obtained by PCR.

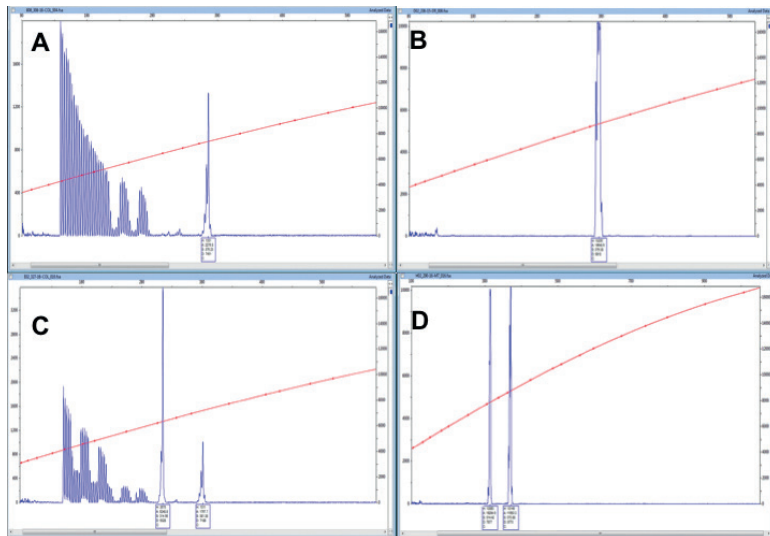
The samples in the plate were denatured at 95 °C for 2 minutes, followed by a cooling with ice before being transferred to CE. The images (electropherograms) produced by CE were analyzed with the ABI Peak Scanner software and (4,15). Examples are shown in Figure 5.2, 5.3, 5.4 and 5.5.





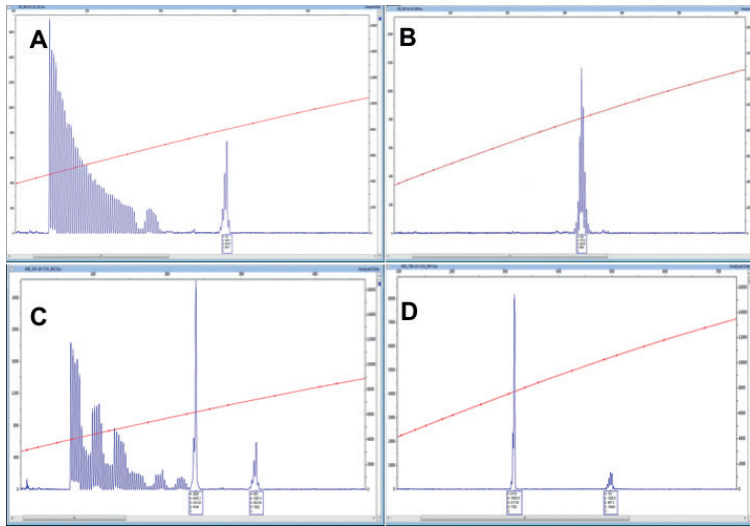
**Figure 5.2** Electropherograms showing alleles of the *FMR1* gene within the normal range.

In the A and C figures images of capillary PCR electrophoresis using F (forward) and R (reverse) primers and CGG linker are observed. In the B and D figures images of capillary PCR electrophoresis was obtained using F (forward) and R (reverse) primers. Figures A and B: male, with the allele of the *FMR1* gene with 30 repetitions and two AGG interruptions. Figures C and D: female, with 29 repetitions in one allele and 30 in the other allele with two AGG interruptions in each allele.



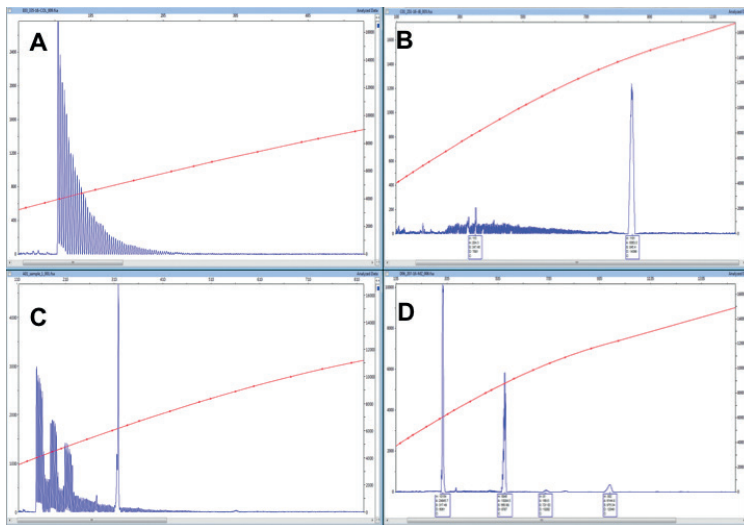
**Figure 5.3** Electropherograms showing alleles of the *FMR1* within the grey zone range

In the A and C peaks were obtained using F (forward), R (reverse) and CGG linker primers. In the B and D figures, images of capillary PCR electrophoresis were obtained using F (forward) and R (reverse) primers. Figures A and B: male, with allele of the *FMR1* gene with 50 CGG and two AGG interruptions. Figures C and D: female, with 29 repetitions in one allele and 51 in the other allele and two AGG interruptions in each allele.



**Figure 5.4 Electropherograms with alleles of the FMR1 gene within a PM range**

In figures A and C peaks were obtained using F (forward), R (reverse) and CGG linker. In primers. Electropherograms in figures B and D were observed using F (forward) and R (reverse) primers. Figures A and B: male, with an FMR1 allele of 55 CGG and one AGG interruption. Figures C and D: female with one allele of 30 CGG and one of 93 CGG in the other allele with two AGG interruptions in each one.



**Figure 5.5 Electropherograms showing alleles of the FMR1 gene in FM range**

Electropherograms depicted in A and C were obtained using F (forward), R (reverse) and CGG linker primers. B and D images were obtained using F (forward) and R (reverse) primers. A and B: male, with an FMR1 allele harboring more than 200 CGG repeats. C and D: female with an allele of 30 CGG repeats and two AGG interruptions and another allele with more than 200 CGG repeats and no AGG interruptions.

The samples that in the CE (capilar electrophoresis) pattern produced suggestive images of expanded alleles (PM or FM) after the first PCR round, were submitted to a second PCR round using the previously described protocol. Only the diluting solution was varied 1  $\mu$ L but with the FMR1 Forward and Reverse with FAM primers; these PCR products were taken again EC with the same protocol and the images (electropherograms) produced are used to quantify the triplet's number. See 5.2 B and D, 5.3 B and D, 5.4 B and D, 5.5 B and D.

The number of CGG repeat within any given allele was calculated from the size in base pair of the amplicon, minus the size of the flanking region (239bp), divided by three and compared with the base pair number of the alleles of controls. The classification of the genotypes followed American College of Medical Genetics guidelines for the "normal" repeat (<45 CGG), intermediates (45-54 CGG repetitions), PM (55-200 CGG repetitions) and FM (>200 CGG repetitions) (7,16,17).

The protocol described here, has been partially published in the referenced articles, however, it has modifications and this protocol is the one currently used for identification of the allelic variants of the FMR1 gene in the molecular biology laboratory of the MIND Institute at the University of California in Davis, USA, led by Dr Flora Tassone.

### **Southern blot**

For confirmatory testing Southern Blot was performed using genomic DNA isolated from 3-5 ml of peripheral blood using standard approach (Qiagen DNA isolation kit).

#### ***Step 1. Digestion of DNA***

7 to 10  $\mu$ g of isolated genomic DNA was digested with the EcoRI enzymes (10 units for each 1  $\mu$ g of DNA) and (NruI 10 units for each 1  $\mu$ g of DNA) plus 1X buffer, for 12 hours in the 37 °C water bath. The digested samples were separated on a 0.8% and Tris acetate EDTA (TAE) agarose gel cell by electrophoresis at 70 volts for 21.5 hours. Agarose gel was removed from the electrophoresis box and placed on a tray in which partial denaturation was performed with HCl (0.4 N) for 15 minutes and neutralization in NaOH (0.5 N) for 30 minutes, with agitation on an orbital shaker.

#### ***Step 2: transfer of DNA***

***from the agarose gel to the nylon membrane.*** The DNA was transferred to a positively charged nylon membrane (Roche Diagnostics, Basel, Switzerland), using a vacuum transfer machine (Vacuum Blotter 785, Bio-Rad,

Hercules, CA). In the vacuum transfer apparatus, paper, gel and nylon membrane were assembled and two plastic membrane were placed around it (blank window gasket) to produce a seal on the edges of the nylon membrane. Standard saline citrate was added (10X SSC) until covering the agarose gel, approximately 100 ml; the transfer process last 90 minutes

### ***Step 3: Washing process***

The nylon membrane is washed for 5 minutes on a tray with buffer (2XSSC) upon an orbital agitator. Subsequently the membrane is put on paper (Whatman Blotting Papers), DNA was cross linked using ultraviolet light chamber (UV Crosslinker, FB-UVXL-1000), at 1200 X100 $\mu$ J/CM<sup>2</sup> for 2 minutes.

### ***Step 4: Hybridization process***

Membrane was hybridized in a hybridization machine (roller shaped bottle, Isotemp, Fisher Scientific) in 20 ml of DIG Easy Hybridization Buffer (Roche Diagnostics) wit rotation in the hybridization incubator (Fisher Scientific, Isotemp Hybridization Incubator) for 2 hours (minimum 30 minutes), at 42 °C. Then, 100  $\mu$ l of Dig-labeled probe StB12.3 was added to the solution contained in the hybridization bottle and incubated for 16 hours at 42 °C.

Removal of excess of the probe excess was obtained washing the membrane twice with 200 ml of washing buffer 2X SSC /1%SDS at 65 °C for 10 minutes and a final wash for 30 minutes with 200 ml of the washing buffer 1 X SDS/0,1% SDS. Membrane was then washed for 2 minutes with washing buffer 1X (DIG wash and block kit, Roche diagnosis) and blocked with a blocking buffer for 180 minutes, with enough to cover the membrane. 20 ml of detection buffer was then added with antibodies anti DIG (anti-DIG: diluted Antidigoxin-AP 1:10.000, 75mU/ml) and incubated with agitation for 30 minutes. Membrane was then washed for 30 minutes with the washing buffer 1X (DIG wash and block kit, Roche diagnosis) an orbital agitator.

### ***Step 5. Detection***

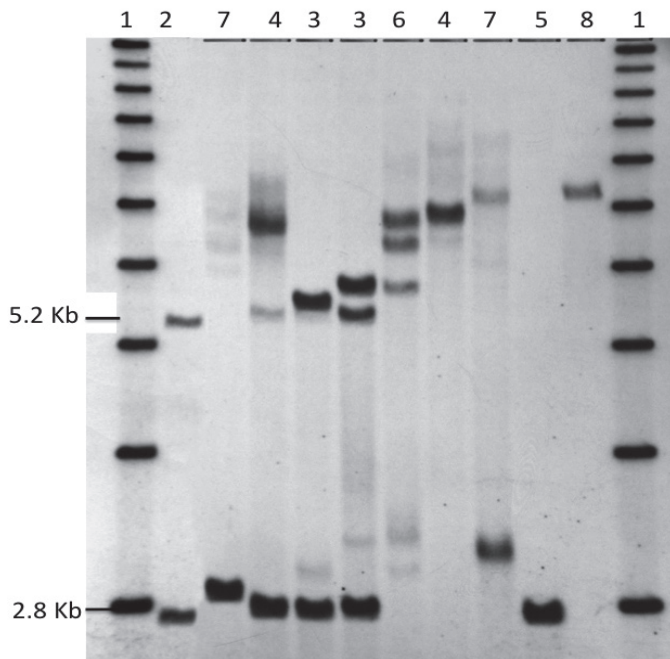
Detection was performed by placing the membrane in detection buffer detection for 5 minutes on an orbital agitator with CDP-Star<sup>®</sup> (Roche diagnostics; 1 ml for a 10 x 10 cm membrane); this solution is a chemiluminescent substrate that makes a chemical reaction with AP (alkaline phosphate) which is part of the antibody against DIG; these reactions produces light.

**Step 6. Develop**

Membrane wrapped in saran wrap was placed between two x-ray films (Premium X-Ray Film, Phenix) and exposed for 2.5 hours to overnight. Film was developed in the developer (Konica Minolta SRX-101. The membrane exposed to an x-ray film (Super RX, Fuji Medical X-Ray, Bedfordshire, United Kingdom) for at least two hours in a dark room yielded a band pattern on an autoradiography. A DNA size ladder (1 kb ladder; Invitrogen, Carlsbad, CA) was used as standard size.

This protocol was developed from the manufacturer's manual (18), and modifications were applied in the molecular biology laboratory of the MIND Institute, directed by Dr. Flora Tassone.

With this methodology both the methylation and the amplification of the CGG triplet CGG can be seen, after a double digestion with EcoRI and NruI (methylation-sensitive enzymes). The digestion with the first enzyme (EcoRI) produces a 5.2 kb band in the Southern blot. The digestion with NruI a methylation-sensitive enzyme cuts the unmethylated within the CpG islands, producing a 2.8 kb band, but it leaves the methylated DNA uncut. Therefore, a normal female produces both a 2.8 kb band, which reflects the X chromosome unmethylated and active, and a 5.2 kb band, which reflects the X chromosome methylated and inactive. PM alleles generally produce bands in the 2.9 to 3.2 range for the X active chromosome, as it presents in the carrier males, and from 5.3 to 5.7 kb for the X inactive chromosome. Carriers generally have two doublets in the analysis. The affected male generally has bands between 5.8 to 9 kb range, reflecting a great amplification with an increase in size in 500 to 6000 base pairs, in comparison to the controls, and corresponding to CGG of approximately 200 to 2000. See figure 5.6 with Southern blot autoradiography with the test performed in patients of Ricaurte, with allelic variants of the FMR1 gene. The main advantage of using the analysis by Southern blot is that it is more reliable highlighting extensive expansions seen in FM alleles. (17)



**Figure 5.6 Southern blot**

Size ladder marker (1 Kb ladder) is shown in Lane 1. Lane 2: normal female control. Lane 3: PM female. Lane 4: female with a FM. Lane 5: normal male. Lane 6 and 7: mosaic males (cells with different CGG repeat alleles methylated and unmethylated). Lane 8: male with a FM.

### **ETHICAL CONSIDERATIONS**

The confidentiality of the participants was protected by using a code in the data collection format, which was the same used on the collected blood spot cards and in the result table handled by researchers. The code and the name of each participant were logged in other documents accessible only to the researchers. The results of the diagnosed PM carriers in this project were not disclosed and the confidentiality of their identities was preserved. During the genetic counseling process, the people's identity was protected and there was a personalized disclosure of the results to the families with subsequent genetic counseling. There are risks for individual counseling, specifically the ones related to reproductive expectations of the carriers who, due to the risks of conceiving a child with FXS, may require special counseling at the time of their lives when they want to become pregnant. At the end of the project, a second genetic counseling session for the PM carriers that wish to have children can be offered and advised on specific prenatal diagnostic techniques for FXS.

## STATISTICAL ANALYSIS

Relative frequencies were constructed in males and females, using as numerator the number of individuals with the allelic variant identified through the tests PCR CGG Linker and/or Southern Blot, grey zone (45-54 CGG), PM (55-200 CGG) and FM (>200 CGG); and as denominator the number of individuals in the analyzed group, A, B, or C:

- A. Males and females for whom sample was obtained.
- B. Native males and females for whom sample was obtained.
- C. Non-native males and females for whom sample was obtained.

Native individuals were defined, during the census, as individuals whose parents, one or both, were residents in Ricaurte at the time of their birthdate.

### Observed frequencies comparison

The observed relative frequencies for PM and FM, calculated from the population from which they were obtained (blood samples in Ricaurte) were compared with the absolute values reported in the meta-analysis carried out by Hunter et al. (2014). Those values were obtained from the sum of the studies included in their analysis of the total population (12); the gray zone frequencies (GZ) were compared with the absolute values reported by Tassone et al. (2012) (7). The odds ratios (OR) obtained for each allele, reflect the relative increase of the frequencies found in Ricaurte in relation with the studies with which they were compared and that represent the accepted global prevalence. Additionally, the confidence intervals were calculated at 95% using the Stata software 14.2<sup>®</sup>.

For this the command “`cci #a #b #c #d, exact`” was used, where the #a corresponded to the individuals that were positive in Ricaurte, #b to the positive individuals in the study to be compared, #c was the total of the sample in Ricaurte and the #d the total population obtained in the studies to be compared. The command “`cci`” was used, because it is a cross-sectional prevalence study and the option “`, exact`” to use Fisher’s exact method for calculating the value of p.

### Prevalence estimation

The prevalence of alleles in the FMR1 gene was estimated for the whole population included in Ricaurte’s census, distinguishing for males, females, total population, native and non-native, groups A, B and C.



Relative frequencies were estimated, but not on the inhabitants of those that were sampled, but on the total of registered males and females, native and non-native.

Given the small number of positive individuals with each allele, it was decided to perform a Bayesian approach to estimate the prevalence of the proportions for FM, PM and GZ.

Punctual Bayesian estimations and their credibility regions (C.R.), that have a different interpretation in comparison with the confidence intervals of the frequency approach were obtained. The credibility regions' limits are the values among which it is possible to find the 95% of the prevalence proportions in a determined population. For the estimation procedures, it was assumed that the natural behavior of the number of individuals belonging to each category of the FMR1 mutations in the population can be modeled using a multinomial distribution, and the previous information on the parameters (vector of prevalence) could be expressed using Dirichlet distribution (the previous distribution).

To establish the parameters of the previous distribution (hyperparameters) empirical Bayes methods were used from the information contained in the Bootstrap intervals, obtained using data from the group of participants whose blood samples were collected (20). The statistical analysis was carried out using the interface between the Open Bugs<sup>®</sup> software and the R software (21).

### **Prevalence comparisons**

The estimated prevalence in Ricaurte and its credibility regions for PM and FM in males and females, were compared with the estimated prevalence and the confidence intervals in the meta-analysis done by Hunter et al. (2014) (12). The prevalence for GZ in males and females in Ricaurte were compared with the prevalence and the confidence intervals reported by Tassone et al. (2012) (7).

Through a simple quotient among the compared prevalence, it was documented how many times each prevalence was higher in Ricaurte, reflecting the increase of the estimated prevalence in Ricaurte in relation with the studies that represent the accepted global prevalence. Additionally, the confidence limits reported were compared in each study to determine if the differences were significant.



## RESULTS

### Ricaurte's census

The total population of Ricaurte, surveyed by the work group of this project was 1,186 inhabitants. 634 were females and 552 males and 419 (75.90%) males and 453 (71.45%) females were natives from Ricaurte.

### Screening and confirmatory test

Informed consent and blood samples were collected on blood spot cards. PCR analysis was carried out on 926 inhabitants, 424 males and 502 females. Of these 329 and 353 were males and females native to Ricaurte respectively. Age of the individuals sampled ranged from 8 days to 96 years.

PCR analysis showed that 57 participants carried an expanded allele of 55 CGG repeat or greater and therefore classified as PM or FM. In addition, four of the inhabitants with negative results were considered non-concordant with the phenotype or family record. For the 61 subjects informed consent to perform the Southern Blot test was requested and they were obtained for 59 of them; for two subjects the consent form and sample couldn't be obtained because one died before PCR results were disclosure and the other one left the country.

The total of studied samples by PCR and Southern blot as confirmatory test, indicated that 33 people has the FM, 25 carried a PM allele and 27 a GZ allele. The absolute frequencies and relatives observed for the A, B and C groups, their confidence intervals (CI) at 95% and the P value calculated with STATA 14 are shown in the table 5.1.

**Table 5.1** Number and relative frequencies of each mutation in the *FMR1* gene among the subjects from Ricaurte according to sex and origin

Females	Full mutation		Premutation		Gray zone	
	N	R.F.	n	R.F.	n	R.F.
Total n= 502	11	1/46	20	1/25	22	1/23
Natives n= 353	11	1/32	19	1/19	19	1/19
Non-natives n= 149	0	0	1	1/49	3	1/50
Males	Full mutation		Premutation		Gray zone	
	N	R.F.	n	R.F.	n	R.F.
Total n= 424	22	1/19	5	1/85	5	1/85
Natives n= 329	22	1/15	5	1/66	2	1/165
Non-natives n= 95	0	0	0	0	3	1/32

N=number; R.F.= Relative frequency of having the mutation among the subjects of whom was obtained sample;  
0= Zero subjects with mutation.

Results show that in Ricaurte, among the inhabitants who had DNA studies, 1 every 19 males and 1 every 45 females had FM; 1 of every 141 males and 1 of every 18 females carried the PM, and 1 of every 85 males and 1 of every 21 females had a gray zone allele.

The observed frequencies in the native group of Ricaurte were for FM 1:32 females and 1:15 in males; while the PM frequencies were of 1:19 in females and 1:66 in males. Finally, the GZ frequency was 1:19 in native females and 1:165 native males.

### Frequency comparisons of the FMR1 gene observed in Ricaurte with the accepted global prevalence

The comparison of relative frequencies observed in Ricaurte of the different allelic categories in group A and of those males and females who were sampled with the absolute reported values by Hunter et al 2014 for FM, and PM and with Tassone et al (2012) for the GZ allele are shown in Table 5.2.

Statistically significant differences were observed for every allele category in both males and females, except for the GZ in males (natives and non-natives). The probabilities in males and females of having an allele with FM in Ricaurte were 323 and 192 times greater than in the meta-analysis of Hunter et al paper. For the PM alleles there was also a significantly higher prevalence in Ricaurte and the odds ratios were 8.8 and 7.1 for males and females respectively.

**Table 5.2 Comparisons among the observed frequency of alleles in Ricaurte compared to the frequency of alleles reported Hunter et al. 2014 for FM and PM, and GZ reported Tassone et al. (2012)**

Allele of the FMR1 gene	Frequency in Ricaurte	Reported frequencies	#a	#b	#c	#d	OR	95% IC	P value
FM males	1/19	1/5879*	22	14	380	78 090	322.93	156.46–686.93	0.000
PM males	1/85	1/730*	5	62	414	45 191	8.80	2.75–21.81	0.000
GZ males	1/85	1/112**	5	65	414	7225	1.34	0.42–3.32	0.431
FM females	1/46	1/8393*	11	9	480	75 530	192.32	72.01–527.07	0.000
PM females	1/25	1/164*	20	539	462	88 134	7.08	4.25–11.17	0.000
GZ females	1/23	1/66**	23	105	455	6784	3.27	1.96–5.23	0.000

#a: Number of positive individuals of Ricaurte (Colombia); #b: Number of positive individuals in each study to compare; #c: Number of screened subjects in Ricaurte with normal alleles; #d: Number of included individuals with normal alleles in each study to compare; OR: Odds Ratio; IC of 95%: confidence interval of 95%; P value: Fisher's exact P; FM: Full mutation; PM: Premutation; GZ: Gray zone; Hunter et al. 2014 (1); \*\* Tassone et al. (2012). (2).

### Estimated population prevalence for the allelic variants of the FMR1 gene in Ricaurte

The prevalence for each of the FMR1 allele, normal, GZ, PM and FM were estimated by Bayesian methods, presented for females and males according to their origin (native, non-native and total) in the Table 2.3. Among the total population, the estimated prevalence of FM was 48.2 per 1,000 males and 20.5 per 1,000 females. The estimated prevalence for FM and PM was greater for native males and females. The prevalence of the allele of the GZ was higher (but marginally significant) in the non-native male in comparison with the native male.

*Table 5.3. Estimated prevalence of each allele of the FMR1 gene in the total population of the Census of Ricaurte according to their sex and origin*

Females	Full mutation			Premutation			Gray zone		
	P	95%	C.R.	P	95%	RC	P	95%	C.R.
Total n= 634	20,5	13,5	28,6	35,8	26,5	46,2	42,2	32,2	53,8
Natives n= 453	31,0	20,4	43,2	51,6	38,2	67,3	51,3	37,7	66,9
Non-natives n=181	16,7	6,3	31,2	8,7	1,9	20,4	18,7	7,3	35,3
Males	Full mutation			Premutation			Gray zone		
	P	95%	C.R.	P	95%	RC	P	95%	C.R.
Total n= 552	48,2	36,3	61,5	14,1	8,0	21,7	13,4	7,4	20,7
Natives n= 419	60,6	45,3	78,2	18,7	10,7	29,4	6,5	2,2	13,3
Non-natives n= 133	15,9	4,8	33,9	12,0	2,6	28,6	30,3	13,0	54,0

N = Number of surveyed subjects; P = Expressed prevalence per 1,000 inhabitants; 95% C.R. = 95% Credibility region.

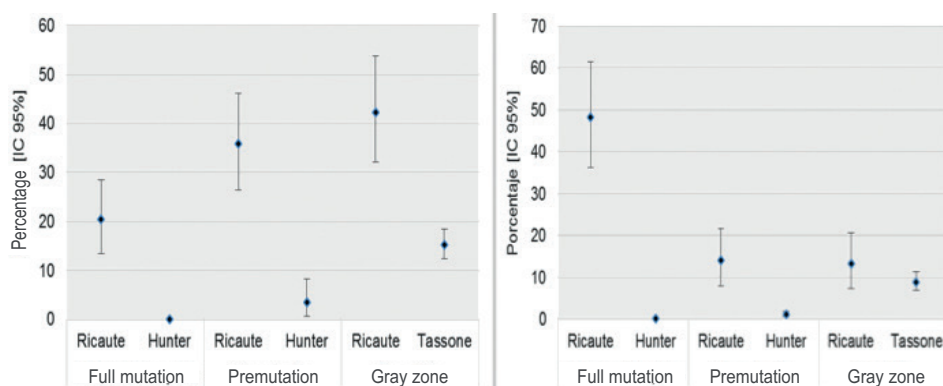
### COMPARISON OF THE ESTIMATED PREVALENCE IN RICAURTE OF THE ALLELES OF FMR1 GENE AND THE REPORTED WORLD-WIDE PREVALENCE

The estimated prevalence in Ricaurte for FM when comparing them with the reported ones by Hunter et al (2014) were 343.3 and 226.7 times higher in males and females respectively in Ricaurte. The PM prevalence were 11.1 and 9.4 times higher in males and females, than what estimated by Hunter et al (2014); likewise, the GZ prevalence in females was 2.78 times more in Ricaurte than in Tassone et al (2012) (7). The prevalence of these alleles is significantly elevated in Ricaurte, given that the confidence limits do not overlap, (Table 5.4 and Figure 5.7), the GZ prevalence in male was 1.81 times higher in Ricaurte than in Tassone et al (2012) (7), but the difference was not significant, given that the confidence limits overlap (Figure 5.7).

**Table 5.4. Estimated prevalence and confidence limits of the alleles of the FMR1 gene for males and females in Ricaurte and the ones established by Hunter et al 2014 (PM and FM) and Tassone et al (2012) (gray zone)**

Females	Full mutation			Premutation			Gray zone		
	P	95%	CL	P	95%	CL	P	95%	CL
Ricaurte (RC)	20,5	13,5	28,6	35,8	26,5	46,2	42,2	32,2	53,8
Hunter*/Tassone** (IC)	0,09*	0,0*	0,29*	3,44*	0,63*	8,33*	15,2**	12,5**	18,5**
Males	Full mutation			Premutation			Gray zone		
	P	95%	CL	P	95%	CL	P	95%	CL
Ricaurte	48,2	36,3	61,5	14,1	8,0	21,7	13,4	7,4	20,7
Hunter* & Tassone**	0,14*	0,01*	0,31*	1,17*	0,6*	1,87*	8,9**	6,9**	11,4**

95% CL= Confidence limits; CR= Credibility region; CI= Confidence interval; P = Expressed prevalence per 1,000 inhabitants.



**Figure 5.7. box-and-whisper plots representing the estimated prevalence and confidence limits of the alleles of the FMR1 gene for males and females in Ricaurte and the ones established by Hunter et al 2014 (PM and FM) and Tassone et al (2012) (gray zone)**

Note that the confidence limits of PM and FM in males and females do not overlap in these studies.

## DISCUSSION

The Fragile X Syndrome prevalence has varied in time related to methodology changes in studies across different Countries. For example, the different diagnostic tests, beginning with the karyotype with folate deficient media, PCR, Q-MSP (Quantitative Methylation-specific PCR), Southern blot and finally the utilization of a combinations of tests has led to more precise results over time. Further, different studies have adopted different ranges in the number of CGG repetitions to define each category which has also made comparisons of the prevalence of the alleles across studies, difficult (7,15,22). Inclusion criteria in the populations studied, for example

individuals with or without ID, couples during preconception evaluation, newborns (NB), have also affected the derived prevalence figures (12).

An example of this problem is evident in the study by Turner et al in 1996 (9) who re-evaluated reported prevalence in two studies of males with FXS, [1:952 (23) and 1:2610] (24) estimated in 1986 using karyotype in individuals with ID and extrapolating to the general population (23,24). Turner et al proposed a new methodology utilizing Southern Blot in the same individuals in whom the karyotype was positive in previous studies, which corrected the prevalence estimation of the FXS to 1:4000 males (9).

With the emergence of low cost DNA testing with elevated sensitivity and specificity, NB screening studies have been performed, contributing with different prevalence figures for each FMR1 allele category (7,8,15,25–27). The study with the highest number of NB samples was carried out by Coffee et al 2009 (8) who screened 36,124 NB males using Q-MSP (Quantitative Methylation-specific PCR) technique. Southern Blot was used to confirm 7 positive cases with the FM and they determined a prevalence of 1:5161 males. With Q-MSP individuals with GZ, PM or females with FM were not identified (8). Tassone et al (2012) published the results of a NB screening study using PCR with a CGG primer, which identified alleles in gray zone, PM and FM range, in both males and females. They screened 14,207 individuals, 7,312 males and 6,895 females. The results of this study showed a prevalence of the PM as 1:430 males and 1:209 females and GZ of 1:112 males and 1:66 females. This study also found a male newborn with the FM. Tassone et al in (2012) used the same PCR technique as in our study in Ricaurte and it is for this reason we decided to compare the gray zone prevalence with Tassone et al (2012) prevalence figures.

Hunter et al 2014, tackles this issue by carrying out a meta-analysis of the FXS prevalence that groups the characteristics of the sample populations. They reviewed 5,582 studies that assessed the prevalence of FXS by utilizing PCR and/or Southern blot testing for the diagnosis of FXS. The frequency data of the allelic variants were assessed in two main groups, one included the total population where individuals with and without ID were studied (mainly screening newborns and pregnant females) and another group, called normal population, including individuals without ID to establish the PM prevalence. The prevalence was estimated through an analysis by randomized effects. In the first group a FM prevalence of 1.4 per 10,000 males (CI of 95%: 0.1-3.1) and 0.9 per 10,000 females (CI of 95%: 0.0-2.9), 1: 7,143 (similar to the result reported in Tassone et al 2012) and 1: 11,111, were calculated respectively. A PM prevalence of 11.7 per 10,000 males (CI of 95%: 6.0-18.7) and 34.4 per 10,000 females (CI of 95%: 6.3-83.3), 1:855 and 1:291

respectively. For the second group the PM prevalence calculus was of 34.4 per 10,000 female (CI 95%: 8.9-60.3), which equals to 1:291, same result as the one estimated in the total population group (12).

In our study in Ricaurte, the estimated prevalence using Bayesian methods for FM was 48.2 (95% CR:36.3-61.50) per 1,000 males and 20.5 (95% CR:13.5-28.6) per 1,000 females (1/21 and 1/49 respectively). The PM prevalence was 13.4 (95% CR:7.4-20.7) per 1,000 males and 35.8 (95% RC:26.5-46.20) per 1,000 females (1/71 males and 1/28 females). The FM prevalence here observed is significantly greater than the prevalence estimated by Hunter et al 2014 (12), given that the confidence limits do not overlap, see table 2.4. for the FM prevalence was 343.3 and 226.7 times higher in males and females respectively in Ricaurte. Likewise, the PM prevalence was 11.1 and 9.4 times more elevated in males and females in Ricaurte, than the prevalence estimated by Hunter et al (12).

Table 5.2. shows that the observed frequencies of the alleles of the FMR1 gene in Ricaurte compared with the absolute frequencies of Hunter's studies et al (2014) with those reported in Tassone et al (2012) for FM alleles and PM in males and females, and GZ in females are dramatically and statistically higher in Ricaurte compared to the other studies.

The prevalence in Ricaurte for the PM in males and females, are, not only significantly elevated when compared to the accepted global prevalence (7,12), but they are the highest when compared to reported comparable studies such as adult screening studies without bias in favor or against a family history of ID, and excluding studies in institutions which involve individuals with ID, given that they are not comparable to Ricaurte's inhabitants (7,8,12,15,26,28-35).

Saul et al in 2008 (32), found a FM prevalence of 1 in 730 in newborn males in the south of California (2 in 1460) and a PM prevalence of 1 in 70 (134 in 9459). Fernández et al in 2009 (15) screened 5267 NB males in Spain and reported a PM prevalence 1:251 (95% IC, 1/164-1/385). All the mentioned prevalence figures are the higher than those reported in the medical literature however, they are lower compared to those found in Ricaurte.

The GZ allelic prevalence in Ricaurte was 13.4 per 1,000 males (95% CR:7.4-20.7) and 42.2 per 1,000 females (95% CR:32.2-53.8), equivalent to 1:75 and 1:24 males and females respectively. When compared to the Tassone et al 2012 prevalence figures, 1:112 (95% IC:1:88-1:145) and 1:66 (95% CI: 1:54-1:80) for males and females respectively, equivalent to 8.9 per 1,000 males (95% CI:6.9-11.4) and 15.1 per 1,000 females (95% CI:12.5-18.5), the prevalence in Ricaurte was 1.51 times elevated in males and 2.79 times elevated in females. The confidence limits of these figures overlap in males and

not in females, so the elevated prevalence in females in Ricaurte was statistically elevated compared to the Tassone data.

The difference in the prevalence figures of the various expanded FMR1 allele categories among studies can also be explained because they come from different ethnic groups (28). For example, two studies performed in Spain, but in different regions showed important differences in the prevalence. The first, a screening study in 5,267 newborn boys (5,000), in the Castile and León region, the GZ region was 1 in 26, PM was 1:251 and FM was 1 in 2,663 (15). In the second study carried out in newborn males (5,000), in the Catalan region, the GZ prevalence was 1 in 449, PM was 1 in 2,663 and FM was 1 in 2,663 (25). These studies present important differences in the GZ allele prevalence and PM in the same country, but with different ethnic groups. Likewise, Tassone and collaborators in 2012 (7), reported differences in the prevalence among the ethnic groups. They performed screening in newborns in 3 States in the United States of America, and observed that the prevalence in black females was higher (1:68) than in Hispanic females (1:570); while the PM in black males was lower (1:780) compared to white males (1:358) and this was lower than Hispanic males (1:595). Although in the study conducted in Ricaurte, it was found that the prevalence figures in FM males and females, and gray zone in females were significantly elevated, these may not represent the country's overall prevalence. Indeed, we observed differences between the native and non-native populations of Ricaurte (see table 2.3). All the identified cases of expanded FMR1 alleles in both males and females, were identified in the native group.

The analysis of this scenario allows us to conclude that there is a genetic cluster of FMR1 mutations in Ricaurte and that the migrants to the village have a lower prevalence that is probably similar to the one in the general population.

## CONCLUSIONS

In Ricaurte, the observed frequencies and the estimated prevalence figures of slyly categories of the FMR1 gene, FM and PM in males and females are significantly elevated when compared with those reported in comparable studies. The estimated prevalence of the gray zone allele in females is also significantly elevated when compared to the world-wide reported prevalence.



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## GENEALOGIES IN THE FAMILIES WITH FRAGILE X SYNDROME IN RICAURTE

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### INTRODUCTION

The genealogy is a tool classically used in population medical genetics in order to approach large families or populations that are suspected or confirmed to have a high frequency of a genetic pathology that has remained through time (1).

The population genealogies in which multiple nuclear families are reunited and the number of linked individuals usually overcomes 100 including those that have died and emigrants must be built through specialized programs with the aim of feeding the information constantly. This allows modifications to be made when they arise and thus integrating the information immediately. This also allows an assessment of a possible founder effect to the data(1,2).

FXS is a genetic disease that is inherited through the X chromosome and it is classified as a trinucleotide repeat disorder. In this case, the triplets CGG located in the 5'UTR region of the FMR1 gene, at Xq27.3 may or may not expand from one generation to another, which produces a particular inheritance pattern that does not fulfill the Mendelian recessive or dominant inherited patterns linked to the X chromosome (3–5).

Ricaurte is a district in Valle del Cauca, where cytogenetic testing in 1999 established a high prevalence of FXS in males and females (6).

During the development of the study “population medical genetics in a district with high prevalence of FXS” carried out in Ricaurte, due to its socio-demographic conditions, foundation and inheritance pattern, it was considered necessary to determine the relationships within families through the construction of the genealogies. This was done at the same time as the results of the PCR screening test were obtained. We could then analyze the inheritance patterns and identify the carriers or probable carriers and the affected individuals with the FM. Through the genealogy studies we could predict who in the emigrant population represented the origin of the founder effect. The aim of this chapter is to determine the relationships within families of the cases with PM or FM of the FMR1 gene and to determine if there is a possible founder effect.

## METHODOLOGY

A transverse descriptive study was carried out, whose population were the individuals with PM or FM of the FMR1 gene, who were identified through the study of the Colombian district with a high FXS prevalence.

### **Pedigree construction**

The construction of the genealogies was done at the same time as the results of the screening test PCR were obtained and also while the individuals and nuclear families with expanded alleles in the range of PM and FM of the FMR1 gene were identified. The purpose of constructing a genealogy is to analyze the inheritance patterns, identify carriers or probable carriers and affected individuals that for several reasons could not have been included in the genealogy during the first visit to their house.

With the collaboration of the staff in Ricaurte, it was agreed to do a home visit to the families with PM or FM of the FMR1 gene.

The following methodology for the construction phase is based on the one described by Poletta et al. (2014) (1).

### ***Initiation phase***

People with whom the database was constructed were defined, giving priority to the oldest family member, preferably females, who were able to give reliable information. The oldest member of the family was consulted with the aim of making an ascending expansion of the pedigrees until the oldest predecessor they can remember and then asking them for relatives with similar physical and behavioral characteristics as the ones shown in individuals diagnosed with FXS in their families. The horizontal family

expansion was also asked with the purpose of evaluating the possible genealogical bonds with other families with FXS cases.

### ***Collection phase***

Genotypic and personal data that wasn't obtained from the first individual was completed with information from other family members that were available to be surveyed, with the district's health promoter and/or other district's elders. With young individuals, the alive family members were specified, allowing a descending expansion of the pedigree, clarifying in the carriers if they were affected or not with FXS and the status of their offspring.

In the interview, personal and genotypic information was collected, guaranteeing confidentiality to the individuals. Interaction between participants is encouraged in order to compare or obtain new data that wasn't detailed initially.

In the beginning, pedigrees on paper were made with the classic form for guiding the interview, but they were immediately transferred to the database in Excel. In the Excel document, a row per individual was used; in every column of this row the following information was registered:

- Individual's identification code.
- Father's code.
- Mother's code.
- Name, including both last names.
- Gender.
- If the individual was alive or death.
- If the individual took the test.
- Test result (categorized in this way: normal, gray zone, PM, FM)
- Whether or not the phenotype was observed by the interviewers or by the interviewee's description of their relative (usually with the deceased ones or with the individuals that didn't live in Ricaurte).
- Comments or peculiar findings.

The pedigrees from these families were made with a genealogical emphasis instead of a demographic one. This entailed that every member that could be registered was included, regardless of their place of residence and their migratory status (immigrated or emigrated). The complete genealogical pedigree allows the identification of possible PM carriers that have emigrated.

### ***Repair phase***

The tabulated genograms that were obtained in the previous phases were corrected in order to avoid omissions and inconsistencies that may result

between different information sources and the new results of the molecular tests were incorporated.

### ***Analysis Phase***

Excel's document was modified in every family visit and the results were included in a format.txt delimited by tabs. This document was then imported into the "Genetic Pedigree Software PROGENY 9: CLINICAL version" program, (Progeny Software LLC, South Bend, Indiana, USA,) which built the diagrammed genogram for each family.

The use of this software allowed the performance of the following tasks electronically:

- Construction of genograms and illustrative graphics that allow storing the collected information in a summarized way.
- Automatic bonding of wide genograms collected in different families, through the identification of shared genealogical triads. A genealogical triad is composed from the mother – father- child bond.
- Establish consanguinity degrees between surveyed families with members who carried the FM or the PM. This can be quantified through the endogamy (f or F) or the relationship index ( $r = 2F$ .)
- Constantly relate the genogram to results of molecular tests, as well as clinical exams that are done, in addition to family medical records and perinatal records or other data of interest.
- Validate through biological logic for the collected information. Including, through understanding the heritability of alleles, results that couldn't be obtained during the study because of the individual's death or because of refusal to participate.

## **RESULTS**

### **Tabulated Pedigree**

Our results include a tabulated pedigree with 484 individuals, 58 with expanded alleles of the FMR1 gene: 33 individuals with a FM, 24 with the PM, 1 with a gray zone allele; and 78 in the normal range, figure 6.1.

It was possible to identify 3 of the 10 founders, I-1 (highlighted in blue circle), II-3 (Highlighted in green circle) and III-1 (highlighted in violet circle), see figure 6.2.

In the violet, blue and green circles founders of Ricaurte; highlighted by violet, blue, yellow and green lines families with cases of interest. In the center of the genealogy in the boxes A, B and C family groups were amplified through the initial pedigree analysis and the FXS inheritance pattern where



possible or certain carriers of PM were identified, and the black arrows show how the pedigree changed.

The individuals with the alleles in the PM and FM belong to 4 family lines of inheritance; each family was differentiated with violet, yellow, blue and green lines in the figure 6.2.

The four families relate each other with political links, marriages or fact unions (domestic partnerships).

No consanguineous unions were found with children with PM or FM.

**Inheritance pattern analysis of the four families with expanded alleles and likely individuals who may have brought the PM or FM to Ricaurte**

12 cases of FM offspring from the founder III-1 and his spouse were found, it is suggested that one of them (III-1 or III-2) had a PM, highlighted in the family in the violet line, see figure 6.2.

In the descendants of II-2, 19 cases with PM and 18 with FM are observed, highlighted family in blue line, see figure 6.2; according to the available information from the descendants that were interviewed, II,2 arrived in Ricaurte near the founding of this town coming from Tambo, Cauca's department district. It is suggested that she had PM.

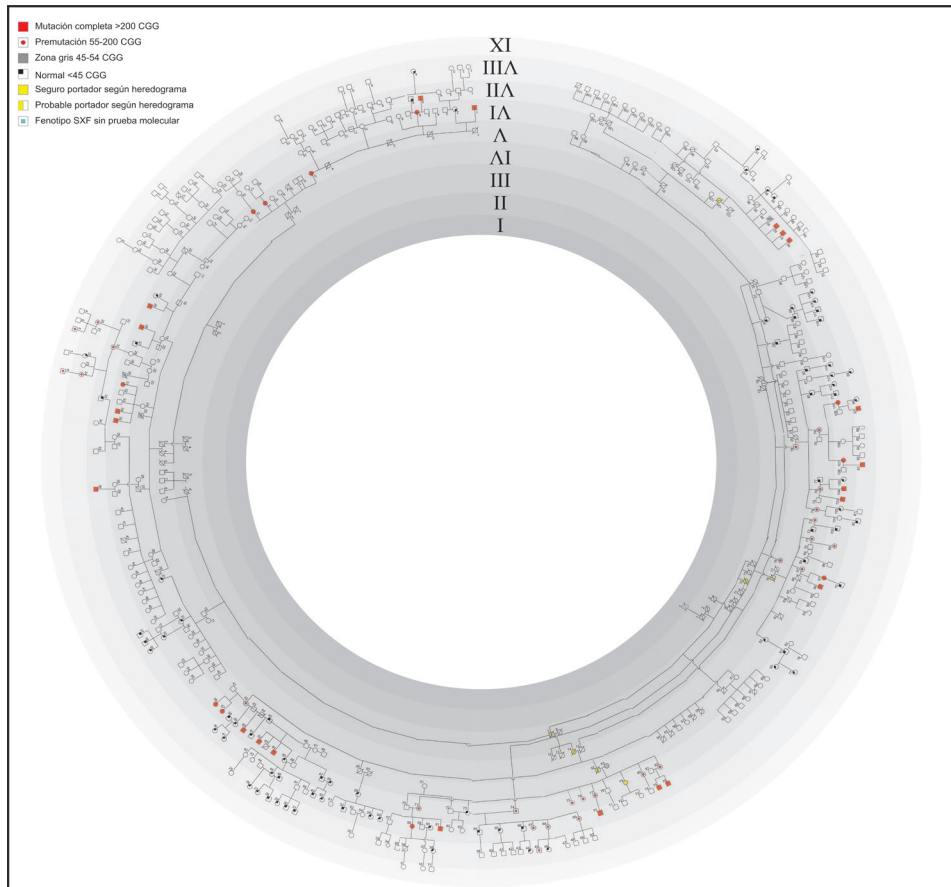
VI-124's descendants were the third identified family nucleus (certain PM carrier), in which 3 cases of FM were diagnosed, highlighted family in green line, see figure 6.2; even though these individuals have the same last name as descendants from II-2 (highlighted family in blue line, see figure 6.2.) and a non-found nexus in the oral traditions can't be discarded.

A fourth familiar line descends from the VII-32 diagnosed as carrier of the PM and contribute to the genetic cluster 5 cases of the PM and none of them with FM, highlighted family in yellow line, see figure 6.2.

**Individuals located in Ricaurte through the pedigree who had expanded allele**

Ten individuals, who had not been previously sampled, were identified as obligatory carriers considering their genealogical information and an active search was made and a sample was obtained. Five females located by having a father or mother or siblings with the PM and molecular PCR tests and Southern blot showed the PM: VII-67, VII, 69, V-74, VI-80, V-73.

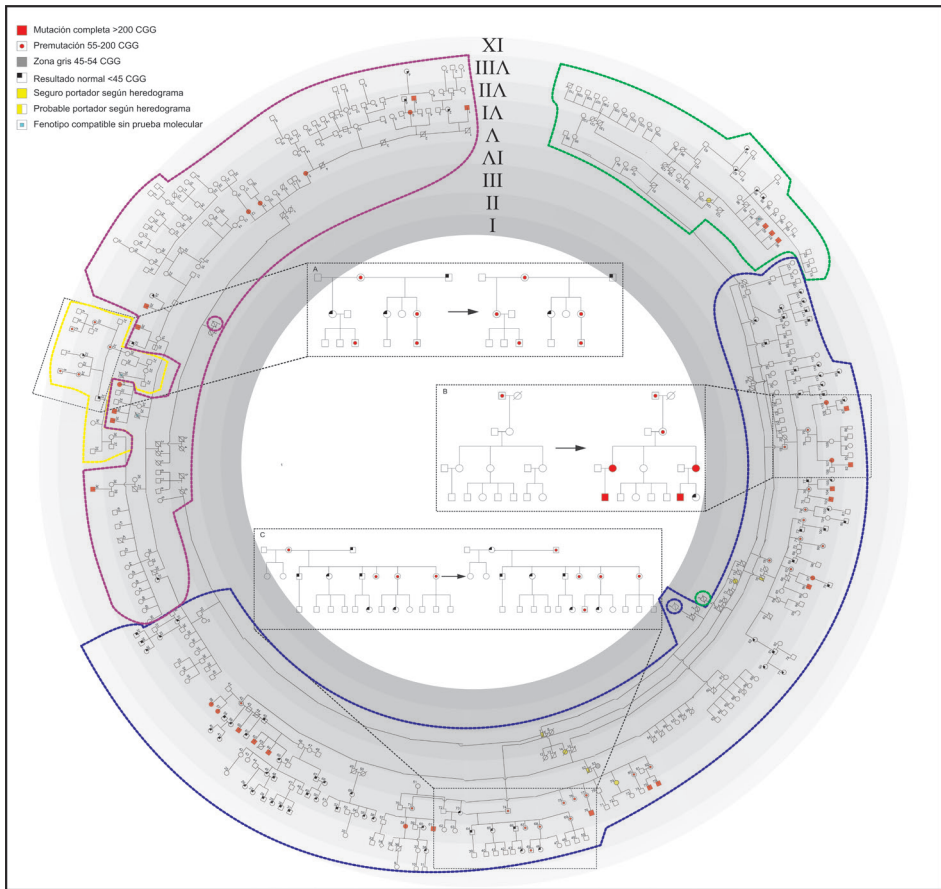
One female was located as being the daughter of a male carrier of the PM. However, the result from the PCR and the Southern blot was normal. However, for being the daughter of a carrier of the PM she should be an obligatory carrier; non-paternity it is postulated.



**Figure 6.1** Genealogy of families with cases of PM and FM of the FMR1 gene in Ricaurte

Two females located for having a mother with PM, the molecular PCR tests and Southern blot showed FM, B box at the center of the pedigree in the figure 6.2.B: VI-107 and VI, 100.

2 males, kids between the age of 6 and 7 years old located by been grandchild of carrier grandmother with PM, the molecular PCR tests and Southern blot showed FM, B box at the center of the pedigree in the figure 6.2.B: VII-82 and VII-88.



*Figure 6.2. Genealogy of families with cases of PM and FM of the FMR1 gene in Ricaurte, with visualization of cases of interest*

**Results: inconsistencies located by the pedigree analysis and rectified with confirmatory testing**

One individual changed from gray zone to PM for having his mother and children with PM, box A in the center of the pedigree in the figure 6.2.B: VIII-20.

VI-74 changed from normal to PM, descendants linked to PM alleles with errors in the packaging of the sample with (his wife’s sample), C box at the center of the pedigree in the figure 6.2.B: VI-74.

VI-73 changed from PM to normal, without descendants with expanded alleles. Error in packaging of the sample with (her husband’s sample): VI-73.

Two changes from PM to normal, without ascendance nor descendants of expanded alleles, not located in the pedigree.

## DISCUSSION

Pedigree construction and its analysis is an important tool to identify individuals with pathological phenotypes and to establish inheritance patterns. These patterns can help to establish the effect of a genetic disease that has not been confirmed yet; a classic example are affected ones that appear after consanguineous unions in the autosomal recessive entities (7).

In the case of families with a clear diagnosis of genetic pathologies with inheritance patterns already established the pedigrees provide information to locate individuals that may have mild phenotypes from diseases with variable expressivity or incomplete penetrance of autosomal dominant diseases. In addition mandatory carriers or probable carriers in recessive autosomal diseases or diseases by triples repetition can be identified (8). Nonetheless, the pedigrees that were made by the geneticist or the genetic counselors have important limitations in terms of data storage, data update and their analysis depending of the interpretation of the social worker that is dealing with the case (This comment is unclear) (1,2).

Tabulated genealogies are a good tool that has been classically used in medical population genetics to engage large families or populations in which it is suspected or confirmed that an elevated frequency of genetic disease exists (1). The population genealogies in which various familiar nuclear families are gathered and the number of linked individuals usually exceeds 100 including individuals who have died or emigrated should be built through specialized programs (1,2).

Tabulated genealogies allow constant information input and can be modified as new laboratory or clinical information becomes available. Also, the systematic compilation of genealogical data during the different phases of a population study allows the expansion of the vertical and horizontal pedigree. This information can ascend and descend through multiple generations in different families. This allows links between independent families through affected individuals similar to the case of the genogram presented here.

In the case of the FXS there is an unusual inheritance pattern characterized by a carrier father passing the PM to all of his daughters who then go on to have children with FXS and the FM from expansion of the CGG repeats to greater than 200. (3,10). Showing that the expansion of the CGG triplet occurs particularly of females with the PM to their children independent from the husband, but boys and males are more affected than females because they only have one X chromosome. Why males with the PM do not pass on the FM in their sperm is not yet known. There is something unique

about the egg and the fertilization process that allows a FM to expand in this offspring from a female. Given this X- linked inheritance pattern with anticipation through the generations related to the degree of CGG expansion, consanguineous unions are not necessary for the appearance of affected people.

On the other hand, as the database was fed continuously with the results of the different allelic variables of the FMR1 gene and the pedigree was analyzed knowing the FXS inheritance pattern, it was possible to infer which of the people who had not been screened or did not live in Ricaurte, could be mandatory carriers or could have a FM. This is how it was possible to give information by different means, then take samples in the population from Ricaurte and diagnose 9 more cases, 5 of them with the PM and 4 with the FM two of whom were minors. We also identified a couple in whom the females had the PM (VI-73) but in the family of the male (VI-74) there were cases of PM and FM and the daughters of the couple had PM. The analysis led us to postulate an error in the samples' packaging; the error was confirmed and rectified with the Southern blot studies in the couple.

The pedigree that is presented here does not limit itself to document graphically the magnitude of the geographic genetic cluster of FXS in Ricaurte. This pedigree also contributed to the location of new cases of PM and FM involvement, and led to the identification of inconsistencies in the results by the screening method allowing us to correct mistakes like the example noted above.

Ricaurte was founded mid XVII century by 10 families and their names are known by a public deed of purchase of the land; some of them come from Spain (11). When there is a high prevalence of individuals with PM and FM the postulation that one of the founders had the PM and his offspring when crossing with the other families had kept and propagated the PM and FM over time, the configuration of a founder effect is a logical hypothesis.

In the pedigree presented here was able to identify 3 of the 10 founders, I-3, II-3 and III-1, figure 6.2. The analysis of their descendants with cases of the PM and FM show us that III-1 must have had the PM; in his descendants 12 cases with the FM were found. Nevertheless, this does not explain all the cases of PM or FM and we observed 3 more family lines with FXS. In the descendants if II-2, there are 19 cases with PM and 18 with the FM. According to the available information from the descendants that were interviewed II-2 arrived in Ricaurte near the founding of the village coming the Tambo Cauca's department district. The third family nucleus that was identified was from VI-124, in which 3 cases of FM were found, however these individuals have the same last name that the descendants from II-2.

What is recognized as isonomy and a nexus in the given information cannot be discarded or ruled out. The fourth family line descended from VII-32 and contributed 5 cases of the PM and none of the FM to the genetic cluster; thus, they did not influence the prevalence of the affected ones and given the number of CGG repetitions in the members of this family with a PM of 55 it was very stable across generations. (12). this stability explains the lack of FM cases in this family and it suggests that this family does not have a connection with other families with FXS where the repeat number was unstable. On the other hand, VII-32 still lives in Ricaurte and his origin is clear from another village of Valle Del Cauca, Roldanillo. This discovery allows us to postulate that the geographic FXS genetic cluster in Ricaurte with the highest prevalence described in literature could be explained by the establishment of many family lines.

Another great genealogy has been built in an isolated population of albinism in Latin America, Aicuña in the north of Argentina. Through oral tradition and documents revising tabulated genealogies scientists utilized specialized software to build 12 generations involving 8000 individuals and a subsequent analysis reached a founder couple in the XVII century. In 14 male descendants from the couple mitochondrial DNA studies and haplotype analysis using 14 specific markers found a consistency of 85% between the oral tradition and the molecular tests results. To explain these differences errors of paternity or legitimacy were postulated when the state registers were made in the tabulated genealogies. The haplotype variants found in this population have a European origin (13).

In Ricaurte's genealogy we found biological inconsistencies with FXS inheritance patterns that can be explained by similar reasons of genealogical inconsistencies similar to the Aicuña genealogy where it is stated that "no socially recognized hidden links cannot be discarded but would not be documented in the pedigree presented here". Thus, we hypothesized that the 3 families in which PM and FM cases were documented depend on just one ancestor is valid and the way to validate it would be by haplotype studies in the contiguous regions of the FMR1 gene in the population of Ricaurte with PM and FM alleles.

Colombian population genotypes and specifically the Vallecaucana ones are a mixture of indigenous, Spanish and African DNA (14). According to documents about the founding of Ricaurte the families of some of the founders came from Spain (11) and these families match with the last names found in the affected families and specifically in the founder that must have the PM located through the pedigree with descendants with PM and FM alleles. Another similar association was demonstrated for Alzheimer di-

sease in Antioquia using haplotype studies and mitochondrial DNA which demonstrate that the anomalous gene was introduced into that population from Spain (15,16). Similarly, we postulated that in Ricaurte a founder effect exists and that the mutation in the FMR1 gene was introduced by Spanish colonists.

### CONCLUSIONS

In population medical genetics systematic pedigrees must be built by feeding the database and carrying out ongoing analyses so probable affected individuals and carriers can be identified. In addition, these pedigrees represent graphically family relationships.

The tabulated genealogy showed that in the FXS genetic cluster that exists in Ricaurte there are 3 nuclear families with PM and FM individuals that are probably descendants of the district founder with Spanish origin.

Haplotypic studies should be conducted around the FMR1 gene in order to demonstrate the founder effect and the origin of the mutation.



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## GENETIC, MEDICAL AND REPRODUCTIVE COUNSELING IN FEMALES WITH FULL MUTATION AND PREMUTATION CARRIERS IN A GENETIC CLUSTER

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### INTRODUCTION

Fragile X syndrome (FXS) is a genetic disease inherited through the X chromosome, caused by the abnormal and unstable expansion of the CGG triplet in the FMR1 gene, therefore it is classified in the group of diseases called triplet repeat disorders (1,2).

There are four ranges of CGG repeats in the FMR1 gene: up to 44 repeats is a normal allele; between 45-54 is gray zone (GZ); 55-200 is a PM; and beyond 200 repetitions is a FM and causes the FXS.(3,4).

Females who have the FM have a 50% chance of passing their expanded FM allele to their children; whereas PM carriers will have different probabilities of passing on the PM or the FM depending on the number of CGG repeats they have, their age and the number of AGG interruptions (after every 9 or 10 CGG repeats) in the 5'UTR region of the FMR1 (5,6). The carriers of the PM can experience fragile X associated disorders that are associated only with the PM including the fragile X-associated primary ovarian insufficiency (FXPOI) and the fragile X-associated tremor/ataxia syndrome (FXTAS) (7,8). A variety of other medical problems are associated with the PM including hypertension, migraine headaches, fibromyalgia, central pain

syndrome, hypothyroidism, sleep apnea, restless legs syndrome, chronic fatigue, anxiety, obsessive compulsive disorder and depression (20).

Genetic counseling is a public health tool with which information is given about natural history of the disease, offspring, chances of having children with FXS or carriers, health implications, probabilities of developing PM disorders like FXTAS or FXPOI, the diagnosis and available treatment. Genetic counseling is a primary prevention intervention that implies individuals make life and reproductive choices with the appropriate information and education. (9,10)

The aim of this study was to provide genetic, medical and reproductive counseling to the Ricaurte inhabitants with the FMR1 gene mutations in the PM or FM range. We also wanted to identify individuals affected with pathologies associated with the PM such as FXPOI and FXTAS.

### METHODOLOGY

A descriptive case series type of study was carried out.

Our study population was defined as Ricaurte inhabitants with PCR and/or Southern blot results for FXS (FM) or PM carriers.

Individuals that met the inclusion criteria were asked to sign an informed consent in order to carry out the verbal and written genetic, medical and reproductive counseling. An individualized document was handed to those that gave their authorization according to their allele and gender. This document summarized the chances of having children with PM and/or affected by FXS; the symptoms and chances of developing FXPOI and FXTAS were also explained. Additionally, specific information about temporary and permanent contraception methods was given to PM carriers and females with the FM. The most convenient contraception method was discussed and agreed on depending on the case, and the patient was encouraged to request this method from their respective health institution and physician.

Those who had an allele in the PM range underwent a medical history and physical examination both related to FXPOI and FXTAS. Within the evaluation, females were asked if they had monthly bleeding, if the answer was negative, the age at which the bleeding stop was asked. Menopause was defined as the absence of menstruation for more than a year. Reproductive and obstetrical history was obtained in all females.

The neurological examination included as assessment for an intention tremor or dysmetria with finger to nose testing and for positional tremor with arm extension for at least 10 seconds. Those with tremor were scored

as slight, moderate or severe, based on the Fahn, Tolosa and Marín tremor scale parameters. (11).

Patients were requested to walk in a straight line, without help, for a 6 meter distance in a flat and regular field and then tandem walk to assess ataxia. Instability or ataxia was assessed based on the 'Scale for the assessment and rating of ataxia (SARA)' parameters. (12)

## **RESULTS**

34 individuals met the inclusion criteria: 20 females with PM, 11 females with FM, 5 males with PM. All of them signed the informed consent form.

### **GENETIC AND REPRODUCTIVE COUNSELING**

Out of the 31 females with PM and FM, 21 (67.74%) were between 49 and 83 years old and were going through menopause or had completed menopause. The 13 female patients left were able to reproduce and besides the genetic counseling, these patients had counseling in family planning methods and in temporary or permanent contraception methods. The contraceptive method was agreed in consensus with the patient, and the health system was induced in order to acquire the method of choice. In seven cases, tubal ligation (permanent) was agreed upon; three females decided to use temporary contraceptive methods: one chose oral contraceptives and two intrauterine devices. In one case, preconception guidance was given and the possibility of FXS prenatal diagnosis was explained. There were two females without a sexual partner; therefore, condoms and oral contraceptives were suggested to them in case they reactivate sexual activities. The five males with the PM were not sexually active, two were over 70 years, the other three were under the age of 18 with no onset of sexual activity, thus, the usage of condom was recommended to the males over 70 years and to one under the age of 18 in case of restarting or beginning sexual activity.

### **FRAGILE X ASSOCIATED PRIMARY OVARIAN INSUFFICIENCY (FXPOI)**

Out of the 20 PM carriers, 17 of them (85%) were over 40 years and were included to carry out a FXPOI analysis. Three of these females (17.6 %) had absence of menstruation before they were 40 years old, fulfilling the absolute FXPOI criteria; one of them had two children affected with FXS and the other one did not have children. Additionally, a female had a hysterectomy at the age of 35 due to persistent vaginal bleedings and two years later she experienced perimenopause symptoms including hot flashes, and profuse night sweating. Another four (23.5%) females over 50 years had an obstetric history without pregnancies even though they had a sexual partner and were looking to reproduce through natural methods. A total of 41.1% (7) females had suggestive of ovarian insufficiency findings.

### **FRAGILE X ASSOCIATED TREMOR ATAXIA SYNDROME**

The 20 female PM carriers underwent a medical/neurological evaluation for signs and symptoms of FXTAS. Eight of these females were over 50 years and two of them presented with ataxia and two had tremor but neither of them had the two symptoms together. The SARA scale and the Fahn, Tolosa and Marin tremor scale was applied to these patients showing that one had moderate tremor and the other one had severe tremor. In conclusion, four females over 50 years (50%), had symptoms and clinical signs of FXTAS.

Of the five males, two were over 50 years; one had distal, slight intensity tremor and had no ataxia, and the other, even though he didn't have tremor nor ataxia, he had behavior changes including aggressiveness, morbid jealousy and disinhibition, signs also described in carriers with FXTAS. (13).

### **DISCUSSION**

Ricaurte is a village with high prevalence of PM carriers and FM individuals. Its population is socially and economically depressed, with limited access to basic health services and even more limitations regarding access to specialized care involving molecular testing for genetic disease or genetic counseling. In 1999, Payan and Saldarriaga reported the FXS endemic focus, through karyotype diagnosis with folate deficit tissue culture media, in 19 people and by clinical characteristics in 16 more. These patients with FXS all came from three families discussed previously. At that time, a prevalence of 1 in 38 males and 1 in 100 females was calculated with FXS which exceeded by more than 100 times the global prevalence reported

for FXS (14). However, after that study there was no specific health system intervention in the population.

In the study shown in this book, a PCR screening using the CGG linker technique was performed in all the Ricaurte inhabitants and the CGG repetition range in the FMR1 gene was confirmed by Southern Blot, 20 females and 5 males were identified as PM carriers and 11 females were identified with a FM.

Females with the PM have different probabilities of CGG expansion in the next generation leading to FXS in the children. When repetitions are in a range between 55 and 99 CGG repetitions, the probability varies between 1.8% and 40%. When there are 100 or more CGG repeats the risk of expanding to the FM 100% in the mutated X that is passed on and since there are 2 X chromosomes the changes of having a child with the FM is 50%. This probability is the same for females with the FM because the X chromosome stays as a FM in the children (2,15). The probability of expansion to the FM in the next generation is increased by maternal age and decreased by the number of AGG interruptions. (6) If the FM is inherited, disease manifestations will depend on the children's gender, if is a boy, he will suffer from FXS, however, if is a girl, she will have a 30% chance of having a normal IQ, and a 70% chance of an IQ less than 85 and features of FXS including anxiety and physical features of FXS. (2,16).

Male carriers of the PM will always pass the PM to their daughters with a relatively stable number of CGG repetitions although some instability in the PM range is common. It is uncertain why this occurs but sperm with the FM do not survive. Sons of a carrier father because they receive the father's Y chromosome and not the X. In males with FM it has been documented that only 1% have reproduced because of social emotional reasons combined with intellectual disability but when they do have children, all of their daughters inherit the PM only. (3,15,17).

Having in mind the socio-demographic conditions of Ricaurte, where the access to health services is limited and the knowledge about contraceptive methods varies but is particularly low in Ricaurte. Besides of identifying the carriers and giving genetic counseling to individuals with mutations in the FMR1 gene, it was necessary to bring birth control counseling and pre-conception counseling as needed in each case. The sum of all our health promotion interventions, including primary and secondary prevention should decrease the number of unplanned pregnancies and reduce the number of new FXS cases.

Multiple types of effectiveness in carriers range from learning problems in children to neurodegeneration in older carriers with FXTAS. (13,19).

FXTAS is characterized by neurological problems including a progressive intention tremor, cerebellar ataxia, parkinsonism, peripheral neuropathy, cognitive deficits, autonomic dysfunction, among others (20). Specific radiological findings make up part of the diagnose criteria, including white matter hyperintensity of the middle cerebellar peduncles, cortical and cerebellar atrophy and hyperintensity of the white matter in different areas of the central nervous system like the pontine nucleus, the insula cortex, the splenium of the corpus callosum and the periventricular region. (21,22).

FXTAS has an estimated prevalence in general population above 55 years old of 1 in 4000 males and 1 in 7800 females, clinical manifestations can appear after the age of 50 with a typical beginning between 60 and 65 years of age, it's frequency increases with the age; it affects approximately the 40% of male PM carriers and among 8-16% of female carriers (23). In Ricaurte, from the males over 50 years with PM, one of them had progressive intention tremor and another one had behavioral changes that suggested there was an alteration in the frontal cortex; this kind of changes have also been described within the psychiatric symptoms of PM n carriers. (13). From the 8 female PM carriers over 50 years of age, 50% had FXTAS symptoms; although the number of PM carriers and FXTAS cases is small, the percentages found in this study exceed what is expected in the literature (13,22).

Exposure to environmental toxins like insecticides, pesticides, general anesthesia, chemotherapy, orange agent, chronic intake of opioids and alcohol abuse, have been reported in patients with appearance of the symptoms at a younger age and/or deterioration of the clinical condition(24)

Opioids and alcohol abuse have been related to cerebral atrophy and white matter disease (25). None of these were found in those affected by FXTAS reported here.

However, residents of Ricaurte, with or without FXTAS, are chronically exposed to pesticides with neurotoxic potential, some for having worked directly in farmlands and others because of manipulating them in their own home garden. The most commonly used pesticides are avermectin (abacmetin), neonicotinoids (imidacloprid, thiocopril) pyrethroid (lambdacialotrin, galacialotrinogamaciolotrin and deltamethrin) and organophosphorus.

Chlorpyrifos and organophosphorus have been related with affective and cognitive disorders after acute and chronic intoxications that haven't caused cholinergic crisis (26–29). The described mechanism of action is related with the neuronal morphogenesis interference in crops (30,31) and in vivo (32), disturbing the axonal and mitochondrial transport as well as the neuronal movement (33–35) Chlorpyrifos leads to in vitro neuronal apoptosis (36) and activates the ryanodine receptor (37), modulating calcium



flow in neurons (38). These studies support the hypothesis that carriers of the FMR1 PM are more susceptible to adverse neurological outcomes associated with neurotoxic pesticides. Therefore, further controlled studies with specific measurements of the pesticides used in the region should be developed to protect all PM carriers and their clinical conditions.

In 20% of females with the PM, FXPOI develops so they go through menopause before the age of 40. They may also have irregular menstrual cycles and fertility reduction, this frequency in carriers is 20 times higher than the frequency in the general population, in which premature ovarian failure is around 1% (7,39,40). It has been shown that in females without Turner Syndrome, the most frequent genetic cause of ovarian insufficiency is the FMR1 gene PM (41); alterations are related with the regulation of the hypothalamic-pituitary-ovaries axis, the number and response of FSH and LH receptors in the granulosa cells, oogenesis regulation, the coordination of the transformation of the germinal cell into later stages and systemic endocrine functions (42,43). Ovarian insufficiency in female carriers is usually accompanied by an alteration in the normal hormonal production with increased FSH values (>40 UI/L) and low estradiol values (<50 pg/ml), also by the FSH/estradiol relation higher than 20 and ovarian dysfunction that decreases the chances of having a successful pregnancy (39,44).

However, a pathophysiological explanation for the occurrence of ovarian failure PM carriers is thought to relate to the RNA toxicity secondary to the high mRNA levels in carriers (45).

In this study, the 17.6% of females fulfilled FXPOIs criteria of menopause before 40 years of age, which complies with the literature. However, it was additionally found that the 23.52% (4 out of 17) had menstrual cycle and reproductive alterations, which allows us to conclude that the 40.12% (7 out of 17) of female carriers over 40 years included in this study had ovarian failure, a higher value than the one described in literature.

Knowledge and technology have to be available in order to obtain benefits in the neediest populations. In this case, molecular tests, genetic counseling and contraceptive methods join together as public health tools in approaching a socially and economically depressed social community with a high prevalence of FMR1 mutations. These tools have helped to decrease new FXS cases and to treat PM and FM individuals and in developing appropriate rehabilitation processes.

With this intervention, it is expected to prevent FXS and stop the perpetuation of the genetic FXS cluster in Ricaurte.

## CONCLUSIONS

Genetic and reproductive counseling was carried out in PM carriers and females with FM. It is expected to reduce the number of new cases with this intervention and also prevent the perpetuation of the high prevalence of FXS.

The FXTAS and FXPOI frequencies are higher when compared to the ones reported in literature. It is hypothesized that chronic exposure to pesticides used in agriculture could be part of the environmental factors that contribute to the appearance of these fragile X-associated disorders.

Molecular testing, genetic and reproductive counseling are public health tools that are used to intervene in communities affected by genetic diseases.

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## RICAURTE: FRAGILE X SYNDROME GENETIC GEOGRAPHIC CLUSTER

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The general goal of this investigational work was to demonstrate that Ricaurte has a genetic cluster of FXS and PM involvement.

Concepts about FXS presented in previous chapters will be integrated into this chapter,

Ricaurte is a genetic geographic cluster of FXS because it fits the established conditions by Castilla et al. (2014) for genetic origin diseases (1,2).

1. Occurrence in a specific geographic territory and geopolitically delimited. Ricaurte is a geographically defined and recognized place by the competent authorities as a village of Bolivar municipality.
2. Prevalence of relevant genotypes of the disease must be greater than the globally accepted prevalence. Given the genomics and inheritance of the FXS the alleles of interest of the FMR1 gene were FM and PM; the gray zone allele was excluded because descendants of the ones who carry it do not have children affected by FXS.

In this study it was found that in Ricaurte 1 of every 21 males and 1 of every 49 females, equivalent to 48.2 (95% RC:36.3-61.50) by 1000 and 20.5 (95% RC:13.5-28.6) by 1000, males and females, respectively, had the FM; FM prevalence was 343,3 and 222,7 times greater than the globally accepted prevalence (3). The prevalence of the PM was 1/71 males and 1/28 females, equally to 13.4 (95% RC:7.4-20.7) by 1000 and 35.8 (95% RC:26.5-46.20) by 1000 males and females respectively, this prevalence is 11.1 and 9.4 times higher in males and females respectively than the globally accepted prevalence. The high prevalence of allelic FM variants and PM alleles has statistical meaning knowing

that the confidence limits of the compared measurements do not overlap each other, see 2.4 chart. Also, this prevalence is the highest in the comparable literature studies (4–16).

3. The prevalence of affected people has been maintained over time. Álvarez Gardeazábal in 1986 in his book *El Divino*, described 1,124 inhabitants with 39 fools (bobos), male adults with intellectual disability and physical characteristics of FXS. (17). In the study conducted by the Universidad del Valle in 1999 it was reported that in a population of 1,128 inhabitants there were 35 people with intellectual disability, 19 of them were FXS confirmed by cytogenetic testing and 16 by phenotype characteristics (18); very similar numbers that the ones described by Álvarez Gardeazábal. However, in the study by the Universidad del Valle 8 individuals under the age of 14 were included as affected by FXS, that could not have been observed by Álvarez Gardeazábal in 1986 (17,18). In the results presented here, there are 1,186 inhabitants in Ricaurte, among them 20 males (including 3 under the age of 8) and 11 females with the FM. Among the individuals diagnosed by cytogenetic in 1999, 6 died before this current study started. So, at least between 1986 and 2016 the prevalence of individuals with intellectual disability and FXS in Ricaurte has remained high. In addition, the FXS most probably reached Ricaurte at the founding of the town, approximately 200 years ago.

### FOUNDER EFFECT

The data suggests that FXS has been in Ricaurte since its founding (first half of the XIX century). The documentation of the purchase of the land where today is Ricaurte by 10 individuals whose names and surnames are known (19), and those today affected by FXS have those same surnames, was described by Álvarez Gardeazábal himself: “the families that have brought fools (bobos) to the town for centuries and centuries”. In the pedigree (figure 6.2) it was observed that the one who probably brought the PM or FM to Ricaurte did it in the first or second generation of those that founded Ricaurte. The high prevalence of PM and FM alleles in the natives are sufficient to conclude that a founder effect happened in Ricaurte.

Based in this discovery we suggest that Ricaurte’s emigrants including their asymptomatic descendants or descendants with intellectual disability that are anywhere in the world should have molecular testing to document the PM or FM.

## FACTORS THAT EXPLAIN THE HIGH PREVALENCE OF FXS IN RICAURTE

The persistent high prevalence of FXS in Ricaurte could be explained by the following factors:

- The arrival of the expanded allele since the founding of Ricaurte allows the dissemination of this allele in a growing population and subsequent establishment in different families.
- Genomic characteristics of inheritance and phenotypic expression in FXS ease the perpetuation of the PM in the population. Given that the carriers of the PM do not usually show specific phenotypical characteristics that suggest that they can have kids affected by FXS (20), this allows the free development of their offspring.. The repeat expansion does not require two carriers to have affected children as in recessive diseases. Therefore, the birth of new affected children does not require consanguineous unions. In addition, being a condition inherited by the X chromosome implies that females affected with the FM can be asymptomatic, and pass on to their offspring the expanded allele perpetuating the allele in the population.(21,22).
- The lack of access to molecular testing and genetic counseling of the inhabitants in the past has led to a lack of education of what perpetuates this disorder.
- Young people migrate to other cities in Colombia or other countries pursuing better life conditions and the ones who have intellectual disability or affected by FXS did not had the possibility to migrate away from the town.
- Popular beliefs could have contributed to the fact that medical causes were not sought for the large number of intellectual disabilities in Ricaurte. These popular beliefs include 4 theories. The first one posits that excessive exposure to magnesium would produce intellectual disability. This theory was supported by the proximity to a mountain rich in that mineral. The second theory was that males would have intellectual disability due to witch craft. The third theory said that the intellectual disability was secondary to consanguineous unions, and the fourth one said that the cause was “bad families”. This last hypothesis matches with the inheritance characteristics of FXS that is a bad gene mutation was in the families

## GENETIC COUNSELING

All the inhabitants of Ricaurte that participated in the study were given the results in a written format and they were asked to sign a record for the receipt. The negative results were given by the project staff, if the person required more information; an appointment was made with one of the researchers to explain the results in more detail. Results in the range of GZ PM and FM were personally delivered by the researcher in charge and the genetic counseling was carried out depending on the case.

Ricaurte's inhabitants with GZ allelic variants, PM and females with FM of the FMR1 gene received reproductive genetic counseling, emphasizing that probability of having children with PM or FM, the last ones with ID thus affected by FXS. Each one signed an informed consent (IC) for receiving genetic counseling.

In addition in the exercise of genetic counseling to the carriers of PM education about signs and symptoms of FXPOI and FXTAS was given. Of the 20 females with the PM, 17 were over 40 years and 4 (23.52%) had a medical history of absent menses before 40 years that corresponded to FXPOI and 3 (17.6%) did not have children despite having a sexual partner. This suggested ovarian insufficiency, also described in carriers of the PM (23). Eight of the females with the PM were more than 50 years old and the 50% had clinical symptoms of FXTAS, one female presented with FXPOI and FXTAS (24); three carriers of the PM who were sisters had more severe phenotypes, one of them had seizures that were difficult to manage and brain atrophy on MRI with contrast, another had severe cerebellar atrophy related with chronic treatment with phenobarbital for seizures and the last one had life-long severe mutism (25). It was also found that from the 20 females with the PM 5 (20%) had chronic seizures, while in the literature this association had a prevalence of 0.55 (1 case in 199) (26). From the five males with PM, two of them were more than 50 years old, one of them had clinic symptoms of FXTAS, had marked intention tremor and the other one presented with behavioral changes of severe frontal deficits, this finding is also described in those with FXTAS (27).

In Ricaurte the frequency and severity of the symptoms of FXTAS, FXPOI and seizures in carriers with the PM are high. This could be explained by a second altered gene or by environmental factors such as pesticides in agriculture

## MEDICAL ATTENTION

As a request by the community, a group of investigators from Congenital Malformations and Perinatal, Medical Genetics and Dysmorphology, UV-HUV (MACOS), organized and conducted medical journeys with pediatric specialists for all of Ricaurte's inhabitants. Carriers of the PM and those affected by the FM received specific recommendations for the treatment of the medical problems such as seizures, aggression, sleep disorders, anxiety, and alcohol and drug consumption, among other conditions. In eight patients with the FM frequent alcohol consumption was found, and this was associated with exacerbation of their behavior problems, such as an increase of impulsivity and aggression, as well as medical problems such as an increase in the frequency of seizures (28). One case was diagnosed with Down syndrome and FXS and she responded well to treatment of anxiety with sertraline (29).

## CONTRIBUTIONS OF THIS INVESTIGATION TO KNOWLEDGE AND SOCIETY

The contributions to knowledge and society of this investigation are based on the development of a methodology for screening and diagnosis of a genetic disease in a whole population. In Ricaurte we demonstrated a high prevalence of FXS and the PM compared to the prevalence elsewhere in the world and a more severe phenotype in many carriers perhaps related to poverty and environmental toxicity.

The reasons for the genetic cluster of fragile X disorders with the highest prevalence of the world lies in the relationship between factors of genetic origin, sociodemographic and environmental circumstances that generated an effect that deteriorates life quality causing intellectual disability in some people, neurodegenerative diseases and endocrine/medical disorders among others, which not only affects individuals, but the families and the entire community.

This study integrates knowledge in the fields of medical population genetics, the FXS, molecular tests, clinic genetics, neurology, obstetrics gynecology, statistics and epidemiology, and applies them in a community that needed help for both diagnosis and treatment.

## CONCLUSIONS

Ricaurte is a fragile X genetic geographic cluster, since it meets all the parameters of the definition of Castilla et al. (2014). It is a geographically defined place, the prevalence of affected people has been high through time, the prevalence of the allelic variants (PM and FM) are significantly elevated compared to the globally accepted prevalence and they are the highest in literature.

The existence of an FXS genetic geographic cluster dramatically affects the health of Ricaurte's inhabitants, not just for the number of individuals with FM and FXS or ID, but also the high frequencies and symptoms severity of the diseases that were found in carriers of the PM like FXTAS, FXPOI and seizures, which influences the social and economic dynamic of all the community.

The fragile X genetic geographic cluster in Ricaurte has been established by a founder effect, sociodemographic conditions of Ricaurte's population, health deficits, popular beliefs and migratory patterns.

With genetic and reproductive counseling carried out at the end of this study, it is expected to mitigate the perpetuation of the fragile X genetic geographic cluster in Ricaurte.

## NEW INVESTIGATION PROPOSALS

We want to perform measurements of pesticide used in agriculture for those with the PM and those affected by FXS and then correlate or findings with age of onset and symptom severity.

Join multicentre studies of treatment to those affected by FXS, FXPOI, and FXTAS will be carried out.

Ricaurte's inhabitants with allelic variables in the PM with seizures deserve genomic studies searching for a second genetic affect that affects the severity of the phenotype.

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## ABOUT THE AUTHORS

### **Wilmar Saldarriaga Gil**

Born in Cali in 1974. Professor of the Department of Morphology, and Department of Gynecology and Obstetrics of the Universidad del Valle; of which he graduated as a doctor and surgeon, and which he represented as a student in research congresses, obtaining several awards, the most important being the "XI Aventis-National Academy of Medicine Award" (2000) for the best research work in the area clinic. Is an obstetrician-gynecologist and holds a Master's degree in Basic Medical Sciences with Emphasis in Embryology and Genetics from Universidad del Valle. In the "V World Congress of Perinatology and XXVI Colombian Congress of Obstetrics and Gynecology" (Cartagena, 2008), it obtained the first place in the category of free works of research in perinatology. And prize for the best research work in the National Congresses of Obstetrics and Gynecology, in the years 2010 and 2016.

Director of the research group on Perinatal Congenital Malformations, Dymorphology and Medical Genetics, MACOS, from Universidad del Valle, category A of Colciencias. It has 80 publications in indexed journals, including: Colombia Médica, Colombian Journal of Obstetrics and Gynecology, British Medical Journal Case Report, Birth Defects, American Journal of Obstetrics & Gynecology, NeuroToxicology, Journal of Human Genetics. Author of the books Fundamentals of Obstetrics and Gynecology and Integrated Human Embryology. In 2014, he received the "Best Medical Educator of Colombia" prize in the "Young Educator" category from Ascofame. In October 2017 he received his PhD in Biomedical Sciences with an emphasis in Medical Genetics from the Universidad del Valle, his thesis received meritorious mention, during the last year of the doctorate he did training at the MIND Institute of the University of California at Davis.

**Randi J. Hagerman**

Distinguished professor of the department of pediatrics and medical director of the MIND institute (medical research of neurodevelopmental disorders) at the University of California Davis (UC-Davis). She is internationally recognized as a doctor and researcher in the field of Fragile X syndrome (SXF) and has a chair dedicated to research in the same syndrome.

The professor Hagerman received her master's degree in medicine from Stanford University, where she also earned her pediatrician degree. He did his subspecialty in learning problems and ambulatory pediatrics at the University of California-San Diego. Subsequently, he spent the next 20 years from 1980 to 2000 at the University of Colorado, where he directed the area of pediatrics of development and behavior. She co-founded the national Fragile X foundation in 1984 and developed a world-renowned research center under SXF treatment. In 2000, Professor Hagerman moved to UC-Davis. Drs. Randi and Paul Hagerman and their team discovered Tremor Syndrome and Ataxia Associated with Fragile X (FXTAS), which is a neurological disorder that affects carriers of the premix of FXS. Dr. Hagerman has published at least 500 articles, most of them in high impact journals, JAMA and Nature, among others; it has an index h of 81 according to SCOPUS. His current research includes targeted treatments for SXF and for those affected by FXTAS; in addition to genotype-phenotype studies in the broad spectrum of FXS.

**Sergio Agilar Glaxiola**

Dr. Aguilar-Gaxiola is an internationally renowned expert on mental health in ethnic populations. As on-site principal investigator of the Mexican American Prevalence and Services Survey ? the largest mental health study conducted in the United States on Mexican Americans ? he identified the most prevalent mental health disorders in the Mexican-origin population in California?s central valley; showed that the rate of disorders increases the longer the individual resides in the United States; and demonstrated that children of immigrants have even greater rates of mental disorders. From this study, he developed a model of service delivery that increased access to mental health services among the Central Valley?s low-income, underserved, rural populations.

Dr. Aguilar-Gaxiola conducts cross-national epidemiologic studies on the patterns and correlates of psychiatric disorders in general population samples. He is the coordinator for Latin America and the Caribbean of the World Health Organization?s Mental Health Survey, and coordinates the work of the National Mental Health Institute surveys in Mexico, Colum-

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### **Carlos Andrés Fandiño Losada**

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### **Jose Vicente Forero Forero**

Senior of Medicine and Surgery program from the Universidad Del Valle. He was born in Cali on December 15 1992 and studied at Colegio Colombo Britanico high school also in Cali, obtaining International Baccalaureate Diploma granted by the Baccalaureate international organization with headquarters in Switzerland. During his undergraduate he participated in 3 medical research congresses, being the coauthor or speaker of original works. He presented the investigation protocol "Description of the expression profile of miRNAs in the serum of newborns with hypoxic-ischemic encephalopathy at the Hospital Universitario del Valle" in the XXV Colombian Student Program of medical research that took place in Armenia in 2014, where he achieved acknowledgement as the second best protocol. Also he is coauthor of the book named "Genetic and reproductive counseling in premutation carriers in a Fragile X Syndrome genetic cluster" presented by

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He has been outstanding during his undergraduate as a student that is interested in medical research, particularly in the subject regarding medical genetics; he is part of the Congenital and Perinatal Malformations, Dysmorphology and Medical Genetics research group (MACOS) from the Universidad Del Valle with COLCIENCIAS A category. He published 5 articles till this date, in indexed magazines including Colmédica, Journal of Pregnancy, Iatreia, Revista Colombiana de Cardiología and the Acta Neurológica Colombiana.

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He was born in Cali in 1984. He is the assistant professor of the morphology department of the Universidad Del Valle. Is part of the Congenital and Perinatal Malformations, Dysmorphology and Medical Genetics research group (MACOS) from the Universidad Del Valle with COLCIENCIAS A category. Has over 30 publications in national and international indexed magazines and is the coauthor of “Integrated Human Embryology” book.

Chronologically his academic educational is the following: Colegio La-cordaire academic bachelor (2001), physician and surgeon from the Uni-

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He has performed as neonatal resuscitator physician of the Hospital Universitario del Valle, dysmorphology and genetics consultant from the same institution, geneticist of the Equipo de Enfermedades Huérfanas de la Secretaría de Salud Municipal de Cali, and as medicine professor in several universities

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### **Carolina Isaza de Lourido**

Physician and surgeon (1981); and basic medical sciences magister (1992) from the Universidad Del Valle (1992), where she worked as main professor in the morphology department until June of 2017, she carried out an investigation in the embryology and genetics areas. Founder and director of the Congenital and Perinatal Malformations, Dysmorphology and Medical Genetics research group (MACOS); vice chancellor of the Uni-

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He published several papers in national and international magazines, such as: Colombia médica, Revista Colombiana de Obstetricia y Ginecología, Bio-médica, Births Defects, American Journal of Medical Genetics. Actually, she is the medical director of the Neuro and cardiovascular clinic “DIME”.

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Dr. Tassone received her B.S. degree in biology from the University of Rome "La Sapienza" and her Ph.D. from the University of Rome UCSC in 1992. She is a Professor in the Department of Biochemistry and Molecular Medicine and a MIND Institute investigator at the University of California, Davis, School of Medicine. Dr. Tassone is a molecular geneticist with a specialty in gene transcriptional and translational regulation and she has carried out a significant work on the molecular characterization of the FMR1 gene at the structure, expression, CGG instability methylation and function levels. She has extensively reported on genotype/phenotype correlations particularly related to gene expression and methylation in patients with fragile X syndrome, with a full mutation and/or mosaicism.

Dr. Tassone has made a number of important observations related to the FMRP expression and to the mechanism of gene expression of the FMR1 gene, especially regarding the effects of expanded alleles (55-200 CGG repeats) on premutation carriers. Her most significant work led to the important discovery of gene dysregulation (increased mRNA activity) among premutation carriers. This discovery has provided the molecular basis for the forms of clinical involvement among carriers, including fragile X-associated tremor/ataxia syndrome (FXTAS), which was described in 2001. Since then she has carried out extensive work on the molecular basis and abnormal molecular phenotype observed in individuals with FXTAS.



Dr. Tassone is the director of a Fragile X project and the Director of the Fragile X Biobank, funded by the National Fragile X Foundation. Dr. Tassone is well known in the international Fragile X community; her work has been presented internationally and she has published extensively on the molecular aspect of both Fragile X and FXTAS and autism.

### **Marisol Silva**

Is a native of Mexico who immigrated at a young age to the United States. She attended the University of California, Davis as an undergraduate where she received her Bachelor of Science in Genetics and Bachelor of Arts in Psychology. As an undergraduate she worked as a research recruiter under Dr. Flora Tassone on the NIH funded pilot study: Newborn Screening in Fragile X Syndrome. For the past few years she has continued working as a Research Associate under Dr. Tassone, providing laboratory support to various research studies and clinical trials in Fragile X syndrome and Autism. She has also been coauthor of some publications in international indexed journals, including: *Brain and Development*, *Iatreia*, *Journal of Human Genetics*, *eNeurologicalSci*, and *NeuroToxicology*. She is a recent graduate from San Francisco State University, Clinical Laboratory Science program and is an American Society for Clinical Pathology (ASCP) certified Clinical Laboratory Scientist.

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**Universidad del Valle**



This book describes how it's possible to demonstrate that Ricaurte is a genetic cluster of fragile X syndrome (FXS) with the highest prevalence in the world through an investigational project cofounded between the Universidad del Valle and the MIND institute of the University of California, Davis.

FXS is described in depth, from genomics, through heritability and FXS phenotype until experimental treatment. Characteristics of Ricaurte are shown; Bolivar's municipality town in Valle del Cauca, including its foundation, social contextualization, its regional display characterized by a religious and national context by El Divino book, written by Gustavo Álvarez Gardeazábal, talks about the intellectual disability of an important number of inhabitants that are described masterfully.

Also, the project "Population medical genetics in a Colombian town with a high prevalence of fragile X syndrome", which was an ambitious proposal, where besides the establishments of grey zone allelic variants prevalences, premutation and full mutation of FMR1 gene and their comparison with the globally accepted prevalence, the execution of a public health investigation with genetic and reproductive counseling to whom may have children affected by FXS or suffer associated diseases like FXTAS or FXPOI was achieved. With the methodology of reaching the houses and every inhabitant, asking for informative consent, taking blood samples, delivering results, establishing interfamilial relationships through a specialized software and performing genetic and reproductive counseling, shows how the investigators integrated day to day with the community and understood the reality of the country in poor families with many members with intellectual disability or the fear of those who may have affected children; it was proven that Ricaurte is a genetic cluster of FXS with the highest prevalence in the world in which there's a family with the highest number of affected members reported in literature and where an intervention that will impact the decrease of new cases in order to prevent the perpetuation of the genetic and secondary social problematic was carried out.

