Patologías asociadas a la premutación del gen *FMR1*

María Isabel Álvarez Mora

ADVERTIMENT. La consulta d'aquesta tesi queda condicionada a l'acceptació de les següents condicions d'ús: La difusió d'aquesta tesi per mitjà del servei TDX (**www.tdx.cat**) i a través del Dipòsit Digital de la UB (**diposit.ub.edu**) ha estat autoritzada pels titulars dels drets de propietat intel·lectual únicament per a usos privats emmarcats en activitats d'investigació i docència. No s'autoritza la seva reproducció amb finalitats de lucre ni la seva difusió i posada a disposició des d'un lloc aliè al servei TDX ni al Dipòsit Digital de la UB. No s'autoritza la presentació del seu contingut en una finestra o marc aliè a TDX o al Dipòsit Digital de la UB (framing). Aquesta reserva de drets afecta tant al resum de presentació de la tesi com als seus continguts. En la utilització o cita de parts de la tesi és obligat indicar el nom de la persona autora.

ADVERTENCIA. La consulta de esta tesis queda condicionada a la aceptación de las siguientes condiciones de uso: La difusión de esta tesis por medio del servicio TDR (**www.tdx.cat**) y a través del Repositorio Digital de la UB (**diposit.ub.edu**) ha sido autorizada por los titulares de los derechos de propiedad intelectual únicamente para usos privados enmarcados en actividades de investigación y docencia. No se autoriza su reproducción con finalidades de lucro ni su difusión y puesta a disposición desde un sitio ajeno al servicio TDR o al Repositorio Digital de la UB. No se autoriza la presentación de su contenido en una ventana o marco ajeno a TDR o al Repositorio Digital de la UB (framing). Esta reserva de derechos afecta tanto al resumen de presentación de la tesis como a sus contenidos. En la utilización o cita de partes de la tesis es obligado indicar el nombre de la persona autora.

WARNING. On having consulted this thesis you're accepting the following use conditions: Spreading this thesis by the TDX (**www.tdx.cat**) service and by the UB Digital Repository (**diposit.ub.edu**) has been authorized by the titular of the intellectual property rights only for private uses placed in investigation and teaching activities. Reproduction with lucrative aims is not authorized nor its spreading and availability from a site foreign to the TDX service or to the UB Digital Repository. Introducing its content in a window or frame foreign to the TDX service or to the UB Digital Repository is not authorized (framing). Those rights affect to the presentation summary of the thesis as well as to its contents. In the using or citation of parts of the thesis it's obliged to indicate the name of the author.

PATOLOGÍAS ASOCIADAS A LA PREMUTACIÓN DEL GEN *FMR1*

Memoria presentada por **Mª Isabel Álvarez Mora** para optar al grado de Doctora por la Universitat de Barcelona

Tesis adscrita al **PROGRAMA DE DOCTORADO EN GENÉTICA,** Departament de Genètica, Facultat de Biologia, Universitat de Barcelona; Tutor: Bru Cormand Rifa

Tesis realizada bajo la dirección de la Dra. Montserrat Milà y la Dra. Laia Rodriguez-Revenga en el Departamento de Bioquímica y Genética Molecular Hospital Clínic de Barcelona

La Co-Directora	La Co-Directora	El tutor	La doctoranda
Dra. M. Milà	Dra. L Rodriguez-Revenga	Dr. B Cormand	Mª Isabel Álvarez

Universitat de Barcelona

er ciberer CENTRO DE INVESTIGACIÓN BIOMÉDICA EN RED DE ENFERMEDADES RARAS

CLÍNIC BARCELONA Hospital Universitari

A mi madre

AGRADECIMIENTOS

En primer lugar quiero agradecer a mi grupo Montse, Laia e Irene por haberme acogido con los brazos abiertos y hacerme sentir como una más desde el primer momento. Gracias a ellas hoy puedo estar aquí y espero poder quedarme mucho más ya que siento que he encontrado el sitio donde quiero estar. De las tres he aprendido muchísimas cosas durante este tiempo y quiero agradecerles de todo corazón la oportunidad que me han dado. No sólo a nivel profesional sino también a nivel personal ya que junto a Loli, Montsita, Joan Anton y Celia me han demostrado su amistad y sin ellos el día a día no sería igual. Gracias por acompañarme en mis miles de cafes/tapers a destiempo y por aguantar mi desorden y mi caos, gracias por todo de verdad!

Especialmente quiero agradecerles a mis directoras Montse y Laia por todo el apoyo que he tenido, la paciencia que han mostrado y el ánimo que me han dado siempre para seguir adelante y poder acabar esta tesis.

A mis compañeras del hospital Estefi, Vero y Alba quiero agradecerles que siempre me han ayudado cuando las he necesitado, muchas gracias chicas!

A todos los MITOS!! y en especial a Gloria, Mariona, Marc y Ester que me han enseñado todo el mundo mitocondrial, han batallado conmigo y se han vuelto un apéndice más de mi día a día.

A mis *bionenes*. Sin vosotras para mí la biología no sería lo mismo, sé que disfruto al hacer lo que hago gracias en parte a todos nuestros recuerdos que han sido maravillosos y por eso quiero agradeceros a vosotras este trabajo. Tenéis la capacidad de convertir mis penas y agobios en risas y buenos momentos, sabéis que os quiero. #ponylove!!

A mi gente, a mi Morago, a mi madre, a mi hermana, a mi tía y a mis niñas por haberme apoyado siempre y soportar los miles de ensayos de mis presentaciones. Sé que estáis orgullosos de mí y os sentís felices de que haya realizado este trabajo. Gracias por confiar en mí y demostrarme lo mucho que me queréis.

Y por último a todos los que me habéis ayudado durante estos 5 años (Juliana, Inés, Petar,….) por vuestro amabilidad y buenos consejos. Quiero agradeceros vuestro granito de arena para que este viaje haya llegado a su término.

Va por ustedes!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!!

LISTADO DE ABREVIATURAS

3'UTR	Región 3' no traducida
5'UTR	Región 5' no traducida
γH2AX	*Del inglés* "fosfo- H2A histone family, member X"
ADN	Ácido desoxirribonucleico
AGG	Trinucleótido adenina-guanina-guanina
AKT	*Del inglés "v-akt murine thymoma viral oncogene homolog 1"*
ApoE	*Del inglés "Apolipoprotein E"*
ARN	Ácido Ribonucleico
ARNm	Ácido Ribonucleico mensajero
ATP	Adenosina Trifosfato
AUG	Adenina-uracilo-guanina
CAG	Trinucleótido citosina-adendina-guanina
caja RGG	dominio con cinco repeticiones Arginina-Glicina-Glicina
CGG	Trinucleótido citosina-guanina-guanina
CNVs	Variaciones en el número de copias
CpG	Citosina fosfo Guanina
CRHR1	*Del inglés "Corticotropin releasing hormone receptor 1"*
CYFIP1	*Del inglés* "Cytoplasmic FMR1 interacting protein 1"
DI	Discapacidad Intelectual
dominio KH	Dominio Lisina-Histidina
DGCR8	*Del inglés* "DiGeorge critical region 8"
extremo C-terminal	Extremo Carboxi-terminal
FDR	*Del inglés* "False Discovery Rate"
FMR1	*Del inglés "Fragile X mental retardation 1*"
FMRP	*Del inglés "*Fragile X Mental Retardation Protein"
FOP	Fallo ovárico prematuro
FRAXA	*Fragile X Site A*
FSH	Hormona folículo estimulante
FXPOI	Insuficiencia ovárica primaria asociada al X-Frágil
FXR1P	*Del inglés* "Fragile-X-related protein 1"
FXR2P	*Del inglés* "Fragile-X-related protein 2"
FXTAS	Síndrome de temblor/ataxia asociado al X-Frágil
GO	*Del inglés* "Gene Ontology"
HD	Corea de Huntington
hnRNP A2	*Del inglés* "Heterogeneous ribonucleoprotein particle A2"
HTT	Huntingtina
Kb	Kilobase

KEGGs	*Kyoto Encyclopedia of Genes and Genomes*
KI	*Knock-in*
KO	*Knock-out*
LH	Hormona luteinizante
LTD	*Long-term depression*
LTP	*Long-term potentation*
MAPK	*Del inglés* "Mitogen Activated Protein Kinases"
MFN2	Mitofusina 2
mGluR	*Del inglés* "Metabotropic glutamat receptors"
miRNAs	microARNs
NES	Señal de Exportación Nuclear
NLS	Señal de Localización Nuclear
O_2	Oxígeno
OMIM	*Del inglés* "Online Mendelian Inheritance in Man"
OPA1	*Del inglés* "Optic atrophy 1"
CMSPs	Células mononucleares de sangre perfiérica
PM	portadores de la premutación en el gen *FMR1*
PI3K	fosfoinositol 3-quinasa
POI	Insuficiencia Ovárica Primaria
poliA	poliAlanina
poliG	poliGlicina
poliQ	poliGlutamina
poliR	poliArginina
RM	Resonancia Magnética
R-loops	Híbridos ADN/ARN
RAN translation	*Del ingés* "Repeat Associated Non-ATG Translation"
ROS	Especies reactivas del oxígeno
RT-qPCR	PCR cuantitativa a tiempo real
señal MCP	señal de los pedúnculos medios cerebelosos
smallRNAs	ARNs de pequeño tamaño
SN	Sistema Nervioso
SNC	Sistema Nervioso Central
SXF	Síndrome X Frágil
VEGF	*Del ingés* "Vascular Endothelial Growth Factor"
XCI	Inactivación cromosoma X

ÍNDICE

Introducción

Capítulo 1: Gen *Fragile X mental retardation 1*…………………......3-15

Factores que median la expansión del trinucleótido CGG….7-11

Características de FMRP…………………………..………11-15

Capítulo 2: Patologías asociadas a la premutación del gen *FMR1*..16-33

Insuficiencia Ovárica Primaria Asociada al X-Frágil……..21-26

Síndrome de Ataxia y Temblor asociado al X-Frágil……..27-33

Capítulo 3: Mecanismos moleculares asociados a la premutación..34-45

Mecanismo de toxicidad del ARNm expandido de *FMR1*..37-39

Traducción no mediada por AUG………………….…..39-40

Factores adicionales……………………………........41-43

Disfunción mitocondrial y FXTAS………………………..44-45

Hipótesis y Objetivos

Hipótesis…………………………………..……………49-50

Objetivos………………………...……...……………..51

Informe de los directores…………………………………………53-58

Resultados

Capítulo 1…………………………..…………………...…………61-69

Mecanismos patogénicos en FXPOI

Resumen Trabajo I..……………….……………………………61-62

Artículo 1……......……………………………………………63-69

Capítulo 2...64-130

Mecanismos patogénicos y factores de riesgo en FXTAS

Resumen Trabajo II..…………..……………………..............70-72

Artículo 2...…………………………………………...……..73-86

Resumen Trabajo III..…………..…………………...………….87-88

Artículo 3..……………………………………........................89-99

Resumen Trabajo IV...100-102

Artículo 4..103-130

Discusión

Bases moleculares asociadas a la premutación en *FMR1*..133-137

Mecanismos patogénicos en FXPOI.........................138-141

Mecanismos patogénicos y factores de riesgo en FXTAS.142-150

Tecnologías de alto rendimiento.............................151-154

Conclusiones...155-158

Bibliografía...159-174

Anexos

Anexo I: "Clinical features associated with FMR1 premutation carriers"..177-196

Anexo II: "High apolipoprotein E4 allele frequency in FXTAS patients"..197-200

Anexo III: "Blood expression profiles of fragile X premutation carriers identify candidate genes involved in neurodegenerative and infertility phenotypes"..................................201-212

INTRODUCCIÓN

Capítulo I

Gen *Fragile X mental retardation 1*

El Síndrome X Frágil (OMIM# 300624; ORPHA908) es la causa más común de discapacidad intelectual (DI) familiar afectando a uno de cada 4000 varones y una de cada 6000 mujeres (Hagerman 2002). Las manifestaciones clínicas de este síndrome son muy variables y dependen en gran manera de la edad y del sexo del individuo afectado. Los principales signos clínicos son la presencia de DI moderada o severa, macroorquidismo, facies alargadas, prognatismo y orejas grandes y aladas. En el caso de las mujeres, la inactivación del cromosoma X (XCI) repercute en la penetrancia del síndrome ya que únicamente un 30% presentan signos clínicos. La expresividad en las mujeres es muy variable y puede ir desde el mismo fenotipo que en los varones hasta únicamente manifestar una DI ligera sin rasgos físicos característicos aunque suelen presentar trastornos de conducta, aislamiento y angustia social (Milà et al. 1996).

En el año 1943 se describió por primera vez las características clínicas de este síndrome nombrado originalmente el “Síndrome Martin-Bell” en honor a los investigadores que lo describieron (Martin & Bell 1943). Aunque dada la herencia se sospechaba de un síndrome ligado al cromosoma X, no fue hasta el año 1969 cuando se detectó la misma

alteración citogenética en una familia con 4 varones afectados con DI. Estos individuos eran portadores de una fragilidad del brazo largo del cromosoma X en la banda Xq27.3, conocido como FRAXA (*Fragile X Site A*). A pesar que esta anomalía se cuestionaba como método diagnóstico puesto que únicamente se detectaba al estudiar metafases obtenidas de linfocitos cultivados en medios deficientes en ácido fólico (Lubs 1969), dio origen al nombre actual "Síndrome X Frágil" (SXF). Finalmente, en el año 1991 tres grupos independientes realizaron la clonación del gen responsable de este síndrome, el *Fragile X mental retardation 1* (*FMR1*) (Oberlé et al. 1991; Verkerk et al. 1991; Yu et al. 1991). El estudio molecular reveló que la fragilidad cromosómica se debe a una expansión anómala del trinucleótido citosina-guanina-guanina (CGG) en la región 5' no traducida (5'UTR) del exón 1 del gen *FMR1*. La proteína sintetizada se conoce como *Fragile X Mental Retardation Protein* (FMRP) y su expresión está regulada por una isla CpG situada en el extremo 5' del gen *FMR1*. Este gen consta de 38kb en la secuencia genómica y contiene 17 exones que dan lugar principalmente a un transcrito de un tamaño de 4.8kb que contiene 632 aminoácidos. Sin embargo, *in vivo* se han observado hasta 12 isoformas más cortas como resultado del *splicing* alternativo de los exones 12, 14, 15 y 17 (revisado en Willemsen et al. 2011).

La región 5' UTR del primer exón contiene una tramo de repeticiones CGG variable, considerado como un polimorfismo en la población general. Según la longitud de la expansión se diferencian 4 clases alélicas: normales (6-44 CGGs), intermedios (45-54 CGGs), premutados (55-200 CGGs), mutados (>200CGGs) (Figura 1).

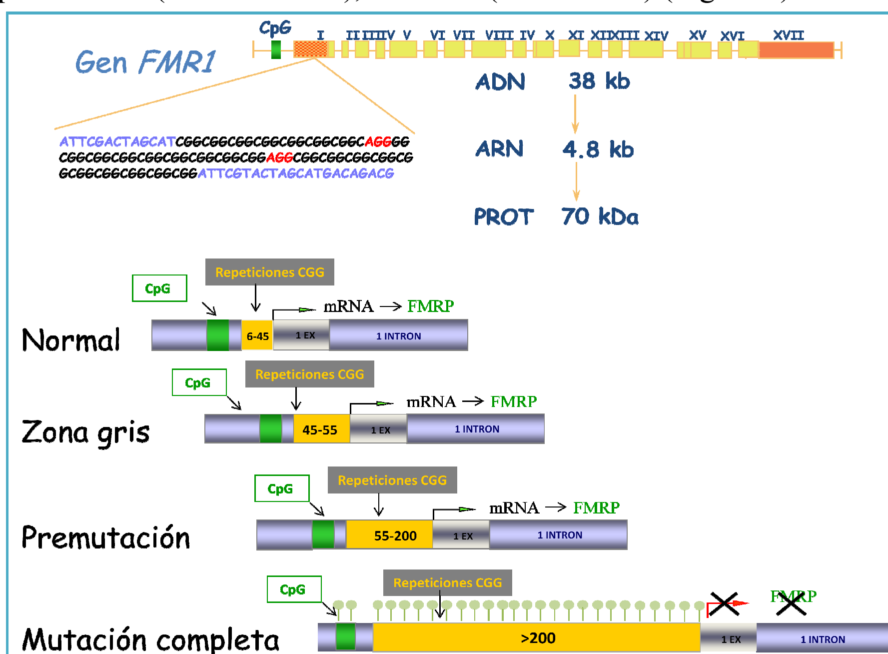


Figura 1. Esquema gen *FMR1*. Descripción molecular de los alelos existentes (modificada de https://fragilex.org/2014/genetics/fmr1-gray-zone-allele-what-do-we-know-about-it/).

En los alelos normales (6-45 CGGs) la isla CpG adyacente no se encuentra metilada, y por tanto el gen *FMR1* está activo y FMRP es sintetizada. Estos alelos se transmiten de manera estable a la siguiente generación, siendo los alelos con 29 y/o 30 repeticiones CGGs los más frecuentes en la población caucásica, representando hasta el 46% de los cromosomas (Milà et al. 1994). La eficiencia en la síntesis de FMRP

depende del número de CGGs, siendo de 30 repeticiones la longitud óptima de traducción proteica, lo que podría explicar la elevada frecuencia de estos alelos en la población (Chen et al. 2003).

Los alelos intermedios (45-54 CGGs) también son conocidos como “zona gris” debido a su proximidad entre la separación de los alelos normales y premutados. La prevalencia de estos alelos es relativamente elevada en la población caucásica estimada en 1 de cada 66 mujeres y 1 de cada 112 hombres (Tassone et al. 2012a). Hasta el momento no se ha establecido una implicación clínica clara de estos alelos (Hall et al. 2011; Madrigal et al. 2011; Nolin et al. 2013). Actualmente, se consideran ligeramente inestables existiendo la posibilidad de expansión al rango de premutación al transmitirse a la siguiente generación e incluso al rango de mutación completa en 2 generaciones dependiendo del número de interrupciones de las secuencia CGG por el trinucleótido adenina-guanina-guanina (AGG) (Terracciano et al. 2004; Zuñiga et al. 2005; Fernandez-Carvajal et al. 2009).

En los alelos premutados (55-200 CGGs) el rango de repeticiones se vuelve altamente inestable tanto a nivel meiótico como mitótico, pudiendo expandir al transmitirse por vía materna a alelos con la mutación completa. En los alelos intermedios y los premutados la isla

CpG del gen *FMR1* no está metilada, y por tanto, hay transcripción del ARNm de *FMR1* y síntesis de FMRP.

Finalmente, los alelos con mutación completa (>200 CGGs) se caracterizan por la hipermetilación tanto de las citosinas de la propia expansión como de la isla CpG adyacente que inhibe la transcripción génica y por consiguiente se inhibe también la expresión de la proteína FMRP. Esta mutación es la causa más común de SXF y se atribuye a la ausencia de FMRP la aparición de las manifestaciones clínicas en los individuos afectados (Oberlé et al. 1991; Verkerk et al. 1991; Yu et al. 1991). Sin embargo, se han detectado muy excepcionalmente individuos SXF como consecuencia de mutaciones puntuales en la secuencia codificante (Grønskov et al. 2011).

Factores que median la expansión del trinucleótido CGG

El riesgo de expansión de los alelos intermedios y premutados se ha correlacionado con el origen parental, con el número de repeticiones CGG y con el número de interrupciones AGG (Tabla 1).

En primer lugar se ha demostrado que a pesar de la inestabilidad en las transmisiones por vía paterna, las expansiones al rango de mutación completa ocurren únicamente en alelos transmitidos por vía materna. En los alelos paternos se han descrito una mayor frecuencia de contracciones que en los alelos maternos (Rifé et al. 2004; Nolin et al.

1996), mientras que las expansiones aunque se producen frecuentemente nunca alcanzan el rango de mutación completa (Nolin et al. 2003). Este tipo de transmisión se cree que es consecuencia de la selección negativa de los gametos con alelos con la mutación completa durante la espermatogénesis (Ashley-Koch et al. 1998; Sullivan et al. 2002).

Tabla 1. Riesgo de expansión de los alelos maternos al rango de mutación completa en función del número de repeticiones CGG y del número de interrupciones AGG (obtenida de Yrigollen et al. 2012).

	$(AGG)_n$	Riesgo expansión
$(55\text{-}59)_n$ CGG	0	2.6%
	1	1.1%
	2	0.1%
$(60\text{-}69)_n$ CGG *calculado para 60 y 65	0	8.2-22.9%
	1	3.5-10.7%
	2	0.4-1.2%
$(70\text{-}79)_n$ CGG *calculado para 70 y 75	0	49.6-76.5%
	1	28.5-56.9%
	2	4-12%
$(80\text{-}99)_n$ CGG *calculado para 80,85, 90 y 95	0	91.5%-99.7%
	1	81.4%-99.4%
	2	31.2-94.3%
$(100\text{-}109)_n$ CGG *calculado para 100 y 105	0	99.9%-100%
	1	99.8%-99.9%
	2	98.2%-99.5%
$(110\text{-}120)_n$ CGG *calculado para 110, 115 y 120	0	100%
	1	100%
	2	99.8-100%

En segundo lugar, el grupo de Rifé y colaboradores (2004) determinó que los alelos con más 80 repeticiones CGG tenían un riesgo de expansión 7 veces superior al de los alelos con 59 repeticiones y en el caso de los alelos con más de 100 CGGs el riesgo de expansión era del 100%. En base a estas observaciones, existe una correlación positiva entre el riesgo de expansión de los alelos transmitidos por vía materna y el número de repeticiones, de tal manera que las mujeres con alelos de mayor tamaño tienen un mayor riesgo de expansión al rango de mutación completa (Nolin et al. 2011).

Por último, hay evidencias que sugieren que las interrupciones AGG tienen un papel importante en el incremento de la estabilidad del tramo CGG ya que mantienen su número y posición al transmitirse por vía materna (Nolin et al. 2015; Yrigollen et al. 2012). En realidad, aproximadamente el 95% de los alelos normales contienen 1 o 2 interrupciones, siendo los alelos más frecuentes los que contienen dos interrupciones, localizadas después de 9 repeticiones CGG [$(CGG)_9AGG(CGG)_9AGG(CGG)_n$] o de 10 repeticiones CGG [$(CGG)_{10}AGG(CGG)_9(CGG)_n$]. Las primeras evidencias de estos alelos datan en las primeras divergencias de las razas humanas hace 200.000 años (Eichler & Nelson 1996), aunque también se han descrito más raramente alelos normales sin ninguna interrupción o bien con más de

dos (Yrigollen et al. 2014). Por otra parte los alelos inestables, ya sean intermedios o premutados, segregados en familias afectadas por el SXF suelen perder una o ambas interrupciones (Eichler et al. 1994), por lo que se confiere el mayor riesgo de expansión a los alelos premutados sin interrupciones (Nolin et al. 2013) (Figura 2).

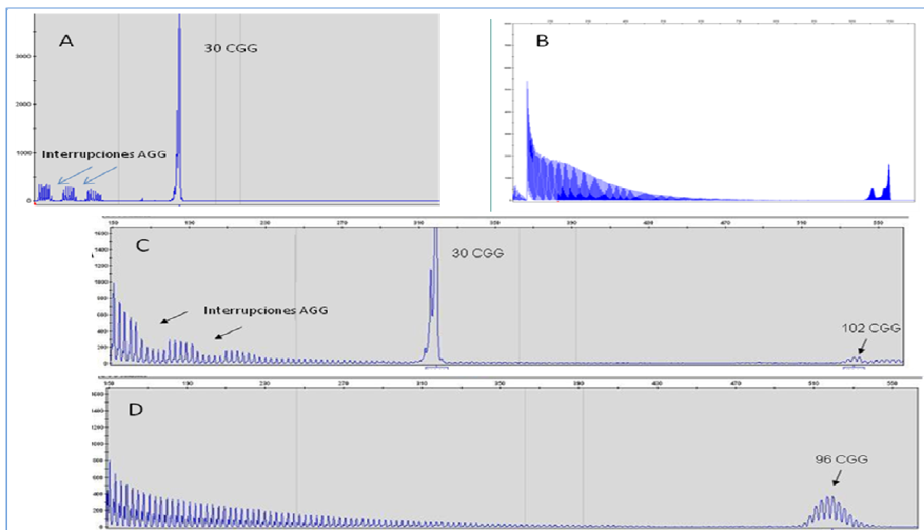


Figura 2. Visualización de la región polimórfica CGG del gen *FMR1* amplificado mediante el kit Amplidex FMR1 PCR (Asuragen). Se indica el tamaño de la expansión y la presencia de interrupciones AGG. **A)** Perfil de un varón portador de un alelo normal y dos interrupciones; **B)** Perfil de un varón portador de un alelo con mutación completa; **C)** Perfil de un mujer portadora de un alelo con la premutación y un alelo normal; **D)** Perfil de un varón portador de la premutación

Actualmente el alelo más pequeño descrito con capacidad de expandir al rango de mutación completa en una única generación es de 56 repeticiones CGG sin ninguna interrupción AGG (Fernandez-Carvajal et al. 2009). Aparentemente, las interrupciones AGG no tienen ningún impacto en los procesos de transcripción y/o traducción del gen *FMR1* (Tassone et al. 2007). Sin embargo, aunque por el momento se

desconocen los mecanismos por los que median la estabilización de la longitud del tramo CGG, se ha sugerido que impedirían y/o desestabilizarían la formación de estructuras secundarias en la región expandida (ej. CGG hairpin loops), disminuyendo de esta manera el riesgo de slippage de la ADN polimerasa durante los procesos de replicación y/o de reparación del ADN (Napierala et al. 2005; Jarem et al. 2010).

Características de FMRP

FMRP es un factor de transcripción que contiene en la parte central de la proteína dos dominios de homología hnRNP K (dominios KH) y en el extremo C-terminal una región enriquecida en aminoácidos arginina y lisina conocido como caja RGG. Ambos tipos de dominios desempeñan un papel crucial ya que promueven la interacción con la vía de los microARNs (miRNAs) (Jin et al. 2004) y le proporcionan la capacidad de reprimir la transcripción génica mediante la unión a estructuras no codificantes de sus ARNm dianas (Darnell et al. 2001; Darnell et al. 2005). De hecho, la mutación puntual más severa de un paciente con el SXF (I304N) se ha descrito en el dominio KH2 (De Boulle et al. 1993). Además FMRP, contiene señales de localización (NLS) y de exportación nuclear (NES) que le permiten actuar como lanzadera entre el núcleo y el citoplasma de la célula (Figura 3).

FMRP se expresa de manera ubicua detectándose en mayor abundancia en espermetagonias y en tejido cerebral, principalmente en cortex, cerebelo e hipocampo (Devys et al. 1993). La expresión predominante en estos tejidos es consistente con la amplia disfunción neurológica y el macroorquidismo característico de los individuos afectos con el SXF (revisado en Hagerman et al. 2014). En las células neuronales, la localización de FMRP es principalmente citoplasmática, detectándose en las dendritas, en el soma y en la zona post-sináptica (Weiler et al. 1997) aunque también se detecta en el núcleo celular gracias a las señales NLS y NES.

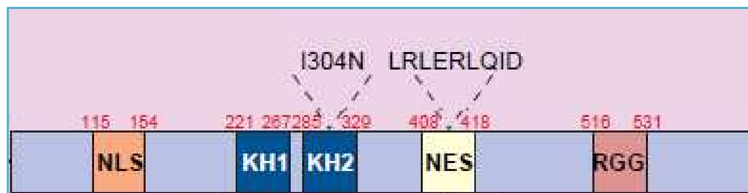


Figura 3. Esquema estructura proteica de FMRP. La mutación puntual más severa y la secuencia conceso de la señal de exportación nuclear se encuentra indicada (modificado de Kooy et al. 2000).

La función principal de FMRP es la inhibición de la traducción de sus ARNm dianas y el transporte de éstos hacia las espinas dendríticas cuya traducción también se ve silenciada (revisado en Gallagher & Hallahan 2012; Hagerman et al. 2014). Concretamente, FMRP tiene como diana entre 400 y 600 transcritos diferentes expresados en el cerebro incluyendo el suyo propio (Adinolfi et al. 1999). En el citoplasma, se encuentra asociada con ribosomas libres y/o unidos al retículo

endoplasmático mediante la interacción con sus ARNms dianas y con varias proteínas (ej. FXR1P, FXR2P, CYFIP1…), formando complejos multiproteicos conocidos como ribonucleoproteínas dependientes de ARNm (revisado en Willemsen et al., 2011). Finalmente, FMRP también se detecta localizada en gránulos citoplasmáticos de ARN que son dirigidos hacia las espinas dendríticas (Figura 4).

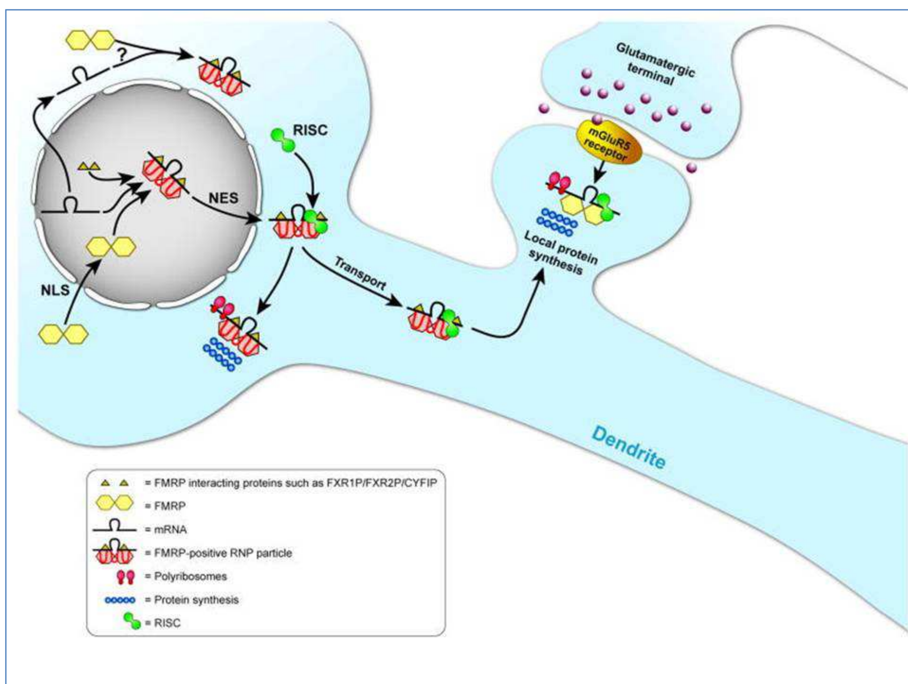


Figura 4. Función FMRP en las neuronas (obtenido de Willemsen et al. 2011).

El gran abanico funcional de FMRP justifica que su ausencia de lugar a las deficiencias intelectuales manifestadas por los pacientes afectados con SXF (revisado en Lozano et al. 2014a). El amplio cuadro clínico presente en los pacientes SXF se atribuye a la desregulación simultanea de varias vías de señalización neuronal (revisado en Hagerman et al.

2014). Todas las evidencias hasta el momento, sugieren que la pérdida de función de *FMR1* promovería la desregulación de la síntesis local de números genes implicados en el desarrollo de las espinas dendríticas y en el proceso de plasticidad sináptica (revisado en Antar & Bassell 2003; Willemsen et al. 2004; Bassell & Warren 2008). A nivel molecular la plasticidad sináptica refleja la capacidad de las neuronas para fortalecer (long-term potentation, LTP) o debilitar (long-term depression, LTD) las conexiones sinápticas individuales de manera continuada. Principalmente, las transmisiones LTD vienen inducidas por receptores mGluR (*metabotropic glutamat receptors*), proceso indispensable para la consolidación de la memoria y el aprendizaje. Actualmente se ha propuesto la "Teoría del mGluR" en el SXF (revisado en Bear et al. 2004) ya que se detecta en el modelo murino knock-out (KO) de *fmr1* una señalización exagerada de esta vía tanto en el hipocampo como en el cerebelo.

Los cambios neurobiológicos que se producen en los pacientes con SXF se centran en alteraciones estructurales detectadas en las sinapsis. En el año 2001, Irwin y colaboradores pusieron de manifiesto mediante el análisis microscópico de tejidos post-mortem que los pacientes afectados con el SXF presentaban espinas dendríticas con un fenotipo más inmaduro y una mayor densidad de protuberancias en algunas

áreas del cerebro en comparación con los individuos normales (Figura 5). Las espinas dendríticas son el punto de contacto en la excitación de la neurona postsináptica, lo que sugiere que la disfunción cognitiva de los pacientes SXF subyace en las alteraciones ya sean a nivel funcional, de fuerza o de desarrollo de la sinapsis (revisado en Pfeiffer & Huber 2009). En consonancia con los hallazgos detectados en pacientes SXF, estudios realizados en el modelo KO de *fmr1* de mosca y de ratón han puesto de manifiesto la implicación de FMRP en la sinapsis ya que, en ausencia de esta proteína, las neuronas presentan una deficiencia en el transporte dendrítico de los ARNm así como una movilidad reducida de los gránulos de ARN (Estes et al. 2008; Dictenberg et al. 2008). Además, el modelo murino KO de *fmr1* presenta tanto las alteraciones en la función sináptica (revisado en Pfeiffer & Huber 2009) como los cambios estructurales presentes en los pacientes SXF.

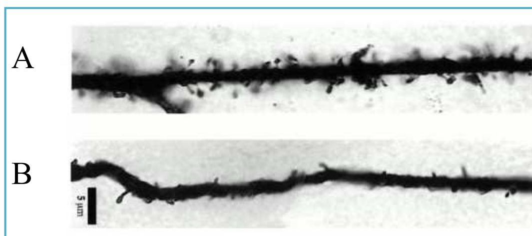


Figura 5. Análisis microscópico de espinas dendríticas procedentes de: **A)** paciente afectado de SXF; **B)** individuo control (obtenido de Iwin et al. 2001).

Capítulo II

Patologías asociadas a la premutación del gen *FMR1*

Desde el descubrimiento del gen *FMR1*, el conocimiento de las patologías asociadas a la premutación ha evolucionado drásticamente ya que durante muchos años la importancia de estos alelos se restringía al elevado riesgo de tener descendencia afectada del SXF. Históricamente, los individuos portadores de alelos premutados se consideraban asintomáticos dado que no manifestaban DI. Sin embargo, desde el año 2000 han aparecido dos patologías de aparición tardía asociadas específicamente a estos individuos: la insuficiencia ovárica primaria (FXPOI; OMIM#311360, ORPHA619) y el síndrome de temblor/ataxia asociado al X-Frágil (FXTAS; OMIM#300623, ORPHA93256). Estas patologías se atribuyen a un mecanismo diferente de la pérdida de función del gen *FMR1*, por lo que representan formas alélicas independientes del SXF. No obstante, cada vez es más evidente que existe un amplio espectro de manifestaciones clínicas que abarca alteraciones cognitivas, psiquiátricas, endocrinas e inmunológicas que afecta en cierto grado a los individuos portadores de la premutación (Tabla 2).

Un factor que tienen en común todas las patologías asociadas a la premutación es la penetrancia reducida, es decir, no todos los

individuos portadores de alelos premutados desarrollaran alguna de estas patologías y en cambio otros pueden llegar a desarrollar varías de ellas. En este contexto, el desarrollo de las tecnologías de alto rendimiento ha proporcionado herramientas científica y metodológicamente prometedoras para el avance del conocimiento sobre las causas y mecanismos involucrados en las enfermedades complejas. Entre ellas, los *microarrays* de expresión son particularmente potentes para proporcionar una visión global del perfil de expresión génica en un único experimento (Wong & Chang 2005). En esta tesis doctoral se presentan los resultados obtenidos mediante el uso de *microarrays* de expresión génica y de miRNAs, con el fin de identificar grupos funcionales de genes con una expresión aberrante en individuos con FXPOI o FXTAS. El **trabajo I** y el **trabajo II** de esta tesis doctoral se centran en la identificación de mecanismos moleculares que puedan estar implicados en la patogénesis de FXPOI y de FXTAS, respectivamente.

Actualmente, la asociación entre la premutación en el gen *FMR1* y el FXPOI y/o el FXTAS se encuentra científicamente documentada. Sin embargo, respecto las manifestaciones clínicas recientemente asociadas con los individuos portadores de la premutación únicamente se ha descrito una mayor prevalencia de estas patologías, especialmente en

los pacientes con FXTAS, que la descrita en la población general. Por lo tanto son necesarios más estudios para esclarecer su relación con la premutación del gen *FMR1*. Recientemente nuestro grupo ha editado un libro centrado en las formas alélicas del gen *FMR1* y a los fenotipos asociados. En el **anexo 1** de esta tesis doctoral se presenta el capítulo donde se recogen las principales manifestaciones clínicas asociadas a la premutación.

Tabla 2. Manifestaciones clínicas asociadas a la premutación del gen *FMR1* (modificado del anexo I)

	Cohorte estudiada*	Referencias
Características reproductivas		
FXPOI	Mujeres PM	(Revisado en Wheeler et al. 2014)
Dificultades obstétricas y perinatales	Mujeres PM	
Manifestaciones asociadas deficiencia de estrógenos	Mujeres PM con FXPOI	
Trastornos Inmunológicos		
Fibromialgia	Mujeres PM	(Berry-Kravis et al. 2007; Coffey et al. 2008; Rodriguez-Revenga et al. 2009a; Leehey et al. 2011; Winarni et al. 2012)
Trastornos tiroideos	Mujeres PM	(Coffey et al. 2008; Rodriguez-Revenga et al. 2009a; Winarni et al. 2012)
Síndrome del intestino irritable	Mujeres PM	(Winarni et al. 2012)
Trastornos del desarrollo		
Deficiencias en la memoria del trabajo	Hombres PM	(Kogan et al. 2008; Cornish et al. 2009)
Tartamudez	Mujeres PM	(Sterling et al. 2013)
Discapacidad del procesamiento espacio-temporal	PM jóvenes-adultos	(Wong et al. 2012)
Debilidad aritmética	Mujeres PM	(Lachiewicz et al. 2006; Semenza et al. 2012)
Retraso del desarrollo	Hombres/Mujeres PM	(Bailey et al. 2008)

Disfunción capacidades autonómicas		
Impotencia	Hombres FXTAS	(Greco et al. 2007)
Hipertensión	PM con FXTAS	(Coffey et al. 2008; Hamlin et al. 2011)
Incontinencia fecal y urinaria	PM con FXTAS	(Jacquemont et al. 2003; revisado en Leehey 2009)
Implicaciones neurocognitivas y psiquiátricas		
FXTAS	Hombres/Mujeres PM	(Hagerman et al. 2001)
Depresión	Mujeres PM	(Rodriguez-Revenga et al. 2008a; Bourgeois et al. 2011; Hunter et al. 2012; Kraan et al. 2014)
Ansiedad	Mujeres PM	
Trastornos del estado de ánimo	Mujeres PM	
Convulsiones	Niños varones PM	(Chonchaiya et al. 2012)
Trastorno del Espectro Autista	Niños varones PM	(Farzin et al. 2006; Chonchaiya et al. 2012)
Déficit de atención con hiperactividad	Hombres/Mujeres PM	(Farzin et al. 2006; Kraan et al. 2014)
Demencia	PM con FXTAS	(Greco et al. 2006; Rodriguez-Revenga et al. 2010)
Neuropatía periférica	Hombres/Mujeres PM	(Hagerman et al. 2007; Coffey et al. 2008)
Otras manifestaciones clínicas		
Migraña	Hombres/Mujeres PM	(Au et al. 2013)

*PM: portadores de la premutación en el gen *FMR1*

Insuficiencia Ovárica Primaria Asociada al X-Frágil

El ovario humano adquiere su capacidad funcional durante el desarrollo embrionario ya que las mujeres al nacer tienen un número finito de folículos primordiales que constituyen la reserva ovárica. Al llegar a la madurez sexual, se establece el proceso cíclico de ovulación regulado complejamente por el eje hipotálamo-hipófisis-ovario (Figura 6). El número de folículos va disminuyendo de manera paulatina a lo largo de la vida reproductiva hasta llegar su agotamiento de dichos folículos primordiales manifestándose en las mujeres la aparición de la menopausia (revisado en Vital-Reyes 2010).

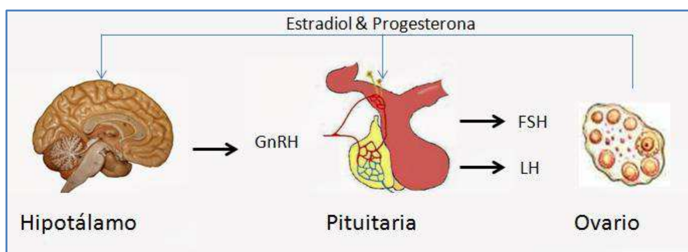


Figura 6.
Eje hipotálamo-hipófisis-ovario.

La insuficiencia ovárica primaria (POI), antes conocida como fallo ovárico prematuro (FOP), se define clínicamente como la cesación de las menstruaciones antes o alrededor de los 40 años. Esta fenómeno viene asociado con un incremento en los niveles en suero de las gonadotropinas FSH (hormona folículo estimulante) y LH (hormona luteinizante), así como con una disminución en los niveles de estrógenos (Nelson 2009). Como consecuencia, la disfunción ovárica

promueve la aparición prematura de problemas de fertilidad y de hipoestrogenismo que conlleva al envejecimiento temprano de numerosos tejidos. De esta manera, se incrementa el riesgo de padecer osteoporosis, enfermedades cardiovasculares o neurodegenerativas (revisado en Sherman et al. 2014).

Aunque en la mayoría de los casos el origen de la POI es desconocido, existen numerosos mecanismos patogénicos que pueden conducir a la menopausia precoz incluyendo tanto alteraciones genéticas como factores ambientales (ej. enfermedades autoinmunes, metabólicas, infecciones o causas iatrogénicas) (revisado en Goswami & Conway 2005). Respecto a los factores genéticos, en aproximadamente el 7% de POI se ha descrito una etiología genética debida tanto a alteraciones cromosómicas como génicas. Independientemente de tratarse de casos familiares o esporádicos, se han detectado principalmente alteraciones en el cromosoma X (revisado en Persani et al. 2010). De hecho, hasta un 20% de mujeres con POI idiopática son portadoras de la premutación del gen *FMR1*, estableciéndose como causa principal de la POI familiar no sindrómica (Sherman 2000; Mallolas et al. 2001). Esta forma es conocida como FXPOI y su asociación con los alelos premutados está bien establecida.

En realidad, las primeras evidencias de esta asociación existen desde antes del descubrimiento del gen *FMR1*. En 1991, el grupo de Cronsiter y colaboradores puso de manifiesto un incremento 20 veces superior de la incidencia de la menopausia precoz en las mujeres portadoras de la fragilidad del cromosoma X respecto a la incidencia de la población general establecida alrededor del 1% (Cronister et al. 1991). Posteriormente, Allinghan-Hawkins y colaboradores (1999) reportaron que el 16% de las portadoras de la premutación padecían disfunción ovárica mientras que ninguna de las portadoras de la mutación completa lo padecía y únicamente detectaron un caso (0.4%) entre las mujeres con alelos normales (Allingham-Hawkins et al. 1999). De esta manera, la premutación en el gen *FMR1* se estableció como factor de riesgo para el desarrollo de POI. Sin embargo, se cree que todas las portadoras de la premutación presentan una disfunción ovárica en un rango continuo de severidad, incluso en aquellas que no llegan a manifestar FXPOI (Sullivan et al. 2011).

Estudios realizados en el modelo murino de FXPOI sugieren que la disfunción ovárica en las mujeres con FXPOI podría ser consecuencia de una aceleración en la pérdida de los folículos (Hoffman et al. 2012) así como defectos en el desarrollo de los folículos en crecimiento (Lu et al. 2012). Sin embargo, los mecanismos moleculares que promueven la

desregulación en los oocitos permanecen sin elucidar (revisado en Wittenberger et al. 2007). No obstante, se han estudiado numerosos factores que podrían incrementar el riesgo de desarrollar FXPOI (Tabla 3). En primer lugar, se ha descrito una asociación con el número de repeticiones, estableciéndose el mayor riesgo de POI a los alelos que contienen entre 80 y 99 repeticiones CGG. Sorprendentemente, está relación no es lineal ya que las mujeres con POI portadoras de alelos entre 55-78CGGs y 100-200CGGs presentan un menor grado de disfunción ovárica (Allen et al. 2007). En segundo lugar, se ha sugerido que el número de interrupciones AGG puede influenciar el riesgo a desarrollar FXPOI. En un estudio realizado por Bodega y colaboradores (2006), detectaron 9 mujeres con POI portadoras de alelos intermedios, las cuales presentaban en todos los casos el tramo CGG ininterrumpido. Sin embargo, para poder asociar la ausencia de interrupciones AGG con la FXPOI se necesitan más estudios. En tercer lugar, aunque no se ha detectado un efecto directo de la inactivación preferencial en el cromosoma X con la FXPOI, existen evidencias que sugieren que este proceso podría estar contribuyendo al fenotipo. Concretamente, se ha detectado un porcentaje superior de inactivación preferencial del alelo normal entre las portadoras con FXPOI que entre las portadoras que no desarrollaban FXPOI (Rodriguez-Revenga

2009b). Por último, se ha descartado la influencia de los niveles de expresión de *FMR1* (Tejada et al. 2008) así como del origen parental del alelo premutado (Murray et al. 2000).

Tabla 3. Estudio de los posibles factores genéticos que medien el desarrollo del FXPOI.

Factor	Asociación	Referencia
Origen parental	No	Murray et al. 2000
Número de repeticiones CGG	Sí	Sullivan et al. 2005
Número de interrupciones AGG	Posible	Bodega et al., 2006
Niveles del mRNA de *FMR1*	No	Tejada et al., 2008
Background genético	Sí	Hunter et al., 2008
Inactivación preferencial cromosomaX	Posible	Rodriguez-Revenga et al. 2009b

Respecto FMRP, *a priori* se había descartado su implicación en la FXPOI dada la ausencia de este fenotipo entre las mujeres afectadas con el SXF. Sin embargo, existen evidencias recientes que han cuestionado el rol de esta proteína en la disfunción ovárica. En primer lugar, se ha demostrado que FMRP se expresa en animales *wild-type* en las células de la granulosa, en el cuerpo lúteo y de manera más llamativa en los oocitos (Ferder et al. 2013). Además, FMRP es detectada principalmente de manera citolplasmática en los oocitos durante todas las etapas de la foliculogénesis (Figura 7). Finalmente,

estudios realizados en el modelo murino de FXPOI han evidenciado una acumulación nuclear aberrante de FMRP en numerosos oocitos con un nivel elevado de ubiquitinación (Hoffman et al. 2012). En base a estas observaciones, se ha sugerido que la disminución de los niveles de FMRP podría contribuir a la FXPOI.

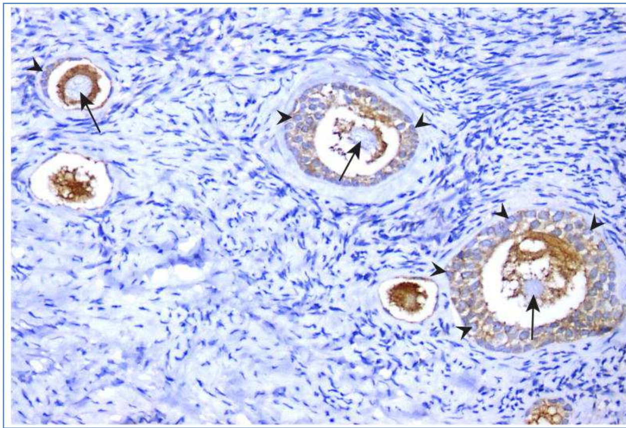


Figura 7. Expresión proteína FMRP durante la foliculogenésis en los ooctios (señalados por flechas) y en las células de granulosa (señalados por punta de flecha) (obtenida de Willemsen et al. 2011).

Síndrome de Temblor/Ataxia asociado al X-Frágil

El grupo liderado por la Dr. Hagerman describió por primera vez en el año 2001 un trastorno del movimiento específico en 5 varones mayores de 50 años portadores de la premutación caracterizado por un temblor de acción progresivo y ataxia asociado a ciertos rasgos radiológicos (Hagerman et al. 2001). Este síndrome fue denominado FXTAS y posteriormente también fue descrito en mujeres portadoras de la premutación aunque con una frecuencia menor, una afectación fenotípica más leve y a una edad de presentación superior (Hagerman et al. 2004).

FXTAS es una enfermedad neurodegenerativa de aparición tardía con penetrancia incompleta dependiente de la edad y del sexo que afecta a los individuos portadores de la premutación en *FMR1*. Sin embargo, se han descrito algunos casos de FXTAS en individuos portadores de alelos intermedios (Liu et al. 2013) e incluso más raramente en individuos portadores de alelos con la mutación completa hipometilada (Loesch et al. 2012; Santa María et al. 2014).

Su prevalencia se estima entre el 40-45% para los varones y en el 8-16% para las mujeres por encima de los 50 años, aunque se ha demostrado que la penetrancia incrementa con la edad afectando

alrededor del 75% de los individuos premutados a partir de los 80 años (Jacquemont et al. 2004; Rodriguez-Revenga et al. 2009a).

Por otra parte, se ha puesto de manifiesto que hasta un 4% de los individuos con alteraciones del movimiento de etiología desconocida son portadores de la premutación en *FMR1*, de esta manera, FXTAS se considera la forma más común de trastornos del movimiento causados por un defecto monogénico (Macpherson et al. 2003; Rodriguez-Revenga et al. 2007; Rodriguez-Revenga et al. 2008b). El diagnóstico temprano de estos pacientes no sólo aporta un beneficio para ellos sino que también tiene un doble interés ya que repercute en el resto de la familia, la cual debe recibir asesoramiento genético sobre el SXF.

Los síntomas clínicos aparecen en los portadores de la premutación a partir de los 50 años de vida y aunque existe una gran variabilidad en la progresión de la disfunción neurológica, normalmente lo primero que se manifiesta es un temblor de intención en la mano dominante. Este temblor es progresivo y a medida que avanza la enfermedad van apareciendo otros síntomas tales como la ataxia que conlleva problemas de equilibrio con frecuentes caídas, la pérdida de sensibilidad en extremidades distales y la aparición de pérdidas en funciones autónomas como son la impotencia, la hipertensión o la pérdida del control de los esfínteres (revisado en Hagerman et al. 2003; Hagerman

& Hagerman 2013). Además, aproximadamente el 64% de los pacientes con FXTAS presenta síntomas parkinsonianos caracterizado por un temblor intermitente en reposo, facies inexpresiva y un tono incrementado (Apartis et al. 2012). Generalmente, los pacientes FXTAS experimentan además síntomas psicológicos como son la ansiedad, la irritabilidad, los cambios de humor y el comportamiento de reclusión, que podrían estar relacionados con la alteración cognitiva y/o con cambios en el área límbica del cerebro (revisado en Hagerman & Hagerman 2004). Finalmente, en estadios más avanzados de la enfermedad algunos pacientes pueden manifestar déficit cognitivo asociado principalmente a problemas de memoria y de la función ejecutiva, que incluso puede llegar a progresar a demencia en algunos casos (Rodriguez-Revenga et al. 2010).

El análisis de las imágenes obtenidas mediante resonancia magnética (RM) cerebral han permitido establecer marcadores específicos de la enfermedad de FXTAS. Concretamente estos pacientes presentan un incremento en la señal, obtenida en las imágenes de secuencia T2, de los pedúnculos medios cerebelosos (señal MCP) así como de la sustancia blanca cerebral adyacente que se consideran características patonomónicas de los pacientes FXTAS (Figura 8). Otros hallazgos neuroradiológicos característicos son la detección de un aumento del

tamaño de los ventrículos cerebrales y de una atrofia cerebral generalizada afectando al cortex, al cerebelo y al tronco cerebral (Jacquemont et al. 2003; Rodriguez-revenga et al. 2012).

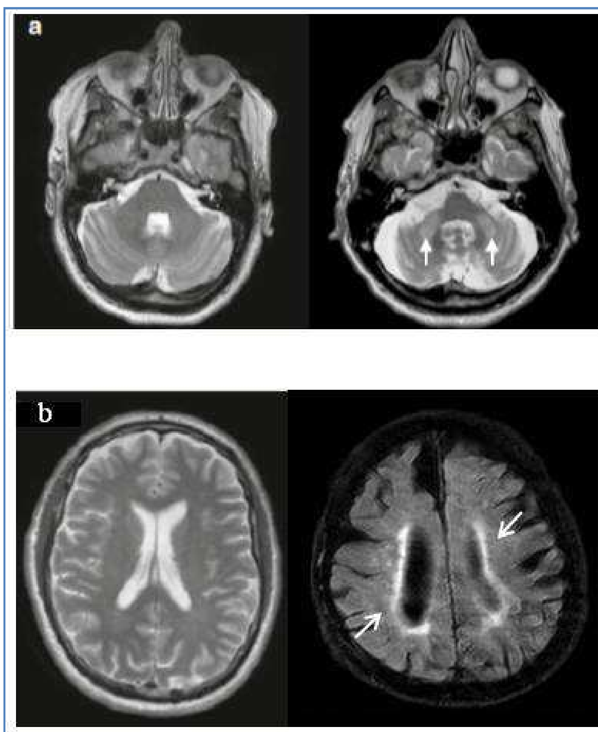


Figura 8. Imágenes de resonancia magnética en secuencia T2, representándose a la izquierda un individuo control (modificadas de Hagerman 2013) y a la derecha un paciente FXTAS (modificadas de Rodriguez-Revenga et al., 2012). Las flechas blancas señalan las hiperintensidades de: **a)** los pedúnculos medios cerebelosos, conocida como semal MCP y **b)** la sustancia blanca cerebral.

Respecto los aspectos neuropatológicos, el estudio inmunohistológico de tejidos cerebrales obtenidos de pacientes FXTAS post-mortem ha permitido descubrir tres características generales. En primer lugar, se ha puesto de manifiesto la afectación de la sustancia blanca cerebral y cerebelar. En segundo lugar, estos pacientes presentan afectación de los astrocitos, sobretodo en la región de la sustancia blanca cerebelar subcortical. Finalmente, se ha identificado la presencia de inclusiones

aisladas en los núcleos de neuronas y astrocitos (Figura 8), distribuidas de manera extensa por el cerebro y el tronco cerebral, con una mayor densidad en el hipocampo y en el córtex frontal (Greco et al. 2002; Greco et al. 2006). Las inclusiones no se detectan en las células de Purkinje, aunque sí que se observa una pérdida importe de este tipo celular así como una degeneración axonal en el cerebelo (Greco et al. 2002).

Las inclusiones representan la característica neuropatológica más llamativa asociada a FXTAS y se ha descrito una asociación significativa entre el número de inclusiones, el número de repeticiones CGG y la edad de fallecimiento del paciente que además correlaciona con el carácter progresivo de la enfermedad (Greco et al. 2006). Por último, estas inclusiones se diferencian inmunohistoquímicamente de las detectadas en otras ataxias asociadas a expansiones del trinucelotido citosina-adenina-guanina (CAG) ya que presentan tinción positiva para hematoxilina-eosina y para ubiquitina mientras que son negativas para poliglutamina (poliQ), α-synucelina y tau (Figura 9).

De la misma manera que se ha propuesto para otras enfermedades neurodegenerativas causadas por expansiones tipo CAG como el Corea de Huntington (HD) o las ataxias espinocerebelosas, las inclusiones halladas en el tejido cerebral de pacientes FXTAS podrían tener un

efecto citotóxico y conducir a la muerte celular. Sin embargo, la presencia de estas inclusiones en algunos portadores de la premutación sin FXTAS ha puesto en controversia su impacto sobre la viabilidad celular (Tassone et al. 2012b) ya que no queda claro si son tóxicas *per se* o simplemente son consecuencia de la disfunción celular subyacente (Hunsaker et al. 2011).

Aunque se han descrito casos de FXTAS en individuos con alelos intermedios o con la mutación completa sin metilar, actualmente el diagnóstico de FXTAS se basa en los criterios establecidos por el grupo de Jacquemont y colaboradores (Tabla 4). De esta manera, en base a la combinación de signos clínicos, radiológicos y neuropatológicos que manifiesten los individuos, los pacientes FXTAS se clasifican como "posible", "probable" o "definitivo".

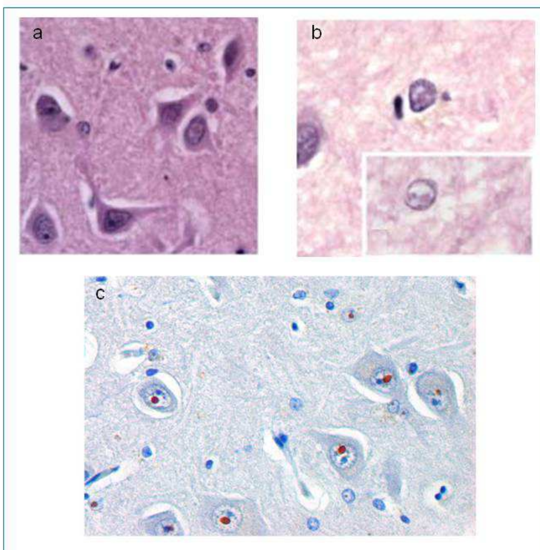


Figura 9. Inclusiones intranucleares en neuronas y astrocitos de pacientes FXTAS. Las secciones de parafina con tinción de hematoxilina-eosina muestran la presencia de las inclusiones en: a) en las células piramidales y b) en astrocitos del córtex frontal (Modificado de Tassone et al., 2012b); c) inclusiones ubiquitin-positivas de región hipocampal (modificado de Willemsen et al., 2011)

Tabla4. Criterios diagnósticos para FXTAS (modificado de Hagerman & Hagerman 2013).

Criterio inclusión	número de repeticiones CGG del gen *FMR1* entre 55 y 200		
Categorías diagnósticas	Signos Radiológicos	Signos Clínicos	Signos Neuropatológicos
DEFINITIVO	1 criterio mayor	1 criterio mayor	
	1 criterio mayor		1 criterio mayor
PROBABLE	1 criterio mayor	1 criterio menor	
		2 criterios mayores	
POSIBLE	1 criterio menor	1 criterio mayor	

Criterios Radiológicos	
Mayor	RM con lesiones en la sustancia blanca en MCPs y/o en el tronco cerebral
Menor	RM con lesiones de la sustancia blanca en el cerebro
Menor	Atrofia moderada-severa generalizada
Signos Clínicos	
Mayor	Temblor de intención
Mayor	Ataxia de la marcha
Menor	Parkinsonismo
Menor	Pérdida de memoria a corto plazo moderada-severa
Menor	Déficit función ejecutiva
Signos Neuropatológicos (post-mortem)	
Mayor	Inclusiones intranucleares

Capítulo III

Mecanismos moleculares asociados a la premutación

Existen un elevado número de enfermedades hereditarias causadas por la expansión de secuencias repetitivas de ADN, la mayoría de las cuales provocan la aparición de trastornos neurológicos y neuromusculares. En el genoma humano se han caracterizado cuatro tipos de trinucleótidos (CGG/GCC, CAG/GTC, CTG/GAC y GAA/CTT) asociados a enfermedad, cuya fisiopatología varía dependiendo no solo de su secuencia sino también en función de su localización ya que pueden detectarse tanto en regiones codificantes como en regiones intrónicas e intergénicas (Figura 10). El SXF fue la primera patología humana descrita originada por en un proceso de mutación dinámica ya que estas expansiones son altamente inestables y se transmite con un inusual patrón de herencia no mendeliano (revisado en Rosales-Reynoso et al. 2009).

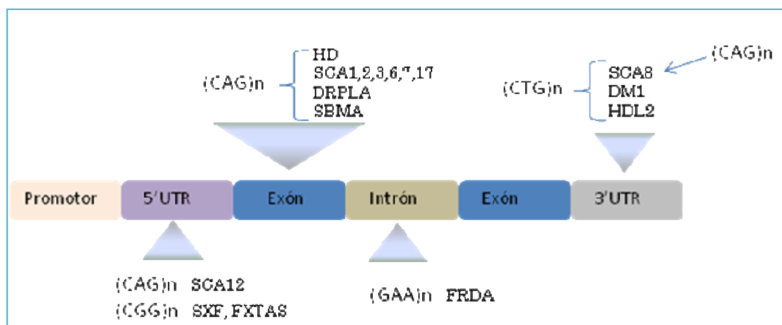


Figura 10. Diagrama de la estructura génica general que señala la localización de las expansiones de trinucleótidos asociadas a enfermedad (modificado de Rosales-Reynoso et al 2009).

Aunque cada enfermedad está causada por una mutación en particular, estos trastornos comparten un elevado número de características como la penetrancia incompleta, la expresividad variable y el fenómeno de anticipación (Ashley & Warren 1995). Las mutaciones en regiones no codificantes, como en el caso del gen *FMR1*, suelen traducirse en enfermedad mediante la expresión de un transcrito expandido (ej. FXTAS) o bien mediante la pérdida de función del gen debido a la disrupción de su transcripción (ej. SFX). Por otra parte las mutaciones en regiones codificantes, como en el gen *HTT*, suelen ser del tipo CAG y promueven la síntesis de proteínas con una región poliQ que se fragmentan en péptidos tóxicos para la célula (ej. HD) (revisado en Nelson et al. 2013).

Con respecto a las patologías asociadas a la premutación del gen *FMR1*, el promotor del gen se encuentra sin metilar, por lo que el gen permanece activo. De hecho, la característica molecular asociada a los individuos portadores de la premutación es el incremento de la expresión del gen *FMR1* de 2 a 8 veces (detectada en linfocitos) y una ligera disminución en los niveles de FMRP respecto a los niveles detectados en individuos con alelos normales (revisado en Hagerman & Hagerman 2004). Los niveles del ARNm parecen estar correlacionados con el número de repeticiones CGG, de manera que cuanto más se

acerca el número de repeticiones al límite superior de la premutación (100-200 CGG) más se elevan los niveles del ARNm de *FMR1* y más baja la síntesis de FMRP (Tassone et al. 2000a; Tassone et al. 2000b). Este incremento no está ligado a una mayor estabilidad del ARNm ya que el proceso de degradación de ARN por mutaciones terminadoras, conocido normalmente como *nonsense mediated decay*, no está alterado en los individuos portadores de la premutación (Tassone et al. 2000a). Por otra parte, existe una correlación negativa entre la longitud de la expansión de los alelos premutados y la eficiencia del proceso de traducción, de manera que las expansiones de menor tamaño permiten una traducción más eficiente, estableciéndose un límite en las 100 repeticiones CGG donde la eficiencia de la traducción se ve reducida. En base a estas observaciones se ha postulado que podría generase un mecanismo de retroalimentación negativa que por una parte resultaría en un incremento de la transcripción génica y por otra en la disminución de la síntesis de FMRP (Tassone et al. 2007; Peprah et al. 2010).

Hasta la fecha, se han propuesto tres posibles mecanismos responsables del incremento de la transcripción del *FMR1* expandido (revisado en Usdin et al. 2014). En primer lugar, se ha puesto de manifiesto que tramos largos de repeticiones CGG impiden la formación de los

nucleosomas *in vitro* (Wang et al. 1996), de manera que si este proceso se ve alterado *in vivo* el promotor de *FMR1* estaría más accesible para su interacción con los factores de transcripción. En segundo lugar, se ha detectado un aumento de la acetilación de las histonas del promotor de *FMR1* que también podría mediar el incremento de la expresión de este gen (Todd et al. 2010). Sin embargo aún está por determinar si este hallazgo es realmente una causa o bien una consecuencia de la transcripción de *FMR1*. Por último, se ha demostrado la formación de híbridos de ADN/ARN en los alelos premutados conocidos como R-loops (Loomis et al. 2014). Estas estructuras promueven el silenciamiento génico en el SXF (Groh et al. 2014) y en los alelos con la premutación podrían promover una mayor transcripción ya que son poco propensos al ensamblaje de los nucleosoma y se cree que bajo estas circunstancias son más propensos a descondensar la cromatina.

Mecanismo de toxicidad del ARNm expandido de *FMR1*

Como se ha descrito anteriormente en otras enfermedades neurodegenerativas (ej. distrofia miotónica), las patologías asociadas a la premutación se basan en un modelo de ganancia de función tóxica del ARNm (revisado en Hagerman 2013; Li & Jin 2012). Bajo este modelo, el evento desencadenante en la patogénesis del FXTAS implica el reclutamiento excesivo de diversas proteínas de unión a

ARN que alteraría la función que ejercen en la célula (revisado en Hagerman & Hagerman 2015). El secuestro proteico se cree que viene mediado por la formación de estructuras secundarias inducidas por la propia expansión (ej. "*Hairpins*") que promovería la unión excesiva de estas proteínas (Figura 11).

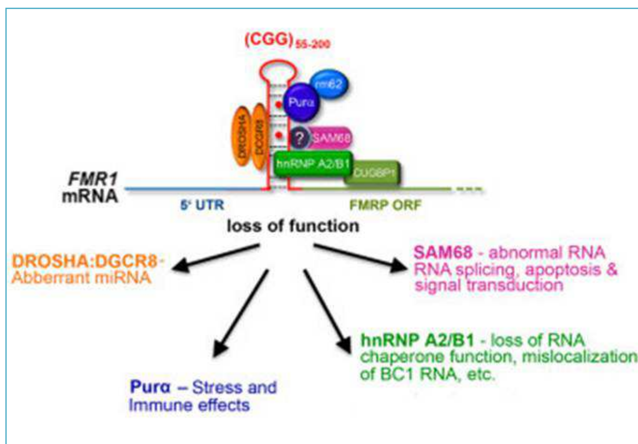


Figura 11. Modelo de ganancia de función tóxica del ARNm de *FMR1* (obtenido de Berman et al. 2014).

En consonancia, los hallazgos observados en tejidos cerebrales post-mortem de pacientes FXTAS apoyan este modelo de toxicidad ya que se han identificado en las inclusiones de los pacientes más de 30 proteínas, aunque no FMRP, imprescindibles para la función normal de la célula además de el ARNm de *FMR1* (Iwahashi et al. 2006, Iwahashi & Hagerman 2008). Entre las proteínas detectadas se encuentran: Sam68, moduladora del *splicing* de los ARNs; Purα, activador transcripcional posiblemente implicado en la replicación del ADN; hnRNP A2, implicada en el metabolismo y transporte de los ARNms y

DGCR8, imprescindible para la biogénesis de los miRNAs (revisado en Hagerman & Hagerman 2015).

Traducción no mediada por AUG (*RAN translation*)

A pesar de las múltiples evidencias que apoyan el modelo de toxicidad del ARNm de *FMR1*, se han propuesto otros mecanismos como posibles procesos desencadenantes de la patogenicidad de los alelos premutados (revisado en Hagerman 2013). Recientemente, la toxicidad mediada por el mecanismo de *RAN (Repeat-Associated Non-AUG-initiated) translation* se ha asociado con la enfermedad de FXTAS y de FXPOI. Este mecanismo promueve la traducción independientemente del codón de inicio AUG, de esta manera, el tramo de repeticiones CGG puede ser traducido en cualquiera de sus posibles pautas de lectura (CGG, GGC o GCC), dando lugar respectivamente a péptidos de poliarginina (poliR), poliglicina (poliG) y polialanina (poliA) que podrían resultar tóxicos para la célula, como se ha descrito en las enfermedades asociadas a la síntesis de poliQ (revisado en Weber et al. 2014). El grupo de Todd y colaboradores (2013) ha puesto de manifiesto la acumulación de péptidos con tramos poliG (FMRpoliG) en las inclusiones ubiquitin-positivas en diversos modelos animales y celulares así como en muestras cerebrales procedentes de pacientes FXTAS post-mortem (Figura 12). Estos autores han identificado en el

modelo animal de *Drosophilla* una correlación positiva entre los niveles de FMRpoliG y la toxicidad celular (Todd et al. 2013). Por otra parte, se ha detectado la presencia citoplasmática de la proteína FMRpoliG en las células de la granulosa que envuelven el oocito en las mujeres portadoras de la premutación (comunicación oral Cohen 2015). Estas evidencias sugieren que el mecanismo de *RAN translation* podría promover un efecto tóxico asociado a síntesis de FMRpoliG en FXTAS y FXPOI.

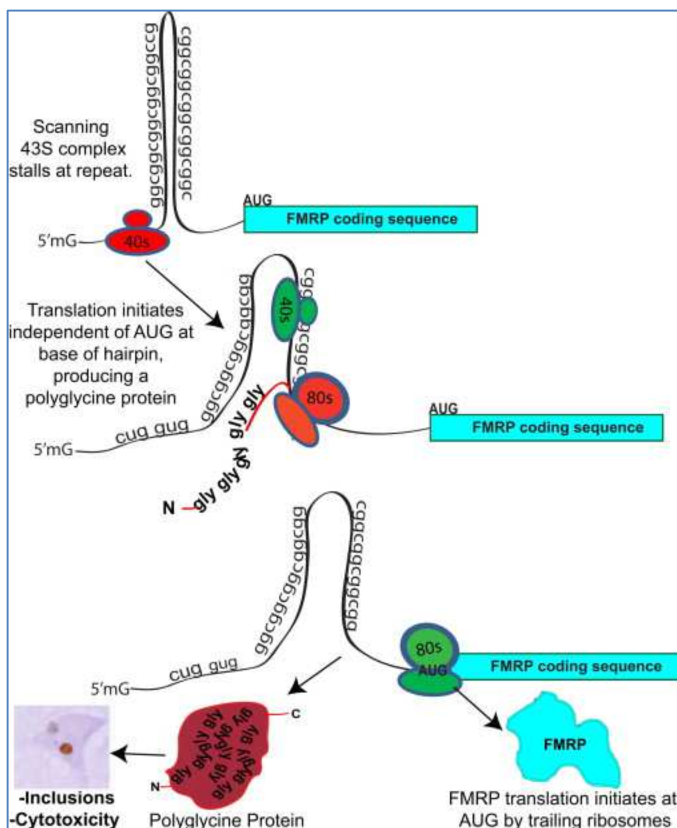


Figura 12. Modelo de toxicidad basado en el mecanismo de *RAN translation* (obtenido de Todd et al. 2013).

Factores adicionales

La penetrancia reducida es una característica común de todas las patologías asociadas a la premutación del gen *FMR1*, por lo que deben existir además de la premutación otros factores implicados en la aparición y desarrollo de estos trastornos (revisado en Hagerman & Hagerman 2015). Como en todas las enfermedades de herencia ligada al cromosoma X, se ha sugerido que la prevalencia del FXTAS en las mujeres portadores de la premutación es menor respecto a los hombres debido a la contribución del alelo normal, aunque no se descarta la implicación de otros factores genéticos sexo-dependientes.

En las mujeres tiene lugar la XCI al azar de uno de los cromosomas X para equiparar la dosis de los genes de este cromosoma respecto a la de los hombres. En la mayoría de los casos cada alelo contribuye con una ratio del 50:50, por lo que cada cromosoma X se encuentra activo en un mismo número de células. Sin embargo, en algunos casos el proceso de XCI se encuentra, detectándose una distribución de XCI que oscila de ratios 100:0 hasta 0:100. Se considera inactivación preferencial severa a partir de una ratio de XCI 90:10 y/o 10:90 (Amos-Landgraf et al. 2006). En las mujeres portadoras de alteraciones cromosómicas o génicas del cromosoma X se ha descrito una mayor frecuencia de este mecanismo, que en el caso del FXTAS tendría lugar la inactivación

preferencial del alelo normal. En el **trabajo III** de esta tesis doctoral se presentan los resultados obtenidos del estudio del proceso de inactivación preferencial en las mujeres portadoras de la premutación con y sin FXTAS.

Existen otros factores genéticos, como polimorfismos y variaciones en el número de copia (CNVs), que podrían modular el riesgo a desarrollar ciertas patologías asociadas a la premutación. En el año 2012 se determinó la asociación entre la presencia del polimorfismo rs7209436 del gen *CRHR1* y un mayor riesgo de depresión y ansiedad entre las mujeres portadoras de la premutación (Hunter et al. 2012). Posteriormente, en el trabajo presentado por Silva y colaboradores (2013) se identificó que alelo 4 de la apoliproteina E (*ApoE*), considerado factor de riesgo en la enfermedad de Alzheimer, podría predisponer también al desarrollo del FXTAS en los individuos portadores de la premutación **(ver anexo 2)**. Recientemente, se ha identificado la presencia de CNVs en aproximadamente el 20% de los portadores de la premutación afectados con trastornos del espectro autista y/o problemas neurológicos que podrían actuar como *second hits* en estos individuos (Lozano et al. 2014b).

Respecto los factores ambientales, existen evidencias que ponen de manifiesto su asociación con algunas patologías asociadas a la

premutación. Si bien es sabido que el efecto del tabaco se ha asociado con la menopausia precoz que afecta a la población general, se ha demostrado que las mujeres portadoras de la premutación fumadoras experimentan la aparición de la FXPOI a edades más avanzadas que las premutadas no fumadoras (Allen et al. 2007; Spath et al. 2011). Por otra parte, en FXTAS se ha evidenciado que la exposición a determinas toxinas (ej. tratamiento del cáncer con quimioterapia) promueve fenotipos más severos y a una edad de debut más temprana (Paul et al. 2010). Del mismo modo, el abuso de drogas o el someterse a procedimientos quirúrgicos que conlleven el uso de anestesia se han descrito como factores de riesgo para FXTAS (revisado en Polussa et al. 2014; Hagerman & Hagerman 2013).

En resumen, el gran desafío de las patologías asociadas a la premutación es poder identificar aquellos portadores que desarrollarán alguno de estos trastornos para ofrecerles un asesoramiento genético preciso. La identificación y caracterización de posibles biomarcadores resultaría de gran importancia puesto que permitiría un diagnóstico presintomático y quizás a un tratamiento precoz que diera lugar a un enlentecimiento de la progresión de la enfermedad en estos individuos.

Disfunción mitocondrial y FXTAS

Desde hace unos años la disfunción mitocondrial se han propuesto como uno de los mediadores centrales de la desregulación y de la disfunción celular en los pacientes FXTAS (revisado en Hagerman & Hagerman 2013). El sistema nervioso central (SNC) depende en gran medida del funcionamiento eficiente de las mitocondrias debido a la elevada demanda energética de las neuronas. Las mitocondrias son la mayor fuente de suministro de adenosina trifosfato (ATP) a través de la fosforilación oxidativa. Mediante este proceso metabólico la energía liberada por la oxidación de nutrientes es utilizada para producir ATP y especies reactivas del oxígeno (ROS) (Figura 13).

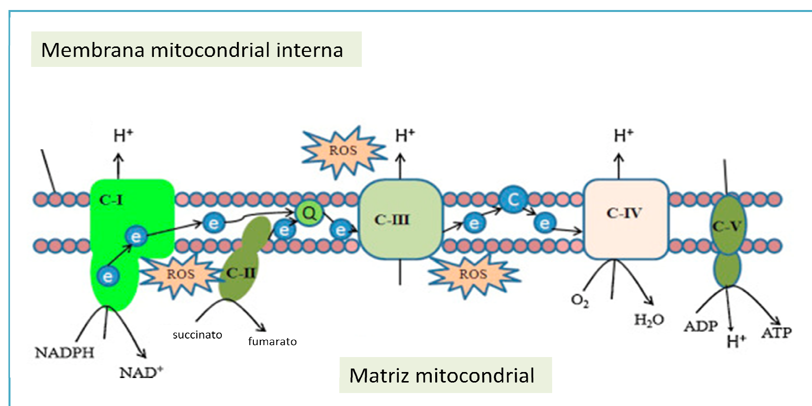


Figura 13. Esquema vía fosforilación oxidativa (modificado de Bhat et al 2015).

La disfunción mitocondrial se ha propuesto como mecanismo patogénico unificador de numerosas enfermedades neurodegenerativas comunes (ej. Alzheimer, Parkinson o Huntington) dado que se ha

demostrado que la disminución de la síntesis de ATP y/o el incremento del estrés oxidativo pueden conducir a la neurodegeneración (revisado en Federico et al. 2012).

En los individuos portadores de la premutación se han publicado recientemente diversos trabajos que ponen de manifiesto la alteración de la función mitocondrial en estos individuos que podría explicar la reducción de la viabilidad neuronal (Ross-Inta et al. 2010; Napoli et al. 2011; Kaplan et al. 2012; Hukema et al. 2014). Con el fin de aportar nuevos datos sobre la asociación entre disfunción mitocondrial y FXTAS, en el **Trabajo IV** de esta tesis doctoral se presentan los resultados obtenidos hasta el momento del estado de las funciones mitocondriales en los pacientes FXTAS.

HIPÓTESIS

OBJETIVOS

HIPÓTESIS

Las patologías asociadas a la premutación en *FMR1* presentan una penetrancia reducida debida a múltiples factores genéticos y ambientales. Los mecanismos subyacentes que explican la asociación entre la disfunción ovárica primaria y la premutación en *FMR1* actualmente se desconocen. El estudio mediante *microarrays* de expresión del perfil de expresión génica en mujeres portadoras de la premutación con y sin FXPOI y en mujeres control puede conducirnos a la identificación de vías potencialmente causales o a funciones bioquímicas alteradas que se encuentren implicadas en la patogénesis del FXPOI.

Por otra parte, la expresión del gen *FMR1* está regulada por diversos miRNAs, FMRP interacciona con la maquinaria de los miRNAs e incluso con algunos directamente y se han identificado a DROSHA y DGCR8 entre las proteínas secuestradas por el ARNm expandido de *FMR1*. En base a estas observaciones, la desregulación de los miRNAs podría tener un papel en la patogénesis del FXTAS. Ante este escenario, la aplicación de la *microarrays* de expresión en el estudio de las patologías asociadas a la premutación puede ser de gran interés ya que puede aportar información sobre las causas genéticas del desarrollo del FXPOI y del FXTAS.

Por último, la disfunción de la actividad mitocondrial podría estar implicada en la patogénesis del síndrome FXTAS, de la misma manera que sucede en otras enfermedades neurodegenerativas, tales como enfermedad de Alzheimer, Parkinson o Huntington. La caracterización de la vía de la fosforilación oxidativa podría identificar alteraciones moleculares que dieran lugar a terapias dirigidas a restablecer los procesos mitocondriales básicos tales como el metabolismo de energía o la generación de radicales libres, los cuales proporcionarían una gran esperanza en el tratamiento del FXTAS.

OBJETIVOS

El objetivo principal de esta tesis doctoral es el de ampliar el conocimiento de las bases moleculares de las patologías asociadas a la premutación, principalmente en FXPOI y FXTAS.

Los objetivos específicos de esta tesis doctoral son los siguientes:

1. Determinar el perfil de expresión génica en sangre periférica en mujeres de la población general y en las portadoras de la premutación con y sin FXPOI.
2. Identificar grupos funcionales cuya expresión se ve desregulada por la premutación en *FMR1* que puedan estar implicados en la FXPOI.
3. Determinar el perfil de expresión de los miRNAs en muestras de sangre periférica en varones de la población general y en portadores de la premutación con FXTAS.
4. Identificar los miRNAs que puedan estar implicados en la aparición y desarrollo de FXTAS.
5. Estudiar vías que puedan repercutir en la viabilidad de la célula como la vía de la fosforilación oxidativa.
6. Estudiar posibles factores que puedan modificar la penetrancia de las patologías asociadas a la premutación del gen *FMR1*.

INFORME DIRECTORES

Título de la tesis:

"PATOLOGÍAS ASOCIADAS A

LA PREMUTACIÓN DEL GEN *FMR1*"

Autora: María Isabel Álvarez Mora

Directores: Montserrat Milà Recasens y Laia Rodriguez-Revenga Bodi

Artículo 1

Título: Deregulation of key signaling pathways involved in oocyte maturation in *FMR1* premutation carriers with Fragile X-associated primary ovarian insufficiency.

Autores: Alvarez-Mora MI, Rodriguez-Revenga L, Madrigal I, Garcia-Garcia F, Duran M, Dopazo J, Estivill X, Milà M.

Publicación: Gene. 2015 Oct 15;571(1):52-7.

Índice de calidad: SCI2013/2014 = 2.138; Cuartil 3 de la categoría GENETICS & HEREDITY.

Aportación de la autora en el artículo: Extracción ARN total de sangre periférica, marcaje e hibridación de los *microarrays* de expresión génica, análisis de datos bioinformáticos y preparación del manuscrito.

Artículo 2

Título: MicroRNA expression profiling in blood from fragile X-associated tremor/ataxia syndrome patients.

Autores: Alvarez-Mora MI, Rodriguez-Revenga L, Madrigal I, Torres-Silva F, Mateu-Huertas E, Lizano E, Friedländer MR, Martí E, Estivill X, Milà M.

Publicación: Genes Brain and Behaviour. 2013 Aug;12(6):595-603.

Índice de calidad: SCI2013/2014 = 3.661; Cuartil 1 de la categoría BEHAVIORAL SCIENCES.

Aportación de la autora en el artículo: extracción de microRNAs, marcaje e hibridación *microarrays* de expresión, análisis de datos *microarrays* de expresión, comparativa resultados *microarrays* y smallRNA sequencing, validación resultados mediante PCR cuantitativa a tiempo real y preparación del manuscrito.

Artículo 3

Título: Skewed X inactivation in women carrying the FMR1 premutation and its relation with FXTAS.

Autores: Alvarez-Mora MI, Rodriguez-Revenga L, Feliu A, Badenas C, Madrigal I, Milà M.

Publicación: Neurodegenerative Diseases. *Accepted Article*.

Índice de calidad: SCI2013/2014 = 3.511; Cuartil 2 de la categoría NEUROSCIENCES

Aportación de la autora en el artículo: selección individuos para el estudio, determinación inactivación preferencial mediante análisis del locus del gen de receptor de andrógenos, análisis estadísticos y preparación del manuscrito.

Artículo 4

Título: Characterization of mitochondria state in FXTAS: Functional study and mitochondrial dynamics analysis.

Autores: Alvarez-Mora MI, Rodriguez-Revenga L, Madrigal I, Guitart-Mampel M, Garrabou G, Milà M.

Publicación: en preparación.

Aportación de la autora en el artículo: extracción células mononucleares de sangre periférica (CMSPs), cuantificación de proteínas en CMSPs y cultivo de fibroblastos, valoración consumo de oxígeno en CMSPs y cultivo de fibroblastos, ensayo de la actividad de la enzima citrato sintasa en CMSPs y cultivo de fibroblastos, cuantificación peroxidación lipídica en CMSPs y cultivo de fibroblastos, obtención imágenes de inmuocitoquímica mediante

microscopia confocal en fibroblastos, valoración de la red mitocondrial mediante software ImageJ/Fiji y preparación del manuscrito.

Barcelona, 27 de Octubre de 2015

Montserrat Milà   Laia Rodriguez-Revenga

RESULTADOS

Capítulo I

Mecanismos patogénicos en FXPOI

TRABAJO I

"DESREGULACIÓN DE VÍAS DE SEÑALIZACIÓN CLAVE INVOLUCRADAS EN LA MADURACIÓN DE LOS OOCITOS EN MUJERES PORTADORAS DE LA PREMUTACIÓN EN EL GEN *FMR1* CON FXPOI"

Resumen:

La POI afecta aproximadamente al 1% de la población general mientras que entre las mujeres portadoras de la premutación del gen *FMR1* la prevalencia se encuentra alrededor de un 20% y se conoce FXPOI. Actualmente se desconoce el mecanismo molecular que da lugar a la reducción de la viabilidad en el ovario; sin embargo, se ha sugerido que podría deberse a una disminución del número inicial de folículos. Estudios recientes en el modelo murino de FXPOI han descartado esta hipótesis ya que se ha demostrado una mayor tasa de depleción de los folículos así como problemas en el desarrollo de los folículos en crecimiento.

El objetivo de este estudio es proporcionar nuevos datos sobre los mecanismos moleculares que conducen a FXPOI. Con este fin se determinó el perfil de expresión génica mediante *microarrays* de expresión en muestras de sangre periférica procedentes de 12 mujeres

portadoras de la premutación en el gen *FMR1* (6 FXPOI y 6 NO-FXPOI) y como grupo control 4 mujeres de la población general con una edad de aparición de la menopausia superior a los 50 años.

Los resultados del análisis de expresión diferencial de los datos obtendios mediante *microarrays* no identificaron diferencias significativas entre grupos. Sin embargo, en el análisis de enriquecimiento funcional se identificaron varías vías de señalización imprescindibles para la maduración de los oocitos desreguladas significativamente en las mujeres portadoras de la premutación con FXPOI. En concreto se detectó una infraregulación de las vías de señalización por VEGF, el metabolismo del inositol fosfato, el ciclo celular y la señalización por las MAPK así como numerosos procesos biológicos relacionados con la supervivencia y muerte celular en las mujeres con FXPOI.

En resumen, estos datos sugieren que la disfunción ovárica asociada a los alelos premutados se debe a la desregulación generalizada de vías de señalización indispensables para la maduración de los oocitos. Por lo tanto, para profundizar en el conocimiento de los mecanismos moleculares del FXPOI e incluso para el desarrollo de posibles tratamientos, se deberían considerar los genes por bloques funcionales en lugar de centrar los estudios en un único gen y su efecto.

Gene 571 (2015) 52–57

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Deregulation of key signaling pathways involved in oocyte maturation in *FMR1* premutation carriers with Fragile X-associated primary ovarian insufficiency

M.I. Alvarez-Mora [a,b,c], L. Rodriguez-Revenga [a,b,c], I. Madrigal [a,b,c], F. Garcia-Garcia [d], M. Duran [e], J. Dopazo [d,f,g], X. Estivill [h,i,j,k,l], M. Milà [a,b,c,*]

[a] CIBER de Enfermedades Raras, Hospital Clínic, 08036 Barcelona, Spain
[b] Biochemistry and Molecular Genetics Department, Hospital Clinic, 08036 Barcelona, Spain
[c] Institut d'Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) 08036 Barcelona, Spain
[d] Computational Genomics Department, Centro de Investigación Príncipe Felipe, 46012 Valencia, Spain
[e] Obstetrics and Gynecology Department Hospital Clinic, 08036 Barcelona, Spain
[f] Functional Genomics Node, INB, Centro de Investigación Príncipe Felipe, Valencia, Spain
[g] CIBER de Enfermedades Raras, Centro de Investigación Príncipe Felipe, 46012 Valencia, Spain
[h] Bioinformatics and Genomics Program, Centre for Genomic Regulation (CRG), 08003 Barcelona, Spain
[i] Universitat Pompeu Fabra (UPF), 08005 Barcelona, Spain
[j] Hospital del Mar Medical Research Institute (IMIM), 08003 Barcelona, Spain
[k] CIBER de Epidemiología y Salud Pública (CIBERESP), Centre for Genomic Regulation, 08003 Barcelona, Spain
[l] Genomics and Personalized Medicine Section, Dexeus Woman's Health, 08028 Barcelona, Spain

ARTICLE INFO

Article history:
Received 2 March 2015
Received in revised form 12 May 2015
Accepted 16 June 2015
Available online 18 June 2015

Keywords:
FMR1 premutation
FXPOI
Female infertility
Oocyte maturation

ABSTRACT

FMR1 premutation female carriers are at risk for Fragile X-associated primary ovarian insufficiency (FXPOI). Insights from knock-in mouse model have recently demonstrated that FXPOI is due to an increased rate of follicle depletion or an impaired development of the growing follicles. Molecular mechanisms responsible for this reduced viability are still unknown. In an attempt to provide new data on the mechanisms that lead to FXPOI, we report the first investigation involving transcription profiling of total blood from *FMR1* premutation female carriers with and without FXPOI. A total of 16 unrelated female individuals (6 *FMR1* premutated females with FXPOI; 6 *FMR1* premutated females without FXPOI; and 4 no-FXPOI females) were studied by whole human genome oligonucleotide microarray (Agilent Technologies). Fold change analysis did not show any genes with significant differential gene expression. However, functional profiling by gene set analysis showed large number of statistically significant deregulated GO annotations as well as numerous KEGG pathways in FXPOI females. These results suggest that the impairment of fertility in these females might be due to a generalized deregulation of key signaling pathways involved in oocyte maturation. In particular, the vasoendotelial growth factor signaling, the inositol phosphate metabolism, the cell cycle, and the MAPK signaling pathways were found to be down-regulated in FXPOI females. Furthermore, a high statistical enrichment of biological processes involved in cell death and survival were found deregulated among FXPOI females. Our results provide new strategic approaches to further investigate the molecular mechanisms and potential therapeutic targets for FXPOI not focused in a single gene but rather in the set of genes involved in these pathways.
© 2015 Elsevier B.V. All rights reserved.

1. Introduction

Primary ovarian insufficiency (POI), previously referred as premature ovarian failure (POF), is defined as the occurrence of amenorrhea for at least 4 months, before the age of 40 in women, accompanied with alteration of specific serum markers levels (follicle-stimulating hormone (FSH) >40 IU/L and estradiol <50 pg/mL) (Nelson, 2009).

Genetic factors affecting the ovary and the uterus are common causes of infertility. In fact it is reported that 7% of POI cases have a genetics etiology (Bachelot et al., 2009), involving genes with various

Abbreviations: POI, primary ovarian insufficiency; POF, premature ovarian failure; FSH, follicle-stimulating hormone; FXPOI, Fragile X-associated primary ovarian insufficiency; FMRP, Fragile X mental retardation protein; FXS, Fragile X syndrome; FXTAS, Fragile X-associated tremor/ataxia syndrome; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; FDR, False Discovery Rate; VEGF, vasoendotelial growth factor.

* Corresponding author at: Biochemistry and Molecular Genetics Department, Hospital Clínic, C/Villarroel, 170, 08036 Barcelona, Spain.
E-mail addresses: misabel_alvarez@hotmail.com (M.I. Alvarez-Mora), lbodi@clinic.ub.es (L. Rodriguez-Revenga), imadbajo@clinic.ub.es (I. Madrigal), fgarcia@cipf.es (F. Garcia-Garcia), mduran@clinic.ub.es (M. Duran), jdopazo@cipf.es (J. Dopazo), xavier.estivill@crg.eu (X. Estivill), mmila@clinic.ub.es (M. Milà).

http://dx.doi.org/10.1016/j.gene.2015.06.039
0378-1119/© 2015 Elsevier B.V. All rights reserved.

biological functions such as regulation of the hypothalamic-pituitary ovarian axis, regulation of oogenesis, coordination of development of germ cell to primordial stage, regulation of development of further stages and participation in systemic endocrinal functions (Dixit et al., 2010).

The most common known genetic cause of 46, XX POI is the expansion of a CGG repeat located in the 5′ UTR region of the *FMR1* gene, referred as Fragile X-associated POI (FXPOI) (Sullivan et al., 2005) or POF1 (MIM # 311360). This CGG repeat element is polymorphic in the general population, ranging from 6 to 54 repeats. However, the CGG track can be unstable upon maternal transmission and the number of repeats can expand in the next generation. Full-mutated alleles (CGG >200 repeats) become transcriptionally silenced through a methylation mechanism, leading to an absence of *FMR1* protein (FMRP) (Oberlé et al., 1991; Verkerk et al., 1991; Yu et al., 1991), which is responsible for Fragile X syndrome (FXS) (OMIM # 300624). Individuals with alleles between 55 and 200 CGG repeats are called *FMR1* premutation carriers. In this premutation condition, the *FMR1* gene is hyperfunctional and the FMRP is at normal levels, and this is why carriers of *FMR1* premutation alleles were assumed to be clinically unaffected. However and contrary to expectation, a subgroup of male and female carriers does show some clinical affectation, which among others includes FXPOI and Fragile X-associated tremor/ataxia syndrome (FXTAS) (reviewed in Hagerman and Hagerman, 2004; Willemsen et al., 2011).

POI occurs in approximately 1% of the general female population (Coulam et al., 1986), whereas up to 20% of the *FMR1* premutation female carriers are affected of this condition (Sherman, 2000). Conversely, 2–14% of women with idiopathic sporadic POI are estimated to carry an *FMR1* premutation allele (Mallolas et al., 2001).

It was found that *FMR1* premutation alleles are transcriptionally up-regulated with some individuals presenting markedly increased (2–8-fold) production of the expanded CGG-repeat mRNA (Tassone et al., 2000). This fact together with the absence of FXTAS or FXPOI cases in the full-mutation range, promoted to propose an RNA "toxic gain-of-function" model for *FMR1* premutation associated pathologies (Hagerman and Hagerman, 2004).

Currently, the mechanism of the impaired ovarian function related to the *FMR1* premutation is unclear. In an attempt to provide new insights on the mechanisms that lead to FXPOI, we report here the first investigation involving transcription profiling of total blood from *FMR1* premutation females carriers with and without FXPOI.

2. Material and methods

2.1. Human samples

A total of 16 unrelated female individuals were selected and divided in three groups: 6 *FMR1* premutated females with FXPOI (Group 1); 6 *FMR1* premutated females without FXPOI (Group 2); and 4 no-POI females with normal *FMR1* alleles (Group 3). All *FMR1* premutation carriers were recruited from FXS families. Data from CGG repeat number and menopause onset of these individuals are summarized in Table 1. All subjects provided written informed consent for testing and for the research use of their phenotypic and genetic data. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona.

2.2. Total RNA isolation

For each individual, 2.5 mL of peripheral venous blood was collected in 5 mL PAXgene tubes (Qiagen, Valencia, CA, USA). Total RNA was isolated and purified with the PAXgene Blood RNA Kit according to manufacturer's instructions (Qiagen, Valencia, CA, USA). The RNA quality and quantity were evaluated on an Agilent 2100 Bioanalyzer with RNA6000 Nano Reagents and Supplies (Agilent Technologies, Santa Clara, CA, USA).

Table 1
Characteristics of *FMR1* premutated and non-premutated females.

	(CGG)n	Amenorrhea onset	Age
G1_1	33, 71	40	45
G1_2	30, 94	40	49
G1_3	30, 82	40	65
G1_4	29, 62	40	57
G1_5	30, 68	39	60
G1_6	30, 154	38	78
G2_1	20, 76	NA	48
G2_2	27, 60	47	67
G2_3	45, 80	52	56
G2_4	29, 70	50	50
G2_5	30, 80	NA	42
G2_6	30, 168	51	61
G3_1	29, 32	53	64
G3_2	28, 29	50	51
G3_3	29, 29	48	55
G3_4	27, 30	56	61

G1 (*FMR1* premutation female carriers with FXPOI).
G2 (*FMR1* premutation female carriers without FXPOI).
G3 (no-POI females with normal *FMR1* alleles).
NA (not applicable).

2.3. Gene expression microarray

Four hundred nanograms of the total RNA was labeled using Quick Amp Labeling kit following the manufacturer's instructions (One-Color Microarray-Based Gene Expression Analysis Quick Amp Labeling Protocol Version 5.7, Agilent Technologies, Santa Clara, CA, USA). Briefly, mRNA was reverse transcribed in the presence of T7-oligo-dT primer to produce cDNA. cDNA was then in vitro transcribed with T7 RNA polymerase in the presence of Cy3-CTP to produce labeled cRNA. The labeled cRNA was hybridized to an Agilent 4x44K Whole Human Genome 60-mer oligonucleotide microarray, according to the manufacturer's protocol (Agilent Technologies, Santa Clara, CA, USA). Afterwards the microarrays were washed and scanned on an Agilent G2565CA Microarray Scanner System (Agilent Technologies, Santa Clara, CA, USA). Data were extracted using Feature Extraction V10.7.3.1 (Agilent Technologies, Santa Clara, CA, USA).

2.4. Data processing and statistical analysis

Agilent Processed Signal (Agilent Feature Extraction Software) was standardized across arrays using *quantile* normalization (Bolstad et al., 2003). Differential gene expression was carried out using the limma package from Bioconductor (http://www.bioconductor.org/) (Smyth, 2004). Multiple testing adjustments of p-values were done according to Benjamini and Hochberg methodology (Benjamini and Hochberg, 1995).

Gene set analysis was carried out for the Gene Ontology (GO) terms and for the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways using FatiScan (Al-Shahrour et al., 2007) in Babelomics (http://babelomics.bioinfo.cipf.es/) (Medina et al., 2010). This method detects significantly up- or down-regulated blocks of functionally related genes in lists of genes ordered by differential expression. Given that many functional terms are simultaneously tested, the results of the test are corrected for multiple testing to obtain an adjusted p-value. Gene set analysis returns adjusted p-values based on False Discovery Rate method (Benjamini and Hochberg, 1995; Benjamini and Yekutieli, 2001). GO annotations for the genes in the microarray were taken from Ensembl 70 release (http://www.ensembl.org) and KEGG pathways from the KEGG web page (http://www.genome.jp/kegg/).

3. Results

Whole genome expression profile from three different unrelated female groups was obtained in an attempt to elucidate FXPOI molecular

signature. Groups 1 and 2 consisted of *FMR1* premutation female carriers with and without FXPOI, respectively. Group 3 consisted of normal *FMR1* alleles female carriers with normal amenorrhea age of onset (Table 1). Fold change analysis did not show any genes with significant differential gene expression (adjusted p-value <0.05) when comparing all different groups (data not shown). However, functional profiling by gene set analysis showed large number of statistically significant deregulated GO annotations as well as numerous KEGG pathways in *FMR1* premutation female carriers with FXPOI (adjusted p-value <0.05) (Supplementary Fig. 1). We identified 22 statistically significant pathways that encompassed metabolism, regulatory and cell signaling (Fig. 1). Among them, several pathways that have been shown to play important roles in fertility such as the VEGF signaling (vasoendotelial growth factor) (hsa04370), the inositol phosphate metabolism (hsa00562), cell cycle (hsa04110), and the MAPK signaling (hsa04010) pathways were found to be down-regulated in *FMR1* premutation female carriers with FXPOI. When analyzing expression levels of genes related to these pathways, although none of them reached a significant p-value, the majority of them presented lower expression levels in *FMR1* premutation female carrier with FXPOI (Fig. 2).

To investigate the biological functions associated with FXPOI, gene set analysis was tested for function enrichment (Fig. 3). A high statistical enrichment of biological processes involved in cell cycle and cell death and survival was found. *FMR1* premutation female carriers with FXPOI showed down-regulation of processes related with apoptotic mechanisms and programmed cell death (Fig. 3).

4. Discussion

The ovary contains follicles at various developmental stages and its function includes the cyclic recruitment, development and regression of the follicles. The pool of primordial follicles is maintained in dormancy as a reserve to provide oocytes throughout the reproductive life. FXPOI involves infertility, irregular menses and an early menopause. Although in theory, FXPOI could arise from the presence of a smaller than normal primordial follicle pool at birth, insights from two different publications using a knock-in mouse model, demonstrated that FXPOI is due to an accelerated loss or an impaired development of the growing follicles (Hoffman et al., 2012; Lu et al., 2012). Regarding the *FMR1* CGG repeat number, it has also been described that normal alleles containing lower CGG repeat expansions (<26 CGGs) are associated with a polycystic ovarian-like phenotype that would lead to a prematurely diminished functional ovarian reserve (Gleicher et al., 2010, 2013).

Up to date, the only molecular signature associated to the *FMR1* premutation allele is the presence of significantly elevated levels of *FMR1* mRNA, which led to propose an RNA "toxic gain-of-function" model, in which the abnormal mRNA (expanded CGG repeat) itself is causative of the *FMR1* premutation-associated disorders. Although this mechanism is well supported for FXTAS, in FXPOI it is still under study (Willemsen et al., 2011).

Besides the RNA-gain-of-function toxicity hypothesis, other potential mechanisms have been explored (reviewed in Sullivan et al., 2011). Factors such as the repeat size tract, the sequence organization of the CGG repeat tract, the parental origin of the premutation, the X-chromosome inactivation pattern as well as familial aggregation and environmental factors have been examined (Murray et al., 2000; Sullivan et al., 2005; Hunter et al., 2008; Rodriguez-Revenga et al., 2009). In addition, FMRP might also contribute in the molecular mechanisms involved in the reduced fertility since it has been shown that controls germline proliferation during oogenesis (Epstein et al., 2009; Ferder et al., 2013). Although somehow all of them might have an impact on the development and severity of FXPOI, additional and as yet unidentified genetic factors might also be implicated.

In this study we present the first investigation involving transcription profiling of total blood from *FMR1* premutation female carriers with FXPOI. In particular, we have compared this group of individuals with two female groups (one of carriers of *FMR1* premutation alleles and the other with normal CGG repeat alleles) presenting amenorrhea at normal age of onset (Table 1). Differential expression analysis did not show any statistically significant difference between the three groups, although a sample size effect or tissue specificity cannot be fully discarded. However, gene set analysis, which search for groups of genes that are functionally related, identified several KEGG pathways (adj. p-value <0.05) that might be relevant in FXPOI pathology (Fig. 1). Remarkably, we found that signaling mechanisms necessary for the maintenance of the survival

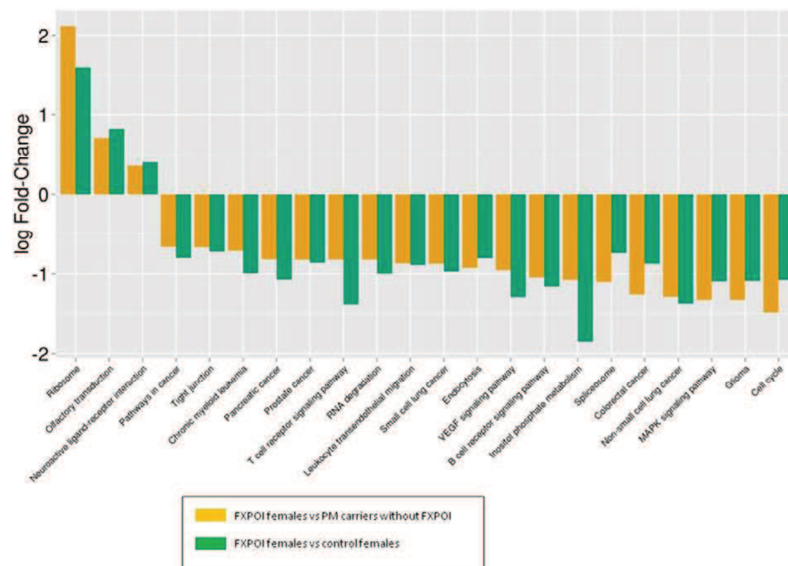


Fig. 1. KEGG pathways significantly deregulated in *FMR1* premutation female carriers with FXPOI. Yellow bars represent the fold change obtained when comparing Group 1 and Group 2. Green bars represent fold change obtained when comparing Group 1 and Group 3.

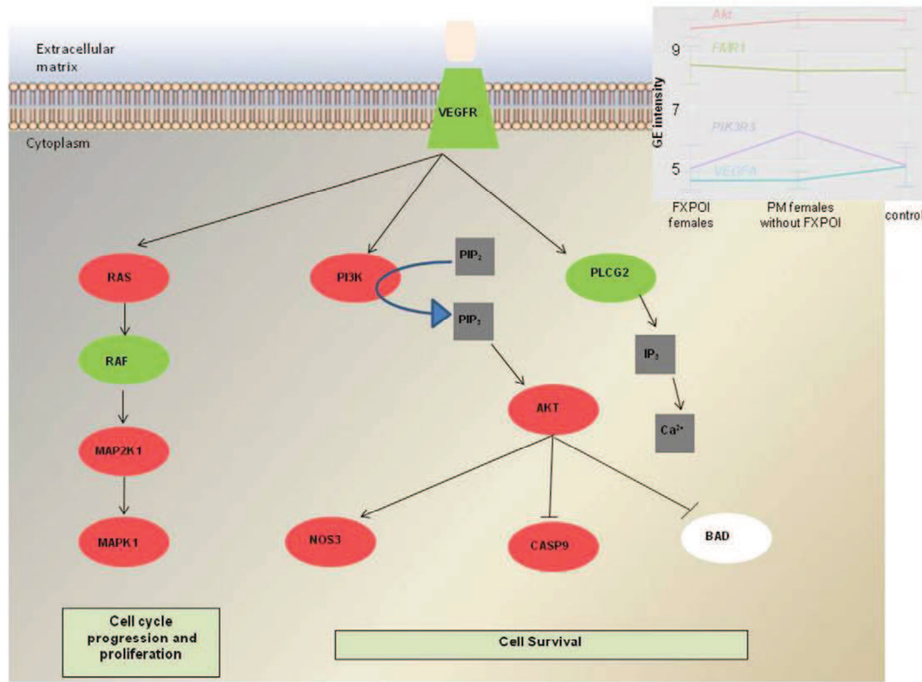


Fig. 2. VEGF, MAPK and PI3K/AKT signaling pathways deregulated in *FMR1* premutation female carriers with FXPOI. In red are represented genes with lower expression levels whereas those in green represent higher expression levels in this group rather than non-POI females. The graphic represents relative expression of *VEGF*, *PI3K3R*, *AKT2* and *FMR1* genes. The Y axis corresponds to gene expression microarray normalized results.

and activation of the primordial follicle as well as oocyte maturation are down-regulated in FXPOI women. In particular, we have found that the VEGF signaling, the inositol phosphate metabolism the MAPK signaling and the cell cycle pathway are down-regulated in blood sample of FXPOI women (adj. p-value <0.05) (Fig. 1). VEGF is a key regulator of physiological angiogenesis during embryogenesis, skeletal growth and reproductive functions. Evidences demonstrate that VEGF and its receptors protect follicle and granulosa cells from apoptosis, suggesting that VEGF functions as a survival factor (Kosaka et al., 2007). In this study we identified under expression of members of VEGF pathway, including *PI3K* and

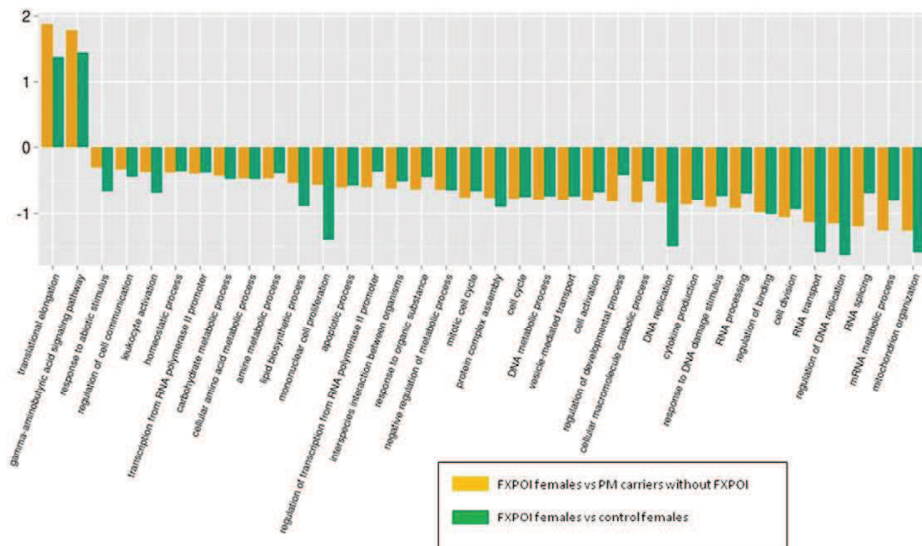


Fig. 3. Biological processes significantly deregulated in *FMR1* premutation female carriers with FXPOI. Yellow bars represent the fold change obtained when comparing Group 1 and Group 2. Green bars represent fold change obtained when comparing Group 1 and Group 3.

AKT (Fig. 2). In consonance with this observation, the inositol phosphate metabolism pathway, which mediated the production of PI3K substrates, is also down-regulated in FXPOI females (adj. p-value <0.05) (Fig. 1). Several lines of evidence have indicated that the *PI3K/AKT* gene family is critical regulator of follicle growth, differentiation and survival (Cecconi et al., 2012). In fact, lack of basal level of PI3K activation leads to the premature depletion of the pool of primordial follicles and to the development of POI (Reddy et al., 2009). Furthermore, *Akt* knockout mice females display reduced fertility and abnormal estrous cyclicity. *Akt* knockout ovaries also have a reduced number of growing antral follicles, significantly larger primary and secondary oocytes and an increase in the number of degenerate oocytes (Brown et al., 2010). In addition, Lu et al. (2012) have recently demonstrated that *FMR1* expanded allele can lead to reduced phosphorylation levels of AKT protein in the ovary of FXPOI mice.

While the VEGF and downstream PI3K pathways are required to activate genes associated with cell growth and differentiation, it has been shown that additional pathways such as MAPK are also necessary (Hunzicker-Dunn and Maizels, 2006). Several members of the MAPK superfamily (ERK1/2, p38MAPK) have been demonstrated to play an important function in LH/FSH signaling pathways, resulting in maturation of ovarian follicles and ovulation (reviewed in Conti et al., 2012; Hunzicker-Dunn and Maizels, 2006).

Finally, when subjecting gene expression microarray results to gene set analysis and tested for function enrichment we found several significantly deregulated biological processes in FXPOI females, including cell-cycle progression/arrest, DNA repair and apoptosis (Fig. 3). These results are in consonance with the deregulation of the PI3K/AKT pathway found in FXPOI females, as it is well known that this pathway can regulate many aspects of cell function (Cecconi et al., 2012). In this scenario, Lu et al. (2012) found an increased apoptosis of follicle cells and an alteration of the AKT signaling cascade in ovaries of the *FMR1* knock-in model.

Blood expression profiling in *FMR1* premutation male carriers has recently identified an abundant gene expression deregulation among these individuals (Mateu-Huertas et al., 2014). Although these results are apparently not in concordance with the ones herein reported, it should be taken into consideration that female carriers have a second non-mutated *FMR1* gene on the other X-chromosome which could compensate the marked gene deregulation detected in *FMR1* premutation male carriers. Nevertheless, when performing gene enrichment analysis the molecular functions and biological pathways found to be deregulated, are consistent in both studies. In agreement with Mateu-Huertas et al. (2014), functions such as cell cycle, DNA repair and cell death and survival are statistically significant deregulated in *FMR1* premutation female carriers. We are aware that this study presents two limitations: tissue-specificity gene expression and the normal X-chromosome which might be masking deregulation promoted by the expanded allele in ovarian cells.

5. Conclusion

The gene expression profile of blood samples from FXPOI females suggested that the impairment of fertility might be due to a generalized deregulation of key signaling pathways involved in oocyte maturation. Our results provide new strategic approaches to further investigate the molecular mechanisms and potential therapeutic targets for FXPOI not focused in a single gene but rather in the set of genes involved in these pathways.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.gene.2015.06.039.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We wish to thank the FXPOI women and FXS families for their cooperation. This work was supported by the Instituto de Salud Carlos III (ISCIII) [PI12/00879], co-financed by Fondo Europeo de Desarrollo Regional (FEDER) “Una manera de hacer Europa” and AGAUR from the Autonomous Catalan Government (2014 SGR603) from Generalitat de Catalunya. This work was partially supported by grants from Spanish Ministry of Economy and Competitiveness (MINECO) [BIO2011-27069, SAF2013-49108-R]. The CIBER de Enfermedades Raras and of Epidemiologia y Salud Pública is an initiative of the ISCIII.

References

Al-Shahrour, F., Arbiza, L., Dopazo, H., Huerta, J., Minguez, P., Montaner, D., Dopazo, J., 2007. From genes to functional classes in the study of biological systems. BMC Bioinforma. 8, 114.

Bachelot, A., Rouxel, A., Massin, N., Dulon, J., Courtillot, C., Matuchansky, C., Badachi, Y., Fortin, A., Paniel, B., Lecuru, F., et al., 2009. Phenotyping and genetic studies of 357 consecutive patients presenting with premature ovarian failure. Eur. J. Endocrinol. 161 (1), 179–187.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B 57 (1), 289–300.

Benjamini, Y., Yekutieli, D., 2001. The control of the false discovery rate in multiple testing under dependency. Ann. Stat. 29 (4), 919–1188.

Bolstad, B.M., Irizarry, R.A., Astrand, M., Speed, T.P., 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19 (2), 185–193.

Brown, C., LaRocca, J., Pietruska, J., Ota, M., Anderson, L., Smith, S.D., Weston, P., Rasoulpour, T., Hixon, M.L., 2010. Subfertility caused by altered follicular development and oocyte growth in female mice lacking PKB alpha/Akt1. Biol. Reprod. 82 (2), 246–256.

Cecconi, S., Mauro, A., Cellini, V., Patacchiola, F., 2012. The role of Akt signalling in the mammalian ovary. Int. J. Dev. Biol. 56 (10-12), 809–817.

Conti, M., Hsieh, M., Zamah, A.M., Oh, J.S., 2012. Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. Mol. Cell. Endocrinol. 356 (1-2), 65–73 (Review).

Coulam, C.B., Adamson, S.C., Annegers, J.F., 1986. Incidence of premature ovarian failure. Obstet. Gynecol. 67 (4), 604–606.

Dixit, H., Rao, L., Padmalatha, V., Raseswari, T., Kapu, A.K., Panda, B., Murthy, K., Tosh, D., Nallari, P., Deenadayal, M., et al., 2010. Genes governing premature ovarian failure. Reprod. Biomed. Online 20, 724–740.

Epstein, A.M., Bauer, C.R., Ho, A., Bosco, G., Zarnescu, D.C., 2009. *Drosophila* Fragile X protein controls cellular proliferation by regulating cbl levels in the ovary. Dev. Biol. 330 (1), 83–92.

Ferder, I., Parborell, F., Sundblad, V., Chiauzzi, V., Gómez, K., Charreau, E.H., Tesone, M., Dain, L., 2013. Expression of fragile X mental retardation protein and FMR1 mRNA during folliculogenesis in the rat. Reproduction 145 (4), 335–343.

Gleicher, N., Weghofer, A., Lee, I.H., Barad, D.H., 2010. FMR1 genotype with autoimmunity-associated polycystic ovary-like phenotype and decreased pregnancy chance. PLoS One 5 (12), e15303.

Gleicher, N., Kim, A., Barad, D.H., Shohat-Tal, A., Lazzaroni, E., Michaeli, T., Lee, H.J., Kushnir, V.A., Weghofer, A., 2013. FMR1-dependent variability of ovarian aging patterns is already apparent in young oocyte donors. Reprod. Biol. Endocrinol. 11, 80.

Hagerman, P.J., Hagerman, R.J., 2004. The fragile-X premutation: a maturing perspective. Am. J. Hum. Genet. 74 (5), 805–816 (Review. Erratum in: Am J Hum Genet. 2004; 75(2):352).

Hoffman, G.E., Le, W.W., Entezam, A., Otsuka, N., Tong, Z.B., Nelson, L., Flaws, J.A., McDonald, J.H., Jafar, S., Usdin, K., 2012. Ovarian abnormalities in a mouse model of fragile X primary ovarian insufficiency. J. Histochem. Cytochem. 60 (6), 439–456.

Hunter, J.E., Epstein, M.P., Tinker, S.W., Charen, K.H., Sherman, S.L., 2008. Fragile X-associated primary ovarian insufficiency: evidence for additional genetic contributions to severity. Genet. Epidemiol. 32 (6), 553–559.

Hunzicker-Dunn, M., Maizels, E.T., 2006. FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. Cell. Signal. 18 (9), 1351–1959 (Review).

Kosaka, N., Sudo, N., Miyamoto, A., Shimizu, T., 2007. Vascular endothelial growth factor (VEGF) suppresses ovarian granulosa cell apoptosis in vitro. Biochem. Biophys. Res. Commun. 363 (3), 733–737.

Lu, C., Lin, L., Tan, H., Wu, H., Sherman, S.L., Gao, F., Jin, P., Chen, D., 2012. Fragile X premutation RNA is sufficient to cause primary ovarian insufficiency in mice. Hum. Mol. Genet. 21 (23), 5039–5047.

Mallolas, J., Duran, M., Sánchez, A., Jiménez, D., Castellví-Bel, S., Rifé, M., Milà, M., 2001. Implications of the *FMR1* gene in menopause: study of 147 Spanish women. Menopause 8 (2), 106–110.

Mateu-Huertas, E., Rodriguez-Revenga, L., Alvarez-Mora, M.I., Madrigal, I., Willemsen, R., Milà, M., Martí, E., Estivill, X., 2014. Blood expression profiles of fragile X premutation carriers identify candidate genes involved in neurodegenerative and infertility phenotypes. Neurobiol. Dis. 65, 43–54.

Medina, I., Carbonell, J., Pulido, L., Madeira, S.C., Goetz, S., Conesa, A., Tárraga, J., Pascual-Montano, A., Nogales-Cadenas, R., Santoyo, J., 2010. Babelomics: an integrative

platform for the analysis of transcriptomics, proteomics and genomic data with advanced functional profiling. Nucleic Acids Res. 38 (Web Server issue), W210–W213.

Murray, A., Ennis, S., MacSwiney, F., Webb, J., Morton, N.E., 2000. Reproductive and menstrual history of females with fragile X expansions. Eur. J. Hum. Genet. 8 (4), 247–252.

Nelson, L.M., 2009. Clinical practice. Primary ovarian insufficiency. N. Engl. J. Med. 360 (6), 606–614.

Oberlé, I., Rousseau, F., Heitz, D., Kretz, C., Devys, D., Hanauer, A., Boué, J., Bertheas, M.F., Mandel, J.L., 1991. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. Science 252, 1097–1102.

Reddy, P., Adhikari, D., Zheng, W., Liang, S., Hämäläinen, T., Tohonen, V., Ogawa, W., Noda, T., Volarevic, S., Huhtaniemi, I., et al., 2009. PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles. Hum. Mol. Genet. 18 (15), 2813–2824.

Rodriguez-Revenga, L., Madrigal, I., Badenas, C., Xunclà, M., Jiménez, L., Milà, M., 2009. Premature ovarian failure and fragile X female premutation carriers: no evidence for a skewed X-chromosome inactivation pattern. Menopause 16 (5), 944–949.

Sherman, S.L., 2000. Premature ovarian failure in the fragile X syndrome. Am. J. Med. Genet. 97, 189–194.

Smyth, G.K., 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3, 3.

Sullivan, A.K., Marcus, M., Epstein, M.P., Allen, E.G., Anido, A.E., Paquin, J.J., Yadav-Shah, M., Sherman, S.L., 2005. Association of *FMR1* repeat size with ovarian dysfunction. Hum. Reprod. 20 (2), 402–412.

Sullivan, S.D., Welt, C., Sherman, S., 2011. *FMR1* and the continuum of primary ovarian insufficiency. Semin. Reprod. Med. 29 (4), 299–307.

Tassone, F., Hagerman, R.J., Taylor, A.K., Gane, L.W., Godfrey, T.E., Hagerman, P.J., 2000. Elevated levels of *FMR1* mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. Am. J. Hum. Genet. 66 (1), 6–15.

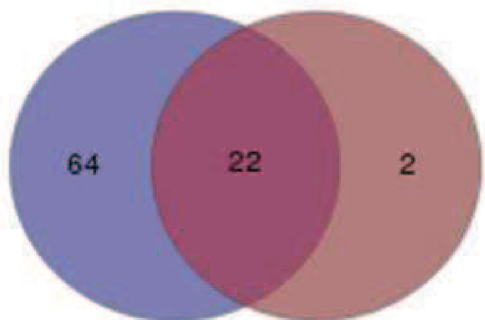
Verkerk, A.J., Pieretti, M., Sutcliffe, J.S., Fu, Y.H., Kuhl, D.P., Pizzuti, A., Reiner, O., Richards, S., Victoria, M.F., Zhang, F.P., et al., 1991. Identification of a gene (*FMR-1*) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65 (5), 905–914.

Willemsen, R., Levenga, J., Oostra, B.A., 2011. CGG repeat in the *FMR1* gene: size matters. Clin. Genet. 80 (3), 214–225.

Yu, S., Pritchard, M., Kremer, E., Lynch, M., Nancarrow, J., Baker, E., Holman, K., Mulley, J.C., Warren, S.T., Schlessinger, D., et al., 1991. Fragile X genotype characterized by an unstable region of DNA. Science 252, 1179–1181.

Supplementary Figure 1.

A. KEGG pathways



B. Biological Processes GO terms

C. Molecular Function GO terms

D. Cellular Component GO terms

Capítulo II

Mecanismos patogénicos y factores de riesgo en FXTAS

TRABAJO II

"PERFIL DE EXPRESIÓN EN SANGRE DE LOS microRNAs EN PACIENTES CON EL SÍNDROME DE TEMBLOR/ATAXIA ASOCIADO AL X-FRÁGIL"

Resumen:

Los miRNAs son moléculas de ARN de cadena simple con un tamaño aproximado entre 18 y 25 nucleótidos cuya función principal es la regulación negativa de la expresión génica a través de su unión a la región 3´UTRs de sus ARNm dianas. Cuando del grado de complementariedad es total disminuyen la expresión génica mediante la degradación del ARNm, mientras que si la complementariedad es parcial promueven el bloqueo del proceso de traducción.

Los miRNAs están implicados en la mayoría de procesos biológicos como en la proliferación, el desarrollo, la apoptosis y la inflamación celular. Desde el descrubrimiento del primer miRNAs, se ha puesto de manifiesto la importancia que estos tienen en el desarrollo del cerebro ya que juegan un papel crucial en aspectos funcionales de las neuronas como el creciemiento de las neuritas y la formación de las sinapsis. De esta manera, no es de extañar que la desregulación de determinados miRNAs haya sido asociada con diversas enfermedades

neurodegenerativas como el Huntington o el Alzheimer. En FXTAS también existen evidencias que sugieren que los miRNAs pueden contribuir a la aparición y a la progresión de la enfermedad.

Con el fin de determinar la posible implicación de los miRNAs en FXTAS, en este trabajo se ha identificado el perfil de expresión de miRNAs en muestras de sangre periférica procedentes de 8 varones FXTAS y de 4 varones de la población general. La caracterización de la expresión de los miRNAs se ha realizado mediante secuenciación masiva de los smallRNAs y mediante *microarrays* de expresión de miRNAs.

Los resultados obtenidos de la secuenciación mostraron una distribución similar de las distintas clases de smallARNs, siendo los miRNAs el grupo más representado en pacientes y controles. Respecto el análisis de expresión diferencial, los datos obtenidos por secuenciación masiva pusieron de manifiesto la expresión significativamente desregulada de 83 miRNAs (49 infraexpresados y 34 sobreexpreados) mientras que los microarrays detectaron de 31 miRNAs significativamente desregulados (25 infraexpresados y 6 sobreexpreados). Finalmente, al comparar los resultados obtenidos mediante ambas tecnologías se detectaron 14 miRNAs con una desregulación común, 12 de los cuales presentaban una expresión a la

alza y 2 a la baja. Estos resutados se validaron mediante RT-qPCR confirmandose el resultado en todos los casos.

Se ha descrito la sobreexpresión del miR-424 en regiones de la sustancia blanca cerebral procedentes de pacientes post-mortem con Alzheimer y curiosamente, la alteración de la sustancia blanca cerebral junto con la atrofia cerebral es uno de los hallazgos caracteristicos en las imágenes obtenidas mediante RM en los pacietnes con FXTAS.

A pesar que se necesitan más estudios para profundizar en la función de los miRNAs en la enfermedad de FXTAS, estas resultados sugieren que su desrgulación puede contribuir a la patogénesis del FXTAS.

Genes, Brain and Behavior
Official publication of the International Behavioural and Neural Genetics Society

Genes, Brain and Behavior (2013) **12:** 595–603 doi: 10.1111/gbb.12061

MicroRNA expression profiling in blood from fragile X-associated tremor/ataxia syndrome patients

M. I. Alvarez-Mora[†,‡,§], L. Rodriguez-Revenga[†,‡,§], I. Madrigal[†,‡,§], F. Torres-Silva[†], E. Mateu-Huertas[¶], E. Lizano[¶], M. R. Friedländer[¶], E. Martí[¶,], X. Estivill[¶,**,††,‡‡] and M. Milà[†,‡,§,*]**

[†] Biochemistry and Molecular Genetics Department, [‡] CIBER de Enfermedades Raras (CIBERER), Hospital Clínic, Barcelona, Spain, [§] IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer), Barcelona, Spain, [¶] Genetic Causes of Disease Group , [**] CIBER in Epidemiology and Public Health (CIBERESP), Center for Genomic Regulation (CRG), Barcelona, Spain, [††] Experimental and Health Sciences Department, Pompeu Fabra University (UPF), Barcelona, Spain, and [‡‡] Research Institute, Hospital del Mar, Barcelona, Spain

**Corresponding author: M. Milà, Biochemistry and Molecular Genetics Department, Hospital Clínic, C/ Villarroel, 170, 08036 Barcelona, Spain. E-mail: mmila@clinic.ub.es*

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late-onset neurodegenerative disorder associated with *FMR1* gene premutation alleles (55–200 CGG repeats). Fragile X-associated tremor/ataxia syndrome clinical core features include action tremor, gait ataxia, cognitive deficits progressing to dementia, and frequently parkinsonism. Although the pathogenic molecular mechanism of FXTAS is not completely understood, the restriction of the phenotype to the *FMR1* premutation range has given rise to a model based on a RNA toxic gain-of-function. Since the identification of the first microRNAs (miRNAs) and their role in normal development, several studies have associated them with neurodegenerative diseases such as Parkinson, Alzheimer and Huntington diseases, suggesting that they play a key role in brain development, as well as in its morphogenesis. Herein, we present the characterization of miRNA expression profiles in FXTAS male patients using deep sequencing-based technologies and microarray technology. Deep sequencing analysis evidenced 83 miRNAs that were significantly deregulated whereas microarray analysis showed 31. When comparing these results, 14 miRNAs were found deregulated in FXTAS patients. MiR-424 and miR-574-3p showed significant fold change adjusted *P*-values in both platforms in FXTAS patients. MiR-424 has been founded substantially and specifically enriched in human cerebral cortical white matter of Alzheimer disease patients, which, together with cerebral atrophy, is a prominent imaging finding in individuals with FXTAS. The study provides the first systematic evidence of differential miRNA expression changes in FXTAS blood samples. Although further studies are necessary to better characterize the miRNA function in FXTAS disorder, our results suggest that they might contribute to its pathogenesis.

Keywords: Expression profile, *FMR1* gene, FXTAS, high--throughput technologies, IsomiR, miRNAs, mitochondrial dysfunction, neurodegeneration, oxidative stress, trimming variants

Received 27 March 2013, revised 12 June 2013 and 21 June 2013, accepted for publication 21 June 2013

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late-onset neurodegenerative disorder that affects older adults who are carriers of an expansion of 55–200 CGG repeats in the 5′ untranslated region (UTR) of the *FMR1* gene (Hagerman *et al*. 2001). The clinical features of FXTAS include action tremor, gait ataxia, dementia and parkinsonism, in which affected females present milder clinical involvement compared with affected males (Jacquemont *et al*. 2003). It is also characterized by cognitive decline with lack of inhibition and executive function deficits, peripheral neuropathy and autonomic dysfunction (Hagerman *et al*. 2001). Fragile X-associated tremor/ataxia syndrome presents incomplete penetrance with approximately 40% of male and 8–16% of female *FMR1* premutation carriers aged above 50 years developing symptoms (Rodriguez-Revenga *et al*. 2009; Hagerman & Hagerman 2004). Although the cause of this incomplete penetrance is not clear, its prevalence has been shown to increase with advanced age and CGG repeat longer sizes (Rodriguez-Revenga *et al*. 2009; Willemsen *et al*. 2011). Magnetic resonance imaging (MRI) in FXTAS patients demonstrates an abnormal brain pattern affecting the periventricular and subcortical regions of the brain. Fragile X-associated tremor/ataxia syndrome characteristic radiological finding is the hyperintensity of the middle cerebellar peduncle, present in 60% of FXTAS patients and seen in T2 and FLAIR acquisitions (Jacquemont *et al*. 2003). Immunocytochemical staining of post-mortem brain tissue from FXTAS patients shows ubiquitin-positive intranuclear inclusions in neurons with a broad distribution throughout the brain (Greco *et al*. 2002).

Although the pathogenic molecular mechanism is still unknown, the restriction of the phenotype to the *FMR1* premutation range has given rise to a model based on a RNA toxic gain-of-function (Hagerman *et al*. 2001; Hagerman & Hagerman 2004). *FMR1* premutation alleles have an increased level of *FMR1* mRNA of 2–8-fold, and normal or slightly lowered FMRP levels compared with normal alleles (Tassone *et al*. 2000; Kenneson *et al*. 2001). The hypothesis of a RNA toxic gain-of-function suggests that CGG repeat

© 2013 John Wiley & Sons Ltd and International Behavioural and Neural Genetics Society

FMR1 mRNA recruits RNA-binding proteins and other proteins through indirect interactions, creating intranuclear inclusions in neurons, astrocytes and the spinal column, with the greatest numbers of neuronal inclusions found in the hippocampus (Greco *et al*. 2002). The sequestration of these proteins is also thought to prevent normal function leading to downstream alterations (Galloway & Nelson 2009; Li & Jin 2012).

Since the identification of the first miRNAs (miRNAs) and the definition of their role in development, several studies have shown that miRNAs have spatiotemporal and cell-specific expression patterns in the nervous system, suggesting that they play a key role in brain development and in its morphogenesis (Li & Jin 2010). In fact, some miRNAs have already been associated with other neurodegenerative diseases such as Parkinson disease (PD), Alzheimer disease (AD) and Huntington disease (HD) (Barbato *et al*. 2009; Enciu *et al*. 2012; Long & Lahiri 2011).

Herein, we present the characterization of the miRNAs expression profiles in FXTAS male patients using microarray technology and deep sequencing-based technologies. An alteration on the miRNA expression may act as a susceptibility factor leading to FXTAS. The study provides new and relevant data that may be valuable to build a better understanding of the contribution of miRNAs in FXTAS pathogenesis.

Materials and methods

Subjects

A total of 12 unrelated individuals were recruited for this study: 8 male FXTAS patients (mean age 66.6 SD + 6.6) and 4 male controls (mean age 71.5 SD + 2.6). Controls present normal *FMR1* alleles and neurodegenerative disorders were excluded. All FXTAS patients were recruited from FXS families and were molecularly diagnosed at the Biochemical and Molecular Genetics Department of the Hospital Clinic of Barcelona. The CGG repeat number, *FMR1* mRNA levels, FMRP protein levels and clinical/neuroradiological findings of FXTAS patients are summarized in Table 1.

All participants provided written informed consent for testing and for the use of their phenotypic and genetic data. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona.

MiRNA extraction

Whole blood was collected from the patients and controls in 4 ml EDTA tubes. Firstly, lymphocytes were isolated using LinfoSep medium (Biomedics, S.L. Madrid, Spain), following the manufacturer's instructions. MiRNAs were extracted using 'Total RNA isolation Procedure', detailed in the mirVana™ miRNA isolation kit protocol (Ambion®, Life Technologies, Austin, TX, USA). The quality and concentration of RNA were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All RNA samples revealed RNA Integrity Number values, ranging from 7.8 to 9.9. MiRNA extractions were stored at −80°C until processing.

MiRNA microarrays

The Unrestricted Human miRNA V16.0 microarray (Human miRNA Microarray Kit Release 16.0, 8× 60K; Agilent Technologies) was used in six FXTAS patients and four controls. This microarray contains 62 976 probes representing a total of 1349 different miRNAs (1205 human miRNAs and 144 human viral miRNAs) and 2167 control probes. Briefly, 100 ng of total RNA were labeled with Cyanine 3-pCp and hybridized according to the manufacturer's instructions. Following careful washing, the arrays were read using the Agilent microarray scanner (Agilent G2565CA Microarray Scanner System, Agilent Technologies) and the data were extracted using Agilent Feature Extraction V10.7.3.1. Technical replicates were hybridized in different arrays discarding the ones which presented more variance. These experiments were performed at the Biochemistry and Molecular Genetics Department of the Hospital Clinic of Barcelona.

Differential expression analysis from microarray technology

Extracted intensities were corrected using the *normexp* method with an offset of 50 (Ritchie *et al*. 2007). To assure comparability across samples we used quantile normalization on background corrected log2-transformed intensities (http://bmbolstad.com/stuff/qnorm.pdf). Microarray probes were collapsed to miRNAs by taking the median intensity of the respective probes per miRNA. For determining differentially regulated miRNAs moderated *t*-tests were applied using limma (Smyth 2004). MiRNAs with adjusted *P*-value $<5\%$ and additionally a fold change (FC) >1.2 were selected as relevant. All statistical analyses were performed with the Bioconductor project in the R statistical environment (Gentleman *et al*. 2004).

Sequencing

Small RNA (sRNA) libraries, corresponding to four controls and eight FXTAS patients, were prepared. From each sample, 1 μg of total RNA was used to prepare indexed libraries according to the 'TruSeq Small RNA Sample Preparation Guide' (Part# 15004197 Rev. A). Pooled libraries (two different indexed samples per lane) were sequenced in one Illumina Genome Analyzer II run using 36 single-read cycles (Illumina, San Diego, CA, USA).

Preanalysis of sequencing data

Recognition and removal of the adapter and annotation of the sequences were performed using a stand-alone version of the Seqbuster tool designed to process and analyze sRNAs sequencing datasets (http://estivill_lab.crg.es/seqbuster) (Pantano *et al*. 2010, 2011). Out of a total of several million reads, we discarded those without a minimum of 8-nt linker subsequences directly adjoining the insert, showing no more than a single mismatch. Following the application of these filters, populations of at least 8 000 000 reads were recovered in the majority of samples. Sequences were then mapped to human miRNA as described previously (Pantano *et al*. 2011) and mRNA, ncRNA and genomic repeats were mapped to genome databases at (http://hgdownload.cse.ucsc.edu/goldenPath/hg18/bogZips/), using Bowtie alignment software. For miRNA annotation different parameters were configured in Seqbuster tool: one mismatch allowed, 3 nt in the 3′ or 5′-trimming variants, 3 nt in the 3′-addition variants.

Analysis of miRNA variability

In order to characterize miRNA variants, several filters in Seqbuster were applied. Firstly, only sequences with a frequency above 3 were considered. Secondly, a 'Contribution Cut-Off' parameter of 10 was chosen, meaning that every sequence variants (IsomiR) contributes by more than 10% to the total number of variants annotated in the same miRNA locus. Finally, the *Z*-score option was applied to exclude sequencing errors as the possible cause of the nucleotide changes observed in some variants (Pantano *et al*. 2010). The different parameters in the Seqbuster tool allowed the annotation of the following types of alignments: (1) perfect match, when the sequence is identical to the reference sequence; (2) trimming at the 3′-end of the reference miRNA sequence, which is a miRNA variant several nucleotides shorter or longer that matches the mature or precursor reference sequence; (3) trimming at the 5′-end of the reference miRNA sequence, an analogous case focused on the 5′-end of the miRNA; (4) nucleotide additions at the 3′-end of the sequence; and (5) nucleotide substitutions, showing nucleotide changes with respect to the reference sequence.

Table 1: Clinical and *FMR1* molecular findings of eight FXTAS patients characterized for miRNA profiles

Subject	(CGG)*n*	*FMR1* mRNA level*	FMRP level (%)†	Clinical findings	MRI findings	Diagnostic‡	FXTAS age of onset (years)
FXTAS 1	106	1.56	67	2 major	1 major + 1 minor	DEF	62
FXTAS 2	113	3.20	88	1 major + 1 minor	1 major	DEF	65
FXTAS 3	71	1.54	75	1 major	1 minor	POS	77
FXTAS 4	65	1.72	100	2 major	1 major + 1 minor	DEF	60
FXTAS 5	59	1.22	94	1 minor	1 major	PROB	64
FXTAS 6	126	2.87	92	2 major	1 major	DEF	64
FXTAS 7	60	1.78	100	2 major	2 minor	PROB	73
FXTAS 8	75	2.06	83	2 minor + 1 major	1 minor	POS	62

DEF, definite; PROB, probable; POS, possible.
*RNA levels are reported as fold elevated over those in normal sex-matched control individuals.
†Percentage of hair roots positive for FMRP by immunohystochemical staining.
‡Diagnostic criteria described by Jacquemont *et al*. (2003).

Differential expression analysis using sequencing technology

The sequencing performance was evaluated using the 'Sequencing Capacity' package in the 'General' module of SeqBuster with default parameters (Pantano *et al*. 2010). The Wilcoxon test was applied to determine statistically significant differences in frequency distribution between samples. For differential expression analysis between control and FXTAS samples, miRNA loci containing all sequences mapping to each locus were considered. Several options and filters were applied. Firstly, for a given miRNA the frequency was normalized to reads per million (RPM), referring to the total of sequences mapping onto miRNAs (Normalized frequency = counts miR-x/counts total miRNAs * 10 E6). Secondly, the *Z*-test (Reinartz *et al*. 2002) was applied to show statistical significance since it is the most restrictive test used for evaluation of differentially expressed sequences in high-throughput sequencing methodologies. Thirdly, the Benjamini and Hochberg method was applied to correct the *P*-value assigned by the *Z*-test (Klipper-Aurbach *et al*. 1995). Fourthly, when analyzing all sequences mapping onto a miRNA locus, 10 was chosen as the 'Contribution Cut-Off' value (Pantano *et al*. 2010). Finally, the *Z*-score option was applied to exclude sequencing errors as the possible cause of the nucleotide changes observed in the IsomiR (Dohm *et al*. 2008).

Real Time-quantitative polymerase chain reaction

Real time-quantitative PCR (RT-qPCR) analysis was performed using TaqMan® MicroRNA Assays (Applied Biosystems, Foster City, CA, CA). Retro Transcribe (RT) reaction was performed following the 'Creating Custom RT and Preamplification Pools' protocol using six 5× RT-primers. The preamplification primer pool was performed according to the manufacturers' protocol (Applied Biosystems) and then diluted 1:2 to have a final concentration of 10× PreAmp primer pool. The preamplification reaction was performed according to manufacturer's instructions and qPCR was carried out using 7300 Real Time PCR System (Applied Biosystems). Two snoRNAs (Z30 and RNU 24) and two endogenous miRNAs (hsa-miR-16, hsa-miRNA-93) were selected as reference miRNAs since they are universally expressed in normal tissues (Benes & Castoldi 2010; Liang *et al*. 2007). All reactions were run in triplicate and data was evaluated following the $\delta\delta$Ct method (Livak & Schmittgen 2001).

Results

Small RNAs profiling of FXTAS by deep sequencing of blood RNA samples

High-throughput sequencing of sRNAs of all blood RNA samples revealed a sequence yield between 12 million and 20 million unfiltered sequence-reads per sample. After removing the reads containing ambiguous base calls (<0.3%), only those longer than 18 nucleotides were considered for mapping. Most reads mapped to the human genome with <6% of the sequences not being identified.

Sequences were annotated on the basis of the overlap with publicly available genome data, including miRNA, tRNAs, rRNAs, protein-coding genes, other sRNAs and genomic repeats. In all samples, miRNAs were the most abundant class of detected sRNAs (around 44%), followed by miscellaneous RNAs (miscRNAs) (29%), which included several classes of cytoplasmic sRNAs (Fig. 1a). Sequences derived from 743 to 967 distinct miRNAs were identified in the different libraries (frequency >1%), showing a sequence length distribution with a sharp peak in the 22-nucleotide (Fig. 1b). Most of the detected miRNAs (>70%) had less than 500 counts (Fig. 1c). The highest expressed miRNAs in the majority of the samples included members of the let-7 family, miR-103a, miR-21, miR-22, miR-26 and miR-30 (Table S1) in concordance with previous observations (Marti *et al*. 2010).

MiRNA variability in blood RNA samples of FXTAS patients

Around 93% of all the sequenced miRNAs presented IsomiRs. The remaining 7%, showed few reads suggesting that the lack of variability is related to its low abundance. An average of 11 000 different IsomiRs were found in every sample. Using the Seqbuster tool (http://code.google.com/p/seqbuster/wiki/SeqBuster) for the analysis of miRNA variability, no major differences were detected in the distribution of the different types of IsomiRs when comparing control individuals and FXTAS.

Regarding the variants, those affecting the 3′-end of the miRNAs, especially variants generated by 3′-trimming, were the most abundant. The frequency of nucleotide substitution in mature miRNAs and 5′-trimming variants was generally low, contributing little to the total number of sequences annotated onto a miRNA locus (Fig. 2a). Although this is the normal behavior of most of the miRNAs (e.g. miR-574-3p and miR-101-3p, Fig. 2b), not all followed the same tendency. For example, most of the IsomiRs from miR-181a-5p corresponded to 5′-trimming whereas scarce 3′trimming variants were detected (Fig. 2b).

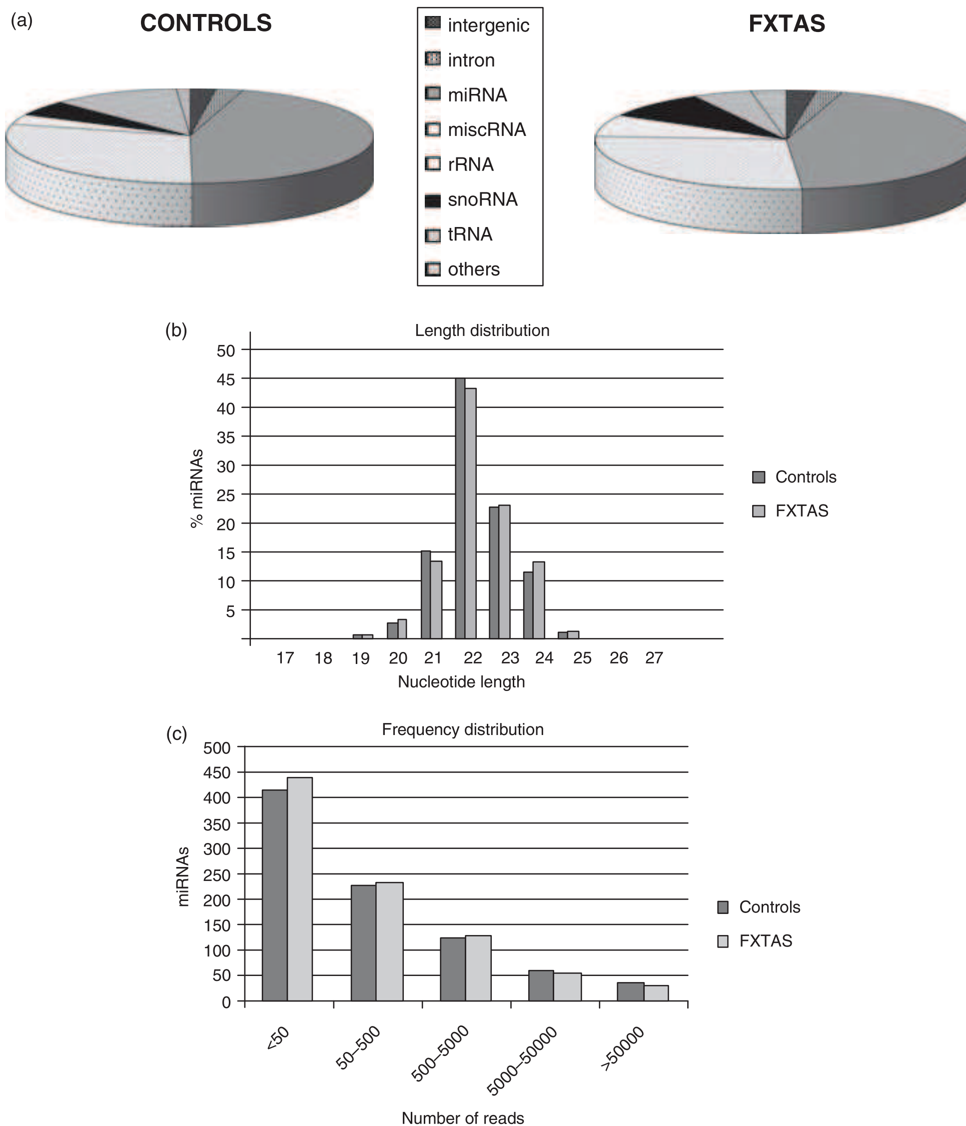
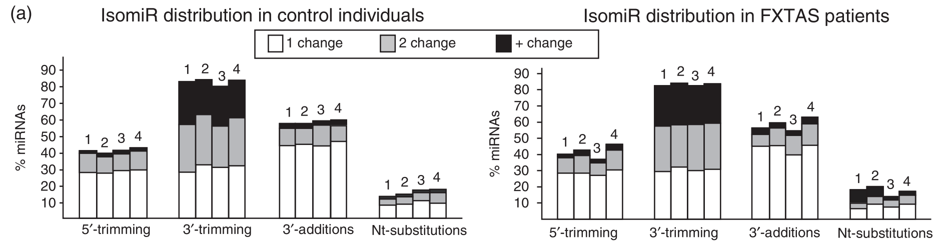


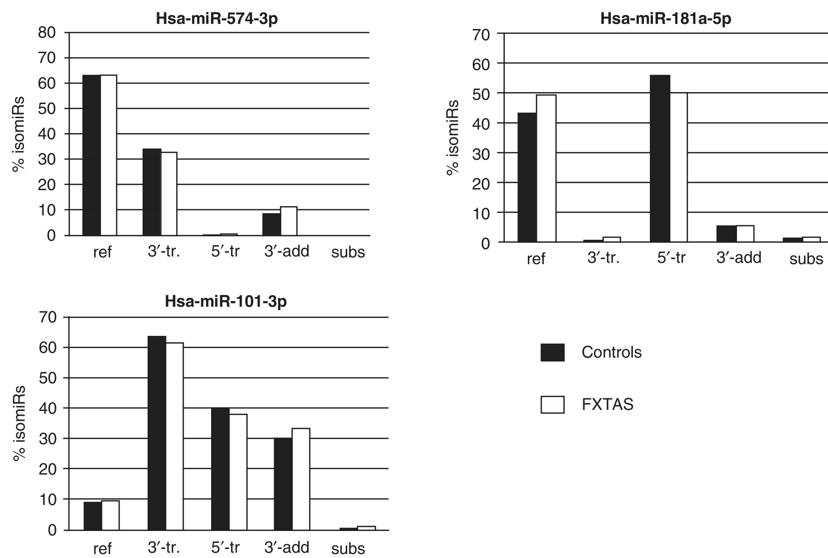
Figure 1: Small RNAs profiling of FXTAS patients and control samples by deep sequencing. (a) Annotation breakdown of small RNA sequences; (b) miRNA sequence length distribution; and (c) miRNA sequence abundance distribution.

MiRNA transcriptome deregulation in blood RNA of FXTAS patients

The analysis of differential miRNA expression in control versus FXTAS patients showed 83 miRNAs significantly deregulated (49 downregulated and 34 upregulated) in the FXTAS cohort (FC >1.2 or <−1.2 adjusted *P*-value <0.05) (Table S2). To compare this expression pattern with an independent and totally different method, miRNA microarray analysis (Agilent Technologies) was performed. Results showed 31 deregulated human miRNA in FXTAS patients (25 downregulated and 6 upregulated) (FC >1.2 or <−1.2 adjusted *P*-value <0.05) (Table S3). When comparing these results, miR-424 and miR-574-3p showed significant FC adjusted *P*-values in both platforms in FXTAS patients. While miR-424 was detected upregulated in both experiments, miR-574-3p showed contradictory results (Tables S2 and S3).



(b) Histogram displaying percentage of IsomiR distribution in control aNd FXTAS patients



reference	**TACAGTACTGTGATAACTGAA**
5‘ trimming variant	**G**TACAGTACTGTGATAACTGAA
3‘ trimming variant	TACAGTACTGTGATAACTGAA**G**
3‘ addtion variant	TACAGTACTGTGATAACTGAA**TG**
NT substitution	TACAGTACTG**C**GATAACTGAA

Figure 2: IsomiRs profiling of FXTAS patients and control samples. (a) Histogram displaying the percentage of miRNAs with different types of IsomiRs (5-trimming, 3-trimming, 3-additions and nucleotide substitutions), in controls and FXTAS patients. In this analysis, IsomiRs with frequencies >10 were considered. For every type of variability, the histogram shows the proportion of miRNAs presenting one (white), two (grey) or more than two (black) IsomiRs; (b) Histogram displaying the percentage of IsomiR distribution for miR-574-3p, miR-181a-5p and miR-101-3p, selected in control samples (black bars) and FXTAS patients (white bars). The total of sequences mapping onto the indicated miRNA in each individual is considered as 100%. For every miRNA the first bar represents the abundance of the corresponding reference miRNA. An example of the IsomiRs from miR-101-3p is detailed in the chart below the arrow.

Table 2: Fold change values for the microRNAs deregulated in blood RNA of FXTAS patients using microarray and deep sequencing technologies. Significant *P*-values are shown in brackets

miRNA name	Fold change sequencing	Fold change microarray
hsa-miR-23a-5p	2.32 ($P \leq 0.01$)	1.20*
hsa-miR-25	2*	1.37
hsa-miR-29a-5p	1.92 ($P \leq 0.01$)	1.39*
hsa-miR-27a-5p	1.72 ($P \leq 0.01$)	1.43*
hsa-miR-30a-5p	1.39 ($P < 0.0001$)	1.28†
hsa-miR-142-3p	1.37 ($P < 0.0001$)	1.70
hsa-miR-101-3p	1.35 ($P < 0.0001$)	1.28
hsa-miR-106b-3p	1.27 ($P < 0.0001$)	1.21
hsa-miR-424-5p	1.23 ($P \leq 0.01$)	1.30 ($P < 0.01$)
hsa-miR-29b-3p	1.22 ($P < 0.0001$)	1.45
hsa-miR-181a-3p	1.22 ($P < 0.0001$)	1.26
hsa-miR-26b-3p	1.20 ($P < 0.0001$)	1.27
hsa-miR-1260b	−1.83 ($P < 0.0001$)	−1.22
hsa-miR-451a	−2.33 ($P < 0.0001$)	−1.69‡

*The corresponding miRNAs is the other strand of the miRNA duplex.
†Microarray technology detected upregulation of miR-30c-3p, 30e-5p and 30e-3p.
‡Microarray technology detected downregulation of all species of miR-451.

Real time-quantitative polymerase chain reaction validation was carried out, confirming miR-424 upregulation and a downregulation for miR-574-3p.

When considering only FC (>1.2 or < −1.2) and not adjusted *P*-values, 14 miRNAs were found deregulated (2 downregulated and 12 upregulated) in FXTAS patients by both approaches (Table 2). Real time-quantitative polymerase chain reaction analysis was performed in half of the upregulated miRNAs, confirming increased expression compared with age-matched controls (Fig. 3).

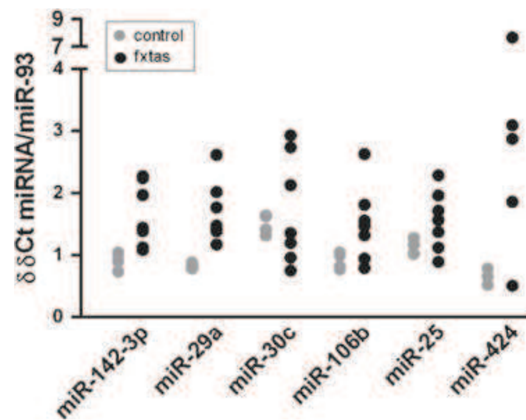


Figure 3: Real time-quantitative PCR analysis of the expression of upregulated miRNAs relative to miR-93 in blood samples of FXTAS (*n* = 7) and age-matched controls (*n* = 4).

Discussion

Fragile X-associated tremor/ataxia syndrome is a leading monogenic neurodegenerative disorder affecting premutation carriers of the *FMR1* gene CGG repeat. However, it has been described that most neurodegenerative disorders are probably not just a 'one-hit' phenomenon, but rather they probably involve a sequential progression of pathological processes that collectively result in neuronal death and/or compromised connectivity (Nelson *et al*. 2008). Several studies have uncovered the crucial role of miRNAs in the fine-tuning of neuronal gene expression during development and have described their implication in neurodegenerative disease (Liu *et al*. 2004; Giraldez *et al*. 2005).

Different clues point to a possible involvement of miRNAs in FXTAS. First of all, *FMR1* premutation CGG repeats have been reported to lead to deregulation of miRNAs in the brain of the FXTAS fly model (Tan *et al*. 2012). The overexpression of miR-277 in a FXTAS *Drosophila* model induced an aggravated eye phenotype, suggesting that this miRNA could be involved in the neurodegeneration of FXTAS. Secondly, miR-125b and miR-132 have been associated with FMRP in the mouse brain, controlling synaptic function and structure (Edbauer *et al*. 2010). Finally, a set of miRNAs has been experimentally identified to target the 3′ UTR region of the human *FMR1* gene (Yi *et al*. 2010; Zongaro *et al*. 2013). We performed a high-throughput deep sequencing and microarray miRNA study using blood in order to define the expression levels of miRNAs sequence variants among FXTAS patients. To our knowledge, this is the first study to describe the miRNA expression profiles in male FXTAS patients.

In agreement with previous studies, including miRNA profiling in neurodegenerative disorders, sRNAs sequencing revealed IsomiRs for virtually all detected the miRNAs (Morin *et al*. 2008; Marti *et al*. 2010). Our results show a similar distribution of the different types of IsomiRs in control and FXTAS samples, suggesting no major affectation in their biogenesis or stability (Fig. 1). Deep sequencing analysis evidenced 83 miRNAs that were significantly deregulated, whereas microarray analysis showed only 31. Microarray technology is based on direct specific hybridization with probes designed according to the miRNA sequences annotated in miRBase (D'Andrade & Fulmer-Smentek 2012). On the other hand, deep sequencing is based on amplification and identification of miRNAs and their IsomiRs, which provides a full dissection of miRNA landscape. In fact, IsomiR analysis showed that the reference sequence for some miRNAs is the least represented, which could provide an explanation for the lack of total concordance among the results provided by each platforms. In addition, the correlation of the miRNA expression levels found using these technologies is moderated, in agreement with previous reports (Git *et al*. 2010; Pradervand *et al*. 2010).

Recently, Sellier *et al*. (2013) has found decreased levels of mature miRNAs in brain tissue from FXTAS patients triggered

by an alteration of the miRNA-processing machinery. They suggested a model in which pathogenic-expanded CGG repeats mimic the structure of primary miRNAs (pri-miRNAs), leading to a partial sequestration of DGCR8-DROSHA which might explain the reduced levels of mature miRNAs, and ultimately, the neuronal cell dysfunction. The methodology used in our study cannot evaluate pri-miRNAs transcripts and therefore, our results cannot contribute with additional data in the scenario proposed by Sellier *et al*. (2013). Regarding, miRNA expression profiling, Sellier *et al*. (2013) found that the majority of the deregulated miRNAs revealed reduced expression levels compared with controls. Herein, the results obtained by microarray analysis seem to be in consonance, although a fewer number of miRNAs were found to be deregulated. Nevertheless, when comparing microarray and sRNAs sequencing data, the expression of deregulated miRNAs is substantially upregulated. In fact, 14 miRNAs were found altered in FXTAS blood samples by both profiling platforms (FC >1.2 or <-1.2 and not adjusted *P*-values), and 12 of them showed increased expression levels relative to control samples (Table 2). Real time-quantitative polymerase chain reaction analysis was performed for half of them, confirming increased expression levels (Fig. 3). Finally, it is well known that miRNAs are tissue-specific and/or temporally regulated in their expression (Bartel 2004), which might contribute to explain the differences observed between the results herein reported and the previously described (Sellier *et al*. 2013).

Among the 14 miRNAs deregulated, the miR-424 showed an overexpression with significant FC adjusted *P*-value by both profiling platforms. MiR-424 has been founded substantially and specifically enriched in human cerebral cortical white matter of AD patients (Wang *et al*. 2011). Interestingly, together with cerebral atrophy, white matter alteration is a prominent imaging finding in individuals with FXTAS (Adams *et al*. 2007). In fact, white matter MRI hyperintensities in the middle cerebellar peduncles represent a major diagnostic criterion for FXTAS (Brunberg *et al*. 2002).

MiR-424 belongs to the miR-15/107 gene group, which has been found to participate in pathways involved in cellular metabolism and stress (Finnerty *et al*. 2010). Recently, it has been reported that hippocampal neurons from *Fmr1* premutation mouse model show abnormalities in the number, mobility and metabolic function of mitochondria which may constitute a risk factor of developing clinical FXTAS (Kaplan *et al*. 2012). Remarkably, evidences of mitochondrial dysfunction and oxidative stress have also been described in human fibroblasts from FXTAS patients (Ross-Inta *et al*. 2010; Napoli *et al*. 2011). As in many other neurodegenerative disorders (Lin & Beal 2006) mitochondrial dysfunction seems to be an incipient pathological process occurring in FXTAS disorder (Ross-Inta *et al*. 2010; Napoli *et al*. 2011) and based on our results, the overexpression of miR-424 might somehow contribute to this process. Apart from miR-424, our results showed other deregulated miRNAs that have also been associated with mitochondrial dysfunction. In particular, the overexpression of miR-23a has been detected in leukemic cells and in skeletal muscle of amyotrophic lateral sclerosis patients, inducing mitochondrial dysfunction and leading to cell death (Rathore *et al*. 2012; Russell *et al*. 2012).

MiR-23a, which belongs to the miR-23a ~ miR-27a ~ miR-24-2 cluster, has been reported to induce cell apoptosis mediated by induction of endoplasmic reticulum stress (Chhabra *et al*. 2011). In particular, it has been published that the overexpression of this miRNA cluster induces endoplasmic reticulum stress, which leads to an increase of cytosolic and mitochondrial calcium levels (Chhabra *et al*. 2011). In this work, it is suggested that the calcium release might cause mitochondrial membrane depolarization which in turn leads to release of apoptosis-inducing factors from mitochondria resulting in cell death (Chhabra *et al*. 2011). On the other hand, it is known that the neuronal activity on the growth of neuronal dendrites is strongly influenced by spatial and temporal changes in intracellular calcium (Lohmann & Wong 2005; Segal *et al*. 2000). A recent study has demonstrated a higher frequency of amplified calcium transients in iPSC-derived *FMR1* premutation-expressing neurons, suggesting that it might be responsible for the neuronal phenotypes found in these cells (Liu *et al*. 2012). These deregulated calcium findings and neuronal network electric activity have also been described in primary hippocampal neuronal cultures from a FXTAS mouse model (Cao *et al*. 2012). Our results showed an upregulation of miR-23a and miR-27a in FXTAS patients (Table 2). Although more studies are required to pinpoint the role of these miRNAs into the mechanisms involved in FXTAS disorder, it might be plausible that the deregulation of miR-23a and miR-27a trigger the generation of reactive oxygen species causing oxidative stress response and mitochondrial dysfunction. To our knowledge miR-574-3p, the other miRNA that also showed significant deregulation in both platforms and was confirmed by RT-qPCR has only been associated with cancer processes (Su *et al*. 2012; Bryant *et al*. 2012; Tatarano *et al*. 2012), and there is no clear relationship with neurodegenerative disorders.

To conclude, our study provides the first systematic evidence of differential miRNA expression changes in FXTAS blood samples. It describes the miRNA expression profile in FXTAS patients and provides data suggesting its implication in the disease. Even though the pathogenesis of FXTAS is thought to involve an RNA toxic gain-of-function mechanism, it is likely that it reflects a much broader process in which miRNAs might have an important role. Better understanding of FXTAS pathogenesis is necessary in order to unravel the molecular causes of the syndrome and to develop targeted therapeutic approaches. Although further studies are necessary to better characterize the miRNA function in FXTAS, our results on abnormal profiles of miRNAs in blood, shed light not only on the FXTAS pathogenesis but also on the other *FMR1* premutation associated disorders.

References

Adams, J.S., Adams, P.E., Nguyen, D., Brunberg, J.A., Tassone, F., Zhang, W., Koldewyn, K., Rivera, S.M., Grigsby, J., Zhang, L., Decarli, C., Hagerman, P.J. & Hagerman, R.J. (2007) Volumetric brain changes in females with fragile X-associated tremor/ataxia syndrome (FXTAS). *Neurology* **69**, 851–859.

Barbato, C., Ruberti, F. & Cogoni, C. (2009) Searching for MIND: microRNAs in neurodegenerative diseases. *J Biomed Biotechnol* **2009**, 871313.

Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function [Review]. *Cell* **116**, 281–297.

Benes, V. & Castoldi, M. (2010) Expression profiling of microRNA using real-time quantitative PCR, how to use it and what is available. *Methods* **50**, 244–249.

Brunberg, J.A., Jacquemont, S., Hagerman, R.J., Berry-Kravis, E.M., Grigsby, J., Leehey, M.A., Tassone, F., Brown, W.T., Greco, C.M. & Hagerman, P.J. (2002) Fragile X premutation carriers: characteristic MR imaging findings of adult male patients with progressive cerebellar and cognitive dysfunction. *AJNR Am J Neuroradiol* **23**, 1757–1766.

Bryant, R.J., Pawlowski, T., Catto, J.W., Marsden, G., Vessella, R.L., Rhees, B., Kuslich, C., Visakorpi, T. & Hamdy, F.C. (2012) Changes in circulating microRNA levels associated with prostate cancer. *Br J Cancer* **106**, 768–774.

Cao, Z., Hulsizer, S., Tassone, F., Tang, H.T., Hagerman, R.J., Rogawski, M.A., Hagerman, P.J. & Pessah, I.N. (2012) Clustered burst firing in FMR1 premutation hippocampal neurons: amelioration with allopregnanolone. *Hum Mol Genet* **21**, 2923–2935.

Chhabra, R., Dubey, R. & Saini, N. (2011) Gene expression profiling indicate role of ER stress in miR-23a~27a~24-2 cluster induced apoptosis in HEK293T cells. *RNA Biol* **8**, 648–664.

D'andrade, P.N. & Fulmer-Smentek, S. (2012) Agilent microRNA microarray profiling system. *Methods Mol Biol* **822**, 85–102.

Dohm, J.C., Lottaz, C., Borodina, T. & Himmelbauer, H. (2008) Substantial biases in ultra-short read data sets from high-throughput DNA sequencing. *Nucleic Acids Res* **36**, e105.

Edbauer, D., Neilson, J.R., Foster, K.A., Wang, C.F., Seeburg, D.P., Batterton, M.N., Tada, T., Dolan, B.M., Sharp, P.A. & Sheng, M. (2010) Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. *Neuron* **65**, 373–384.

Enciu, A.M., Popescu, B.O. & Gheorghisan-Galateanu, A. (2012) MicroRNAs in brain development and degeneration. *Mol Biol Rep* **39**, 2243–2252.

Finnerty, J.R., Wang, W.X., Hebert, S.S., Wilfred, B.R., Mao, G. & Nelson, P.T. (2010) The miR-15/107 group of microRNA genes: evolutionary biology, cellular functions, and roles in human diseases. *J Mol Biol* **402**, 491–509.

Galloway, J.N. & Nelson, D.L. (2009) Evidence for RNA-mediated toxicity in the fragile X-associated tremor/ataxia syndrome. *Future Neurol* **4**, 785.

Gentleman, R.C., Carey, V.J., Bates, D.M. *et al*. (2004) Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* **5**, R80.

Giraldez, A.J., Cinalli, R.M., Glasner, M.E., Enright, A.J., Thomson, J.M., Baskerville, S., Hammond, S.M., Bartel, D.P. & Schier, A.F. (2005) MicroRNAs regulate brain morphogenesis in zebrafish. *Science* **308**, 833–838.

Git, A., Dvinge, H., Salmon-Divon, M., Osborne, M., Kutter, C., Hadfield, J., Bertone, P. & Caldas, C. (2010) Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. *RNA* **16**, 991–1006.

Greco, C.M., Hagerman, R.J., Tassone, F., Chudley, A.E., Del Bigio, M.R., Jacquemont, S., Leehey, M. & Hagerman, P.J. (2002) Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. *Brain* **125**, 1760–1771.

Hagerman, P.J. & Hagerman, R.J. (2004) The fragile-X premutation: a maturing perspective. *Am J Hum Genet* **74**, 805–816.

Hagerman, R.J., Leehey, M., Heinrichs, W., Tassone, F., Wilson, R., Hills, J., Grigsby, J., Gage, B. & Hagerman, P.J. (2001) Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. *Neurology* **57**, 127–130.

Jacquemont, S., Hagerman, R.J., Leehey, M., Grigsby, J., Zhang, L., Brunberg, J.A., Greco, C., Des Portes, V., Jardini, T., Levine, R., Berry-Kravis, E., Brown, W.T., Schaeffer, S., Kissel, J., Tassone, F. & Hagerman, P.J. (2003) Fragile X premutation tremor/ataxia syndrome: molecular, clinical, and neuroimaging correlates. *Am J Hum Genet* **72**, 869–878.

Kaplan, E.S., Cao, Z., Hulsizer, S., Tassone, F., Berman, R.F., Hagerman, P.J. & Pessah, I.N. (2012) Early mitochondrial abnormalities in hippocampal neurons cultured from Fmr1 premutation mouse model. *J Neurochem* **123**, 613–621.

Kenneson, A., Zhang, F., Hagedorn, C.H. & Warren, S.T. (2001) Reduced FMRP and increased FMR1 transcription is proportionally associated with CGG repeat number in intermediate-length and premutation carriers. *Hum Mol Genet* **10**, 1449–1454.

Klipper-Aurbach, Y., Wasserman, M., Braunspiegel-Weintrob, N., Borstein, D., Peleg, S., Assa, S., Karp, M., Benjamini, Y., Hochberg, Y. & Laron, Z. (1995) Mathematical formulae for the prediction of the residual beta cell function during the first two years of disease in children and adolescents with insulin-dependent diabetes mellitus. *Med Hypotheses* **45**, 486–490.

Li, X. & Jin, P. (2010) Roles of small regulatory RNAs in determining neuronal identity. *Nat Rev Neurosci* **11**, 329–338.

Li, Y. & Jin, P. (2012) RNA-mediated neurodegeneration in fragile X-associated tremor/ataxia syndrome. *Brain Res* **1462**, 112–117.

Liang, Y., Ridzon, D., Wong, L. & Chen, C. (2007) Characterization of microRNA expression profiles in normal human tissues. *BMC Genomics* **8**, 166.

Lin, M.T. & Beal, M.F. (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* **443**, 787–795.

Liu, C.G., Calin, G.A., Meloon, B., Gamliel, N., Sevignani, C., Ferracin, M., Dumitru, C.D., Shimizu, M., Zupo, S., Dono, M., Alder, H., Bullrich, F., Negrini, M. & Croce, C.M. (2004) An oligonucleotide microchip for genome-wide microRNA profiling in human and mouse tissues. *Proc Natl Acad Sci USA* **101**, 9740–9744.

Liu, J., Koscielska, K.A., Cao, Z., Hulsizer, S., Grace, N., Mitchell, G., Nacey, C., Githinji, J., Mcgee, J., Garcia-Arocena, D., Hagerman, R.J., Nolta, J., Pessah, I.N. & Hagerman, P.J. (2012) Signaling defects in iPSC-derived fragile X premutation neurons. *Hum Mol Genet* **21**, 3795–3805.

Livak, K.J. & Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. *Methods* **25**, 402–408.

Lohmann, C. & Wong, R.O. (2005) Regulation of dendritic growth and plasticity by local and global calcium dynamics. *Cell Calcium* **37**, 403–409.

Long, J.M. & Lahiri, D.K. (2011) MicroRNA-101 downregulates Alzheimer's amyloid-beta precursor protein levels in human cell cultures and is differentially expressed. *Biochem Biophys Res Commun* **404**, 889–895.

Marti, E., Pantano, L., Banez-Coronel, M., Llorens, F., Minones-Moyano, E., Porta, S., Sumoy, L., Ferrer, I. & Estivill, X. (2010) A myriad of miRNA variants in control and Huntington's disease brain regions detected by massively parallel sequencing. *Nucleic Acids Res* **38**, 7219–7235.

Morin, R.D., O'connor, M.D., Griffith, M., Kuchenbauer, F., Delaney, A., Prabhu, A.L., Zhao, Y., Mcdonald, H., Zeng, T., Hirst, M., Eaves, C.J. & Marra, M.A. (2008) Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells. *Genome Res* **18**, 610–621.

Napoli, E., Ross-Inta, C., Wong, S., Omanska-Klusek, A., Barrow, C., Iwahashi, C., Garcia-Arocena, D., Sakaguchi, D., Berry-Kravis, E., Hagerman, R., Hagerman, P.J. & Giulivi, C. (2011) Altered zinc transport disrupts mitochondrial protein processing/import in fragile X-associated tremor/ataxia syndrome. *Hum Mol Genet* **20**, 3079–3092.

Nelson, P.T., Wang, W.X. & Rajeev, B.W. (2008) MicroRNAs (miRNAs) in neurodegenerative diseases. *Brain Pathol* **18**, 130–138.

Pantano, L., Estivill, X. & Marti, E. (2011) A non-biased framework for the annotation and classification of the non-miRNA small RNA transcriptome. *Bioinformatics* **27**, 3202–3203.

Pantano, L., Estivill, X. & Marti, E. (2010) SeqBuster, a bioinformatic tool for the processing and analysis of small RNAs datasets, reveals

ubiquitous miRNA modifications in human embryonic cells. *Nucleic Acids Res* **38**, e34.
Pradervand, S., Weber, J., Lemoine, F., Consales, F., Paillusson, A., Dupasquier, M., Thomas, J., Richter, H., Kaessmann, H., Beaudoing, E., Hagenbuchle, O. & Harshman, K. (2010) Concordance among digital gene expression, microarrays, and qPCR when measuring differential expression of microRNAs. *Biotechniques* **48**, 219–222.
Rathore, M.G., Saumet, A., Rossi, J.F., De Bettignies, C., Tempe, D., Lecellier, C.H. & Villalba, M. (2012) The NF-kappaB member p65 controls glutamine metabolism through miR-23a. *Int J Biochem Cell Biol* **44**, 1448–1456.
Reinartz, J., Bruyns, E., Lin, J.Z., Burcham, T., Brenner, S., Bowen, B., Kramer, M. & Woychik, R. (2002) Massively parallel signature sequencing (MPSS) as a tool for in-depth quantitative gene expression profiling in all organisms. *Brief Funct Genomic Proteomic* **1**, 95–104.
Ritchie, M.E., Silver, J., Oshlack, A., Holmes, M., Diyagama, D., Holloway, A. & Smyth, G.K. (2007) A comparison of background correction methods for two-colour microarrays. *Bioinformatics* **23**, 2700–2707.
Rodriguez-Revenga, L., Madrigal, I., Pagonabarraga, J., Xuncla, M., Badenas, C., Kulisevsky, J., Gomez, B. & Mila, M. (2009) Penetrance of FMR1 premutation associated pathologies in fragile X syndrome families. *Eur J Hum Genet* **17**, 1359–1362.
Ross-Inta, C., Omanska-Klusek, A., Wong, S., Barrow, C., Garcia-Arocena, D., Iwahashi, C., Berry-Kravis, E., Hagerman, R.J., Hagerman, P.J. & Giulivi, C. (2010) Evidence of mitochondrial dysfunction in fragile X-associated tremor/ataxia syndrome. *Biochem J* **429**, 545–552.
Russell, A.P., Wada, S., Vergani, L., Hock, M.B., Lamon, S., Leger, B., Ushida, T., Cartoni, R., Wadley, G.D., Hespel, P., Kralli, A., Soraru, G., Angelini, C. & Akimoto, T. (2012) Disruption of skeletal muscle mitochondrial network genes and miRNAs in amyotrophic lateral sclerosis. *Neurobiol Dis* **49C**, 107–117.
Segal, I., Korkotian, I. & Murphy, D.D. (2000) Dendritic spine formation and pruning: common cellular mechanisms? *Trends Neurosci* **23**, 53–57.
Sellier, C., Freyermuth, F., Tabet, R., Tran, T., He, F., Ruffenach, F., Alunni, V., Moine, H., Thibault, C., Page, A., Tassone, F., Willemsen, R., Disney, M.D., Hagerman, P.J., Todd, P.K. & Charlet-Berguerand, N. (2013) Sequestration of DROSHA and DGCR8 by expanded CGG RNA repeats alters microRNA processing in fragile X-associated tremor/ataxia syndrome. *Cell Rep* **3**, 869–880.
Smyth, G.K. (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**, Article 3.
Su, Y., Ni, Z., Wang, G., Cui, J., Wei, C., Wang, J., Yang, Q., Xu, Y. & Li, F. (2012) Aberrant expression of microRNAs in gastric cancer and biological significance of miR-574-3p. *Int Immunopharmacol* **13**, 468–475.
Tan, H., Poidevin, M., Li, H., Chen, D. & Jin, P. (2012) MicroRNA-277 modulates the neurodegeneration caused by Fragile X premutation rCGG repeats. *PLoS Genet* **8**, e1002681.
Tassone, F., Hagerman, R.J., Taylor, A.K., Gane, L.W., Godfrey, T.E. & Hagerman, P.J. (2000) Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. *Am J Hum Genet* **66**, 6–15.
Tatarano, S., Chiyomaru, T., Kawakami, K., Enokida, H., Yoshino, H., Hidaka, H., Nohata, N., Yamasaki, T., Gotanda, T., Tachiwada, T., Seki, N. & Nakagawa, M. (2012) Novel oncogenic function of mesoderm development candidate 1 and its regulation by MiR-574-3p in bladder cancer cell lines. *Int J Oncol* **40**, 951–959.
Wang, W.X., Huang, Q., Hu, Y., Stromberg, A.J. & Nelson, P.T. (2011) Patterns of microRNA expression in normal and early Alzheimer's disease human temporal cortex: white matter versus gray matter. *Acta Neuropathol* **121**, 193–205.
Willemsen, R., Levenga, J. & Oostra, B.A. (2011) CGG repeat in the FMR1 gene: size matters. *Clin Genet* **80**, 214–225.
Yi, Y.H., Sun, X.S., Qin, J.M., Zhao, Q.H., Liao, W.P. & Long, Y.S. (2010) Experimental identification of microRNA targets on the 3′ untranslated region of human FMR1 gene. *J Neurosci Methods* **190**, 34–38.
Zongaro, S., Hukema, R., D'Antoni, S., Davidovic, L., Barbry, P., Catania, M.V., Willemsen, R., Mari, B. & Bardoni, B. (2013) The 3′ UTR of FMR1 mRNA is a target of miR-101, miR-129-5p and miR-221: implications for the molecular pathology of FXTAS at the synapse. *Hum Mol Genet* **22**, 1971–1982.

Acknowledgments

This work was supported by the following sources: FIS 09-00413 and PI12/00879 from Instituto de Salud Carlos III and Fondo Europeo de Desarrollo Regional (Fondos FEDER), and AGAUR (SGR1337) from Generalitat de Catalunya. The CIBER de Enfermedades Raras is an initiative of the ISCIII. We also wish to thank FSE (SFRH/BD/81271/2011). M.R.F. is supported by EMBO Long-Term fellowship ALTF 225-2011. We wish to thank the FXTAS patients and FXS families for their cooperation and Dr. Salazar and Dr. Tizzano from Hospital Sant Pau, Barcelona, Spain for their contribution in the RT-qPCR experiments. The authors declare no conflict of interest.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Table S1: Number of miRNA and IsomiRs, and the 15 top-expressed miRNAs.

Table S2: Upregulated miRNAs in FXTAS patients by Deep Sequencing technology.

Table S3: Human miRNA deregulated in FXTAS patients obtained by microarray.

Supplementary Table 1. Number of miRNA and isomiRs, and the 15 top-expressed miRNAs

	CONTROL INDIVIDUALS				FXTAS PATIENTS							
	1	2	3	4	1	2	3	4	5	6	7	8
Nº of miRNAs	880	786	903	886	840	967	765	974	860	886	856	
Nº ≠ reads	12105	12280	16956	15226	14888	20679	9893	18129	14905	14170	13479	8956
Top expressed miRNAs	let-7a-5p	let-7a-5p	let-7a-5p	let-7a-5p	let-7a-5p	let-7a-5p	let-7a-5p	let-7a-5p	let-7a-5p	let-7a-5p	let-7a-5p	let-7a-5p
	let-7f-5p	let-7f-5p	let-7f-5p	let-7f-5p	let-7f-5p	let-7f-5p	let-7f-5p	let-7f-5p	let-7f-5p	let-7f-5p	let-7f-5p	let-7f-5p
	let-7g-5p	let-7g-5p	let-7g-5p	let-7g-5p	miR-103a-3p	let-7g-5p	let-7g-5p	let-7g-5p	miR-103a-3p	let-7g-5p	let-7g-5p	miR-101-3p
	miR-103a-3p	miR-103a-3p	let-7i-5p	miR-103a-3p	miR-126-5p	let-7i-5p	miR-126-5p	miR-101-3p	miR-126-5p	miR-101-3p	miR-103a-3p	miR-126-5p
	miR-126-5p	miR-126-5p	miR-101-3p	miR-126-5p	miR-142-5p	miR-101-3p	miR-142-5p	miR-103a-3p	miR-142-5p	miR-103a-3p	miR-126-5p	miR-142-5p
	miR-142-5p	miR-142-5p	miR-103a-3p	miR-142-5p	miR-143-3p	miR-103a-3p	miR-150-5p	miR-126-5p	miR-143-3p	miR-126-5p	miR-142-5p	miR-143-3p
	miR-150-5p	miR-16-5p	miR-126-5p	miR-150-5p	miR-16-5p	miR-126-5p	miR-181a-5p	miR-142-5p	miR-150-5p	miR-142-5p	miR-150-5p	miR-181a-5p
	miR-16-5p	miR-181a-5p	miR-142-5p	miR-151a-3p	miR-181a-5p	miR-142-3p	miR-191-5p	miR-146b-5p	miR-16-5p	miR-146b-5p	miR-16-5p	miR-191-5p
	miR-181a-5p	miR-191-5p	miR-146b-5p	miR-16-5p	miR-191-5p	miR-142-5p	miR-21-5p	miR-148a-3p	miR-181a-5p	miR-181a-5p	miR-181a-5p	miR-21-5p
	miR-191-5p	miR-21-5p	miR-148a-3p	miR-181a-5p	miR-21-5p	miR-150-5p	miR-22-3p	miR-150-5p	miR-191-5p	miR-191-5p	miR-191-5p	miR-22-3p
	miR-21-5p	miR-22-3p	miR-150-5p	miR-191-5p	miR-22-3p	miR-16-5p	miR-26a-5p	miR-16-5p	miR-21-5p	miR-21-5p	miR-21-5p	miR-223-3p
	miR-22-3p	miR-223-3p	miR-151a-3p	miR-21-5p	miR-223-3p	miR-181a-5p	miR-26b-5p	miR-181a-5p	miR-22-3p	miR-22-3p	miR-22-3p	miR-26a-5p
	miR-26a-5p	miR-26a-5p	miR-16-5p	miR-22-3p	miR-26a-5p	miR-186-5p	miR-30d-5p	miR-186-5p	miR-223-3p	miR-26a-5p	miR-223-3p	miR-26b-5p
	miR-26b-5p	miR-26b-5p	miR-181a-5p	miR-223-3p	miR-26b-5p	miR-191-5p	miR-30e-5p	miR-191-5p	miR-26a-5p	miR-26b-5p	miR-26a-5p	miR-30d-5p
	miR-30e-5p	miR-486-5p	miR-186-5p	miR-26a-5p	miR-30e-5p	miR-21-5p	miR-92a-3p	miR-21-5p	miR-26b-5p	miR-30d-5p	miR-26b-5p	miR-30e-5p

Supplementary Table 2
Upregulated miRNAs in FXTAS patients by Deep Sequencing technology

miRNA	Norm freq (controls)	Norm freq (FXTAS)	FC	p-value	adj. p-value
miR-5701	28	139	5	0	0
miR-23a-5p	10	23	2,3256	0,02	0,01
miR-423-5p	217	425	1,9608	0	0
miR-29a-5p	13	25	1,9231	0,04	0,01
miR-450a-5p	21	37	1,7544	0,02	0,01
miR-3615	100	171	1,7241	0	0
miR-27a-5p	22	38	1,7241	0,02	0,01
miR-132-3p	38	61	1,6129	0,01	0
miR-574-3p	60	92	1,5385	0	0
miR-361-3p	215	324	1,5152	0	0
miR-29c-3p	792	1170	1,4706	0	0
miR-505-3p	49	72	1,4706	0,01	0
miR-181c-5p	1026	1464	1,4286	0	0
miR-29a-3p	1738	2474	1,4286	0	0
miR-660-5p	192	270	1,4085	0	0
miR-30a-5p	492	685	1,3889	0	0
miR-142-3p	4232	5803	1,3699	0	0
miR-199b-5p	104	142	1,3699	0	0
miR-625-5p	64	88	1,3699	0,01	0
miR-101-3p	8112	10957	1,3514	0	0
miR-1280	135	182	1,3514	0	0
miR-130b-3p	170	230	1,3514	0	0
miR-423-3p	2202	2932	1,3333	0	0
let-7b-5p	1961	2574	1,3158	0	0
miR-92a-3p	36627	48390	1,3158	0	0
miR-106b-3p	296	373	1,2658	0	0
miR-769-5p	504	639	1,2658	0	0
miR-339-3p	251	313	1,2500	0	0
miR-424-5p	96	119	1,2346	0,02	0,01
let-7e-5p	334	407	1,2195	0	0
miR-181a-3p	400	490	1,2195	0	0
miR-19b-3p	1546	1892	1,2195	0	0
miR-29b-3p	194	237	1,2195	0	0
miR-26b-3p	142	171	1,2048	0,01	0

Downregulated miRNA in FXTAS patients by deep sequencing technology

miRNA	Norm freq (controls)	Norm freq (FXTAS)	FC	p-value	adj. p-value
miR-103a-3p	16970	13820	-1,23	0	0
miR-148b-3p	5864	4778	-1,23	0,02	0,01
let-7g-5p	18294	14679	-1,25	0	0
miR-21-3p	2206	1704	-1,29	0	0
miR-28-3p	3770	2931	-1,29	0	0
miR-17-5p	1794	1357	-1,32	0	0
miR-22-3p	60473	45795	-1,32	0	0
miR-340-5p	5344	4005	-1,33	0	0
let-7f-5p	48624	35817	-1,36	0	0
miR-15b-5p	1130	800	-1,41	0	0
miR-16-5p	27582	19419	-1,42	0	0
miR-27b-3p	10987	7646	-1,44	0	0
miR-125a-5p	760	522	-1,46	0	0
miR-26b-5p	27520	18876	-1,46	0	0
miR-652-3p	518	352	-1,47	0	0
miR-126-5p	45424	30526	-1,49	0	0
miR-185-5p	220	146	-1,51	0,04	0,01
miR-191-5p	60975	39597	-1,54	0	0
miR-181a-2-3p	416	268	-1,55	0	0
miR-301b	176	113	-1,56	0,04	0,01
miR-144-5p	543	344	-1,58	0	0
miR-20a-5p	3586	2264	-1,58	0	0
miR-151a-5p	7070	4384	-1,61	0	0
miR-99b-5p	400	248	-1,61	0	0
miR-222-3p	1080	662	-1,63	0	0
miR-374a-5p	248	150	-1,65	0	0
miR-199a-5p	548	331	-1,66	0	0
miR-20b-5p	130	78	-1,67	0,03	0,01
miR-10a-5p	5912	3485	-1,7	0	0
let-7i-5p	9742	5659	-1,72	0	0
miR-369-3p	161	90	-1,79	0	0
miR-1260b	170	93	-1,83	0	0
miR-584-5p	1312	718	-1,83	0	0
miR-98	7956	4049	-1,96	0	0
miR-144-3p	544	276	-1,97	0	0
miR-224-5p	75	36	2,08	0,01	0
miR-486-5p	35969	16906	-2,13	0	0
miR-7-5p	45	21	-2,14	0,05	0,02
miR-126-3p	3967	1824	-2,17	0	0

miR-190a	204	93	-2,19	0	0
miR-146b-5p	8231	3658	-2,25	0	0
miR-151a-3p	8988	3996	-2,25	0	0
miR-451a	8308	3564	-2,33	0	0
miR-182-5p	1113	467	-2,38	0	0
miR-335-3p	450	179	-2,51	0	0
miR-146a-5p	5029	1795	-2,8	0	0
miR-486-3p	68	23	-2,96	0	0
miR-4446-3p	73	23	-3,17	0	0
miR-379-5p	59	15	-3,93	0	0

Supplementary Table3.Human miRNA deregulated in FXTAS patients obtained by microarray

miRNA	FC	p-value	adjusted p-value
miR-574-3p	-2,4004	2,02E-04	7,35E-03
miR-574-5p	-1,8496	4,24E-04	1,10E-02
miR-4290	-1,5314	1,50E-05	2,95E-03
miR-4254	-1,5246	4,58E-05	3,49E-03
miR-3149	-1,4552	2,04E-05	3,44E-03
miR-1281	-1,3958	4,97E-04	1,20E-02
miR-4324	-1,3909	1,44E-05	2,95E-03
miR-3935	-1,3731	9,07E-04	1,63E-02
miR-411*	-1,3339	3,37E-06	2,95E-03
miR-466	-1,3110	2,00E-04	7,35E-03
miR-1825	-1,2882	1,10E-03	1,91E-02
miR-32*	-1,2825	5,21E-05	3,58E-03
miR-483-3p	-1,2810	3,77E-04	1,03E-02
miR-485-3p	-1,2792	8,19E-04	1,60E-02
miR-595	-1,2681	9,18E-05	5,02E-03
miR-1909*	-1,2664	9,31E-05	5,02E-03
miR-34b	-1,2593	1,18E-03	1,97E-02
miR-885-5p	-1,2509	1,34E-04	6,00E-03
miR-758	-1,2310	2,86E-03	3,24E-02
miR-744*	-1,2208	1,00E-05	2,95E-03
miR-1306	-1,2153	1,92E-03	2,68E-02
miR-1910	-1,2150	1,52E-05	2,95E-03
miR-1228*	-1,2132	4,11E-05	3,46E-03
miR-449b*	-1,2082	2,21E-03	2,83E-02
miR-3613-3p	-1,2054	2,98E-04	8,92E-03
miR-342-3p	1,2892	4,64E-03	4,10E-02
miR-424	1,3055	2,25E-04	7,96E-03
miR-590-5p	1,3413	3,14E-03	3,35E-02
miR-25	1,3786	2,89E-03	3,24E-02
miR-15a	1,4059	3,76E-03	3,61E-02
miR-140-5p	1,5835	3,30E-03	3,35E-02

TRABAJO III

"INACTIVACIÓN PREFERENCIAL DEL CROMOSOMA X EN LAS MUJERES PORTADORES DE LA PREMUTACIÓN DEL GEN *FMR1* Y SU RELACIÓN CON FXTAS"

Resumen:

FXTAS es una enfermedad neurodegenerativa de aparición tardía que afecta a los portadores de la premutación del gen *FMR1*. Como en la mayoría de las enfermedades con herencia ligada al cromosoma X, en las mujeres se ha descrito un penetrancia inferior respecto a la de los varones portadores de la premutación. Este fenómeno está relacionado parcialmente con el mecanismo de compensación de dosis génica que se produce mediante la inactivación al azar en el embrión de uno de los dos cromosomas X. Sin embargo, en ocasiones se produce una inactivación preferencial de uno de los cromosomas X silenciándose el mismo en un mayor número de células. Este fenómeno ocurre en mayor frecuencia en las mujeres portadoras de alteraciones/mutaciones con herencia ligada al X, en cuyo caso suele inactivarse el cromosoma portador de la alteración, evitándose la aparición de la enfermedad.

Con el fin de determinar la implicación del mecanismo de XCI preferencial en la penetrancia del FXTAS, se ha estudiado el patrón de XCI preferencial en 31 mujeres portadoras de la premutación (10 FXTAS y 21 sin FXTAS).

Los resultados de este estudio pusieron de manifiesto un incremento significativo en la frecuencia de XCI severa en ambos grupos de portadoras de la premutación respecto el descrito en la población normal ($P=0.015$), considerada para ratios de XCI ≥90:10 o ≥10:90. Además se detectaron diferencias significativas en el patrón de XCI severa entre FXTAS y no FXTAS ($P=0.018$). Concretamente, todas las mujeres con XCI preferencial (3/10) en el grupo con FXTAS expresaban únicamente el cromosoma con la premutación. Sin embargo, todas las portadoras de la premutación asintomáticas con XCI severa (5/21) expresaban únicamente el cromosoma no expandido.

En resumen, a pesar del tamaño reducido de la muestra y teniendo en cuenta que los patrones de inactivación preferencial son tejido-dependientes, estos resultados sugieren que la inactivación preferencial del alelo expandido puedo ejercer un efecto protector en las mujeres portadoras de la premutación.

Skewed X inactivation in women carrying the *FMR1* premutation and its relation with FXTAS.

Alvarez-Mora MI[1-3], Rodriguez-Revenga L [1-3], Feliu A[1], Badenas C [1,2], Madrigal I[1-3], Milà M[1-3]

[1] Biochemistry and Molecular Genetics Department. Hospital Clinic, Barcelona, Spain

[2] CIBER of rare diseases (CIBERER), Barcelona, Spain

[3] IDIBAPS, Barcelona, Spain

Running title: Skewed X inactivation in FXTAS

Corresponding author:

Montserrat Milà Recasens

Biochemistry and Molecular Genetics Department

Hospital Clínic

C/ Villarroel, 170

08036 Barcelona, Spain

Tel. +34 93 227 54 00 Ext.2784

Fax. +34 93 451 52 72

e-mail: mmila@clinic.ub.es

Keywords: FXTAS; *FMR1* premutation; X-chromosome inactivation.

ABSTRACT

Background: Fragile-X associated Tremor/Ataxia Syndrome (FXTAS) is a late onset multisystem neurological disorder characterized by intention tremor and cerebellar ataxia. **Objective:** We hypothesized that in *FMR1* premutation females with FXTAS a normal X chromosome might more frequently be inactivated; therefore, the aim of this study was to determine the relationship between skewed X chromosome inactivation (XCI) and FXTAS disease. **Methods:** We studied the XCI patterns of 10 cases of *FMR1* premutation in women with FXTAS and 21 without FXTAS. **Results:** The distribution of XCI patterns in the FXTAS and no-FXTAS groups showed differences regarding the allele presenting severe skewed XCI. In the FXTAS group, all cases preferentially inactivated the non-expanded X chromosome whereas in the no-FXTAS group, all inactivated the expanded X chromosome. Nevertheless no significant differences were found on comparing XCI frequencies among *FMR1* premutation carriers with and without FXTAS. As expected, we found statistically significant differences in the skewed XCI on comparing *FMR1* premutation women and controls. **Conclusion:** Although the reduced sample size and blood XCI patterns are two limitations of this study, our results suggest that the skewed XCI of the normal *FMR1* allele may be a risk factor for the development of FXTAS. Furthermore, our findings also support the protective effect of the expression of a normal *FMR1* allele.

48 **INTRODUCTION**

49 Fragile-X associated Tremor/Ataxia Syndrome (FXTAS) is a late onset
50 multisystem neurodegenerative disorder characterized by intention
51 tremor and cerebellar ataxia which appear after 50-60 years of age in
52 carriers of a pathological expansion in the *FMR1* gene. The molecular
53 defect is a CGG expansion mainly in the premutation range (55-200
54 CGG), although some cases have been described in individuals
55 carrying alleles in the grey zone (45-54 CGG) or even in full mutation
56 range (>200 CGG), albeit non methylated, but always with *FMR1*
57 expression [1]. Clinical symptoms also include cerebellar brain
58 atrophy, Parkinsonism, memory loss, cognitive loss and bowel
59 incontinence, with women generally presenting milder symptoms than
60 men.

61 On the other hand, it is well known that genetic dosage compensation
62 leads to inactivation of one X-chromosome during embryonary
63 development in women. X-chromosome inactivation (XCI) is required
64 for female development and has implications for human disease. This
65 process is a complex trait determined by the combinatorial effect of
66 stochastic events and selection biases [2]. In somatic cells XCI is
67 generally random, with the maternal and paternal X having an equal
68 probability of inactivation, but in some cases there is a bias or

69 preferential inactivation (80:20 or 90:10) of one of the two X-
70 chromosomes. This phenomenon is more frequent in women carrying
71 an X-linked disease since skewed XCI generally protects females for
72 developing the disease. However, XCI can also result in increasing the
73 severity of disease symptoms, if the X-chromosome with a gene
74 mutation remains preferentially active [3]. In FXTAS, few affected
75 women preferentially present an inactivated normal X chromosome [4-
76 6].
77 In this study, we hypothesized that in *FMR1* premutation females with
78 FXTAS a normal X chromosome might more frequently be inactivated.
79 Therefore, XCI inactivation patterns were determined in 10 cases of
80 women with FXTAS and 21 without FXTAS.

81 **MATERIAL AND METHODS**

82 **Participants**

83 A total of 31 *FMR1* premutation female carriers (10 with FXTAS and
84 21 without FXTAS), clinically and radiological evaluated for FXTAS
85 were recruited for this study. No significant differences were observed
86 in age between the two groups (FXTAS 57 ± 13 years and no-FXTAS
87 52 ± 12; P=0.3). All individuals were recruited from Fragile X
88 Syndrome families and were molecularly diagnosed at the Biochemical
89 and Molecular Genetics Department of the Hospital Clinic of

90 Barcelona. All the samples analyzed had the same ethnic and racial
91 background.
92 All participants provided written informed consent for testing and for
93 the use of their phenotypic and genetic data. The Ethics Committee of
94 the Hospital Clinic of Barcelona approved the study.

95 **Determination of X-chromosome inactivation patterns**

96 Molecular analysis of the *FMR1* CGG repeats and methylation status
97 was performed by Southern blot after double-digestion with EcoRI and
98 EagI using the Stb12.3 probe as described elsewhere [7]. We also
99 analyzed the androgen receptor (AR) locus digested with the *HpaII*
100 enzyme as described previously [8]. Skewed XCI status was considered
101 with a threshold value greater than 90-10% [9].

102 **Statistical analysis**

103 Data was analyzed with the Fisher exact test using commercially
104 available software (SPSS-PC, Version 18.0; SPSS Inc, Chicago, IL,
105 USA) and significance was accepted with a P-value <0.05.

106 **RESULTS AND DISCUSSION**

107 The results of the present study are summarized in Table 1. Regarding
108 which allele was inactivated we found statistically significant
109 differences between women with and without FXTAS (P=0.018). In the
110 FXTAS group, all cases with skewed XCI showed the non-expanded X

111 chromosome preferentially inactivated, whereas in all of the women in
112 the no-FXTAS group the expanded X chromosome was inactivated.
113 However, comparison between *FMR1* premutation carriers with and
114 without FXTAS did not reveal significant differences in the frequency
115 of skewed XCI (P=0.517), with both groups exceeding 20% of skewed
116 XCI. On the other hand, statistical analysis showed significant
117 differences on comparing *FMR1* premutation carriers and controls
118 (P=0.015).
119 Skewed XCI has been suggested to be a risk factor for the development
120 of FXTAS (4-6). In agreement with this hypothesis, we found
121 significant differences as to which allele underwent XCI. Preferential
122 inactivation of the normal X chromosome was observed in FXTAS
123 patients whereas the expanded X chromosome was inactivated in all the
124 patients in the no-FXTAS group. In addition, none of the women
125 predominantly expressing the normal *FMR1* allele developed FXTAS.
126 Furthermore, this study detected similar skewed XCI frequencies in
127 both groups. Furthermore in accordance with the higher frequency of
128 XCI in X-linked diseases, a statistically significant increase (P=0.015)
129 was also observed in the frequency of skewed XCI on comparing the
130 *FMR1* premutation groups (~25%) with the general population which is
131 estimated to be around 9% [7]. In a previous report, the percentage of

132 adult population with an XCI ratio of 90:10 was found to be 3.6%. [9].
133 These differences might be a consequence of sample age, since our
134 previous study was based on females over the age of 40 years [7].
135 Supporting these results, Liu and collaborators (2012) generated iPSCs-
136 derived neuronal cells of a *FMR1* premutation female carrier in which
137 each line exclusively expressed either the normal or the premutation
138 *FMR1* allele. These authors demonstrated that neurons harboring the
139 expanded allele presented synaptic alterations as well as function
140 abnormalities compared to neurons with a normal X allele [10].
141 Although the reduced sample size and blood XCI patterns are two
142 limitations of this study, our results suggest that the skewed XCI of the
143 normal *FMR1* allele may be a risk factor for the development of
144 FXTAS. Furthermore, our findings also support the protective effect of
145 the expression of a normal *FMR1* allele.
146

147 **ACKNOWLEDGMENTS**

148 This work was supported by the Instituto de Salud Carlos III (ISCIII)
149 [PI12/00879], co-financed by Fondo Europeo de Desarrollo Regional
150 (FEDER) "una manera de hacer Europa" and AGAUR from the
151 Autonomous Catalan Government (2014 SGR603). The CIBER de
152 Enfermedades Raras and is an initiative of the ISCIII.

153

154 **REFERENCES**

155 1. Hagerman PJ, Hagerman RJ (2015). Fragile X-associated

156 tremor/ataxia syndrome. Ann N Y Acad Sci *1338*: 58-70. Review.

157 2. Bolduc V, Chagnon P, Provost S, Dubé MP, Belisle C, Gingras

158 M, Mollica L , Busque L (2008). No evidence that skewing of X

159 chromosome inactivation patterns is transmitted to offspring in humans.

160 J Clin Invest *118*: 333-341.

161 3. Valencia K , Wutz A (2015). Recent insights into the regulation

162 of X-chromosome inactivation. Advances in genom and Genet *5*: 227-

163 238

164 4. Berry-Kravis E, Potanos K, Weinberg D, Zhou L , Goetz CG

165 (2005). Fragile X-associated tremor/ataxia syndrome in sisters related

166 to X-inactivation. Ann Neurol *57*:144-147

167 5. Al-Hinti JT, Nagan N , Harik SI (2007). Fragile X premutation

168 in a woman with cognitive impairment, tremor, and history of

169 premature ovarian failure. Alzheimer Dis Assoc Disord *21*: 262-264.

170 6. Rodriguez-Revenga L, Pagonabarraga J, Gómez-Anson B,

171 López-Mourelo O, Madrigal I, Xunclà M, Kulisevsky J , Milà M

172 (2010). Motor and mental dysfunction in mother-daughter transmitted

173 FXTAS. Neurology *75*, 1370-1376

174 7. Rodriguez-Revenga L, Madrigal I, Badenas C, Xunclà M,

175 Jiménez L, Milà M (2009). Premature ovarian failure and fragile X
176 female premutation carriers: no evidence for a skewed X-chromosome
177 inactivation pattern. Menopause *16*; 944-949.
178 8. Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont
179 JW (1992) Methylation of HpaII and HhaI sites near the polymorphic
180 CAG repeat in the human androgen-receptor gene correlates with X
181 chromosome inactivation. Am J Hum Genet *51*, 1229-1239
182 9. Amos-Landgraf JM, Cottle A, Plenge RM, Friez M, Schwartz
183 CE, Longshore J , Willard HF (2006). X chromosome-inactivation
184 patterns of 1,005 phenotypically unaffected females. Am J Hum Genet
185 *79*, 493-499.
186 10. Liu J, Koscielska KA, Cao Z, Hulsizer S, Grace N, Mitchell G,
187 Nacey C, Githinji J, McGee J, Garcia-Arocena D, Hagerman RJ, Nolta
188 J, Pessah IN , Hagerman PJ (2012) Signaling defects in iPSC-derived
189 fragile X premutation neurons. Hum Mol Genet *21*, 3795-3805.

Table 1. Comparison of X-chromosome inactivation ratios in the different groups of *FMR1* premutation carriers and controls.

	Total	Random XCI	Skewed XCI	Normal X Inactivated‡	Expanded X Inactivated‡
FMR1 premutation carriers with FXTAS	10	70% (7/10)	30% (3/10)	100%(3/3)	0%
FMR1 premutation carriers without FXTAS	21	76.19% (16/21)	23.81% (5/21)	0%	100% (5/5)

FMR1 premutation carriers	31	74.19% (23/31)	25.81% (8/31)*
CONTROLS[∞]	220	90.45% (199/220)	9.55% (21/220)

[*] Statistically significant ($P<0.05$ by Fisher Exact Test) compared to controls.

[‡] Statistically significant ($P<0.05$ by Fisher Exact Test) between *FMR1* premutation carriers with and without FXTAS.

[∞] Data from Rodriguez-Revenga *et al*., 2009 [7].

TRABAJO IV (Manuscrito en preparación)

"CARACTERIZACIÓN DEL ESTADO DE LA MITOCONDRIA EN FXTAS: ESTUDIO FUNCIONAL Y ANÁLISIS DE LA DINÁMICA MITOCONDRIAL"

Resumen:

En el sistema nervioso las mitocondrias son esenciales para la producción de energía, la regulación del calcio, el mantenimiento del potencial de membrana, el plegamiento de las proteínas mediante las chaperones, el transporte axonal y dendrítico, y la liberación y recaptación de los neurotransmisores en las sinapsis. En la mayoría de enfermedades neurodegenerativas la disfunción mitocondrial y el estrés oxidativo se dan de forma temprana y existen fuertes evidencias que sugieren que esta desregulación tiene un papel causal en la patogénesis de las enfermedades.

Las mitocondrias forman una red interconectada y altamente dinámica, cuya estructura y biogénesis se encuentra sujeta a las necesidades de la célula. Las mitocondrias son capaces de variar su morfología desde redes elongadas e interconectadas hasta compartimentos fragmentados y desconectados mediante una estricta regulación permanente y balanceada de los procesos de fusión y fisión, englobados bajo el término "dinámica mitocondrial". Recientemente, alteraciones en la

dinámica mitocondrial se ha realizado con diversas enfermedades neurodegenerativas como el Parkinson o el Alzheimer. Existen varias evidencias que indican que la disfunción mitocondrial contribuye a la patogénesis del FXTAS, sin embargo los procesos de dinámica mitocondrial han sido escasamente estudiados.

En este trabajo se realizó el estudio funcional de la capacidad oxidativa de las mitocondrias, la determinación del contenido mitocondrial, la cuantificación del estrés oxidativo y el análisis de la morfología de la red mitocondrial.

En primer lugar, los resultados de este estudio demostraron una disminución significativa en la capacidad oxidativa de los fibroblastos de pacientes con FXTAS mientras que la valoración del consumo de O_2 en sangre no detectó diferencias significativas entre grupos. Los cambios observados durante la respiración celular en fibroblastos fueron específicos de la cadena respiratoria mitocondrial y no pueden atribuirse a un menor número de mitocondrias ya que el análisis de la actividad de la enzima citrato sintasa, parámetro ampliamente utilizado como indicador del contenido mitocondrial, mostró niveles similares entre pacientes y controles en ambos tejidos.

En segundo lugar, se identificó un incremento en los niveles de estrés oxidativo mediante la cuantificación de lípidos peroxidados en sangre

periférica y en cultivo de fibroblastos, aunque este incremento no fue estadísticamente significativo.

Por último, las imágenes obtenidas mediante microscopia confocal pusieron de manifiesto que los fibroblastos procedentes de pacientes FXTAS presentan una red mitocondrial significativamente más circular y menos interconectada que en el grupo control.

La función y la dinámica mitocondrial son dos eventos que se encuentran estrictamente regulados para satisfacer temporal y espacialmente las necesidades bioenergéticas de cualquier tipo celular y especialmente en las neuronas ya que dependen casi exclusivamente de la fosforilación oxidativa como fuente de energía. En este trabajo se ha puesto de manifiesto la alteración de la función y de la dinámica mitocondrial, por lo que ambas podrían estar implicadas en la fisiopatología del FXTAS y podrían contribuir en la aparición de los síntomas neurológicos.

Characterization of mitochondria state in FXTAS: Functional study and mitochondrial dynamics analysis

Alvarez-Mora MI[1-3], Rodriguez-Revenga L[1-3], Madrigal I[1-3], Guitart-Mampel M[3,4], Garrabou G[2-4], Milà M[1-3]

[1] Biochemistry and Molecular Genetics Department. Hospital Clinic, Barcelona, Spain

[2] CIBER of rare diseases (CIBERER), Barcelona, Spain

[3] IDIBAPS, Barcelona, Spain

4 Muscle Research and Mitochondrial Function Laboratory, CELLEX, Faculty of Medicine-University of Barcelona, Internal Medicine Department, Hospital Clinic, Barcelona, Spain

Corresponding author:
Montserrat Milà Recasens
Biochemistry and Molecular Genetics Department
Hospital Clínic
C/ Villarroel, 170
08036 Barcelona, Spain
Tel. +34 93 227 54 00 Ext.2784
Fax. +34 93 451 52 72
e-mail: mmila@clinic.ub.es

ABSTRACT

Neurodegenerative diseases are a heterogeneous group of disorders characterized by gradually progressive, selective loss of anatomically or physiologically related neuronal systems, in which mitochondrial involvement is likely to be an important common theme. FXTAS is a late-onset neurodegenerative disorder that affects *FMR1* premutation (55-200CGG repeats) alleles carriers. In an attempt to provide new insights onto the mechanisms involved in FXTAS pathogenesis, in this study we report the characterization of mitochondrial function and dynamics by the assessment of oxidative respiratory chain function, mitochondrial content, oxidative stress levels and mitochondrial network complexity. Results from mitochondrial function revealed that mitochondrial respiratory capacity is compromised in skin fibroblasts whereas in blood no major differences were observed between FXTAS and control groups. Fibroblasts from FXTAS patients showed a significant decrease in the basal mitochondrial respiration and a reduction of oxidative activities related to the stimulation of mitochondrial respiratory chain complexes I and II. This alteration was not attributable to lower mitochondria content as confirmed by the analysis of CS activity in both tissues. Furthermore, ROS production was enhanced in FXTAS patients. Finally, fibroblasts from FXTAS patients presented more circular and less interconnected mitochondria. Mitochondrial function and dynamics are two events that must be tightly regulated in order to temporally and spatially satisfy the bioenergetic needs of any cell type, especially neurons. Herein, we demonstrated that mitochondrial respiration as well as architecture is altered in skin fibroblasts from FXTAS patients, indicating that both are implicated in the pathophysiology and/or aetiology of neurological symptoms.

Keywords: FXTAS; oxygen uptake; ROS production; mitochondrial dynamics; mitochondrial dysfunction

INTRODUCTION

Fragile X-associated tremor/ataxia syndrome (FXTAS, OMIM #300623) is a late-onset neurodegenerative disorder that appears in at least one-third of adult carriers of a premutation (55-200 CGG repeats) in the fragile X mental retardation 1 gene (*FMR1*) (reviewed in Hagerman & Hagerman 2004). It has been shown that FXTAS penetrance depends on age and sex, exceeding 50% for men aged 70-90 years, and that there is significant variability in the progression of neurological dysfunction (Jacquemont et al. 2004; Rodriguez-Revenga et al. 2009). FXTAS is characterized by intention tremor, cerebellar gait ataxia, and parkinsonism, as well as brain atrophy and often middle cerebellar peduncle hyperintensities on magnetic resonance imaging (MRI) scans (Hagerman et al. 2001; Jacquemont et al. 2003). The neuropathological hallmark of FXTAS is the presence of ubiquitin-positive eosinophilic intranuclear inclusions in neurons and astrocytes throughout the brain (Greco et al. 2002; Greco et al. 2006).

Neurodegenerative diseases are a heterogeneous group of disorders characterized by gradually progressive, selective loss of anatomically or physiologically related neuronal systems. Despite this heterogeneity, mitochondrial involvement is likely to be an important common theme in these diseases (Lin & Beal 2006). Mitochondria are key regulators of

cell survival and death that have a central role in ageing. Mutations in genes involved in mitochondrial quality control and dynamics are known to cause genetic forms of neurodegenerative diseases (Rugarli & Langer 2012). There are several evidences supporting a role for mitochondrial dysfunction in the FXTAS pathogenesis (Ross-Inta et al. 2010; Napoli et al. 2011; Kaplan et al. 2012; Cao et al. 2013; Hukema et al. 2014). First of all, cultured neurons carrying the *FMR1* premutation exhibit significant oxidative stress and mitochondrial dysfunction (Kaplan et al. 2012; Cao et al. 2013). Mitochondrial dysfunction is more severe in those patients with FXTAS compared to carriers without FXTAS, and there is documented iron dysregulation at the mitochondria in Patients with FXTAS (Ross-Inta et al. 2010; Ariza et al. 2015). Secondly, deregulation of the oxidative phosphorylation (OXPHOS) pathway has been described in blood profiling from patients with FXTAS (Alvarez-Mora et al. 2013; Mateu-Huertas et al. 2014). In an attempt to provide new insights onto the mechanisms involved in FXTAS pathogenesis, in this study we report the characterization of mitochondrial function by the assessment of oxidative respiratory chain function, mitochondrial content, oxidative stress levels and mitochondrial dynamics in patients with FXTAS and control individuals.

MATERIAL AND METHODS

Subjects

Patients with FXTAS and control subjects with normal CGG repeat size (≤40CGGs) were recruited for this study. All patients with FXTAS belong to Fragile X syndrome families and were molecularly diagnosed at the Biochemical and Molecular Genetics Department of the Hospital Clinic of Barcelona, Spain and clinically evaluated at the Neurology Service of the Hospital Sant Pau and Santa Creu of Barcelona. FXTAS group encompasses patients who met criteria in any of the three categories of involvement: definite, probable, and possible (Jacquemont et al. 2003).

All participants provided written informed consent for testing and for the use of their phenotypic/clinical and genetic data. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona.

Blood cells isolation

Peripheral blood mononuclear cells (PBMC) were obtained by a Ficoll density gradient centrifugation procedure as described elsewhere (Prilutskiĭ et al. 1990). PBMCs were further divided and stored into aliquots at -80ºC until analysis.

Fibroblasts culture

Skin biopsies were obtained using a 3-mm punch from 3 patients with FXTAS and 3 control individuals recruited from the Dermatology Department of the Hospital Clínic of Barcelona. The biopsy was diced under sterile conditions and then plated in T25 flasks in Minimum Essential Media (MEM) 13% containing 500 ml MEM (Gibco®) and 75 ml Newborn Calf Serum (Gibco®) supplement with 0,30 ml Penicillin (Gibco®) and 0,30 Streptomycin (Gibco®) at 37ºC with a 5% CO_2 atmosphere.

Mitochondrial respiratory function in PMBCs

Global and specific MRC Complex I stimulated oxygen consumption was measured by polarography at 37°C in fresh PMBCs from 6 patients with FXTAS and 6 control individuals using a Clark oxygen electrode (Hansatech Instruments Limited, UK) as previously described (Barrientos 2002). Manual titration of inhibitors and uncouplers was performed using Hamilton syringes (Hamilton Company, Reno, NV, USA). Data recording was performed using the O_2view software version 1.0.0.1. (Hansatech Instruments Limited, UK). The oxygen uptake rates of mitochondria were evaluated in PBMCs as described previously (Moren et al. 2013). Results were expressed as nanomoles of oxygen per minute and milligram of protein (nmol of O_2/minute*mg protein).

Mitochondrial respiratory function in fibroblasts

To determine oxygen consumption of skin fibroblasts from FXTAS and control individuals, high-resolution respirometry was performed at 37°C by polarographic oxygen sensors in a two-chamber Oxygraph-2k system according to manufacturer's instructions (OROBOROS Instruments, Innsbruck, Austria). Manual titration of inhibitors and uncouplers was performed using Hamilton syringes (Hamilton Company, Reno, NV, USA). Data recording was performed using the DatLab software v5.1.1.9 (Oroboros Instruments, Austria). The oxygen uptake rates of mitochondria were evaluated in fibroblasts as described previously (Pesta & Gnaiger 2012). Briefly, a million of living fibroblast cells were obtained from control and FXTAS patients and resuspended in cold respiration media medium (0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose and 0.1% (w/v) bovine serum albumin, pH 7.1). Cell suspension was added to a 2 ml chamber to assess oxygen flux by measuring Routine respiration (endogenous substrate usage). Afterwards, cells were permeabilized by digitonine (0.004 % (w/v)) and Leak respiration was measured by adding glutamate (final concentration 10mM) and malate (2 mM), in the absence of ADP. Oxidative phosphorylation due to complex I substrates was quantified

by the addition of ADP (5 mM, GMox), followed by addition of pyruvate (5mM, GMPox), and to measure convergent electron flow through both complex I and II, succinate (10 mM, Sox) was added. Subsequently, carbonyl cyanide m-chlorophenylhydrazone (CCCP) was titrated to achieve maximum flux through the electron transfer system (0.5 μM). Finally, respiration was inhibited by the sequential addition of rotenone (1 μM), malonate (5mM) and antimycin A (2.5 μM), respectively. Specific oxygen uptakes rates were obtaining by subtracting the rates from antimicine as is considered unspecific oxygen consumption following manufacture's recommendations. Oxygen consumption were normalized for the number of cells, thus, results are expressed as picomoles of oxygen per millilitre (pmol O_2/s*mL).

Mitochondrial content

Citrate synthase (CS) activity was determined as it is considered a reliable marker of mitochondrial content (reviewed in Rustin, 1994). The assay was performed at 412 nm following the reduction in 5mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) in the presence of 10μL of cell suspension and 10mM acetyl-CoA in a medium with 1M Tris/HCl (pH 8.1) and 10% Triton X-100. The reaction was started by adding 50μL of 10mM oxaloacetic acid. The absorbance changes were

followed in a HITACHI U2900 spectrophotometer using the UV-Solution software version 2.2. Data points were measured every 15 seconds during 4 minutes. Rates were calculated from the linear part of ΔAbs/min against mg of protein plots and using a molar absorption coefficient of 3676.5M−1·cm−1. Citrate synthase activity was expressed as nanomoles of product per minute and milligram of protein (nmol/minute·mg protein).

Measurement of reactive oxygen species (ROS) production

Lipid peroxidation was measured as an indicator of oxidative damage of ROS in cellular lipid compounds in PMBCs from 6 patients with FXTAS and 6 controls as well as in skin fibroblasts using the BIOXYTECH® LPO-586™ by the spectrophotometric measurement of malondialdehyde (MDA) and 4-hydroxyalkenal (HAE) levels, according to the manufacturer's instruction (Oxis International Inc., CA, USA). Fatty acid peroxide decomposition products were considered as indicators of ROS production. Results were normalized by protein content and expressed as micromolar of MDA and HAE per milligram of protein (µM MDA+HAE/mg protein).

Mitochondrial network complexity analysis in cultured skin fibroblasts

Cultured skin fibroblasts were washed with PBS before fixation with 4% paraformaldehyde for 15 minutes. Fixed cells were washed, permeabilized with 0.1% Triton X-100 incubated in blocking solution (1% Bovine serum albumin). Immunochemistry for the mitochondrial network complexity was performed using three different labelling. Firstly, cells were incubated during 1hour at 37ºC with the TOM20 rabbit polyclonal primary antibody (Santa Cruz Biotechnology, Ref. FL-145) diluted 1:100 that specifically binds to outer mitochondrial membrane. Secondly, cells were simultaneously incubated during 45 minutes at 37ºC with Alexa Fluor 488 donkey anti-rabbit secondary antibody (Life Technologies, Ref. A21206) diluted 1:400 and with the Wheat Germ Aglutinin (WGA) Alexa Fluor 594 conjugate probe (Life Technologies, Ref. W11262) diluted 1:200 to stain plasmatic membrane. Finally, cells were incubated 5 minutes at room temperature with the TOPRO3 IODIDE probe (Life Technologies, Ref. T3605) to stain cell nuclei. After mounting of the coverslips onto microscopy slides with ProLong Gold (Life Technologies, Ref. P36930), samples were analyzed by confocal microscopy. Images were acquired using a Leica TCS SL laser scanning confocal spectral microscope (Leica Microsystems Heidelberg GmbH, Manheim, Germany) with Argon and HeNe lasers attached to a Leica DMIRE2 inverted microscope.

Different combinations of fluorochromes were used for labelling and emissions were acquired sequentially as follows: (i) Alexa Fluor 488: Excitation at 488 nm, triple dichroic beam-splitter (TD 488/543/633) and emission detection ranges: 500- 535 nm; (ii) Alexa Fluor 594: Excitation at 543 nm, triple dichroic beam-splitter (TD 488/543/633) and emission detection ranges: 555- 700 nm; (iii) TOPRO3: Excitation at 633 nm, triple dichroic beam-splitter (TD 488/543/633) and emission detection ranges: 640- 785 nm. All images were obtained using 63x PL APO oil immersion objective lens (NA 1.32) equipped with phase contrast optics, confocal pinhole set at 1 Airy unit, 1024x1024 pixels, and electronic zoom (2.5). A minimum of 10 fibroblasts from each individual were visualized.

A macro of instructions was written to be executed in the open source software ImageJ (www.fiji.sc) (Wayne Rasband, NH, USA), to perform semi-automatic quantitation. Briefly, WGA labelling was used to evaluate whole cell area and Tom20 to label the mitochondrial network. WGA labelled cell was median filtered (radius 2), intensity thresholded and segmented to measure whole cell area. Mitochondria image was background subtracted, Gaussian blur filtered (radius 0.8), local thresholded and finally segmented. Mitochondria network of each cell was then subjected to particle analysis and the following parameters

were measured: aspect ratio (AR=major axis/minor axis) and circularity (4π·area/ perimeter2). Form factor (FF) was calculated as the inverse of circularity. An AR value of 1indicates a perfect circle, and as mitochondria elongates and become more elliptical AR increases. A FF value of 1 corresponds to a circular unbranched mitochondrion, while higher FF values indicate a longer more branched mitochondrion.

Statistical analysis

Results were expressed as mean ± standard error means (SEM). Statistical analysis were performed using the non-parametric Kruskall-Wallis test and the Mann–Whitney U test, when required, using commercially available software (SPSS-PC, Version 18.0; SPSS Inc, Chicago, IL, USA). Significance was accepted for asymptotic bilateral p-values below 0.05.

RESULTS

All subjects included in this study were unrelated *FMR1* premutation carriers ascertained through fragile X syndrome families that presented with FXTAS symptoms, including tremor and/or ataxia and controls in a similar age group range. Table 1 summarizes clinical and molecular data of individuals recruited in this study.

In order to assess mitochondrial function, mitochondria respiration was determined in PBMCs and skin fibroblasts from controls and patients with FXTAS. While results obtained from PBMCs did not showed statistically significant differences between groups, data from fibroblasts revealed a significant decline in the OXPHOS capacity in patients with FXTAS (Table 2). In particular, the basal oxygen consumption of all complexes in intact cells were statistically significant reduced in patients with FXTAS relative to controls when using endogenous substrates (P=0.05). Furthermore, the oxidative capacities under stimulation of mitochondrial complex I with NAD-linked substrates (GMPox, glutame/malalte/piruvate oxidation) as well as under the combined stimulation of mitochondrial complexes I and II by the addition of the FAD-linked substrate succinate (GMPSox, glutame/malalte/piruvate/succinate oxidation) were also statistically significant reduced in patients with FXTAS compared with controls

(P=0.05). Finally, the inhibition of the complex I with rotenone, indicative of complex II stimulated oxygen consumption, showed a significant reduction in the oxygen uptake in patients with FXTAS compared to controls (P=0.05) whereas the residual oxygen consumption determined by the inhibition of both complex I and II showed similar rates in both groups (Table 2).

The activity of CS enzyme was determined in PMBCs and in cultured skin fibroblasts. Results showed that patients with FXTAS have similar mitochondrial content relative to controls in both tissues (Supplementary Table 1); thus, the reduction in the OXPHOS capacity detected in skin fibroblasts could not be explained by lower mitochondria content.

Oxidative stress measured by the rate of lipid peroxidation did not show statistically significant differences between patients and controls. However, ROS production was 20% enhanced in FXTAS PMBCs and 30% in cultured skin fibroblasts from patients with FXTAS with respect to the control group (Figure 1).

Finally, mitochondrial network complexity was assessed using confocal microscopy in cultured skin fibroblasts. Statistical analysis revealed that average circularity was significantly higher in patients with FXTAS (P=0.05), indicating that mitochondria in these individuals are

less elongated than those of controls (Figure 2A). Furthermore, AR and FF parameters associated with mitochondria length and branching, respectively, were decreased in fibroblasts from patients with FXTAS when compared to controls (Figure 2B and 2C). The positive significant correlation between mitochondria AR and FF detected in control fibroblasts, indicative of mitochondria health status, was lost in FXTAS patients (Figure 2D).

DISCUSSION

In this study, we assessed mitochondrial function and dynamics in FXTAS patients and controls. Our results showed that mitochondrial respiratory capacity and mitochondrial dynamics are compromised in skin fibroblasts whereas in blood no major differences were observed between groups. Since blood is not a highly energy-dependent tissue it may be argued that this cell type is not suitable for assessing metabolic mitochondrial function. Additionally, dermal tissue and neural system share common embryonic origin (ectoderm), which may explain enhanced mitochondrial impairment in skin fibroblasts.

In cultured skin fibroblasts from patients with FXTAS, mitochondrial function characterization evidenced a significant decrease in the basal mitochondrial respiration and a reduction of oxidative activities related to the stimulation of mitochondrial complexes I and II (Table 2). These changes were not attributable to a lower density of mitochondria per cell as confirmed by the analysis of CS activity (Suppl. Table1). Our results are in agreement with those previously described, in which a lower OXPHOS capacity was reported in cultured skin fibroblasts from FXTAS patients (Ross-Inta et al., 2010; Napoli et al., 2011). Moreover, we demonstrated a reduced capacity of basal mitochondrial respiration and no significant alterations in residual oxygen consumption (Table

2). On the basis of these results, we can conclude that the changes observed in mitochondrial oxygen uptake in cultured skin fibroblasts from FXTAS patients are specifically mitochondrial and not due to alteration of other cellular oxygen consuming processes such as catalase or autophagy activities. Collectively, our data point to a compromised bioenergetic capacity in skin fibroblasts of FXTAS patients. This, in turn, might lead to a lack of energy in times of higher demand as for example under stress conditions, especially in tissues highly dependent of oxidative metabolism as neurons (Hall et al. 2012). Inefficiencies of OXPHOS capacity enhance ROS generation, which are a collection of highly unstable molecules that attack cellular structures and cause oxidative stress damage, triggering apoptotic mechanisms (Federico et al. 2012). The measurement of ROS production by rates of lipid peroxidation in PBMCs and skin fibroblasts evidenced oxidative stress in patients with FXTAS, albeit it did not reach statistically significance in none of the analyzed tissues (Figure 1). In consonance, previous studies have described an enhanced ROS production in skin fibroblasts from patients with FXTAS by measuring other oxidative stress markers (Ross-Inta et al., 2010; Napoli et al., 2011). The imbalance of net ROS production can cause oxidative damage which is known to have a major role in the cognitive decline

related to normal aging as well as in other neurodegenerative diseases (reviewed in Bhat et al. 2015).

Mitochondria are highly dynamic organelles able to form interconnected networks whose biogenesis and structure are highly influenced by the needs of the cell. In this sense mitochondria are tightly regulated to continuously adapt shape to functional and anatomical requirements ranging from small fragmented compartments to complex filamentous networks in large part by the conserved activities of mitochondria fission, fussion, motility and tethering (reviewed in Lackner 2014). Through these mechanisms, mitochondria continuously merge and divide to share solutes, metabolites, and proteins as a strategy to promote mitochondrial removal and turn-over. Understanding how mitochondrial dynamics is linked to cellular pathophysiology is currently the subject of intense study in a wide range of disorders (Nikolaisen et al. 2014). Impairments in the regulation and function of mitochondria have been associated with aging and disease, including metabolic disorders, cancer and neurodegeneration. Mitochondrial dynamics in FXTAS has been poorly investigated. To our knowledge only Kaplan and collaborators (2012) have described a deficit in the dynamics of mitochondrial movement in hippocampal neurons from a mouse model of FXTAS. These deficits

were found in early development, suggesting that they might contribute as a risk factor for developing clinical FXTAS. Our study showed that skin fibroblasts from patients with FXTAS present an alteration of mitochondrial network, with more circular and thus less elongated mitochondria, suggestive of reduced fusion processes and defective mitochondrial communication. Moreover, the lost of positive correlation between mitochondria elongation and branching in FXTAS evidenced less interconnected mitochondria (Figure 2). Therefore, on the basis of our results, we can conclude that alterations in the dynamic mitochondrial network are involved in the pathophysiology of FXTAS.

We are aware of the limitations of this study. Firstly, the limited sample size which might explain lack of significance in some of the experiments. However, FXTAS is a rare disease with a very low prevalence in the general population. Secondly, skin fibroblasts might not constitute an appropriate model system due to clonal selection and drift in culture, so it is likely that those cells with lower viability are less represented. On the other hand, skin fibroblasts form dynamic cell-cell contacts, similar to neurons and also reflect age-dependent cumulative cell damage. Regarding FXTAS, it has been demonstrated that fibroblast exhibit overexpression of *FMR1* transcript mimicking the levels observed in neurons (Napoli et al., 2011).

Mitochondrial function and dynamics are two events that must be tightly regulated in order to temporally and spatially satisfy the bioenergetic needs of any cell type, especially neurons. Herein, we demonstrated that mitochondrial respiration as well as architecture is altered in skin fibroblasts from FXTAS patients, indicating that both are implicated in the pathophysiology and/or aetiology of neurological symptoms.

Table1. Characteristics of individuals recruited in this study

		CONTROLS	FXTAS
PBMCs	AGE (years)‡	61.17 ± 1.96	67.83 ± 5.62
	Simple size (n)	6	6
	N females	3	3
	N males	3	3
	(CGG)n ‡	≤35	109.5 ± 12.96
Fibroblasts	AGE (years)	75 ± 9.07	60.67 ± 5.84
	Simple size (n)	3	3
	N females	1	2
	N males	2	1
	(CGG)n ‡	≤30	103 ± 16.64

‡ Results are expressed as mean values ± standard error mean

Table 2. Rates of oxygen uptake in skin fibroblasts. Results are expressed as mean values ± standard error mean.

	Oxygen consumption ($pmolO_2/s*ml$)				
	Routine Repiration	**Estimulated by complex I**	**Estimulated by complex I+II**	**Inhibiting complex I**	**Inhibiting complex I+II**
Control	43.01±5	22.45±6.2	34.42±9.2	25.39±4.6	3.48±1.7
FXTAS	13.98±6	2.81±1.0	3.43±3.3	5.39±5.1	2.80±1.4
p-valor*	**P=0.05**	**P=0.05**	**P=0.05**	**P=0.05**	-

* Significance was accepted for asymptotic bilateral p-values ≤0.05

Supplemententary Table 1. Determination of mitochondria number by means of Citrate Synthase (CS) activity in PMBCs and fibroblasts from FXTAS patients and control individuals. Results are expressed mean values ± SEM.

		Sample size (n)	**CS activity (nmol/min*mg proteins)**
PBMCs	**Control**	6	93.93 ± 15.26
	FXTAS	6	108.03 ±16.29
Skin fibroblasts	**Control**	3	67.07±6.98
	FXTAS	3	60.59±13.78

FIGURE LEGENDS

Figure 1. Quantification of ROS production in patients with FXTAS and control individuals from: **A)** PBMCs and **B)** Skin fibroblasts.

Figure 2. Mitochondrial Network Complexity in skin fibroblasts from patients with FXTAS and control individuals.

REFERENCES

Alvarez-Mora, M.I. et al., 2013. MicroRNA expression profiling in blood from fragile X-associated tremor/ataxia syndrome patients. *Genes, Brain and Behavior*, 12(6), pp.595–603.

Ariza, J. et al., 2015. Dysregulated iron metabolism in the choroid plexus in fragile X-associated tremor/ataxia syndrome. *Brain Research*, 1598, pp.88–96.

Barrientos, A., 2002. In vivo and in organello assessment of OXPHOS activities. *Methods*, 26(4), pp.307–316.

Bhat, A.H. et al., 2015. Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight. *Biomedicine & pharmacotherapy = Biomédecine & pharmacothérapie*, 74, pp.101–10.

Cao, Z. et al., 2013. Enhanced asynchronous Ca2+ oscillations associated with impaired glutamate transport in cortical astrocytes expressing Fmr1 gene premutation expansion. *Journal of Biological Chemistry*, 288(19), pp.13831–13841.

Federico, A. et al., 2012. Mitochondria, oxidative stress and neurodegeneration. *Journal of the Neurological Sciences*, 322(1-2), pp.254–262.

Greco, C.M. et al., 2002. *Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers.*,

Greco, C.M. et al., 2006. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). *Brain*, 129(1), pp.243–255.

Hagerman, P.J. & Hagerman, R.J., 2004. The fragile-X premutation: a maturing perspective. *American journal of human genetics*, 74(5), pp.805–816.

Hagerman, R.J. et al., 2001. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. *Neurology*, 57(1), pp.127–130.

Hukema, R.K. et al., 2014. Induced expression of expanded CGG RNA causes mitochondrial dysfunction in vivo. *Cell Cycle*, 13(16), pp.2600–2608.

Hall, C.N. et al., 2012. Oxidative phosphorylation, not glycolysis, powers presynaptic and postsynaptic mechanisms underlying brain information processing. *The Journal of Neuroscience*, 32(26),pp.8940-8951.

Jacquemont, S. et al., 2003. Fragile X premutation tremor/ataxia syndrome: molecular, clinical, and neuroimaging correlates. *American journal of human genetics*, 72(4), pp.869–878.

Jacquemont, S. et al., 2004. Penetrance of the Fragile X – Associated Tremor / Ataxia Syndrome in a. *Jama*, 291(4), p.10.

Kaplan, E.S. et al., 2012. Early mitochondrial abnormalities in hippocampal neurons cultured from Fmr1 pre-mutation mouse model. *Journal of Neurochemistry*, 123(4), pp.613–621.

Lackner, L.L., 2014. Shaping the dynamic mitochondrial network. *BMC biology*, 12(1), p.35.

Lin, M.T. & Beal, M.F., 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, 443(7113), pp.787–795.

Mateu-Huertas, E. et al., 2014. Blood expression profiles of fragile X premutation carriers identify candidate genes involved in neurodegenerative and infertility phenotypes. *Neurobiology of Disease*, 65, pp.43–54.

Moren, C. et al., 2013. Study of oxidative, enzymatic mitochondrial respiratory chain function and apoptosis in perinatally HIV-infected pediatric patients. *Drug and Chemical Toxicology*, 36(4), pp.496-500.

Napoli, E. et al., 2011. Altered zinc transport disrupts mitochondrial protein processing/import in fragile X-associated tremor/ataxia syndrome. *Human Molecular Genetics*, 20(15), pp.3079–3092.

Nikolaisen, J. et al., 2014. Automated quantification and integrative analysis of 2D and 3D mitochondrial shape and network properties. *PLoS ONE*, 9(7), pp.1–16.

Pesta D. & Gnaiger E., 2012. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Methods in Molecular Biology*, 810, pp. 25-58.

Prilutskiĭ, A.S., Khodakovskiĭ, A. V & Maĭlian, E.A., 1990. [A method of separating mononuclears on a density gradient]. *Laboratornoe delo*, (2), pp.20–3.

Rodriguez-Revenga, L. et al., 2009. Penetrance of FMR1 premutation associated pathologies in fragile X syndrome families. *European journal of human genetics : EJHG*, 17(10), pp.1359–1362.

Ross-Inta, C. et al., 2010. Evidence of mitochondrial dysfunction in fragile X-associated tremor/ataxia syndrome. *The Biochemical journal*, 429(3), pp.545–552.

Rugarli, E.I. & Langer, T., 2012. Mitochondrial quality control: a matter of life and death for neurons. *The EMBO Journal*, 31(6), pp.1336–1349.

Figure 1.

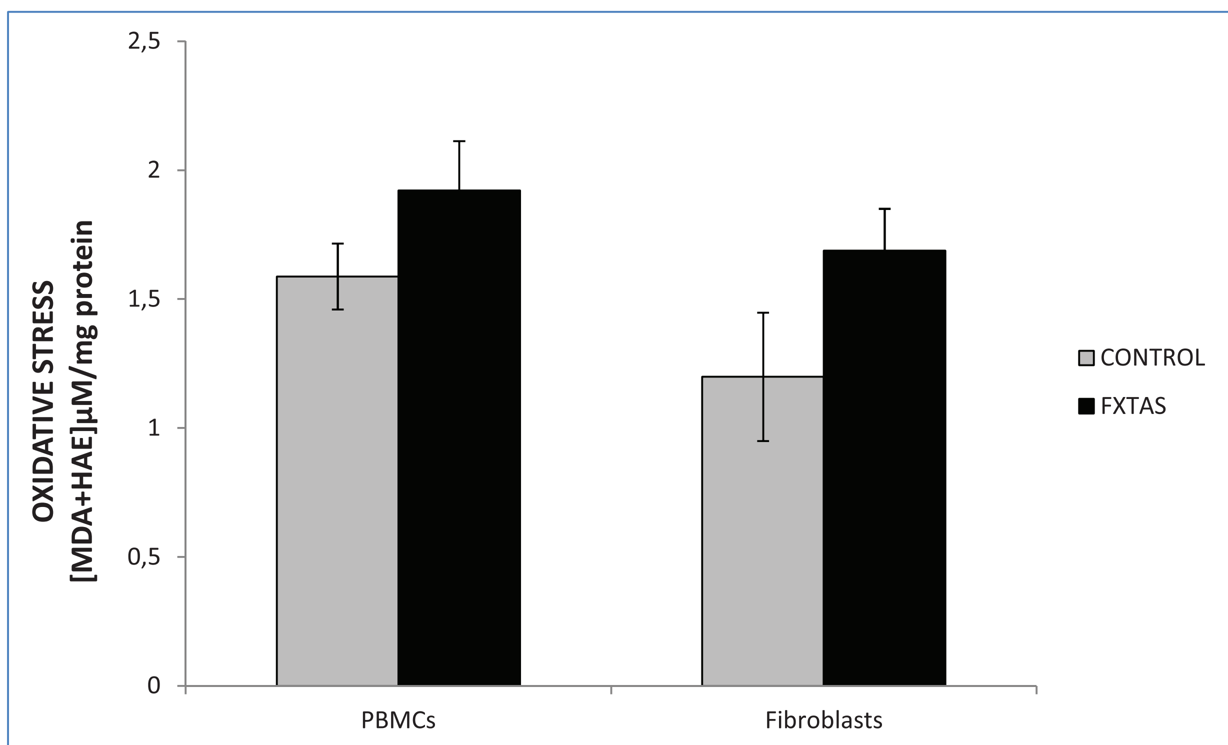
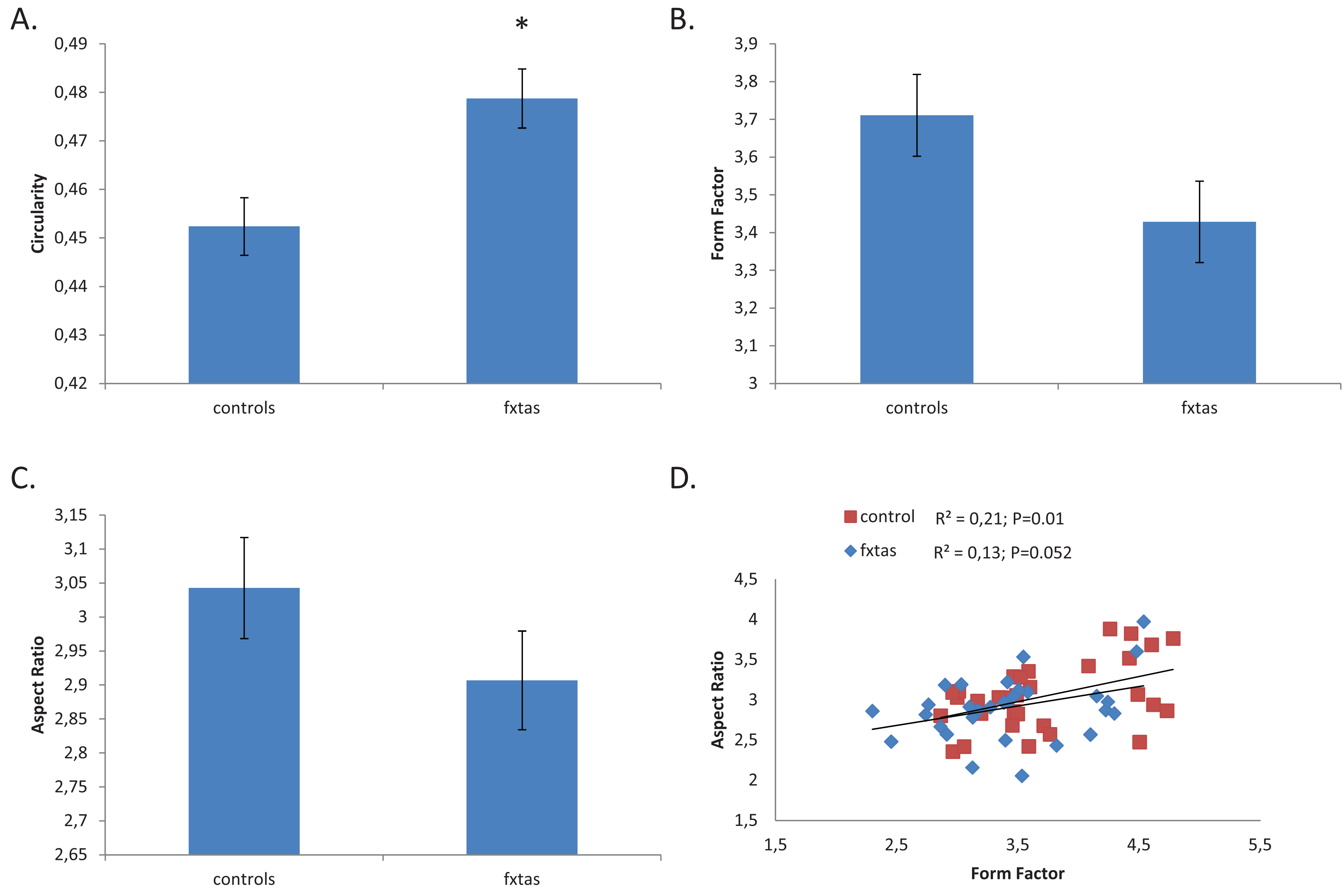


Figure 2.



DISCUSIÓN

Bases moleculares asociadas a la premutación en *FMR1*

La descripción y caracterización del impacto clínico de los alelos con la premutación de *FMR1* es de gran importancia dada su elevada prevalencia en la población general, estimada en 1/130-250 mujeres y en 1/250-810 hombres (revisado en Tassone et al. 2014). En los últimos años se ha descrito un creciente espectro clínico a parte del FXTAS y del FXPOI asociado con estos individuos que incluye hipertensión, fibromialgia, trastornos tiroideos y psicológicos (ver anexo I). Aunque por el momento la relación de estos fenotipos con la premutación se desconoce, la revisión bibliográfica realizada por Wheeler y colaboradores (2014) ha permitido clasificarlos como “definitivamente relacionado”, “probablemente relacionado”, “posiblemente relacionado” e “improbablemente relacionado” en función de su presencia o ausencia con FXPOI y/o FXTAS.

Existen numerosos factores que podrían estar implicados en la aparición de las patologías asociadas a la premutación. En primer lugar, se ha sugerido que la deficiencia en la síntesis de FMRP puede estar implicada en la alteración neuroconductual y en los fenotipos cognitivos asociados. Sin embargo, la ausencia de FXTAS y/o FXPOI en los individuos con la mutación completa sugiere que el déficit de

FMRP tendría un papel secundario en estas patologías (revisado en Hagerman & Hagerman 2015).

En segundo lugar, numerosas evidencias apoyan la toxicidad del ARNm de *FMR1* como evento desencadenante de la patogenicidad de los alelos premutados. Una de ellas, es la detección de inclusiones intranucleares no sólo en el SNC sino también en el SN periférico y en tejidos no cerebrales, afectando órganos que se ven comprometidos en los individuos portadores de la premutación como son, entre otros, la tiroides, la glándula pituitaria y los testículos (Greco et al. 2007; Hunsaker et al. 2011). El secuestro proteico que median los alelos con la premutación conlleva la deficiencia funcional de estas proteínas que en consecuencia promueve la desregulación de la célula. Los resultados presentados en el trabajo I y en el anexo III pusieron de manifiesto la desregulación de la expresión génica en pacientes con FXPOI y FXTAS, respectivamente.

En tercer lugar, hay evidencias de la traducción del tramo expandido de la región 5'UTR del gen *FMR1* a partir del mecanismo de *RAN translation.* Se ha demostrado la síntesis de péptidos poliG que colocalizan con las inclusiones intranucleares en los pacientes FXTAS. También se han detectado a nivel citoplasmático en las células de la granulosa que envuelven el ovario (Todd et al 2013, Cohen Y,

comunicación personal). En base a estos hallazgos, actualmente se considera que el mecanismo de *RAN translation* contribuye a la toxicidad celular asociada a los alelos premutados.

En cuarto lugar, se han descrito otros mecanismos implicados en las patologías asociadas a la premutación como son la transcripción del ARNm *antisense* de *FMR1*, y la desregulación de la vía de los miRNAs (revisado en Hagerman & Hagerman 2015). Los resultados del trabajo II apoyan las evidencias previas de la implicación de los miRNAs en la patogénesis del FXTAS. No obstante, ni la desregulación de los miRNAs ni la toxicidad del ARNm expandido de *FMR1* proporcionan una explicación concreta de la reducción en la viabilidad celular. Para esto, sería necesario identificar los mecanismos moleculares que se encuentran alterados en estos individuos.

En los últimos años se ha propuesto la alteración de la homeostasis del calcio y la disfunción mitocondrial como mediadores centrales de la desregulación celular (revisado en Hagerman & Hagerman 2013). En este contexto, los resultados del trabajo IV pusieron de manifiesto la disfunción mitocondrial en tejidos no relacionados con el SNC y la alteración de la morfología de la red mitocondrial. Estos hallazgos proporcionan nuevas líneas de investigación para esclarecer la relación entre la premutación y la disfunción mitocondrial.

Todos los mecanismos descritos hasta este punto tienen como requisito la transcripción del ARNm expandido de *FMR1*, sin embargo se ha propuesto recientemente la implicación de un mecanismo co-transcirpcional en la patogénesis de los alelos premutados. Cuando existen deficiencias en el procesamiento del ARNm, la formación de R-loops que tiene lugar durante la transcripción de regiones rica en GCs (Reddy et al. 2011), puede promover la activación de la respuesta ante el daño en el ADN que tienen como consecuencia la acumulación de la proteína γH2AX fosforilada (Loomis et al. 2014). Esta proteína ha sido detectada en las inclusiones intranucleares de pacientes FXTAS (Iwahashi et al. 2006), por lo que recientemente se ha propuesto que la maquinaria de reparación del ADN también podría contribuir a la toxicidad celular (revisado en Hagerman & Hagerman 2015).

En conjunto, todas estas evidencias ponen de manifiesto la complejidad de las patologías asociadas a la premutación y la necesidad de profundizar en el conocimiento de las bases moleculares que subyacen a todos estos fenotipos (Figura 18). Únicamente el avance en el conocimiento de los mecanismos moleculares comunes y específicos en que se basan estas patologías permitirá elucidar la relación entre los alelos premutados y el desarrollo de estas enfermedades.

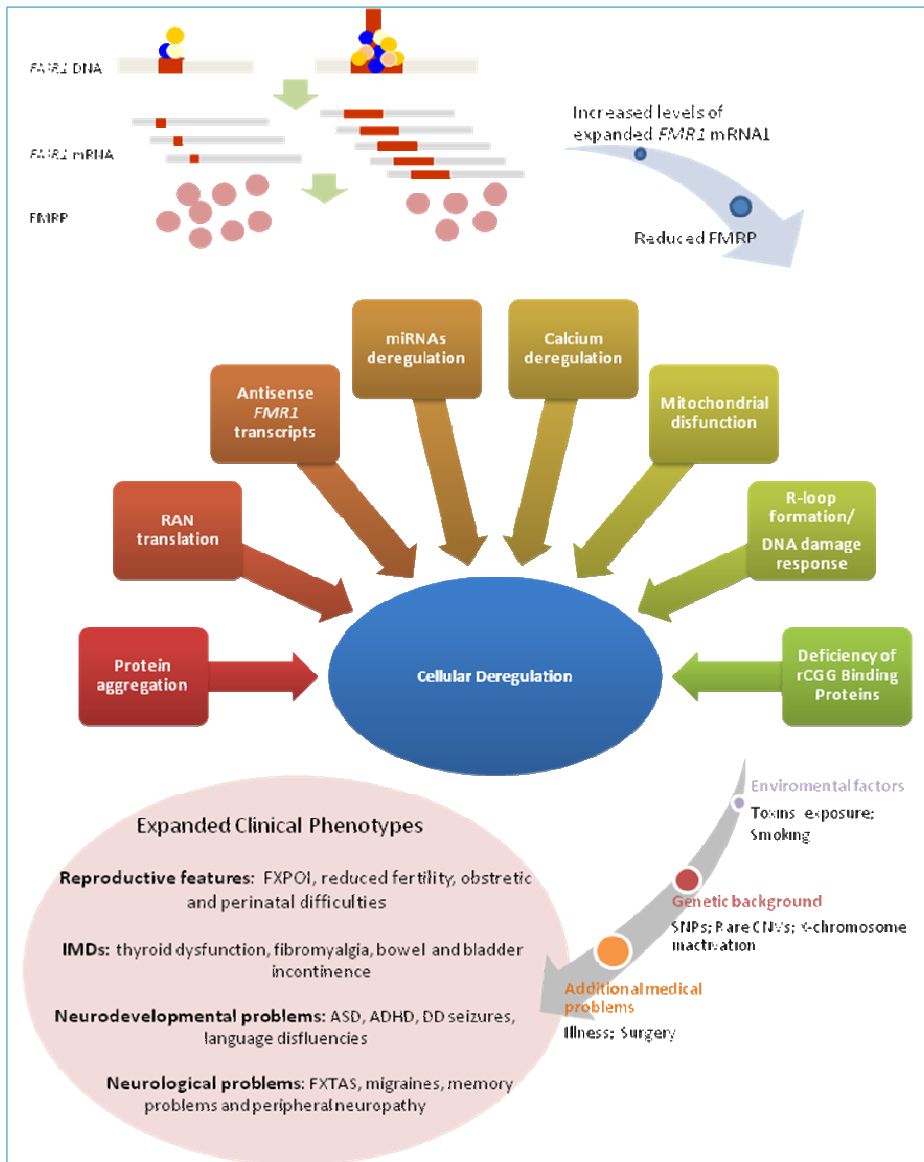


Figura 18. Diagrama de los posibles mecanismos implicados en el impacto clínico de los alelos premutados (modificada de anexo 1)

Mecanismos patogénicos en FXPOI

El ovario es un órgano heterogéneo que contiene folículos en distintas etapas de desarrollo. El número de folículos primordiales constituye la reserva ovárica y está establecido desde el nacimiento. Estos folículos se encuentran en latencia para poder proporcionar regularmente los oocitos durante la etapa reproductiva de las mujeres. El ovario regula el reclutamiento cíclico, el desarrollo y la regresión de los folículos que contiene. La menopausia se debe al agotamiento de la reserva ovárica que suele aparecer entre los 45-50 años.(revisado en Reyes 2010).

Las mujeres con FXPOI se caracterizan por manifestar ciclos menstruales irregulares, problemas de fertilidad y por la aparición de la menopausia antes de los 40 años. El desarrollo de modelos animales ha sido de gran utilidad para el estudio de la etiología del FXPOI, aunque actualmente los mecanismos moleculares que promueven la aparición de este fenotipo permanecen sin esclarecer (revisado en Sherman et al. 2014). Se había sugerido que la FXPOI podría ser consecuencia de una disminución del número de folículos primordiales al nacer, sin embargo, estudios recientes en modelos murinos de FXPOI han demostrado que la premutación de *FMR1* no altera la reserva ovárica (Hoffman et al. 2012; Lu et al. 2012). Los modelos animales han puesto de manifiesto que se debe a un problema intrínseco del ovario ya que

existe una aceleración en la pérdida de todos los tipos de folículos (Hoffman et al. 2012) y un deterioro del desarrollo de los folículos en crecimiento (Lu et al. 2012).

En el trabajo I de esta tesis doctoral se ha presentado el estudio del perfil de expresión génica en sangre periférica de mujeres portadoras de la premutación con y sin FXPOI. Estos resultados mostraron que las mujeres portadoras de la premutación con FXPOI presentan una infraexpresión de numerosos genes implicados en el ciclo celular, en el metabolismo del inositol fosfato y en la señalización mediante VEGF y MAPK. Estas vías constituyen mecanismos de señalización indispensables para la activación de los folículos primordiales, el mantenimiento de su supervivencia y la maduración de los oocitos.

El VEGF es un regulador crucial de las funciones reproductivas. Se ha demostrado que la cascada de señalización promovida por este factor de crecimiento protege a los folículos y a las células de la granulosa de la apoptosis, por lo que se considera que actúa como un factor de supervivencia en el ovario (Kosaka et al. 2007). Los resultados del trabajo I se encuentran en consonancia con estas observaciones ya que detectaron una expresión a la baja en las mujeres con FXPOI de varios genes implicados en la señalización vía VEGF como el gen *PI3K* y el

gen *AKT* y en la vía del metabolismo del inositol fosfato, la cual media la producción de substratos de la PI3K.

Existen numerosas evidencias que indican que los genes relacionados con la familia PI3K/AKT son reguladores cruciales del crecimiento, del desarrollo y de la supervivencia de los folículos (Cecconi et al. 2012). De hecho, está demostrado que la falta de activación mediante la vía de la AKT promueve la depleción prematura de los folículos y el desarrollo de POI (Reddy et al. 2009). Además, el modelo murino KO de AKT presenta una reducción de la fertilidad, anomalías del ciclo estral y un incremento del número de oocitos degenerados (Brown et al. 2010). En FXPOI también existen evidencias de que AKT está implicada en la disfunción ovárica. En el modelo murino KI de *FMR1* se ha puesto de manifiesto que la premutación promueve la reducción de los niveles de fosforilación de AKT en el ovario y el incremento de la apoptosis de los folículos (Lu et al. 2012). En consonancia, en el trabajo I se detectaron numerosos procesos biológicos desregulados en las mujeres con FXPOI como son la progresión/arresto del ciclo celular, la reparación del ADN y la apoptosis y es bien sabido que la vía de la PI3K/AKT regula varios aspectos del ciclo celular.

Finalmente, en el trabajo I se detectó la regulación a la baja de la vía de las MAPK y se ha demostrado que varios miembros de esta

superfamilia están implicados en la señalización mediante FSH/LH, por lo que tienen un papel importante en la maduración de los folículos y en el proceso de ovulación (revisado en Conti et al. 2012; Hunzicker-Dunn & Maizels 2006).

En resumen, el perfil de expresión génica en sangre periférica de mujeres portadoras de la premutación con FXPOI sugiere que la disfunción ovárica en estas mujeres se debe a una desregulación generalizada de las vías de señalización implicadas en la maduración de los oocitos. Estos resultados proporcionan nuevos enfoques estratégicos no sólo para estudios posteriores sobre los mecanismos implicados en FXPOI sino también para el desarrollo de posibles dianas terapéuticas que se centren en el conjunto de genes implicados en estas vías en vez de un único gen y su efecto.

Mecanismos patogénicos y factores de riesgo en FXTAS

El FXTAS es una enfermedad neurodegenerativa monogénica que afecta a los individuos portadores de la premutación. Sin embargo, se ha descrito que la mayoría de enfermedades neurodegenerativas no se deben a un evento genético único sino que implican la progresión secuencial de varios procesos patológicos que colectivamente comprometen la conectividad de las neuronas y/o resultan en una muerte neuronal (Nelson et al. 2008). En este contexto, es bien sabido que los miRNAs tienen un papel crucial en la regulación de la expresión génica durante el desarrollo, por lo que la alteración de los miRNAs se ha asociado con diversas enfermedades neurodegenerativas (revisado en Maciotta et al. 2013).

Existen varias evidencias que sugieren que los miRNAs podrían estar implicados en la patogénesis del FXTAS. En primer lugar, se ha detectado que la expresión aberrante de miR-277 agrava el fenotipo neurodegenerativo en el modelo de FXTAS en *Drosophilla* (Tan et al. 2012). En segundo lugar, se ha descrito la asociación entre el miR-125b y el miR-132 con FMRP en el cerebro de ratón para la regulación de la estructura de la sinapsis y en el control de la función sináptica (Edbauer et al. 2010). Finalmente, se han descrito varios miRNAs que están

implicados en la regulación de la expresión de *FMR1* (Zongaro et al. 2013).

En el trabajo II de esta tesis doctoral se detectó una desregulación del perfil de expresión de los miRNAs en muestras de sangre periférica de pacientes con FXTAS. Durante el transcurso de este estudio, Sellier y colaboradores (2013) identificaron a DROSHA y DGCR8 entre las proteínas parcialmente secuestradas por los alelos premutados. Estas proteínas forman parte de la maquinaria necesaria para la biogénesis de los miRNAs por lo que su depleción resulta en una reducción del procesamiento de los miRNAs y por tanto una en una disminución de los miRNAs maduros (Sellier et al. 2013). Los resultados del trabajo II se encuentran en consonancia con estas observaciones ya que los resultados obtenidos mediante *microarrays* mostraban que el 80% de los miRNAs significativamente desregulados se encontraban infra-expresados en los pacientes FXTAS. De manera similar, el 60% de los miRNAs identificados mediante secuenciación presentaban una regulación a la baja de su expresión. Sin embargo, la expresión de los miRNAs identificados al comparar ambas tecnologías se encontraba mayoritariamente incrementada.

De los 14 miRNAs comúnmente desregulados, el miR-424 presentaba una regulación a la alza estadísticamente significativa mediante ambas

tecnologías. La sobreexpresión de este miRNA ha sido evidenciada previamente en regiones de la sustancia blanca cerebral de muestras post-mortem procedentes de pacientes con Alzheimer (Wang et al. 2011). Curiosamente, la alteración de la sustancia blanca cerebral es uno de hallazgos destacados de las imágenes obtenidas por resonancia magnética en los pacientes FXTAS (Brunberg et al. 2002).

El miR-424 pertenece a la família miR-15/107 cuya función se ha asociado a la regulación del metabolismo y al estrés celular. En los ultimos años se ha puesto de manifiesto que los pacientes FXTAS presentan signos de disfunción mitocondrial y un incremento en los niveles de estrés oxidativo. Los resultados del trabajo II sugieren que la sobreexpresión del miR-424 podría contribuir de algún modo con este proceso. Además, en este trabajo se detectó la sobreexpresión de los miR-23a y miR-27a que también han sido implicados en el proceso de disfunción mitocondrial. En la esclerosis lateral amiotrófica se ha demostrado que el miR-23a induce la disfunción mitocondrial provocando la muerte celular en el músculo esquelético de los pacientes (Russell et al. 2013).

El miR-23a forma parte del cluster miR-23a~miR27a~miR24-2 localizado en el cromosoma 8, cuya sobreexpresión induce estrés celular en el retículo endoplasmático resultando en el incremento de los

niveles de calcio que en consecuencia despolariza las membranas mitocondriales y promovue la apoptosis (Chhabra et al. 2011). Por otra parte, se conoce que los cambios espaciotemporales del calcio intracelular influyen en gran manera en el crecimiento neuronal de las dendritas (Lohmann & Wong 2005). En FXTAS, se han detectado oscilaciones en los niveles de calcio en neuronas derivadas de células madre pluripotentes inducidas que albergan la premutación del gen *FMR1* que podrían ser los responsables de las alteraciones sinápitcas que presentan estas células (Liu et al. 2012). Las alteraciones de la actividad eléctrica y del calcio se detectan también en las neuronas hipocampales del modelo murino de FXTAS (Cao et al. 2013). En base a estas observaciones, los resultados presentados en el trabajo II sugieren que la sobreexpresión de los miR-23a y miR-27a puede contribuir a la desregulación del calcio promivida por la premutación de *FMR1* en FXTAS.

Por otra parte, en el anexo III de esta tesi doctoral se determinó el perfil de expresión génica en muestras de sangre periférica en pacientes FXTAS. A diferencia de los resultados obtenidos en las mujeres portadoras de la premutación con y sin FXPOI, en los varones con FXTAS se dectectaron más de 1600 genes con un *fold change* $\geq|1.5|$ y un *false discovery rate* (FDR) $\leq 1\%$. Estos genes están implicados en

vías relevantes en la patogénesis del FXTAS como son la respuesta inflamatoria o la homeostasis y la viabilidad neuronal. Además, se analizó la expresión de 12 de estos genes en distintas areas cerebrales del modelo murino de FXTAS confirmandose, en todos los casos, el resultado obtenido mediante *microarrays*. Dado que la función principal de los miRNAs es la regulación de la expresión génica, la desregulación del perfil de expresión de miRNAs en los pacietnes FXTAS podría contriubiur a la fuerte desregulación génica detectada en estos individuos. En resumen, estos resultados sugieren que los mecanismos moleculares que contribuyen a la patogénesis del FXTAS implican a parte de la toxicidad del ARNm de *FMR1* un proceso mucho más complejo en el cual la desregulación de los miRNAS podría desempeñar un papel importante.

Los resultados obtenidos en estos dos estudios pusieron de manifiesto la alteración de genes y de miRNAs implicados en la regulación de la vía de la fosforilación oxidativa en los pacientes FXTAS. Por lo tanto, se ha estudiado de manera exhaustiva la función mitocondrial en sangre periférica y en fibroblastos procedentes de pacientes FXTAS. Las mitocondrias son reguladores centrales de la supervivencia y muerte celular. La disfunción mitocondrial ha sido relacionada ampliamente

con el proceso de neurodegeneración y el envejecimiento normal de los individuos (revisado en Lin & Beal 2006).

Las neuronas son particularmente sensibles a las alteraciones mitocondriales debido a sus altos requerimientos de energía para el mantenimiento y la transmisión sináptica (Hall et al. 2012). Las funciones principales de las mitocondrias incluyen la producción de energía en forma de ATP, la homeostasis del calcio, la síntesis de numerosos metabolitos necesarios para el funcionamiento celular, la respuesta al estrés y el fomento de la apoptosis. Cuando estos orgánulos no funcionan correctamente son los responsable de generar estrés oxidativo y desencadenar la muerte celular por apoptosis (Federico et al. 2012). Los resultados presentados en el trabajo IV de esta tesis doctoral detectaron la reducción de la capacidad oxidativa de la cadena respiratoria en los fibroblastos de los pacientes con FXTAS mientras que en sangre no se detectaron diferencias significativas en el consumo de O_2. Además, se detectó un incremento del 20% en sangre y del 30% en fibroblastos de los niveles de estrés oxidativo. Estos resultados están en consonancia con las evidencias previas que detectaban en fibroblastos de pacientes FXTAS una disminución de la fosforilación oxidativa (Ross-Inta et al. 2010; Napoli et al. 2011). Los datos de este trabajo sugieren que los pacientes FXTAS presentan una reducción de

la eficiencia de la producción de ATP que a su vez podría llevar a la falta de energía en los momentos de mayor demanda como por ejemplo en condiciones de estrés.

En los últimos años se ha puesto de manifiesto que las mitocondrias conforman una red altamente interconectada y dinámica mediante procesos de fusión y fisión (revisado en van der Bliek et al 2013). El balance de los estos procesos determina la longitud y el grado de conectividad de las mitocondrias. La morfología y distribución de las mitocondrias en las células, y especialmente los procesos de dinámica mitocondrial, permiten el intercambio esencial de material genético y estructural para el reciclaje y "*turn-over*" mitocondrial. El hallazgo más llamativo de los resultados presentados en el trabajo IV de esta tesis doctoral ha sido la detección por primera vez de una alteración de los procesos de dinámica mitocondrial en pacientes FXTAS mediante el estudio por microscopia confocal de la red mitocondrial en fibroblastos. Concretamente, las mitocondrias de los pacientes FXTAS se encuentran menos interconectadas, son más circulares y pierden la correlación positiva entre los parámetros de elongación y ramificación mitocondrial, asociada al estado de salud mitocondrial. Recientemente, alteraciones de la dinámica mitocondrial se han asociado con varias enfermedades neurodegenerativas (revisado en Itoh et al. 2013). Se han

identificado varias proteínas que regulan la morfología de la red mitocondrial, entre las más estudiadas se encuentran OPA1 y MFN2 implicadas en los procesos de fusión mitocondrial. Las mutaciones deletéreas en estos genes son responsables, respectivamente, de la atrofia óptica autosómica dominante y de la enfermedad Charcot–Marie–Tooth tipo 2. Estos trastornos se caracterizan por afectar principalmente a neuronas especializadas que requieren el transporte de las mitocondrias en distancias largas por los axones neuronales (revisado en Burté et al. 2015).

La función y la dinámica mitocondrial son dos eventos que se encuentran estrictamente regulados para cubrir las necesidades bioenergéticas no sólo de las neuronas sino de cualquier tipo celular. Recientemente, se ha sugerido que las proteínas implicadas en la dinámica mitocondrial pueden estar implicadas en la regulación del acoplamiento entre la cadena respiratoria mitocondrial y la fosforilación oxidativa (Mishra et al. 2014). En base a estas observaciones, los resultados del trabajo IV sugieren que las alteraciones detectadas en la arquitectura de la red mitocondrial podrían contribuir a la disminución de la capacidad oxidativa detectada en los fibroblastos de pacientes FXTAS. En conjunto, la alteración de la fosforilación oxidativa y de la arquitectura de la red mitocondrial en

pacientes FXTAS, indica que ambos procesos podrían estar implicados en la fisiopatología del FXTAS y en la etiología de los síntomas neurológicos.

Finalmente se han identificado otros factores genéticos y ambientales que pueden modular el riesgo a desarrollar FXTAS (revisado en Hagerman & Hagerman 2013). Los resultados presentados en el trabajo III mostraron que la XCI contribuye significativamente en el riesgo a desarrollar FXTAS, ya que el patrón de XCI preferencial severa era contrario entre portadoras de la premutación con y sin FXTAS. De manera que todas las mujeres FXTAS con XCI severa inactivaban preferencialmente el alelo normal mientras que las asintomáticas inactivaban preferencialmente el alelo expandido. Por último, existen polimorfismos que podrían contribuir a la penetrancia del FXTAS (revisado en Hagerman & Hagerman 2013). En el anexo II de esta tesis doctoral se identificó un incremento significativo de la frecuencia del alelo ε4 del gen *ApoE* en los pacientes FXTAS respecto los portadores de la premutación sin FXTAS. Esta proteína es crucial en el metabolismo del colesterol y el alelo ε4 se considera un factor de riesgo para el desarrollo de Alzheimer. Los resultados del anexo II sugieren que la presencia del alelo ApoE ε4 podría incrementar el riesgo a desarrollar FXTAS entre los individuos portadores de la premutación.

Tecnologías de alto rendimiento

Los avances en el campo de la biología molecular y de la bioinformática han resultado en el desarrollo de "La era Ómica" que ha permitido una nueva visión global de los procesos biológicos. Entre las múltiples disciplinas que han emergido bajo este concepto se encuentra la genómica funcional centrada en los aspectos dinámicos de los genes, como su transcripción. Existen varias metodologías de alta resolución para estudiar el transcriptoma de las células como los *microarrays* de expresión o la secuenciación de nueva generación. Estas herramientas determinan la expresión de miles de tránscritos de manera simultánea por lo que es indispensable un análisis computacional para la interpretación de este tipo de datos. Este tipo de herramientas son muy útiles por ejemplo para la determinación de cambios significativos en los niveles de expresión de genes o miRNAs específicos. Por este motivo, estas técnicas se han utilizando para el estudio de las bases moleculares del FXTAS (Trabajo II y Anexo III).

Sin embargo, se debe tener en cuenta que una de las limitaciones de estas tecnologías es la elevada tasa de falsos positivos. Por este motivo se aplica la corrección de los p-valores crudos bajo el modelo de FDR que genera un "p-valor ajustado" disminuyendo al máximo la aparición de este tipo de errores aunque tiene como consecuencia inmediata el

incremento de la tasa de falsos negativos (Pawitan et al. 2005). Un ejemplo de esta corrección estricta se ve reflejado en los resultados del trabajo II donde únicamente la sobreexpresión del miR-424 alcanzó un valor estadísticamente significativo por ambas tecnologías. No obstante, se identificaron un total de 14 miRNAs con una desregulación común que fue posteriormente confirmada mediante RT-qPCR.

Por otra parte, puede darse el caso de que múltiples genes pertenecientes a una vía molecular determinada se encuentren ligeramente desregulados sin llegar a niveles estadísticamente significativos. Esto no significa que su alteración conjunta no pueda tener un impacto biológico en el organismo. Por este motivo se ha desarrollado un análisis de enriquecimiento funcional para los datos de *microarrays*, conocido como *Gene Set Analysis* (Subramanian et al. 2005), que permite detectar bloques funcionales de genes que presentan un patrón común de expresión. Existen dos tipos de aproximaciones para este análisis, el enriquecimiento por vías moleculares utilizando bases de datos como la *Kyoto Encyclopedia of Genes and Genomes* (KEGG, http://www.genome.jp/kegg/) o en base a la ontología genómica (GO) que clasifica jerárquicamente los genes en función al proceso biológico, la función molecular y/o el compartimento celular al que se encuentran asociados (http://geneontology.org/). Este es el caso

de los resultados presentados en el trabajo I, en el cual el análisis de expresión diferencial no detectó diferencias significativas entre grupos mientras que el análisis de enriquecimiento funcional puso de manifiesto la desregulación específica en las mujeres con FXPOI de varias vías moleculares indispensables para la maduración de los oocitos así como la desregulación de varios procesos biológicos implicados en la supervivencia y muerte celular.

Cabe mencionar que todos los trabajos presentados en esta tesis doctoral se han realizado estudiando principalmente sangre periférica como indicador de los procesos patológicos en FXPOI y FXTAS. Aunque no se trata del tejido diana, la sangre refleja la actividad fisiológica y patológica de otros órganos y sistemas del cuerpo y además es fácil de obtener y sin riesgo para el paciente, por lo que actualmente supone una fuente atractiva para el descubrimiento y el desarrollo de biomarcadores (Thambisetty & Lovestone 2010, Cristalli et al. 2012). Asimismo, numerosos estudios han demostrado su uso satisfactorio en la detección de cambios de la expresión génica asociada a condiciones fisiológicas/patológicas del ovario y en enfermedades neurodegenerativas (Tang et al. 2001; Twine et al. 2003; Flores et al. 2006; Gielchinsky et al. 2008; Lunnon et al. 2013; Mutez et al. 2011).

En los últimos años la revolución en las tecnologías de alto rendimiento ha supuesto un gran avance en el conocimiento de la base genética de enfermedades complejas. Las disciplinas "ómicas" han hecho factible la identificación de alteraciones moleculares y el desarrollo de biomarcadores para el diagnóstico, pronóstico y predicción de la respuesta a los tratamientos. La identificación de un biomarcador biológico para el diagnóstico presintomático de los individuos portadores de la premutación posibilitaría un mejor consejo genético, un tratamiento precoz y un enlentecimiento de la progresión de las patologías asociadas a estos individuos.

CONCLUSIONES

Los resultados obtenidos de los estudios que comprenden esta tesis doctoral permiten extraer las siguientes conclusiones:

1. El estudio del perfil de expresión génica en sangre periférica procedente de mujeres portadoras de la premutación con y sin FXPOI no ha detectado ningún gen con una expresión significativamente alterada.
2. El análisis de enriquecimiento funcional ha detectado la desregulación significativa de de vías cruciales para la maduración de los oocitos en muestras de sangre periférica procedentes de mujeres portadoras de la premutación con FXPOI.
3. El estudio del perfil de expresión de miRNAs en sangre periférica procedente de varones portadores de la premutación con FXTAS ha detectado 14 miRNAs con una expresión diferencial respecto a los individuos control.
4. Existe una distribución similar para todos los tipos de IsomiRs en FXTAS, por lo que el proceso de biogénesis de los miRNAs y/o su estabilidad no parecen estar alterados en la sangre de los pacientes FXTAS.
5. La premutación del gen *FMR1* promueve una fuerte desregulación génica en los varones afectados de FXTAS, ya

que se han detectado más de 1600 genes diferencialmente expresados mediante el perfil de expresión génica en sangre periférica.

6. El análisis de enriquecimiento funcional de los genes alterados y el análisis de las dianas de los miRNAs diferencialmente expresados en FXTAS han puesto de manifiesto la desregulación de la vía de la fosforilación oxidativa en FXTAS.
7. El estudio del estado de las mitocondrias en pacientes FXTAS pone de manifiesto la alteración de las funciones mitocondriales y de la arquitectura mitocondrial en muestras procedente cultivo de fibroblastos, no así en sangre periférica.
8. El proceso de inactivación preferencial del cromosoma X es un factor modulador de la penetrancia del FXTAS.

Adinolfi, S. et al., 1999. Dissecting FMR1, the protein responsible for fragile X syndrome, in its structural and functional domains. *RNA (New York, N.Y.)*, 5(9), pp.1248–1258.

Allen, E.G. et al., 2007. Examination of reproductive aging milestones among women who carry the FMR1 premutation. *Human Reproduction*, 22(8), pp.2142–2152.

Allingham-Hawkins, D.J. et al., 1999. Fragile X premutation is a significant risk factor for premature ovarian failure: the International Collaborative POF in Fragile X study--preliminary data. *American journal of medical genetics*, 83(4), pp.322–325.

Amos-Landgraf, J.M. et al., 2006. X chromosome-inactivation patterns of 1,005 phenotypically unaffected females. *American journal of human genetics*, 79(3), pp.493–9.

Antar, L.N. & Bassell, G.J., 2003. Sunrise at the synapse: The FMRP mRNP shaping the synaptic interface. *Neuron*, 37(4), pp.555–558.

Apartis, E. et al., 2012. New insights and the need for revised diagnostic criteria. *Neurology*, 79(18), pp.1898–1907.

Ashley, C.T. & Warren, S.T., 1995. Trinucleotide repeat expansion and human disease. *Annual review of genetics*, 29, pp.703–728.

Ashley-Koch, A.E. et al., 1998. Examination of factors associated with instability of the FMR1 CGG repeat. *American journal of human genetics*, 63(3), pp.776–785.

Au, J. et al., 2013. Prevalence and risk of migraine headaches in adult fragile X premutation carriers. *Clinical Genetics*, 84(6), pp.546–551.

Bailey, D.B. et al., 2008. Co-occurring conditions associated with FMR1 gene variations: Findings from a national parent survey. *American Journal of Medical Genetics, Part A*, 146(16), pp.2060–2069.

Bassell, G.J. & Warren, S.T., 2008. Fragile X Syndrome: Loss of Local mRNA Regulation Alters Synaptic Development and Function. *Neuron*, 60(2), pp.201–214.

Bear, M.F., Huber, K.M. & Warren, S.T., 2004. The mGluR theory of fragile X mental retardation. *Trends in Neurosciences*, 27(7), pp.370–377.

Berman, R.F. et al., 2014. Mouse models of the fragile X premutation and fragile X-associated tremor/ataxia syndrome. *Journal of Neurodevelopmental Disorders,* 6(1), pp.25.

Berry-Kravis, E. et al., 2007. Fragile X-associated tremor/ataxia syndrome: clinical features, genetics, and testing guidelines. *Movement disorders : official journal of the Movement Disorder Society*, 22(14), pp.2018–30, quiz 2140.

Bhat, A.H. et al., 2015. Oxidative stress, mitochondrial dysfunction and neurodegenerative diseases; a mechanistic insight. *Biomedicine & pharmacotherapy = Biomédecine & pharmacothérapie*, 74, pp.101–10.

Van der Bliek, A.M., Shen, Q. & Kawajiri, S., 2013. Mechanisms of mitochondrial fission and fusion. *Cold Spring Harbor perspectives in biology*, 5(6).

Bodega, B. et al., 2006. Influence of intermediate and uninterrupted FMR1 CGG expansions in premature ovarian failure manifestation. *Human Reproduction*, 21(4), pp.952–957.

De Boulle, K. et al., 1993. *A point mutation in the FMR-1 gene associated with fragile X mental retardation.*,

Bourgeois, J.A. et al., 2011. Lifetime prevalence of mood and anxiety disorders in fragile X premutation carriers. *The Journal of clinical psychiatry*, 72(2), pp.175–82.

Brown, C. et al., 2010. Subfertility caused by altered follicular development and oocyte growth in female mice lacking PKB alpha/Akt1. *Biology of reproduction*, 82(2), pp.246–56.

Brunberg, J.A. et al., 2002. Fragile X premutation carriers: Characteristic MR imaging findings of adult male patients with progressive cerebellar and cognitive dysfunction. *American Journal of Neuroradiology*, 23(10), pp.1757–1766.

Burté, F. et al., 2015. Disturbed mitochondrial dynamics and neurodegenerative disorders. *Nature reviews. Neurology*, 11(1), pp.11–24.

Cao, Z. et al., 2013. Enhanced asynchronous Ca2+ oscillations associated with impaired glutamate transport in cortical astrocytes expressing Fmr1 gene premutation expansion. *Journal of Biological Chemistry*, 288(19), pp.13831–13841.

Cecconi, S. et al., 2012. The role of Akt signalling in the mammalian ovary. *International Journal of Developmental Biology*, 56(10-12), pp.809–817.

Coffey, S.M. et al., 2008. Expanded clinical phenotype of women with the FMR1 premutation. *American Journal of Medical Genetics, Part A*, 146(8), pp.1009–1016.

Conti, M. et al., 2012. Novel signaling mechanisms in the ovary during oocyte maturation and ovulation. *Molecular and Cellular Endocrinology*, 356(1-2), pp.65–73.

Cornish, K.M. et al., 2009. Lifespan changes in working memory in fragile X premutation males. *Brain and cognition*, 69(3), pp.551–558.

Cristalli, D.O. et al., 2012. Peripheral markers in neurodegenerative patients and their first-degree relatives. *Journal of the neurological sciences*, 314(1-2), pp.48–56.

Cronister, A. et al., 1991. Heterozygous fragile X female: Historical, physical, cognitive, and cytogenetic features. In *American Journal of Medical Genetics*. pp. 269–274.

Chen, L.S. et al., 2003. The (CGG)n repeat element within the 5′ untranslated region of the FMR1 message provides both positive and negative cis effects on in vivo translation of a downstream reporter. *Human Molecular Genetics*, 12(23), pp.3067–3074.

Chhabra, R., Dubey, R. & Saini, N., 2011. Gene expression profiling indicate role of ER stress in miR-23a~27a~24-2 cluster induced apoptosis in HEK293T cells. *RNA Biology*, 8(January 2015), pp.648–664.

Chonchaiya, W. et al., 2012. Increased prevalence of seizures in boys who were probands with the FMR1 premutation and co-morbid autism spectrum disorder. *Human genetics*, 131(4), pp.581–589.

Darnell, J.C. et al., 2001. Fragile X mental retardation protein targets G quartet mRNAs important for neuronal function. *Cell*, 107(4), pp.489–499.

Darnell, J.C. et al., 2005. Kissing complex RNAs mediate interaction between the Fragile-X mental retardation protein KH2 domain and brain polyribosomes. *Genes and Development*, 19(8), pp.903–918.

Devys, D. et al., 1993. The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. *Nature genetics*, 4(4), pp.335–340.

Dictenberg, J.B. et al., 2008. A Direct Role for FMRP in Activity-Dependent Dendritic mRNA Transport Links Filopodial-Spine Morphogenesis to Fragile X Syndrome. *Developmental Cell*, 14(6), pp.926–939.

Edbauer, D. et al., 2010. Regulation of Synaptic Structure and Function by FMRP-Associated MicroRNAs miR-125b and miR-132. *Neuron*, 65(3), pp.373–384.

Eichler, E.E. et al., 1994. Length of uninterrupted CGG repeats determines instability in the FMR1 gene. *Nature genetics*, 8(1), pp.88–94.

Eichler, E.E. & Nelson, D.L., 1996. Genetic variation and evolutionary stability of the FMR1 CGG repeat in six closed human populations. *American Journal of Medical Genetics*, 64(1), pp.220–225.

Estes, P.S. et al., 2008. Fragile X protein controls the efficacy of mRNA transport in Drosophila neurons. *Molecular and Cellular Neuroscience*, 39(2), pp.170–179.

Farzin, F. et al., 2006. Autism spectrum disorders and attention-deficit/hyperactivity disorder in boys with the fragile X premutation. *Journal of developmental and behavioral pediatrics : JDBP*, 27(2 Suppl), pp.S137–144.

Federico, A. et al., 2012. Mitochondria, oxidative stress and neurodegeneration. *Journal of the Neurological Sciences*, 322(1-2), pp.254–262.

Ferder, I. et al., 2013. Expression of fragile X mental retardation protein and Fmr1 mRNA during folliculogenesis in the rat. *Reproduction (Cambridge, England)*, 145(4), pp.335–343.

Fernandez-Carvajal, I. et al., 2009. Expansion of an FMR1 grey-zone allele to a full mutation in two generations. *The Journal of molecular diagnostics : JMD*, 11(4), pp.306–310.

Flores, I. et al., 2006. Identification of molecular markers for endometriosis in blood lymphocytes by using deoxyribonucleic acid microarrays. *Fertility and sterility*, 85(6), pp.1676–83.

Gallagher, A. & Hallahan, B., 2012. Fragile X-associated disorders: A clinical overview. *Journal of Neurology*, 259(3), pp.401–413.

Gielchinsky, Y. et al., 2008. Gene expression in women conceiving spontaneously over the age of 45 years. *Fertility and sterility*, 89(6), pp.1641–50.

Goswami, D. & Conway, G.S., 2005. Premature ovarian failure. *Human reproduction update*, 11(4), pp.391–410.

Greco, C.M. et al., 2002. *Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers.*,

Greco, C.M. et al., 2006. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). *Brain*, 129(1), pp.243–255.

Greco, C.M. et al., 2007. Testicular and pituitary inclusion formation in fragile X associated tremor/ataxia syndrome. *The Journal of urology*, 177(4), pp.1434–7.

Groh, M. et al., 2014. R-loops Associated with Triplet Repeat Expansions Promote Gene Silencing in Friedreich Ataxia and Fragile X Syndrome. *PLoS Genetics*, 10(5).

Grønskov, K. et al., 2011. A nonsense mutation in FMR1 causing fragile X syndrome. *European journal of human genetics*, 19(4), pp.489-491

Hagerman, P., 2013. Fragile X-associated tremor/ataxia syndrome (FXTAS): Pathology and mechanisms. *Acta Neuropathologica*, 126(1), pp.1–19.

Hagerman, P.J. & Hagerman, R.J., 2004. The fragile-X premutation: a maturing perspective. *American journal of human genetics*, 74(5), pp.805–816.

Hagerman, R. & Hagerman, P., 2013. Advances in clinical and molecular understanding of the FMR1 premutation and fragile X-associated tremor/ataxia syndrome. *The Lancet Neurology*, 12(8), pp.786–798.

Hagerman, P.J. & Hagerman, R.J., 2015. Fragile X-associated tremor/ataxia syndrome. *Annals of the New York Academy of Sciences*, 1338(1), pp.58–70.

Hagerman, P.J., Greco, C.M. & Hagerman, R.J., 2003. A cerebellar tremor/ataxia syndrome among fragile X premutation carriers. *Cytogenetic and Genome Research*, 100(1-4), pp.206–212.

Hagerman, R., 2002. The Physical and behavioural phenotype. In R.J. Hagerman & P.J. Hagerman (Eds.) Fragile X Syndrome: Diagnosis, Treatment and Research, The Johns Hopkins University Press, Baltimore, pp. 3–109

Hagerman, R.J. et al., 2001. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. *Neurology*, 57(1), pp.127–130.

Hagerman, R.J. et al., 2004. *Fragile-X-associated tremor/ataxia syndrome (FXTAS) in females with the FMR1 premutation.*,

Hagerman, R.J. et al., 2007. Neuropathy as a presenting feature in fragile X-associated tremor/ataxia syndrome. *American journal of medical genetics. Part A*, 143A(19), pp.2256–60.

Hagerman, R.J. et al., 2014. Translating molecular advances in fragile x syndrome into therapy: A review. *Journal of Clinical Psychiatry*, 75(4).

Hall, C.N. et al., 2012. Oxidative phosphorylation, not glycolysis, powers presynaptic and postsynaptic mechanisms underlying brain information processing. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 32(26), pp.8940–51.

Hamlin, A. et al., 2011. Sleep apnea in fragile X premutation carriers with and without FXTAS. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*, 156B(8), pp.923–8.

Hoffman, G.E. et al., 2012. Ovarian abnormalities in a mouse model of fragile X primary ovarian insufficiency. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 60(6), pp.439–56.

Hukema, R.K. et al., 2014. Induced expression of expanded CGG RNA causes mitochondrial dysfunction in vivo. *Cell Cycle*, 13(16), pp.2600–2608.

Hunsaker, M.R. et al., 2011. Widespread non-central nervous system organ pathology in fragile X premutation carriers with fragile X-associated tremor/ataxia syndrome and CGG knock-in mice. *Acta Neuropathologica*, 122(4), pp.467–479.

Hunter, J. E. et al., 2008. Fragile X-associated primary ovarian insufficiency: evidence for additional genetic contributions to severity. *Genetic Epidemiology*, 32(6), pp.553-559.

Hunter, J.E. et al., 2012. Depression and anxiety symptoms among women who carry the FMR1 premutation: Impact of raising a child with fragile X syndrome is moderated by CRHR1 polymorphisms. *American Journal of Medical Genetics, Part B: Neuropsychiatric Genetics*, 159 B(5), pp.549–559.

Hunzicker-Dunn, M. & Maizels, E.T., 2006. FSH signaling pathways in immature granulosa cells that regulate target gene expression: Branching out from protein kinase A. *Cellular Signalling*, 18, pp.1351–1359.

Irwin, S.A. et al., 2001. Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: A quantitative examination. *American Journal of Medical Genetics*, 98(2), pp.161–167.

Itoh, K. et al., 2013. Mitochondrial dynamics in neurodegeneration. *Trends in cell biology*, 23(2), pp.64–71.

Iwahashi, C. & Hagerman, P.J., 2008. Isolation of pathology-associated intranuclear inclusions. *Methods in Molecular Biology*, 463, pp.181–190.

Iwahashi, C.K. et al., 2006. Protein composition of the intranuclear inclusions of FXTAS. *Brain*, 129(1), pp.256–271.

Jacquemont, S. et al., 2004. Aging in individuals with the FMR1 mutation. *American journal of mental retardation : AJMR*, 109(2), pp.154–164.

Jacquemont, S. et al., 2003. Fragile X premutation tremor/ataxia syndrome: molecular, clinical, and neuroimaging correlates. *American journal of human genetics*, 72(4), pp.869–878.

Jarem, D.A., Huckaby, L. V. & Delaney, S., 2010. AGG interruptions in (CGG)n DNA repeat tracts modulate the structure and thermodynamics of non-b conformations in vitro. *Biochemistry*, 49(32), pp.6826–6837.

Jin, P. et al., 2004. Biochemical and genetic interaction between the fragile X mental retardation protein and the microRNA pathway. *Nature neuroscience*, 7(2), pp.113–117.

Kaplan, E.S. et al., 2012. Early mitochondrial abnormalities in hippocampal neurons cultured from Fmr1 pre-mutation mouse model. *Journal of Neurochemistry*, 123(4), pp.613–621.

Kogan, C.S. et al., 2008. Impact of the Fragile X mental retardation 1 (FMR1) gene premutation on neuropsychiatric functioning in adult males without fragile X-associated Tremor/Ataxia syndrome: a controlled study. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*, 147B(6), pp.859–72.

Kooy, R.F., Willemsen, R. & Oostra, B. a, 2000. Fragile X syndrome at the turn of the century. *Molecular medicine today*, 6(5), pp.193–8.

Kosaka, N. et al., 2007. Vascular endothelial growth factor (VEGF) suppresses ovarian granulosa cell apoptosis in vitro. *Biochem Biophys Res Commun*, 363(3), pp.733–737.

Kraan, C.M. et al., 2014. Impaired response inhibition is associated with self-reported symptoms of depression, anxiety, and ADHD in female FMR1 premutation carriers. *American journal of medical genetics. Part B, Neuropsychiatric genetics : the official publication of the International Society of Psychiatric Genetics*, 165B(1), pp.41–51.

Lachiewicz, A.M. et al., 2006. Arithmetic difficulties in females with the fragile X premutation. *American journal of medical genetics. Part A*, 140(7), pp.665–72.

Leehey, M. a, 2009. Fragile X-associated tremor/ataxia syndrome: clinical phenotype, diagnosis, and treatment. *Journal of investigative medicine : the official publication of the American Federation for Clinical Research*, 57(8), pp.830–836.

Leehey, M. a. et al., 2011. Fibromyalgia in fragile X mental retardation 1 gene premutation carriers. *Rheumatology*, 50(12), pp.2233–2236.

Lin, M.T. & Beal, M.F., 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, 443(7113), pp.787–795.

Li, Y. & Jin, P., 2012. RNA-mediated neurodegeneration in fragile X-associated tremor/ataxia syndrome. *Brain Research*, 1462, pp.112–117.

Liu, J. et al., 2012. Signaling defects in iPSC-derived fragile X premutation neurons. *Human Molecular Genetics*, 21(17), pp.3795–3805.

Liu, Y. et al., 2013. Fragile X-associated tremor/ataxia syndrome (FXTAS) in grey zone carriers. *Clinical Genetics*, 84(1), pp.74–77.

Loesch, D.Z. et al., 2012. Fragile X-associated tremor/ataxia phenotype in a male carrier of unmethylated full mutation in the FMR1 gene. *Clinical genetics*, 82(1), pp.88–92.

Lohmann, C. & Wong, R.O.L., 2005. Regulation of dendritic growth and plasticity by local and global calcium dynamics. *Cell calcium*, 37(5), pp.403–9.

Loomis, E.W. et al., 2014. Transcription-Associated R-Loop Formation across the Human FMR1 CGG-Repeat Region. *PLoS Genetics*, 10(4).

Lozano, R., Rosero, C.A. & Hagerman, R.J., 2014a. Fragile X spectrum disorders. *Intractable & Rare Diseases Research*, 3(4), pp.134–146.

Lozano, R. et al., 2014b. Genomic studies in fragile X premutation carriers. *Journal of Neurodevelopmental Disorders*, 6(1), p.27.

Lu, C. et al., 2012. Fragile X premutation RNA is sufficient to cause primary ovarian insufficiency in mice. *Human Molecular Genetics*, 21(23), pp.5039–5047.

Lubs, H.A., 1969. A marker X chromosome. *American journal of human genetics*, 21(3), pp.231–244.

Lunnon, K. et al., 2013. A blood gene expression marker of early Alzheimer's disease. *Journal of Alzheimer's disease : JAD*, 33(3), pp.737–53.

Macpherson, J. et al., 2003. Observation of an excess of fragile-X premutations in a population of males referred with spinocerebellar ataxia. *Human genetics*, 112(5-6), pp.619–620.

Maciotta, S., Meregalli, M. & Torrente, Y., 2013. The involvement of microRNAs in neurodegenerative diseases. *Frontiers in cellular neuroscience*, 7(December), p.265.

Madrigal, I. et al., 2011. Intermediate FMR1 alleles and cognitive and/or behavioural phenotypes. *European journal of human genetics : EJHG*, 19(8), pp.921–923.

Mallolas, J. et al., 2001. Implications of the FMR1 gene in menopause: study of 147 Spanish women. *Menopause (New York, N.Y.)*, 8(2), pp.106–110.

Martin, J.P. & Bell, J., 1943. A PEDIGREE OF MENTAL DEFECT SHOWING SEX-LINKAGE. *Journal of neurology and psychiatry*, 6(3-4), pp.154–157.

Milà, M. et al., 1994. Molecular analysis of the (CGG)n expansion in the FMR-1 gene in 59 Spanish fragile X syndrome families. *Human Genetics*, 94(4), pp.395–400.

Milà Recasens, M. et al., 1996. [A genetic and molecular study of 85 families affected with the fragile X syndrome]. *Anales españoles de pediatría*, 44(3), pp.250–6.

Mishra, P. et al., 2014. Proteolytic cleavage of Opa1 stimulates mitochondrial inner membrane fusion and couples fusion to oxidative phosphorylation. *Cell metabolism*, 19(4), pp.630–41.

Mutez, E. et al., 2011. Transcriptional profile of Parkinson blood mononuclear cells with LRRK2 mutation. *Neurobiology of aging*, 32(10), pp.1839–48.

Murray, A., Ennis, S. & Morton, N., 2000. No evidence for parent of origin influencing premature ovarian failure in fragile X premutation carriers. *American journal of human genetics*, 67(1), pp.253–254; author reply 256–258.

Napierala, M. et al., 2005. Facile FMR1 mRNA structure regulation by interruptions in CGG repeats. *Nucleic Acids Research*, 33(2), pp.451–463.

Napoli, E. et al., 2011. Altered zinc transport disrupts mitochondrial protein processing/import in fragile X-associated tremor/ataxia syndrome. *Human Molecular Genetics*, 20(15), pp.3079–3092.

Nelson, D.L., Orr, H.T. & Warren, S.T., 2013. The unstable repeats-Three evolving faces of neurological disease. *Neuron*, 77(5), pp.825–843.

Nelson, L.M., 2009. Clinical practice. Primary ovarian insufficiency. *The New England journal of medicine*, 360(6), pp.606–614.

Nelson, P.T., Wang, W.X. & Rajeev, B.W., 2008. MicroRNAs (miRNAs) in neurodegenerative diseases. *Brain Pathology*, 18(1), pp.130–138.

Nolin, S.L. et al., 2003. Expansion of the fragile X CGG repeat in females with premutation or intermediate alleles. *American journal of human genetics*, 72(2), pp.454–464.

Nolin, S.L. et al., 1996. Familial transmission of the FMR1 CGG repeat. *American journal of human genetics*, 59(6), pp.1252–1261.

Nolin, S.L. et al., 2013. Fragile X AGG analysis provides new risk predictions for 45-69 repeat alleles. *American Journal of Medical Genetics, Part A*, 161(4), pp.771–778.

Nolin, S.L. et al., 2011. Fragile X analysis of 1112 prenatal samples from 1991 to 2010. *Prenatal Diagnosis*, 31(10), pp.925–931.

Nolin, S.L. et al., 2015. Fragile X full mutation expansions are inhibited by one or more AGG interruptions in premutation carriers. *Genetics in medicine : official journal of the American College of Medical Genetics*, 17(5), pp.358–64.

Oberlé, I. et al., 1991. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science (New York, N.Y.)*, 252(5010), pp.1097–1102.

Paul, R. et al., 2010. Early onset of neurological symptoms in fragile X premutation carriers exposed to neurotoxins. *NeuroToxicology*, 31(4), pp.399–402.

Pawitan, Y. et al., 2005. False discovery rate, sensitivity and sample size for microarray studies. *Bioinformatics*, 21(13), pp.3017–3024.

Peprah, E. et al., 2010. Examination of FMR1 transcript and protein levels among 74 premutation carriers. *Journal of human genetics*, 55(1), pp.66–68.

Persani, L., Rossetti, R. & Cacciatore, C., 2010. Genes involved in human premature ovarian failure. *Journal of Molecular Endocrinology*, 45(5), pp.257–279.

Pfeiffer, B.E. & Huber, K.M., 2009. The state of synapses in fragile X syndrome. *The Neuroscientist : a review journal bringing neurobiology, neurology and psychiatry*, 15(5), pp.549–567.

Polussa, J., Schneider, A. & Hagerman, R., 2014. Molecular Advances Leading to Treatment Implications for Fragile X Premutation Carriers. *Brain Disorders & Therapy*, 03(02), pp.997–1003.

Reddy, K. et al., 2011. Determinants of R-loop formation at convergent bidirectionally transcribed trinucleotide repeats. *Nucleic acids research*, 39(5), pp.1749–62.

Reddy, P. et al., 2009. PDK1 signaling in oocytes controls reproductive aging and lifespan by manipulating the survival of primordial follicles. *Human Molecular Genetics*, 18(15), pp.2813–2824.

Reyes, V., 2010. Artículo de revisión Evaluación de la reserva ovárica. *Revista Mexicana de Medicina de la Reproducción*, 2(4), pp.89–95.

Rifé, M. et al., 2004. Analysis of CGG variation through 642 meioses in Fragile X families. *Molecular Human Reproduction*, 10(10), pp.773–776.

Rodriguez-Revenga, L. et al., 2007. FXTAS in Spanish patients with ataxia: Support for female FMR1 premutation screening. *Molecular Neurobiology*, 35(3), pp.324–328.

Rodriguez-Revenga, L., et al., 2008a. Evidence of depressive symptoms in fragile-X syndrome premutated females. *Psychiatric genetics*, 18(4), pp.153–155.

Rodriguez-Revenga, L., Santos, M.M., et al., 2008b. Screening for FXTAS in 95 Spanish patients negative for Huntington disease. *Genetic testing*, 12(1), pp.135–138.

Rodriguez-Revenga, L., et al., 2009a. Penetrance of FMR1 premutation associated pathologies in fragile X syndrome families. *European journal of human genetics : EJHG*, 17(10), pp.1359–1362.

Rodriguez-Revenga, L., et al., 2009b. Premature ovarian failure and fragile X female premutation carriers: no evidence for a skewed X-chromosome inactivation pattern. *Menopause (New York, N.Y.)*, 16(5), pp.944–949.

Rodriguez-Revenga, L. et al., 2010. Motor and mental dysfunction in mother-daughter transmitted FXTAS. *Neurology*, 75(15), pp.1370–1376.

Rodriguez-revenga, L. et al., 2012. Neuroimaging in Fragile X-Associated. In P. Prof. Bright, ed. *Neuroimaging - Clinical Applications*. Croatia: InTech, pp. 423–440.

Rosales-Reynoso, M. a., Ochoa-Hernández, a. B. & Barros-Núñez, P., 2009. Enfermedades causadas por expansión de tripletes. *Revista de Neurologia*, 49(2), pp.79–87.

Ross-Inta, C. et al., 2010. Evidence of mitochondrial dysfunction in fragile X-associated tremor/ataxia syndrome. *The Biochemical journal*, 429(3), pp.545–552.

Russell, A.P. et al., 2013. Disruption of skeletal muscle mitochondrial network genes and miRNAs in amyotrophic lateral sclerosis. *Neurobiology of Disease*, 49(1), pp.107–117.

Santa María, L. et al., 2014. FXTAS in an unmethylated mosaic male with fragile X syndrome from Chile. *Clinical genetics*, 86(4), pp.378–82.

Sellier, C. et al., 2013. Sequestration of DROSHA and DGCR8 by expanded CGG RNA Repeats Alters microRNA processing in fragile X-associated tremor/ataxia syndrome. *Cell Reports*, 3(3), pp.869–880.

Semenza, C. et al., 2012. Genetics and mathematics: FMR1 premutation female carriers. *Neuropsychologia*, 50(14), pp.3757–3763.

Sherman, S.L., 2000. Premature ovarian failure in the fragile X syndrome. *Am J Med Genet*, 97(3), pp.189–194.

Sherman, S.L. et al., 2014. Use of model systems to understand the etiology of fragile X-associated primary ovarian insufficiency (FXPOI). *Journal of Neurodevelopmental Disorders*, 6(1), p.26.

Silva, F. et al., 2013. High apolipoprotein E4 allele frequency in FXTAS patients. *Genetics in medicine*, 15(8), pp.639–42.

Spath, M.A. et al., 2011. Predictors and risk model development for menopausal age in fragile X premutation carriers. *Genetics in medicine : official journal of the American College of Medical Genetics*, 13(7), pp.643–650.

Sterling, A.M. et al., 2013. Language dysfluencies in females with the FMR1 premutation. *Brain and cognition*, 82(1), pp.84–9.

Subramanian, A. et al., 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proceedings of the National Academy of Sciences of the United States of America*, 102(43), pp.15545–50.

Sullivan, A.K. et al., 2002. Paternally transmitted FMR1 alleles are less stable than maternally transmitted alleles in the common and intermediate size range. *American journal of human genetics*, 70(6), pp.1532–1544.

Sullivan, A.K. et al., 2005. Association of FMR1 repeat size with ovarian dysfunction. *Human Reproduction*, 20(2), pp.402–412.

Sullivan, S., Welt, C. & Sherman, S., 2011. FMR1 and the Continuum of Primary Ovarian Insufficiency. *Seminars in Reproductive Medicine*, 29(04), pp.299–307.

Tan, H. et al., 2012. MicroRNA-277 modulates the neurodegeneration caused by Fragile X premutation rCGG repeats. *PLoS genetics*, 8(5), p.e1002681.

Tang, Y. et al., 2001. Blood genomic responses differ after stroke, seizures, hypoglycemia, and hypoxia: blood genomic fingerprints of disease. *Annals of neurology*, 50(6), pp.699–707.

Tassone, F. et al., 2000a. Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. *American journal of human genetics*, 66(1), pp.6–15.

Tassone, F. et al., 2000b. Clinical involvement and protein expression in individuals with the FMR1 premutation. *American Journal of Medical Genetics*, 91(2), pp.144–152.

Tassone, F. et al., 2007. Elevated FMR1 mRNA in premutation carriers is due to increased transcription. *RNA (New York, N.Y.)*, 13(4), pp.555–562.

Tassone, F. et al., 2012a. FMR1 CGG allele size and prevalence ascertained through newborn screening in the United States. *Genome medicine*, 4(12), p.100.

Tassone, F. et al., 2012b. Neuropathological, clinical and molecular pathology in female fragile X premutation carriers with and without FXTAS. *Genes, Brain and Behavior*, 11(5), pp.577–585.

Tassone, F., Hagerman, P.J. & Hagerman, R.J., 2014. Fragile x premutation. *Journal of neurodevelopmental disorders*, 6(1), p.22.

Tejada, M.-I. et al., 2008. Analysis of the molecular parameters that could predict the risk of manifesting premature ovarian failure in female premutation carriers of fragile X syndrome. *Menopause (New York, N.Y.)*, 15(5), pp.945–949.

Terracciano, A. et al., 2004. Expansion to full mutation of a FMR1 intermediate allele over two generations. *European journal of human genetics : EJHG*, 12(4), pp.333–336.

Thambisetty, M. & Lovestone, S., 2010. Blood-based biomarkers of Alzheimer's disease: challenging but feasible. *Biomarkers in medicine*, 4(1), pp.65–79.

Todd, P.K. et al. 2010. Histone deacetylases suppress CGG repeat-induced neurodegeneration via transcriptional silencing in models of fragile X tremor ataxia syndrome. *PLOS Genetics*, 6(12), e1001240

Todd, P.K. et al., 2013. CGG repeat-associated translation mediates neurodegeneration in fragile X tremor ataxia syndrome. *Neuron*, 78(3), pp.440–455.

Twine, N.C. et al., 2003. Disease-associated expression profiles in peripheral blood mononuclear cells from patients with advanced renal cell carcinoma. *Cancer research*, 63(18), pp.6069–6075.

Usdin, K. et al., 2014. Repeat-mediated genetic and epigenetic changes at the FMR1 locus in the Fragile X-related disorders. *Frontiers in Genetics*, 5(July), pp.1–16.

Verkerk, A.J. et al., 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell*, 65(5), pp.905–914.

Wang, Y.H. et al., 1996. Long CCG triplet repeat blocks exclude nucleosomes: a possible mechanism for the nature of fragile sites in chromosomes. *Journal of molecular biology*, 263(4), pp.511–516.

Wang, W.-X. et al., 2011. Patterns of microRNA expression in normal and early Alzheimer's disease human temporal cortex: white matter versus gray matter. *Acta neuropathologica*, 121(2), pp.193–205.

Weber, J.J. et al., 2014. From Pathways to Targets: Understanding the Mechanisms behind Polyglutamine Disease. *BioMed Research International*, 2014, pp.1–22.

Weiler, I.J. et al., 1997. Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. *Proceedings of the National Academy of Sciences of the United States of America*, 94(10), pp.5395–5400.

Wheeler, A.C. et al., 2014. Associated features in females with an FMR1 premutation. *Journal of neurodevelopmental disorders*, 6(1), p.30.

Willemsen, R. et al., 2004. The Fragile X Syndrome: From Molecular Genetics to Neurobiology. *Mental Retardation and Developmental Disabilities Research Reviews*, 10(1), pp.60–67.

Willemsen, R., Levenga, J. & Oostra, B., 2011. CGG repeat in the FMR1 gene: Size matters. *Clinical Genetics*, 80(3), pp.214–225.

Winarni, T.I. et al., 2012. Immune-mediated disorders among women carriers of fragile X premutation alleles. *American journal of medical genetics. Part A*, 158A(10), pp.2473–81.

Wittenberger, M.D. et al., 2007. The FMR1 premutation and reproduction. *Fertility and Sterility*, 87(3), pp.456–465.

Wong, D.J. & Chang, H.Y., 2005. Learning more from microarrays: Insights from modules and networks. *Journal of Investigative Dermatology*, 125(2), pp.175–182.

Wong, L.M. et al., 2012. Young adult male carriers of the fragile X premutation exhibit genetically modulated impairments in visuospatial tasks controlled for psychomotor speed. *Journal of neurodevelopmental disorders*, 4(1), p.26.

Yrigollen, C.M. et al., 2012. AGG interruptions within the maternal FMR1 gene reduce the risk of offspring with fragile X syndrome. *Genetics in Medicine*, 14(8), pp.729–736.

Yrigollen, C.M. et al., 2014. Distribution of AGG interruption patterns within nine world populations. *Intractable & Rare Diseases Research*, 3(4), pp.153–161.

Yu, S. et al., 1991. Fragile X genotype characterized by an unstable region of DNA. *Science (New York, N.Y.)*;252(5009), pp.1179-1181.

Zongaro, S. et al., 2013. The 3' UTR of FMR1 mRNA is a target of miR-101, miR-129-5p and miR-221: Implications for the molecular pathology of FXTAS at the synapse. *Human Molecular Genetics*, 22(10), pp.1971–1982.

Zuñiga, A. et al., 2005. Expansion of an intermediate allele of the FMR1 gene in only two generations [2]. *Clinical Genetics*, 68(5), pp.471–473.

ANEXOS

In: Allelic Forms of the FMR1 Gene
Editor: Montserrat Milà ISBN: 978-1-63321-914-4
© 2015 Nova Science Publishers, Inc.

Chapter 5

CLINICAL FEATURES ASSOCIATED WITH *FMR1* PREMUTATION CARRIERS

MI. Alvarez-Mora[1,2] and L. Rodriguez-Revenga*[1-3]
[1] Centre for Biomedical Network Research of Rare Disease (CIBERER), Barcelona, Spain
[2] Biochemistry and Molecular Genetics Department, Hospital Clinic, Barcelona, Spain
[3] IDIBAPS (Institut d'Investigacions Biomèdiques August Pi i Sunyer). Barcelona, Spain.

ABSTRACT

The expansion of the CGG trinucleotide located within the 5'UTR of the *FMR1* gene is involved in a growing number of diseases; the most well-established are Fragile X Syndrome (FXS), Fragile X Tremor/Ataxia Syndrome (FXTAS) and Fragile X Primary Ovarian Insufficiency (FXPOI). Whereas full mutation alleles (>200CGGs) are responsible for the FXS, smaller expansions called premutation alleles (55-200CGGs) are associated with FXTAS and FXPOI. Numerous evidence have currently been reported suggesting that premutation alleles give rise to an increased risk for carriers of these alleles in relation to additional medical, psychiatric and cognitive features which occur at a greater frequency than what would be expected for the general population. In this chapter, we review the clinical features including peripheral neuropathy, immune-mediated disorders,

*Laia Rodriguez-Revenga
lbodi@clinic.ub.es

migraines and neurocognitive involvement which have been suggested to be associated with premutation alleles. In addition, the current understanding of the pathogenic molecular mechanisms that give rise to the spectrum of *FMR1* premutation associated disorders is also reviewed. Although further research is needed in order to shed light on the factors underlying the common incomplete penetrance applicable to all phenotypes associated with the premutation, it is likely that a combination of environmental and genetic factors with differences in intrinsic susceptibility may modulate the appearance and the severity of these disorders.

Keywords: *FMR1* premutation, fibromyalgia, thyroid disease, peripheral neuropathy

INTRODUCTION

In the last years, there has been intense interest in identifying and characterizing the Fragile X premutation-associated phenotypes from the perspective not only of basic science but also of public health given its high prevalence affecting 1:250 females and 1:800 males among the general population [1]. Historically, carriers of *FMR1* premutation (PM) alleles were considered to be clinically unaffected, since the gene is generally not methylated and these individuals do not present intellectual disabilities (ID). The significance of these alleles was generally thought to be their propensity for expansion to the full mutation range (>200 CGG repeats) during maternal transmission resulting in transcriptional silencing of *FMR1* gene, absence of the encoding fragile X mental retardation 1 protein (FMRP) and manifestation of fragile X syndrome (FXS) [2,3]. Although, the features of this expansion are not fully understood, it is currently accepted that they depend on the size of the maternal allele and also on the number of the AGG interruptions within the CGG-repeat track [4]. The AGG interruptions are likely to have stabilizing effects during transmission by decreasing the risk of DNA polymerase slippage during DNA replication [5]. In PM male carriers there is a relative stable transmission, and thus, the risk of expansion to a full mutation is negligible [reviewed in 6].

Despite the belief that PM carriers do not present signs of clinical involvement, prior to the

discovery of the gene *FMR1* Cronister and collaborators (1991) [7] reported, higher rates of premature ovarian failure (POF) among women heterozygous for X-chromosome fragility. Later, Allinghan-Hawkins and colleagues (1999) [8] established PM alleles as a significant risk factor for POF based on the study of 760 women in which 16% of PM carriers were affected with POF whereas none of the full mutation carriers and just one (0.4%) of the controls presented with POF [8]. Currently, the association between ovarian deficiency and PM female carriers, namely Fragile X-Primary ovarian Insufficiency (FXPOI), is well-established, presenting an incidence of around 20% in these women and estimated at around 1% among the general population. Chapter 2 describes the features of FXPOI. Ten years after, the first description of a neurodegenerative disorder named Fragile X Tremor Ataxia Syndrome (FXTAS) associated to older adults PM carriers was made by Hagerman and colleagues (2001) [9]. This syndrome is characterized by white matter changes and global brain atrophy, presenting with core features of intention tremor and gait ataxia. Details of FXTAS are described in Chapter 3.

Over the past 10-15 years, an increasingly broad spectrum of clinical manifestations has been related to individuals who are carriers of PM alleles (Table 1). Domains of clinical involvement seen in some, but not all carriers of PM encompass the presence of medical, emotional and cognitive manifestations which have been widely reported to occur more frequently among PM carriers than in the general population. Some of these features have recently been classified as being 'definitely related', 'probably related', 'possibly related' or 'not likely related' to the molecular changes associated with an *FMR1* expansion based on clinical and previously reported data [reviewed in 10]. Coffey and collaborators (2008) [11] reported the first evidence of an expanded clinical phenotype of women with the PM. In this study, PM female carriers without core features of FXTAS showed significantly more complaints of chronic muscle pain, persistent paraesthesias in the extremities, and a history of tremor than controls. Furthermore, a significantly greater presence of medical co-morbidity was detected in females with definite or probable FXTAS, with an increased prevalence of thyroid disease, hypertension, seizures, peripheral neuropathy, fibromyalgia compared with

controls [11]. In addition, some of the comorbidites associated with FXTAS beyond central nervous system involvement, specifically peripheral neuropathy [11,12] and neuroendocrine dysfunction [11,13-15] have also been associated with PM carriers without FXTAS. Moreover, there is increasing evidence that young PM carriers present increased rates of neurodevelopmental phenotypes such as attention-deficit hyperactivity disorder (ADHD), autism spectrum disorder (ASD) and seizures [reviewed in 16]. Furthermore, increased rates of psychiatric involvement, particularly depression and anxiety have also been associated with FXTAS among adult PM carriers [reviewed in 17]. Neuropsychiatric aspects are discussed in Chapter 4.

Table 1. Clinical manifestations associated with some *FMR1* premutation carriers.

	Cohort studied*	**References**
Immune mediated disorders		
Fibromyalgia	PM females	[11-15]
Thyroid disease	PM females	[11,13,15]
Irritable bowel syndrome	PM females	[15]
Neurodevelopmental Phenotypes		
Working memory deficiencies	PM males	[27,28]
Language dysfluencies	PM females	[29]
Spatiotemporal processing impairment	Young-adult PM carriers	[32]
Arithmetic weaknesses	PM females	[30,31]
Developmental Delay	PM carriers	[75]
Reproductive features		
Ovarian Insufficiency (FXPOI)	PM females	[reviewed in 10]
Obstetric and perinatal difficulties	PM females	
Estrogen-deficiency related conditions	FXPOI PM females	
Autonomic dysfunction		
Impotence	FXTAS PM males	[56]
Hypertension	FXTAS PM both	[11,67]
Bowel and bladder incontinence	FXTAS PM both	[18,68]
Neurocognitive and phychiatric Involvement		
Depression	PM females	[69-71]
Anxiety disorders	PM females	
Mood disorders	PM females	
Seizures and ASD	PM male children	[72,73]
ADHD	PM females	[74]
Other clinical manifestations		
Migraine	PM carriers both	[25]
Peripheral neuropathy	PM carriers both	[11,76]

CLINICAL FEATURES ASSOCIATED WITH *FMR1* PREMUTATION CARRIERS

Neuropathy

Peripheral neuropathy is characterized by a damaging of peripheral nerves which carry information to and from the brain as well as to and from the spinal cord to the rest of the body. Depending on the type of nerve affected it may promote impaired sensation, movement, gland or organ function. The association between neuropathy and *FMR1* alleles was first reported in PM carriers with FXTAS [9,18]. Thereafter, Berry-Kravis and collaborators (2007) [12] reported the first evidence that signs of neuropathy on clinical examination are associated with PM carrier status based on neurological examination data from 207 unrelated individuals. Results from this study revealed that the degree of clinical involvement strongly correlated with the CGG repeat length in males since these individuals presented significantly higher mean scores in the neuropathy screening scale score (P=0.0014), the vibration score (P=0.0015), and the reflex score (P=0.0014) than sex-matched controls suggesting that PM male carriers present higher impairment of both distal vibratory sense and reflexes [12]. The lack of significant differences among PM female carriers presumably reflects the broad variation in clinical involvement among carriers as a result of variation in the X-chromosome activation ratio as well as the decreased penetrance of the clinical manifestation due to the protective effect of the second non-mutated X chromosome [12]. Afterwards, Coffey and collaborators (2008) [11] demonstrated that females with PM alleles also presented significantly higher signs of neuropathy based on findings from 128 PM female carriers without FXTAS. In this study it was shown that these women presented a significant rate of numbness and tingling and muscle pain in the extremities, albeit evidence of neuropathy is increased in PM female carriers presenting with FXTAS [11]. It has been suggested that neuropathic symptoms in PM female carriers are manifested together with the emergence of FXTAS disease [reviewed in 10].

Immune-mediated disorders

Numerous reports support elevated rates of immune-mediated disorders (IMD) in PM female carriers, particularly regarding hypothyroidism and fibromyalgia [11, 13-15]. In contrast, IMDs

have not been evidenced among males with the premutation, likely due to the relative rarity of these disorders among the general male population. In a recent study, Winarni and colleagues (2012) [15] examined the relative likelihood of large clinical manifestations among 344 female carriers of PM alleles and 72 controls including autoimmune thyroid disorder, multiple sclerosis, Sjögren syndrome, rheumatoid arthritis, systemic lupus erythematosus, Raynaud's phenomenon, irritable bowel syndrome and optic neuritis. The results of this study evidenced that among women over 40 years of age 46.54% of PM females without FXTAS experienced one or more of the IMDs surveyed, and the prevalence increased to about 72.73% for those with FXTAS compared to 31.58% for the control group [15]. With respect to FXPOI, both groups of PM females carriers present higher odds ratios of IMDs compared to controls, and similarly, when considering FXTAS symptoms, the odds ratio of IMDs among PM female carriers presenting with FXPOI is about 2.4-fold higher when compared to those without FXPOI. Moreover, these authors found that an autoimmune thyroid disorder was the most common IMD followed by fibromyalgia and irritable bowel syndrome [15].

Increased penetrance for both thyroid disease and fibromyalgia has been broadly reported among PM female carriers [13], although the penetrance of these disorders is highly variable among the general population since it increases with age. For thyroid disease, it has been suggested that the association with PM alleles may be more relevant in older women [10] due to the lack of statistical significance when considering women between 18 and 50 years of age [19]. Nonetheless, in the general population the penetrance estimated for thyroid disorders is of around 10% [20] and around 2-4% for fibromyalgia whereas it has been estimated to be around 15.9% and 24.4%, respectively, among PM female carriers [13]. Conversely, in 700 unrelated Spanish patients with fibromyalgia the frequency of PM alleles did not significantly differ from the estimated rate in the general population [21]. In contrast, another study found a higher incidence of PM alleles among a Spanish female fibromyalgia cohort. Indeed, the incidence of PM alleles was 1 of 88, being 1 of 250 in females in the general population [22]. These controversial results are likely to be caused by a sample size

effect since the data reported by Martorell and colleagues (2012) [22] were based on the screening of 353 females whereas the data presented by Rodriguez-Revenga and coworkers (2013) [21] were based on 700 samples. Nonetheless, it has been shown that the pathophysiology of fibromyalgia involves hyperexcitability of central neurons through several synaptic and neurotransmitter/neurochemical mechanisms [reviewed in 23] suggesting that it could arise through an alteration of pain neurotransmissor mechanisms among PM female carriers [14].

Finally, it has been recently demonstrated that individual carriers of PM alleles present an immune dysregulation and decreased immune responses when compared with healthy controls [24]. Moreover, it has been found that PM carriers present a reduction in the levels of cytokine production which is negatively associated with CGG repeat length, mainly with IL-12 production [24]. Furthermore, these women have also been shown to present a decrease in the relative levels of the surface marker CD25 in T cells suggesting potential differences in the activation of T-cells that regulate immune response [24].

Migraines

Au and collaborators (2013) [25] have recently reported that PM carriers show increased rates in the prevalence of migraines based on physical and medical examination of 315 PM carriers (203 females and 112 males) and 154 controls (83 females and 71 males). Migraine is a neurologic disorder characterized by light and sound sensitivity and pulsatile pain, which is thought to have a polygenic and mutlifactorial etiology. The prevalence of migraine among the general population is estimated around 27.3% in women and 9.7% in men [26], reaching up to 54.2% and 26.79% among female and male PM carriers, respectively, both resulting in statistical significant differences [25]. In addition, this significance was obtained considering both those affected with and also those without FXTAS, adjusted for age. However, the risk of migraine headaches was not correlated with either CGG repeats or *FMR1* mRNA expression [25].

Neurocognitive features

The expanded range of clinical involvement associated with PM carriers also includes an alteration

of various cognitive domains including executive function, working memory and arithmetic skills which become apparent even in young individuals, with a usually more progressive course in PM individuals than in the general population [reviewed in 10]. However, neurocognitive deficits are reportedly more frequent in male than in female carriers of PM alleles. Results reported by Kogan and collaborators (2008) [27] revealed that the CGG expansion confers a significant risk for working memory difficulties based on a controlled study of 40 PM male carriers without manifest symptoms of FXTAS. In addition, Cornish and colleagues (2009) [28] reported neuropsychological measures in PM males regarding core subcomponents of working memory such as verbal memory, visual–spatial memory, and central executive memory revealing that PM males present specific vulnerability in executive control of memory including tasks requiring simultaneous manipulation and storage of new information, regardless of the presence of FXTAS symptoms. These authors revealed an impairment of the central executive working memory among PM male carriers without FXTAS, which was significantly correlated with larger CGG repeat expansions, whereas FXTAS patients demonstrated a more general impairment in terms of phonological working memory in addition to central executive working memory [28]. Moreover, language dysfluencies associated with deficits in organization and planning have been evidenced among PM female carriers [29]. Past research has demonstrated that language dysfluencies are an indicator of executive functioning deficits which are characteristic of other neurodegenerative disorders such as Parkinson and Alzheimer diseases.

Regarding arithmetic skills it has been suggested that PM female carriers show weaknesses in mathematical tasks [30] and this has recently been supported by other groups [31]. Furthermore, it has been demonstrated that PM carriers from 19 to 45 years of age show impairment in spatiotemporal processing which may underlie the impairments observed in arithmetic skills among these individuals since the representations of space and time provide the foundation for an understanding of numbers [32].

Although further research is needed, it has been suggested that determining whether cognitive

impairments are detectable in PM carriers without FXTAS should be prudent since it may not only be an early indicator of cognitive decline in PM carriers [29] but could also be used as a biomarker of disease progression if these features precede motor impairment [32].

CURRENT UNDERSTANDING OF THE MOLECULAR MECHANISMS UNDERLYING PREMUTATION-ASSOCIATED PATHOLOGIES

Premutation-associated phenotypes have been mainly attributed to a pathogenic mechanism involving a gain-of-function toxicity of the expanded *FMR1* mRNA, a process entirely distinct from the FMRP deficiency responsible for the FXS phenotype [reviewed in 33]. This observation was based on the restriction of these clinical phenotypes to the premutation range in which the molecular signature is a 2-8 fold increase in the expression of the PM mRNA and, paradoxically, a slight reduction in Fragile X Mental Retardation Protein (FMRP) levels [34]. The mechanisms underlying the increased transcriptional activity of the PM alleles remain unclear, however, it has been reported that these alleles use differential transcriptional start sites leading to different expression compared to non-expanded *FMR1* alleles [35]. On other hand, it has also been suggested that the reduction of FMRP in PM carriers may promote an added contribution to the clinical involvement observed in both children and adults related to phenotypes associated with reduced cognition and disturbed behavior [36]. However, the FMRP deficiency cannot be a driving factor in the PM-associated disorders since FXTAS and FXPOI are not experienced by full mutation carriers. Despite wide reports that the levels of FMRP expression are only slightly decreased in patients with FXTAS, most of these measurements have been performed using lymphocytes or whole blood samples rather than brain tissue, in which changes are likely to be more robust [reviewed in 37]. The efficiency of FMRP translation is associated with the length of the CGG expansion in light of impairment in translation of larger CGG tracks by interference with ribosomal scanning through the 5'UTR, thereby preventing appropriate loading of the expanded *FMR1* mRNA into polyribosomal complexes [38-40].

It is currently considered that the dual mechanism of involvement, the excess of PM mRNA

expression and the decrease in FMRP translation, is a double hit which may promote phenotypic features of FXS and PM associated disorders [reviewed in 33, 41]. Notwithstanding, the reduction of FMRP synthesis, the phenotype of PM carriers, is different from carriers of full mutation alleles since milder protein deficiency among PM carriers usually leads to mild developmental problems with these individuals having higher IQs and less severe behavioral problems than those with FXS [reviewed in 42]. A FMRP deficit has been correlated with lowered activity of the amygdala among PM male carriers compared to controls on functional magnetic resonance imaging whereas increased levels of expanded mRNA have been strongly associated with obsessive–compulsive symptoms and psychoticism in PM male carriers [43,44]. It has also been described that RNA toxicity leads to the up-regulation of the heat shock proteins Hsp70 and αB-crystallin, which may stimulate immune dysregulation [15]. Regarding migraines, their association with mitochondrial dysfunction is well established. Interestingly, there is evidence pointing to a deregulation of mitochondrial function among PM carriers [45-47], therefore the increased prevalence of migraines in PM carriers may be the result of RNA toxicity leading to mitochondrial deregulation [25]. Likewise, RNA toxicity has also been proposed to shed light on the high rate of thyroid dysfunction among females with the PM by causing a direct effect on the hypothalamic-pituitary-adrenal axis or on the thyroid gland. Additionally, RNA toxicity has also been proposed to promote an autoimmune mechanism or apoptosis in thyroid cells [11]. However, data reported by Cunningham and coworkers (2011) [48] show that the presence of PM alleles promotes migration defects in the neocortex and altered expression of neuronal lineage markers among embryonic PM mice. These results support the hypothesis that the role of the RNA toxicity may be restricted to the initial triggering events since many features of the neuronal and astrocytic cellular phenotype observed in FXTAS patients are already present in the neonatal period, suggesting that the clinical involvement among children carriers of PM alleles may be manifestations of this early, non-degenerative process [reviewed in 33].

The sequestration hypothesis of RNA toxicity was first proposed and established for myotonic

dystrophy type 1 caused by an expansion of a CTG repeat in the 3'UTR of *DMPK* gene [reviewed in 49]. Particularly, in Fragile X PM the model hypothesized that expanded CGG repeats form hairpin loops which are sticky and recruit an excess of specific RNA-binding proteins, resulting in a functional insufficiency of the sequestered proteins and leading to cell dysfunction and death [50]. Although the initial triggering events in these disorders are based on RNA toxicity, this model does not provide evidence regarding cell sickening and death. In this way, there are several candidate downstream pathways, although alterations of both mitochondrial function and calcium regulation are emerging as core mediators of cellular deregulation and dysfunction [reviewed in 31, 39] The increased expression of the expanded *FMR1* mRNA is thought to be the main cause of clinical involvement in PM carriers since the *FMR1* mRNA and the sequestered proteins form aggregates leading to intranuclear inclusions present in several tissues. Interestingly, the expanded mRNA of *FMR1* is detected within the inclusion whereas FMRP is not present [51]. Furthermore, proteomic analysis of these inclusions revealed a large number of proteins presented in the aggregates, including the RNA binding protein hnRNP A2/B1, the nuclear envelope protein lamin A/C, the small heat shock protein αB-crystallin [52], the splicing factor Sam68 [53] and part of the microRNA processor complex DGCR8 [50]. Indeed, it has recently been demonstrated that the sequestration of DGCR8 promotes the deregulation of microRNAs biogenesis, suggesting a central role of DGCR8 as an inductor of RNA toxicity by leading to cell dysfunction and cell loss [50].

Intranuclear inclusions, which represent the neuropathological hallmark of FXTAS [52], have been also detected among PM carriers through different cell types including the central and peripheral nervous system and other tissue including the adrenal glands, the testes, pancreas and heart [54-58]. Recently, Hunsaker and colleagues (2011) [58] reported autopsy findings from ten PM carriers with FXTAS in which intranuclear inclusions were detected throughout multiple tissues including the hypothalamic-pituitary-adrenal axis, pineal gland, cardiac conduction system, peripheral nerves and autonomic ganglia, the thyroid gland, the digestive system, the testes and pancreas. The broad distribution of these inclusions suggests that many organ systems may be affected by RNA toxicity.

Nevertheless, it is necessary to study the processes underlying inclusion formation in depth to address whether they themselves are toxic or reflect cellular dysfunction [58].

Furthermore, additional mechanisms have been proposed as possible triggering events in the PM-associated disorders, although most evidence support CGG-repeat mediated protein sequestration [reviewed in 33,37]. These mechanisms include a RNA-mediated protein aggregation model whereby the CGGs contained within the *FMR1* mRNA might promote a conformational transition in proteins with prion-like domains that may initiate a cascade of protein aggregation similar to what occurs in amyloid plaque formation in Alzheimer's disease. Moreover, the production of a toxic polyglycine peptide has also been proposed as an alternative toxicity model by a non-AUG-initiated (RAN) translation [59]. In addition, a specific splicing isoform is detected exclusively with transcripts of PM alleles suggesting a possible role of antisense transcripts generated at the *FMR1* locus [60].

Otherwise very little attention has been focused on the other end of the spectrum, the so-called "low-normal" numbers of CGG repeats, up to 23 trinucleotide repeats. In this framework, data based on genotype–phenotype correlations have recently been reported suggesting that this range of CGG repeats may have substantial implications for cognitive functioning, cancer, and the odds of having children with neurodevelopmental or neuropsychiatric conditions [61]. Despite these range of CGG number not being associated with altered FMRP synthesis, Chen and co-workers (2003) [62] reported that the efficiency of FMRP translation was based on the number of CGG repeats, conferring the greatest efficiency of protein synthesis to the allele of 30 repeats. Thus, inefficient translation may be related to the clinical manifestations associated with the low numbers of CGG repeats. Likewise, Ramocki and Zoghbi (2008) [63] suggested that imbalances in homeostatic controls of multiple genes including *FMR1* may partially promote the appearance of neurodevelopmental and neuropsychiatric disorders.

At this point, the difficulty in understanding PM-associated pathologies lies in the inability of prediction of which PM carriers will develop any of these phenotypes. The incomplete penetrance

across the phenotypic spectrum is likely to be associated with a combination of genetic and environmental factors which may confer specific vulnerability to PM carriers (Figure 1).

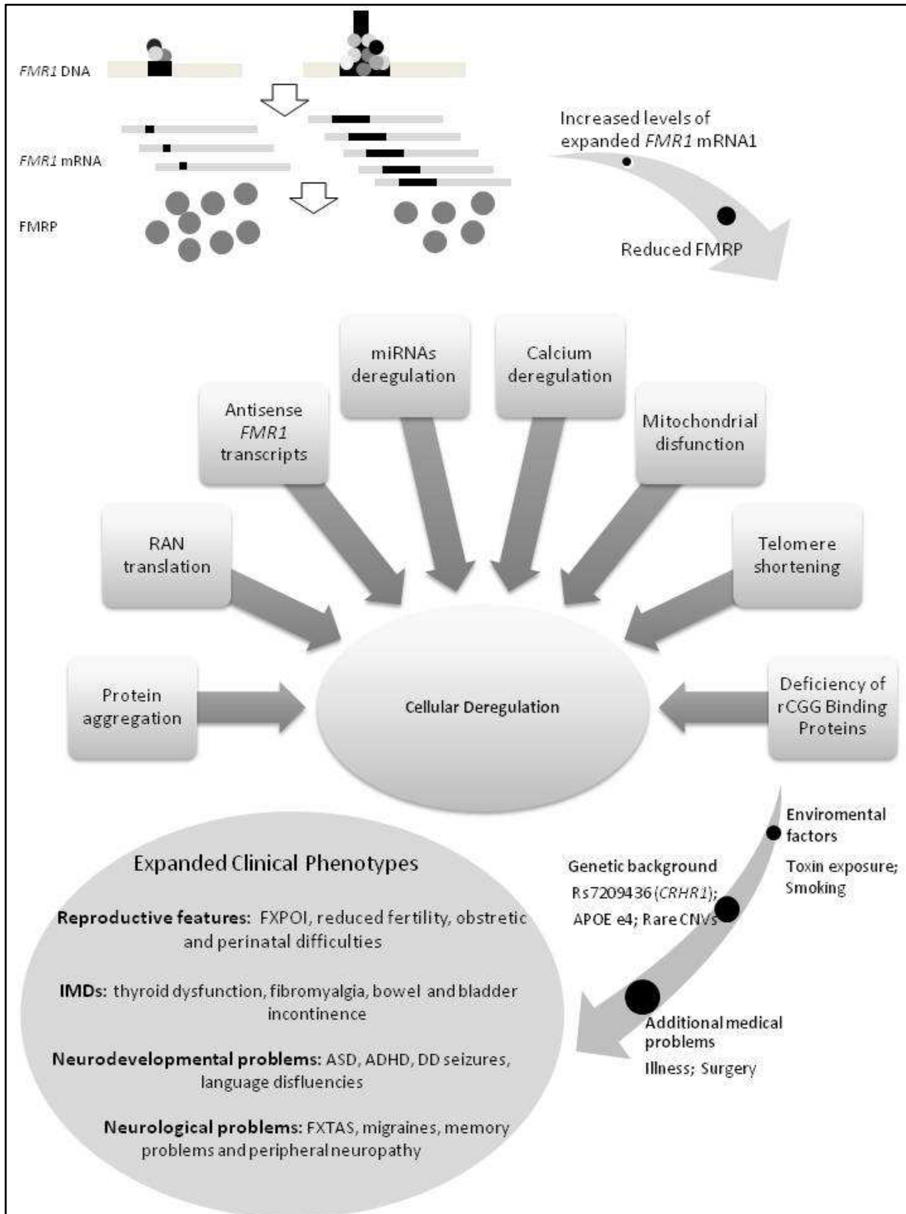


Figure 1. Diagram of the potential players contributing to the clinical involvement associated to PM carriers

Genetic factors that may contribute to the PM-associated disorders include CGG repeat length, expression levels of the expanded *FMR1* mRNA, aberrant translation of the repeat sequence as well as genomic changes in other regions of the genome. Within this framework, specific polymorphisms of the *CRHR1* gene have been associated with female clinical involvement (rs7209436), particularly with depression and anxiety mainly as this gene regulates the expression and release of ACTH from the anterior pituitary gland which, in turn, stimulates the release of cortisol from the adrenal cortex [64]. Furthermore, risk factors for other neurodegenerative disorders such as allele ε4 of the *APOE* gene may also influence the risk of FXTAS as a higher frequency of these alleles has been reported in PM carriers with compared to those without FXTAS [65]. Moreover, it has recently been suggested that individuals who are carriers of PM alleles presenting with ID, seizures or ASD are likely to have a second hit since PM carriers show a significant enrichment (P=2.27e-07) of CNVs compared to controls [66]. Furthermore, these authors found an association between the presence of rare CNVs (not detected in 8000 controls) among PM carriers with either autistic traits or neurological involvement, suggesting that they may have a possible role in this phenotypic variability [66]. Regarding environmental factors, it has been suggested that smoking, prolonged surgery with anesthesia, drug and alcohol abuse or the stress of carrying a child with FXS are also puzzling factors which may act as additional determinants for the phenotypic variability among PM individuals [reviewed in 41,42]. Finally, further longitudinal studies are required to determine the context in which any of the PM-associated phenotypes are developed and what protective factors might reduce the risks of more negative outcomes [reviewed in 10].

CONCLUSION

Overall, it is currently accepted that PM alleles led to multiple distinct clinical features which are present at a greater frequency among PM carriers than what would be expected in the general population. However, the association of the PM with the phenotypes reviewed in this chapter is less well established than its association with FXTAS and FXPOI. Although further research is needed in order to shed light on the factors underlying the common incomplete penetrance applicable to all

phenotypes associated with the PM, a combination of environmental and genetic factors with differences in intrinsic susceptibility likely modulate the appearance and the severity of these disorders.

REFERENCES

1. Hagerman PJ. The fragile X prevalence paradox. J Med Genet. 2008 Aug;45(8):498-9. Erratum in: J Med Genet. 2008 Nov;45(11):768.
2. Rousseau F, Heitz D, Biancalana V, Blumenfeld S, Kretz C, Boué J, Tommerup N, Van Der Hagen C, DeLozier-Blanchet C, Croquette MF, et al. Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. N Engl J Med. 1991 Dec 12;325(24):1673-81.
3. Nolin SL, Lewis FA 3rd, Ye LL, Houck GE Jr, Glicksman AE, Limprasert P, Li SY, Zhong N, Ashley AE, Feingold E, Sherman SL, Brown WT. Familial transmission of the FMR1 CGG repeat. Am J Hum Genet. 1996 Dec;59(6):1252-61.
4. Yrigollen CM, Tassone F, Durbin-Johnson B, Tassone F. The role of AGG interruptions in the transcription of PM alleles. PLoS One. 2011;6(7):e21728.
5. Gacy AM, Goellner G, Juranić N, Macura S, McMurray CT. Trinucleotide repeats that expand in human disease form hairpin structures in vitro. Cell. 1995 May 19;81(4):533-40.
6. Brouwer JR, Willemsen R, Oostra BA. Microsatellite repeat instability and neurological disease. Bioessays. 2009 Jan;31(1):71-83. doi: 10.1002/bies.080122.
7. Cronister A, Schreiner R, Wittenberger M, Amiri K, Harris K, Hagerman RJ. Heterozygous fragile X female: historical, physical, cognitive, and cytogenetic features. Am J Med Genet. 1991 Feb-Mar;38(2-3):269-74.
8. Allingham-Hawkins DJ, Babul-Hirji R, Chitayat D, Holden JJ, Yang KT, Lee C, Hudson R, Gorwill H, Nolin SL, Glicksman A, Jenkins EC, Brown WT, Howard-Peebles PN, Becchi C, Cummings E, Fallon L, Seitz S, Black SH, Vianna-Morgante AM, Costa SS, Otto PA, Mingroni-Netto RC, Murray A, Webb J, Vieri F, et al. Fragile X premutation is a significant risk factor for premature ovarian failure: the International Collaborative POF in Fragile X study--preliminary data. Am J Med Genet. 1999 Apr 2;83(4):322-5.
9. Hagerman RJ, Leehey M, Heinrichs W, Tassone F, Wilson R, Hills J, Grigsby J, Gage B, Hagerman PJ. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology. 2001 Jul 10;57(1):127-30.
10. Wheeler AC, Bailey DB Jr, Berry-Kravis E, Greenberg J, Losh M, Mailick M, Milà M, Olichney JM, Rodriguez-Revenga L, Sherman S, Smith L, Summers S, Yang JC, Hagerman R. Associated features in females with an PM. J Neurodev Disord. 2014;6(1):30. Review.
11. Coffey SM, Cook K, Tartaglia N, Tassone F, Nguyen DV, Pan R, Bronsky HE, Yuhas J, Borodyanskaya M, Grigsby J, Doerflinger M, Hagerman PJ, Hagerman RJ. Expanded

clinical phenotype of women with the PM. Am J Med Genet A. 2008 Apr 15;146A(8):1009-16

12. Berry-Kravis E, Goetz CG, Leehey MA, Hagerman RJ, Zhang L, Li L, Nguyen D, Hall DA, Tartaglia N, Cogswell J, Tassone F, Hagerman PJ. Neuropathic features in fragile X premutation carriers. Am J Med Genet A. 2007 Jan 1;143A(1):19-26.
13. Rodriguez-Revenga L, Madrigal I, Pagonabarraga J, Xunclà M, Badenas C, Kulisevsky J, Gomez B, Milà M. Penetrance of FMR1 premutation associated pathologies in fragile X syndrome families. Eur J Hum Genet. 2009 Oct;17(10):1359-62.
14. Leehey MA, Legg W, Tassone F, Hagerman R. Fibromyalgia in fragile X mental retardation 1 gene premutation carriers. Rheumatology (Oxford). 2011 Dec;50(12):2233-6.
15. Winarni TI, Chonchaiya W, Sumekar TA, Ashwood P, Morales GM, Tassone F, Nguyen DV, Faradz SM, Van de Water J, Cook K, Hamlin A, Mu Y, Hagerman PJ, Hagerman RJ. Immune-mediated disorders among women carriers of fragile X premutation alleles. Am J Med Genet A. 2012 Oct;158A(10):2473-81.
16. Besterman AD, Wilke SA, Mulligan TE, Allison SC, Hagerman R, Seritan AL, Bourgeois JA. Towards an Understanding of Neuropsychiatric Manifestations in Fragile X Premutation Carriers. Future Neurol. 2014 Mar;9(2):227-239.
17. Hunter JE, Abramowitz A, Rusin M, Sherman SL. Is there evidence for neuropsychological and neurobehavioral phenotypes among adults without FXTAS who carry the PM? A review of current literature. Genet Med. 2009 Feb;11(2):79-89.
18. Jacquemont S, Hagerman RJ, Leehey M, Grigsby J, Zhang L, Brunberg JA, Greco C, Des Portes V, Jardini T, Levine R, Berry-Kravis E, Brown WT, Schaeffer S, Kissel J, Tassone F, Hagerman PJ. Fragile X premutation tremor/ataxia syndrome: molecular, clinical, and neuroimaging correlates. Am J Hum Genet. 2003 Apr;72(4):869-78.
19. Hunter JE, Rohr JK, Sherman SL. Co-occurring diagnoses among FMR1 premutation allele carriers. Clin Genet. 2010 Apr;77(4):374-81.
20. Canaris GJ, Manowitz NR, Mayor G, Ridgway EC. The Colorado thyroid disease prevalence study. Arch Intern Med. 2000 Feb 28;160(4):526-34.
21. Rodriguez-Revenga L, Madrigal I, Blanch-Rubió J, Elurbe DM, Docampo E, Collado A, Vidal J, Carbonell J, Estivill X, Mila M. Screening for the presence of PM alleles in women with fibromyalgia. Gene. 2013 Jan 10;512(2):305-8.
22. Martorell L, Tondo M, Garcia-Fructuoso F, Naudo M, Alegre C, Gamez J, Genovés J, Poo P. Screening for the presence of FMR1 premutation alleles in a Spanish population with fibromyalgia.Clin Rheumatol. 2012 Nov;31(11):1611-5.
23. Yunus MB. Role of central sensitization in symptoms beyond muscle pain, and the evaluation of a patient with widespread pain. Best Pract Res Clin Rheumatol. 2007 Jun;21(3):481-97. Review.
24. Careaga M, Rose D, Tassone F, Berman RF, Hagerman R, Ashwood P. Immune dysregulation as a cause of autoinflammation in fragile X premutation carriers: link between FMRI CGG repeat number and decreased cytokine responses. PLoS One. 2014

Apr 9;9(4):e94475

25. Au J, Akins RS, Berkowitz-Sutherland L, Tang HT, Chen Y, Boyd A, Tassone F, Nguyen DV, Hagerman R. Prevalence and risk of migraine headaches in adult fragile X premutation carriers. Clin Genet. 2013 Dec;84(6):546-51

26. Lipton RB, Stewart WF, Diamond S, Diamond ML, Reed M. Prevalence and burden of migraine in the United States: data from the American Migraine Study II. Headache 2001: 41 (7): 646–657.

27. Kogan CS, Turk J, Hagerman RJ, Cornish KM. Impact of the Fragile X mental retardation 1 (FMR1) gene premutation on neuropsychiatric functioning in adult males without fragile X-associated Tremor/Ataxia syndrome: a controlled study. Am J Med Genet B Neuropsychiatr Genet. 2008 Sep 5;147B(6):859-72.

28. Cornish KM, Kogan CS, Li L, Turk J, Jacquemont S, Hagerman RJ. Lifespan changes in working memory in fragile X premutation males. Brain Cogn. 2009 Apr;69(3):551-8.

29. Sterling AM, Mailick M, Greenberg J, Warren SF, Brady N. Language dysfluencies in females with the PM. Brain Cogn. 2013 Jun;82(1):84-9.

30. Lachiewicz AM, Dawson DV, Spiridigliozzi GA, McConkie-Rosell A. Arithmetic difficulties in females with the fragile X premutation. Am J Med Genet A. 2006 Apr 1;140(7):665-72.

31. Semenza C, Bonollo S, Polli R, Busana C, Pignatti R, Iuculano T, Maria Laverda A, Priftis K, Murgia A. Genetics and mathematics: FMR1 premutation female carriers. Neuropsychologia. 2012 Dec;50(14):3757-63

32. Wong LM, Goodrich-Hunsaker NJ, McLennan Y, Tassone F, Harvey D, Rivera SM, Simon TJ. Young adult male carriers of the fragile X premutation exhibit genetically modulated impairments in visuospatial tasks controlled for psychomotor speed. J Neurodev Disord. 2012 Nov 13;4(1):26.

33. Hagerman P. Fragile X-associated tremor/ataxia syndrome (FXTAS): pathology and mechanisms. Acta Neuropathol. 2013 Jul;126(1):1-19.

34. Tassone F, Beilina A, Carosi C, Albertosi S, Bagni C, Li L, Glover K, Bentley D, Hagerman PJ. Elevated FMR1 mRNA in premutation carriers is due to increased transcription. RNA. 2007 Apr;13(4):555-62.

35. Tassone F, De Rubeis S, Carosi C, La Fata G, Serpa G, Raske C, Willemsen R, Hagerman PJ, Bagni C. Differential usage of transcriptional start sites and polyadenylation sites in FMR1 premutation alleles. Nucleic Acids Res. 2011 Aug;39(14):6172-85.

36. Tassone F, Hagerman RJ, Taylor AK, Mills JB, Harris SW, Gane LW, Hagerman PJ (2000) Clinical involvement and protein expression in individuals with the FMR1 premutation. Am J Med Genet 91(2):144–152

37. Renoux AJ, Todd PK. Neurodegeneration the RNA way. Prog Neurobiol. 2012 May;97(2):173-89.

38. Primerano B, Tassone F, Hagerman RJ, Hagerman P, Amaldi F, Bagni C. Reduced FMR1 mRNA translation efficiency in fragile X patients with premutations. RNA. 2002 Dec;8(12):1482-8.

39. Ludwig AL, Raske C, Tassone F, Garcia-Arocena D, Hershey JW, Hagerman PJ. Translation of the FMR1 mRNA is not influenced by AGG interruptions. Nucleic Acids Res. 2009 Nov;37(20):6896-904.

40. Ludwig AL, Hershey JW, Hagerman PJ. Initiation of translation of the FMR1 mRNA Occurs predominantly through 5'-end-dependent ribosomal scanning. J Mol Biol. 2011 Mar 18;407(1):21-34

41. Hagerman R, Hagerman P. Advances in clinical and molecular understanding of the FMR1 premutation and fragile X-associated tremor/ataxia syndrome. Lancet Neurol. 2013 Aug;12(8):786-98.

42. Polussa J, Schneider A, Hagerman R. (2014) Molecular Advances Leading to Treatment Implications for Fragile X Premutation Carriers. Brain Disord Ther 3:2.

43. Hessl D, Tassone F, Loesch DZ, Berry-Kravis E, Leehey MA, Gane LW, Barbato I, Rice C, Gould E, Hall DA, Grigsby J, Wegelin JA, Harris S, Lewin F, Weinberg D, Hagerman PJ, Hagerman RJ. Abnormal elevation of FMR1 mRNA is associated with psychological symptoms in individuals with the fragile X premutation. Am J Med Genet B Neuropsychiatr Genet. 2005; 139B (1):115–121.

44. Hoem G, Raske CR, Garcia-Arocena D, Tassone F, Sanchez E, Ludwig AL, Iwahashi CK, Kumar M, Yang JE, Hagerman PJ. CGG-repeat length threshold for FMR1 RNA pathogenesis in a cellular model for FXTAS. Hum Mol Genet. 2011; 20 (11):2161–2170.

45. Ross-Inta C, Omanska-Klusek A, Wong S, Barrow C, Garcia-Arocena D, Iwahashi C, Berry-Kravis E, Hagerman RJ, Hagerman PJ, Giulivi C. Evidence of mitochondrial dysfunction in fragile X-associated tremor/ataxia syndrome. Biochem J. 2010 Aug 1;429(3):545-52.

46. Napoli E, Ross-Inta C, Wong S, Omanska-Klusek A, Barrow C, Iwahashi C, Garcia-Arocena D, Sakaguchi D, Berry-Kravis E, Hagerman R, Hagerman PJ, Giulivi C. Altered zinc transport disrupts mitochondrial protein processing/import in fragile X-associated tremor/ataxia syndrome. Hum Mol Genet. 2011 Aug 1;20(15):3079-92.

47. Mateu-Huertas E, Rodriguez-Revenga L, Alvarez-Mora MI, Madrigal I, Willemsen R, Milà M, Martí E, Estivill X. Blood expression profiles of fragile X premutation carriers identify candidate genes involved in neurodegenerative and infertility phenotypes. Neurobiol Dis. 2014 May;65:43-54.

48. Cunningham CL, Martínez Cerdeño V, Navarro Porras E, Prakash AN, Angelastro JM, Willemsen R, Hagerman PJ, Pessah IN, Berman RF, Noctor SC. Premutation CGG-repeat expansion of the Fmr1 gene impairs mouse neocortical development. Hum Mol Genet. 2011 Jan 1;20(1):64-79.

49. Todd PK, Paulson HL. RNA-mediated neurodegeneration in repeat expansion disorders.

Ann Neurol. 2010 Mar;67(3):291-300. Review.

50. Sellier C, Freyermuth F, Tabet R, Tran T, He F, Ruffenach F, Alunni V, Moine H, Thibault C, Page A, Tassone F, Willemsen R, Disney MD, Hagerman PJ, Todd PK, Charlet-Berguerand N. Sequestration of DROSHA and DGCR8 by expanded CGG RNA repeats alters microRNA processing in fragile X-associated tremor/ataxia syndrome. Cell Rep. 2013 Mar 28;3(3):869-80.

51. Tassone F, Iwahashi C, Hagerman PJ. FMR1 RNA within the intranuclear inclusions of fragile X-associated tremor/ataxia syndrome (FXTAS). RNA Biol. 2004 Jul;1(2):103-5.

52. Iwahashi CK, Yasui DH, An HJ, Greco CM, Tassone F, Nannen K, Babineau B, Lebrilla CB, Hagerman RJ, Hagerman PJ. Protein composition of the intranuclear inclusions of FXTAS. Brain. 2006 Jan;129(Pt 1):256-71.

53. Sellier C, Rau F, Liu Y, Tassone F, Hukema RK, Gattoni R, Schneider A, Richard S, Willemsen R, Elliott DJ, Hagerman PJ, Charlet-Berguerand N. Sam68 sequestration and partial loss of function are associated with splicing alterations in FXTAS patients. EMBO J. 2010 Apr 7;29(7):1248-61.

54. Louis E, Moskowitz C, Friez M, Amaya M, Vonsattel JP. Parkinsonism, dysautonomia, and intranuclear inclusions in a fragile X carrier: a clinical-pathological study. Mov Disord. 2006 Mar;21(3):420-5.

55. Greco CM, Berman RF, Martin RM, Tassone F, Schwartz PH, Chang A, Trapp BD, Iwahashi C, Brunberg J, Grigsby J, Hessl D, Becker EJ, Papazian J, Leehey MA, Hagerman RJ, Hagerman PJ. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). Brain. 2006 Jan;129(Pt 1):243-55.

56. Greco CM, Soontrapornchai K, Wirojanan J, Gould JE, Hagerman PJ, Hagerman RJ. Testicular and pituitary inclusion formation in fragile X associated tremor/ataxia syndrome. J Urol. 2007 Apr;177(4):1434-7.

57. Gokden M, Al-Hinti JT, Harik SI. Peripheral nervous system pathology in fragile X tremor/ataxia syndrome (FXTAS). Neuropathology. 2009 Jun;29(3):280-4.

58. Hunsaker MR, Greco CM, Spath MA, Smits AP, Navarro CS, Tassone F, Kros JM, Severijnen LA, Berry-Kravis EM, Berman RF, Hagerman PJ, Willemsen R, Hagerman RJ, Hukema RK. Widespread non-central nervous system organ pathology in fragile X premutation carriers with fragile X-associated tremor/ataxia syndrome and CGG knock-in mice. Acta Neuropathol. 2011 Oct;122(4):467-79.

59. Todd PK, Oh SY, Krans A, He F, Sellier C, Frazer M, Renoux AJ, Chen KC, Scaglione KM, Basrur V, Elenitoba-Johnson K, Vonsattel JP, Louis ED, Sutton MA, Taylor JP, Mills RE, Charlet-Berguerand N, Paulson HL. CGG repeat-associated translation mediates neurodegeneration in fragile X tremor ataxia syndrome. Neuron. 2013 May 8;78(3):440-55

60. Ladd PD, Smith LE, Rabaia NA, Moore JM, Georges SA, Hansen RS, Hagerman RJ, Tassone F, Tapscott SJ, Filippova GN. An antisense transcript spanning the CGG repeat region of FMR1 is upregulated in premutation carriers but silenced in full mutation

individuals. Hum Mol Genet. 2007 Dec 15;16(24):3174-87

61. Mailick MR, Hong J, Rathouz P, Baker MW, Greenberg JS, Smith L, Maenner M. Low-normal FMR1 CGG repeat length: phenotypic associations.Front Genet. 2014 Sep 9;5:309

62. Chen LS, Tassone F, Sahota P, Hagerman PJ. The (CGG)n repeat element within the 5' untranslated region of the FMR1 message provides both positive and negative cis effects on in vivo translation of a downstream reporter. Hum Mol Genet. 2003 Dec 1;12(23):3067-74. Epub 2003 Sep 30.

63. Ramocki MB, Zoghbi HY. Failure of neuronal homeostasis results in common neuropsychiatric phenotypes. Nature. 2008 Oct 16;455(7215):912-8. Review.

64. Hunter JE, Leslie M, Novak G, Hamilton D, Shubeck L, Charen K, Abramowitz A, Epstein MP, Lori A, Binder E, Cubells JF, Sherman SL. Depression and anxiety symptoms among women who carry the PM: impact of raising a child with fragile X syndrome is moderated by CRHR1 polymorphisms. Am J Med Genet B Neuropsychiatr Genet. 2012 Jul;159B(5):549-59.

65. Silva F, Rodriguez-Revenga L, Madrigal I, Alvarez-Mora MI, Oliva R, Milà M. High apolipoprotein E4 allele frequency in FXTAS patients. Genet Med. 2013 Aug;15(8):639-42.

66. Lozano R, Hagerman RJ, Duyzend M, Budimirovic DB, Eichler EE, Tassone F. Genomic studies in fragile X premutation carriers. J Neurodev Disord. 2014;6(1):27.

67. Hamlin A, Liu Y, Nguyen DV, Tassone F, Zhang L, Hagerman RJ. Sleep apnea in fragile X premutation carriers with and without FXTAS. Am J Med Genet B Neuropsychiatr Genet. 2011;156B (8):923–928.

68. Leehey MA. Fragile X-associated tremor/ataxia syndrome: clinical phenotype, diagnosis, and treatment. J Investig Med. 2009 Dec;57(8):830-6. Review.

69. Rodriguez-Revenga L, Madrigal I, Alegret M, Santos M, Milà M. Evidence of depressive symptoms in fragile-X syndrome premutated females. Psychiatr Genet. 2008 Aug;18(4):153-5.

70. Bourgeois JA, Seritan AL, Casillas EM, Hessl D, Schneider A, Yang Y, Kaur I, Cogswell JB, Nguyen DV, Hagerman RJ. Lifetime prevalence of mood and anxiety disorders in fragile X premutation carriers. J Clin Psychiatry. 2011 Feb;72(2):175-82.

71. Hunter JE, Leslie M, Novak G, Hamilton D, Shubeck L, Charen K, Abramowitz A, Epstein MP, Lori A, Binder E, Cubells JF, Sherman SL. Depression and anxiety symptoms among women who carry the FMR1 premutation: impact of raising a child with fragile X syndrome is moderated by CRHR1 polymorphisms. Am J Med Genet B Neuropsychiatr Genet. 2012 Jul;159B(5):549-59.

72. Chonchaiya W, Au J, Schneider A, Hessl D, Harris SW, Laird M, Mu Y, Tassone F, Nguyen DV, Hagerman RJ. Increased prevalence of seizures in boys who were probands with the FMR1 premutation and co-morbid autism spectrum disorder. Hum Genet. 2012; 131 (4):581–589.

73. Farzin F, Perry H, Hessl D, Loesch D, Cohen J, Bacalman S, Gane L, Tassone F, Hagerman P, Hagerman R. Autism spectrum disorders and attention-deficit/hyperactivity disorder in boys with the fragile X premutation. J Dev Behav Pediatr. 2006; 27 (2 Suppl):S137–144.

74. Kraan CM, Hocking DR, Georgiou-Karistianis N, Metcalfe SA, Archibald AD, Fielding J, Trollor J, Bradshaw JL, Cohen J, Cornish KM. Impaired response inhibition is associated with self-reported symptoms of depression, anxiety, and ADHD in female FMR1 premutation carriers. Am J Med Genet B Neuropsychiatr Genet. 2014 Jan;165(1):41-51.

75. Bailey DB Jr, Raspa M, Olmsted M, Holiday DB. Co-occurring conditions associated with FMR1 gene variations: findings from a national parent survey. Am J Med Genet A. 2008; 146A (16): 2060–2069.

76. Hagerman RJ, Coffey SM, Maselli R, Soontarapornchai K, Brunberg JA, Leehey MA, Zhang L, Gane LW, Fenton-Farrell G, Tassone F, Hagerman PJ. Neuropathy as a presenting feature in fragile X-associated tremor/ataxia syndrome. Am J Med Genet A. 2007 Oct 1;143A(19):2256-60.

© American College of Medical Genetics and Genomics

ORIGINAL RESEARCH ARTICLE | Genetics inMedicine

High apolipoprotein E4 allele frequency in FXTAS patients

Francisca Silva, MSc[1,2], Laia Rodriguez-Revenga, PhD[1,3], Irene Madrigal, PhD[1,3], Maria Isabel Alvarez-Mora, MSc[1,3], Rafael Oliva, MD, PhD[1,4] and Montserrat Milà, PhD[1–3,5]

Purpose: Fragile X-associated tremor/ataxia syndrome is a late-onset neurodegenerative disorder that occurs in *FMR1* premutation carriers. It is well known that the apolipoprotein E ε4 allele is a risk factor for neurodegenerative disease. The main goal of this work was to evaluate the apolipoprotein E genotypes and allelic distribution among patients with fragile X-associated tremor/ataxia syndrome.

Methods: A total of 44 unrelated *FMR1* premutation carriers (22 presenting with fragile X-associated tremor/ataxia syndrome and 22 without fragile X-associated tremor/ataxia syndrome) were genotyped.

Results: All the apolipoprotein E ε4/4 genotype carriers detected (100%), and six of the seven apolipoprotein E ε4/3 genotype carriers (85.7%) are patients presenting with fragile X-associated tremor/ataxia syndrome symptoms, whereas only 40% of the apolipoprotein E ε3/3 genotype carriers belong to the fragile X-associated tremor/ataxia syndrome group. The results showed that the presence of the apolipoprotein E ε4 allele increases the risk of developing fragile X-associated tremor/ataxia syndrome (odds ratio = 12.041; $P = 0.034$).

Conclusion: On the basis of these results, we conclude that the presence of at least one apolipoprotein E ε4 allele might act as a genetic factor predisposing individuals to develop fragile X-associated tremor/ataxia syndrome.

Genet Med 2013:15(8):639–642

Key Words: apolipoprotein E; association; FXTAS; neurodegeneration

Fragile X-associated tremor/ataxia syndrome (FXTAS, OMIM no. 300623) is a late-onset neuropsychiatric degenerative disorder that occurs in *FMR1* premutation carriers (55–200 CGG repeats). Clinical symptoms, which appear in patients in their 50s or later, include action tremor, progressive cerebellar ataxia, peripheral neuropathy, autonomic dysfunction, cognitive decline, and dementia.[1–4] Magnetic resonance imaging in patients with FXTAS demonstrates mild to moderate cerebellar and brain atrophy, as well as white matter hyperintensities. In addition, hyperintensities in the middle cerebellar peduncles on T2 have been described as a characteristic finding in patients with FXTAS and therefore constitute a major diagnostic feature of the disorder.[3,5] It has been estimated that at least one-third of all *FMR1* premutation carriers will develop an FXTAS syndrome, although there is significant variability in the progression of neurological dysfunction.[2,6,7]

Apolipoprotein E (ApoE) is a lipoprotein that transports cholesterol and other lipids and lipid-soluble molecules into the central nervous system.[8–11] ApoE also modulates the inflammatory response to cellular damage in the brain.[12] The human *ApoE* gene shows polymorphic variation, and three alleles, designated as ApoE ε2, ε3, and ε4, are common in the general population.[13] Variant distribution of these alleles has been shown to be associated with a number of age-related diseases including atherosclerosis, cardiovascular disease, and neurodegenerative disorders.[14–16] Although the pathogenic mechanism involving ApoE in these diseases is still unclear, it has been demonstrated that the ApoE ε4 allele is a well-established genetic risk factor for neurodegenerative disorders including Alzheimer disease (AD), Parkinson disease, and other disorders in which dementia is present.[17–19] On the basis of this observation, we have evaluated the ApoE genotypes and allelic distribution among a *FMR1* premutation carrier cohort presenting with FXTAS. These data might contribute to uncover a new genetic risk factor for FXTAS and might be useful to identify new genes involved in the disease onset and progression.

METHODS

Subjects

A total of 44 unrelated *FMR1* premutation carriers (22 presenting with FXTAS symptoms and 22 without FXTAS clinical symptoms) were included. Samples from subjects belong to the Hospital Clinic of Barcelona and were molecularly diagnosed in the genetics laboratory of the same hospital. All participants were enrolled from families with members known to be affected with fragile X syndrome, and all of them are of Caucasian ethnicity. A classification on the basis of the gender and the age of the participants is summarized in **Table 1**. Although clinical data is scarce for some of the patients and we did not diagnose dementia in all of them, none of the cases included in the study had a diagnosis of AD. Overall, FXTAS encompasses patients who meet criteria in any of the three categories of involvement: definite, probable, and possible.[3] ApoE allele frequencies were compared with those of the control population reported by

[1]Department of Biochemistry and Molecular Genetics, Hospital Clinic, Barcelona, Spain; [2]Fundació Clínic per a la Recerca Biomèdica, Barcelona, Spain; [3]CIBER de Enfermidades Raras (CIBERER), Barcelona, Spain; [4]Faculty of Medicine, University of Barcelona, Barcelona, Spain; [5]IDIBAPS (Institut d'Investigacions Biomèdiques August Pi I Sunyer), Barcelona, Spain. Correspondence: Montserrat Milà (mmila@clinic.ub.es)

Submitted 4 December 2012; accepted 18 January 2013; advance online publication 14 March 2013. doi:10.1038/gim.2013.12

Adroer et al.[20] The study was accomplished in compliance with the Hospital Clinic ethics committee. Written informed consent was obtained from all the subjects before their participation.

Molecular analysis

ApoE genotyping was performed by polymerase chain reaction amplification as described in previous studies.[21] Allele frequencies were estimated by counting the alleles.

Statistical analyses

Differences in age, gender, CGG repeat number, and the presence of the ApoE ε4 allele among patients with and without FXTAS were evaluated by multivariate logistic regression analysis. A nonparametric test (Mann–Whitney *U* test) was applied to examine whether the presence of the ApoE ε4 allele is related to the age of onset of the disease. Moreover, ApoE ε4 allele frequency in FXTAS was compared with those previously described in AD patients and control population.[20] *P* values <0.05 were considered statistically significant. Statistical analyses were performed using commercially available software (SPSS SmartViewer, version 18.0; SPSS, Chicago, IL).

Table 1 Classification of the *FMR1* premutated individuals enrolled in the study

	FXTAS	Non-FXTAS	Total
Men (*n*)	14	7	21
Age			
Mean	70.1	71	
SD	9.1	16.7	
CGG repeat			
Mean	81.3	79.6	
SD	20.3	20.1	
Women (*n*)	8	15	23
Age			
Mean	61.6	55.1	
SD	10.8	12.5	
CGG repeat			
Mean	90.9	80.9	
SD	36.5	24.8	

FXTAS, fragile X-associated tremor/ataxia syndrome.

RESULTS

A total of 22 patients with FXTAS and 22 patients without FXTAS were genotyped for the ApoE locus. The mean ± SD of age and the mean ± SD of CGG repeat number for the FXTAS group were 67 ± 10.4 and 84.8 ± 26.9, respectively. The mean age for the non-FXTAS group was 60 years ±15.5, and the mean value for CGG repeat number was 80.5 ± 22.9 for the non-FXTAS group. When comparing the two groups, there were no significant differences in age (odds ratio (OR) = 1.048; *P* = 0.069) or in CGG repeat number (OR = 1.013; *P* = 0.313). However, significant differences were found in gender (OR = 4.46; *P* = 0.042; 95% confidence interval (CI) = 1.14–17.5) (**Table 1**) and the presence of the ApoE ε4 allele (OR = 12.041; *P* = 0.034; 95% confidence interval = 1.21–119.7) (**Table 2**). ApoE genotypes from *FMR1* premutation carriers (FXTAS and non-FXTAS) are given in **Table 2**. The only ApoE 4/4 genotype carrier detected, and six of the seven ApoE 4/3 genotype carriers were patients presenting with FXTAS symptoms (31.8% of all patients with FTXAS). By contrast, 95.5% of patients without FXTAS were ApoE 3/3 genotype carriers as compared with 62.8% of patients with FXTAS. The ApoE allele frequencies for patients with and without FXTAS are given in **Table 3**. With respect to age of disease onset, Mann–Whitney *U* test showed no statistically significant differences among patients with FXTAS carrying ApoE ε4 allele (65.8 ± 11.9) and those not carrying this allele (67.5 ± 6.3; *P* = 0.596), ruling out an early age of onset on the basis of the presence of the ApoE ε4 allele.

ApoE ε4 allele frequencies were then compared with those previously reported for patients with AD (*n* = 88) and age-matched controls (*n* = 147) (**Table 3**). Of note, no significant differences were found when comparing FXTAS ApoE allele ε4 frequencies with those detected in patients with AD ($\chi^2 = 1.858$; degrees of freedom = 1; *P* = 0.2). By contrast, the comparison with the control population group showed significant differences ($\chi^2 = 7.78$; degrees of freedom = 1; *P* = 0.013).

Table 2 ApoE allele genotype and allele frequencies in *FMR1* premutation carriers

	FXTAS			Non-FXTAS		
Gender (*n*)	Women	Men	No. of subjects (*n*)	Women	Men	No. of subjects (*n*)
	8	14	22	15	7	22
Genotype						
4/4	1	0	1 (4.5)	0	0	0 (0)
4/3	2	5	6 (27.3)	1	0	1 (4.5)
4/2	0	0	0 (0)	0	0	1 (0)
3/3	4	10	14 (63.6)	14	7	21 (95.5)
3/2	1	0	1 (4.5)	0	0	0 (0)

Numbers in parentheses indicate frequencies.

ApoE, apolipoprotein E; FXTAS, fragile X-associated tremor/ataxia syndrome.

Table 3 Allele frequencies in patients with FXTAS, patients with AD, and age-matched controls

ApoE	FXTAS	Non-FXTAS	AD[a] (*n* = 88)	Control[a] (*n* = 147)
ε2 Allele	0.023	0.000	0.022	0.068
ε3 Allele	0.795	0.977	0.687	0.870
ε4 Allele	0.182	0.023	0.289	0.061

AD, Alzheimer disease; ApoE, apolipoprotein E; FXTAS, fragile X-associated tremor/ataxia syndrome.

[a]Data taken from ref. 20.

DISCUSSION

The ApoE ε4 allele is a well-known genetic risk factor for AD.[22] Some studies have shown that ε4 allele frequency is significantly increased among patients with AD and that this association might be related to a cognitive decline and a faster disease progression, contributing to the reduction of the median age for AD onset.[9,14,23] FXTAS is a late-onset neurodegenerative disorder molecularly characterized by increased levels of abnormal (expanded CGG repeat) *FMR1* mRNA and slightly reduced fragile X mental retardation protein levels. The presence of these elevated levels of *FMR1* mRNA led to the proposal of an RNA "toxic gain-of-function" model for FXTAS, in which the mRNA itself, with the abnormal CGG repeat tract, is causative of the neurological disorder.[2,3,24,25] Although AD and FXTAS have different clinical and neuropathological features, both disorders show protein aggregates, with a cytotoxic effect that leads to cell death or a disordered synaptic transmission.[25,26] AD is characterized by senile plaques that are predominantly composed of β-amyloid, an amino acid peptide cleaved from the amyloid precursor protein.[27] Considering the central role of ApoE, which includes transporting cholesterol into the central nervous system and helping to remove amyloid-β protein from the brain,[12] together with the fact that amyloid precursor protein mRNA is a target for fragile X mental retardation protein–mediated translational repression at the synapse,[27] it would seem likely that there is a biological connection among ApoE, the *FMR1* gene, and the FXTAS syndrome. Furthermore, the fact that ApoE variants have been associated with a large number of age-related and neurodegenerative disease and that no reports were available on the ApoE allelic frequencies in FXTAS patients, we found it necessary to investigate the relationship of ApoE with FXTAS. We therefore examined ApoE genotypes in relation to FXTAS among 44 *FMR1* premutation carriers (22 presenting with FXTAS and 22 without FXTAS). The comparison of the two groups (considering the CGG repeat number, age, and gender) showed no significant differences except with respect to gender (**Table 1**). This observation is in concordance with the fact that FXTAS penetrance has been found to be lower in female *FMR1* premutation carriers than in male carriers.[28]

Similar to what has previously reported in other neurodegenerative diseases,[9,14,29] we have found an association between the presence of ApoE ε4 and the risk of having FXTAS, evidencing a significantly higher risk for FXTAS among ε4 carriers (OR = 12.041; $P = 0.034$; 95% confidence interval = 1.21–119.7). By contrast, the ApoE ε2 allele, the allele that protects against AD, was not significantly found among individuals without FXTAS (**Table 2**). Furthermore, the ApoE genotype distribution detected among individuals with FXTAS resembles those found among Spanish patients with AD (**Table 3**).[20] Contrary to what has been published for AD,[14] no association has been found between the presence of the ApoE ε4 allele and the age of disease onset. Although the sample size is small, to our knowledge and considering that FXTAS is a rare disease and still poorly recognized among specialists, the cohort herein studied corresponds to one of the largest Spanish cohorts ever published.

In summary, on the basis of these results, we conclude that the presence of at least one ApoE ε4 allele, together with other factors, might act as a genetic factor predisposing individuals to develop FXTAS. However, further studies are required in order to clarify if this association is also found in other populations. The data herein reported provide a first approach that might help in unraveling other genes related to FXTAS pathology. A better understanding of the molecular underpinnings of FXTAS should shed light on therapeutic approaches that will combat neurodegeneration and improve cognitive and motor performance.

ACKNOWLEDGMENTS

We thank the fragile X families, the "Associació Catalana Síndrome X fràgil", and the Federación Española del Síndrome X Frágil for their cooperation. This work was supported by FIS (PS09/00413) from Instituto de Salud Carlos III, AGAUR SGR1337 from Generalitat de Catalunya, Fondo Europeo de Desarrollo Regional (Fondos FEDER), and FSE (Fundo Social Europeu). F.S. is a recipient of a PhD grant (SFRH/BD/81271/2011) from Fundação Ciência e Tecnologia. The CIBER de Enfermedades Raras is an initiative of the ISCIII.

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

1. Bourgeois JA, Cogswell JB, Hessl D, et al. Cognitive, anxiety and mood disorders in the fragile X-associated tremor/ataxia syndrome. *Gen Hosp Psychiatry* 2007;29:349–356.
2. Hagerman PJ, Hagerman RJ. The fragile-X premutation: a maturing perspective. *Am J Hum Genet* 2004;74:805–816.
3. Jacquemont S, Hagerman RJ, Leehey M, et al. Fragile X premutation tremor/ataxia syndrome: molecular, clinical, and neuroimaging correlates. *Am J Hum Genet* 2003;72:869–878.
4. Rodriguez-Revenga L, Pagonabarraga J, Gómez-Anson B, et al. Motor and mental dysfunction in mother-daughter transmitted FXTAS. *Neurology* 2010;75:1370–1376.
5. Brunberg JA, Jacquemont S, Hagerman RJ, et al. Fragile X premutation carriers: characteristic MR imaging findings of adult male patients with progressive cerebellar and cognitive dysfunction. *AJNR Am J Neuroradiol* 2002;23:1757–1766.
6. Jacquemont S, Farzin F, Hall D, et al. Aging in individuals with the FMR1 mutation. *Am J Ment Retard* 2004;109:154–164.
7. Jacquemont S, Hagerman RJ, Leehey MA, et al. Penetrance of the fragile X-associated tremor/ataxia syndrome in a premutation carrier population. *JAMA* 2004;291:460–469.

8. Ferreira DC, Costa TF, Aguiar SL, et al. Association of apoliprotein E polymorphisms and metabolic syndrome in subjects with extreme obesity. *Clin Chim Acta* 2011;412:1559–1562.
9. Leoni V. The effect of apolipoprotein E (ApoE) genotype on biomarkers of amyloidogenesis, tau pathology and neurodegeneration in Alzheimer's disease. *Clin Chem Lab Med* 2011;49:375–383.
10. Pulkes T, Papsing C, Mahasirimongkol S, Busabaratana M, Kulkantrakorn K, Tiamkao S. Association between apolipoprotein E genotypes and Parkinson's disease. *J Clin Neurosci* 2011;18:1333–1335.
11. Strittmatter WJ, Saunders AM, Schmechel D, et al. Apolipoprotein E: high-avidity binding to beta-amyloid and increased frequency of type 4 allele in late-onset familial Alzheimer disease. *Proc Natl Acad Sci USA* 1993;90:1977–1981.
12. Eisenstein M. Genetics: finding risk factors. *Nature* 2011;475:S20–S22.
13. Weisgraber KH, Shinto LH. Identification of the disulfide-linked homodimer of apolipoprotein E3 in plasma. Impact on receptor binding activity. *J Biol Chem* 1991;266:12029–12034.
14. Helisalmi S, Linnaranta K, Lehtovirta M, et al. Apolipoprotein E polymorphism in patients with different neurodegenerative disorders. *Neurosci Lett* 1996;205:61–64.
15. McCarron MO, Delong D, Alberts MJ. APOE genotype as a risk factor for ischemic cerebrovascular disease: a meta-analysis. *Neurology* 1999;53:1308–1311.
16. Nebes RD, Vora IJ, Meltzer CC, et al. Relationship of deep white matter hyperintensities and apolipoprotein E genotype to depressive symptoms in older adults without clinical depression. *Am J Psychiatry* 2001;158:878–884.
17. Bertram L, Tanzi RE. Thirty years of Alzheimer's disease genetics: the implications of systematic meta-analyses. *Nat Rev Neurosci* 2008;9:768–778.
18. Kim JM, Stewart R, Kim SY, et al. Synergistic associations of depression and apolipoprotein E genotype with incidence of dementia. *Int J Geriatr Psychiatry* 2011;26:893–898.
19. Vemuri P, Wiste HJ, Weigand SD, et al.; Alzheimer's Disease Neuroimaging Initiative. Effect of apolipoprotein E on biomarkers of amyloid load and neuronal pathology in Alzheimer disease. *Ann Neurol* 2010;67:308–316.
20. Adroer R, Santacruz P, Blesa R, López-Pousa S, Ascaso C, Oliva R. Apolipoprotein E4 allele frequency in Spanish Alzheimer and control cases. *Neurosci Lett* 1995;189:182–186.
21. Gené M, Moreno P, Ezquerra M, et al. Low apolipoprotein E epsilon4 allele frequency in the population of Catalonia (Spain) determined by PCR-RFLP and Laser fluorescent sequencer. *Eur J Epidemiol* 1997;13:841–843.
22. Wagner T, Pietrzik CU. The role of lipoprotein receptors on the physiological function of APP. *Exp Brain Res* 2012;217:377–387.
23. D'Onofrio G, Panza F, Seripa D, et al. The APOE polymorphism in Alzheimer's disease patients with neuropsychiatric symptoms and syndromes. *Int J Geriatr Psychiatry* 2011;26:1062–1070.
24. Hagerman RJ, Leehey M, Heinrichs W, et al. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. *Neurology* 2001;57:127–130.
25. Greco CM, Hagerman RJ, Tassone F, et al. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. *Brain* 2002;125(Pt 8):1760–1771.
26. Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science* 2002;297:353–356.
27. Westmark CJ, Malter JS. FMRP mediates mGluR5-dependent translation of amyloid precursor protein. *PLoS Biol* 2007;5:e52.
28. Rodriguez-Revenga L, Madrigal I, Pagonabarraga J, et al. Penetrance of FMR1 premutation associated pathologies in fragile X syndrome families. *Eur J Hum Genet* 2009;17:1359–1362.
29. Gao J, Huang X, Park Y, et al. Apolipoprotein E genotypes and the risk of Parkinson disease. *Neurobiol Aging* 2011;32:2106.e1–2106.e6.

Neurobiology of Disease 65 (2014) 43–54

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Blood expression profiles of fragile X premutation carriers identify candidate genes involved in neurodegenerative and infertility phenotypes

Elisabet Mateu-Huertas [a,b,c,d], Laia Rodriguez-Revenga [e,f], Maria Isabel Alvarez-Mora [e,f], Irene Madrigal [e,f], Rob Willemsen [g], Montserrat Milà [e,f], Eulàlia Martí [a,b,c,d,*,1], Xavier Estivill [a,b,c,d,*,1]

[a] Genomics and Disease Group, Bioinformatics and Genomics Programme, Centre for Genomic Regulation (CRG), Barcelona 08003, Catalonia, Spain
[b] Universitat Pompeu Fabra (UPF), Barcelona 08003, Catalonia, Spain
[c] Hospital del Mar Medical Research Institute (IMIM), Barcelona 08003, Catalonia, Spain
[d] CIBER de Epidemiología y Salud Pública (CIBERESP), CRG, Barcelona 08003, Catalonia, Spain
[e] Biochemistry and Molecular Genetics Department, Hospital Clínic and IDIBAPS, Barcelona 08036, Catalonia, Spain
[f] CIBER de Enfermedades Raras (CIBERER), Hospital Clínic, Barcelona 08003, Catalonia, Spain
[g] Department of Clinical Genetics, Erasmus MC, Rotterdam 3000 DR, The Netherlands

ARTICLE INFO

Article history:
Received 7 August 2013
Revised 16 December 2013
Accepted 30 December 2013
Available online 10 January 2014

Keywords:
Fragile X Tremor/Ataxia Syndrome
Fragile X associated Premature Ovarian Insufficiency
Early at menopause 1 (EAP1)
Histone deacetylase 5 (HDAC5)

ABSTRACT

Male premutation carriers presenting between 55 and 200 CGG repeats in the Fragile-X-associated (*FMR1*) gene are at risk of developing Fragile X Tremor/Ataxia Syndrome (FXTAS), and females undergo Premature Ovarian Failure (POF1). Here, we have evaluated gene expression profiles from blood in male *FMR1* premutation carriers and detected a strong deregulation of genes enriched in FXTAS relevant biological pathways, including inflammation, neuronal homeostasis and viability. Gene expression profiling distinguished between control individuals, carriers with FXTAS and carriers without FXTAS, with levels of expanded *FMR1* mRNA being increased in FXTAS patients. In vitro studies in a neuronal cell model indicate that expression levels of expanded *FMR1* 5′-UTR are relevant in modulating the transcriptome. Thus, perturbations of the transcriptome may be an interplay between the CGG expansion size and *FMR1* expression levels. Several deregulated genes (*DFFA*, *BCL2L11*, *BCL2L1*, *APP*, *SOD1*, *RNF10*, *HDAC5*, *KCNC3*, *ATXN7*, *ATXN3* and *EAP1*) were validated in brain samples of a FXTAS mouse model. Downregulation of *EAP1*, a gene involved in the female reproductive system physiology, was confirmed in female carriers. Decreased levels were detected in female carriers with POF1 compared to those without POF1, suggesting that *EAP1* levels contribute to ovarian insufficiency. In summary, gene expression profiling in blood has uncovered mechanisms that may underlie different pathological aspects of the premutation. A better understanding of the transcriptome dynamics in relation with expanded *FMR1* mRNA expression levels and CGG expansion size may provide mechanistic insights into the disease process and a more accurate FXTAS diagnosis to the myriad of phenotypes associated with the premutation.

© 2014 Published by Elsevier Inc.

Abbreviations: ACTB, Beta Cytoskeletal Actin; AKT1, v-akt murine thymoma viral oncogene homolog 1; APP, amyloid beta (A4) precursor protein; ATXN3, spinocerebellar ataxia type 3 protein; ATXN7, spinocerebellar ataxia type 7 protein; BCL-X, Bcl-2-like protein 1; BMF, Bcl-2-modifying factor; CASP3, apoptosis-related cysteine peptidase 3; CDKN1B, cyclin-dependent kinase inhibitor 1B; COX6C, cytochrome c oxidase subunit VIc; COX7B, cytochrome c oxidase subunit VIIb; DFFA, DNA fragmentation factor subunit alpha; EAP1, early at menopause 1; FMR1, Fragile X mental retardation 1; FMRP, FMR1 protein; FXPOI, Fragile X-associated Premature Ovarian Insufficiency; FXTAS, Fragile X associated Tremor/Ataxia Syndrome; GFAP, glial fibrillary acidic protein; HDAC5, histone deacetylase 5; hnRNPA2, heterogeneous nuclear ribonucleoprotein A2; HPRT1, hypoxanthine phosphoribosyltransferase 1; HSP, heat shock protein; IPA, Ingenuity Pathway Analysis; KCNC3, potassium voltage-gated channel member 3; KI, knock-in; lncRNA, long non-coding RNAs; MBNL1, muscle blind-like protein 1; MEG3, maternally expressed 3; NEAT1, Nuclear Enriched Abundant Transcript 1; POF1, Premature Ovarian Failure; RNF10, ring finger protein 10; RTPCR, reverse transcription PCR; SAM68, Src-associated in mitosis 68 kDa protein; SOD1, superoxide dismutase 1; TBP, TATA box binding protein; TUG1, taurine up-regulated gene 1; UTR, Untranslated region; ZNF815, zinc finger protein 815; V21, 5′FMR1-UTR Harboring 21 CGG repeats; V79, 5′FMR1-UTR Harboring 79 CGG repeats.

* Corresponding authors at: Genomics and Disease Group, Bioinformatics and Genomics Programme, Centre for Genomic Regulation (CRG), Barcelona 08003, Catalonia, Spain.
E-mail addresses: eulalia.marti@crg.cat (E. Martí), xavier.estivill@crg.cat (X. Estivill).

Available online on ScienceDirect (www.sciencedirect.com).

[1] These authors contributed equally to the work.

0969-9961/$ – see front matter © 2014 Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.nbd.2013.12.020

Introduction

Trinucleotide repeat disorders are a set of genetic diseases caused by expansions of three nucleotides in selective genes, which are mainly connected to neurological diseases (Orr and Zoghbi, 2007). This is the case of the fragile X mental retardation 1 (*FMR1*) gene (OMIM 309550), which contains a highly polymorphic CGG repeat at the 5′ untranslated region (5′-UTR) (Fu et al., 1991; Verkerk et al., 1991). In the general population, the length of the *FMR1* CGG repeat ranges from 5 to 55 copies, and 30 repeats are the most commonly found allele (Peprah, 2012). *FMR1* CGG repeats ranging from 55 to 200 trinucleotides constitute the premutation alleles, while full mutation alleles are characterized by more than 200 repeats. In fully mutated alleles, the gene is generally hypermethylated and thus silenced, resulting in Fragile X Syndrome (FXS; OMIM 300624), the most common form of inherited mental retardation in childhood (Tassone et al., 1999). Yet, premutation alleles are characterized by a 2 to 8 fold increase of the production of *FMR1* mRNA compared with normal individuals, despite normal or slightly reduced levels of the *FMR1* protein (FMRP) (Hessl et al., 2005). Interestingly, premutation alleles are associated with a large range of different disorders, such as Fragile X associated Tremor/Ataxia Syndrome (FXTAS; OMIM 300623) (Hagerman et al., 2001), Premature Ovarian Failure (POF1; OMIM 311360) (Sullivan et al., 2005), thyroid dysfunction, hypertension, fibromyalgia, and chronic muscle pain (Coffey et al., 2008; Hagerman et al., 2004), and have often an adult onset of development.

FXTAS is a neurodegenerative disorder that develops mainly in men over 50 years of age (Hagerman and Hagerman, 2004). FXTAS is characterized by a progressive action tremor, gait ataxia and other frequent variable features of cognitive decline; especially executive dysfunction, parkinsonism, neuropathy, and autonomic dysfunction (Grigsby et al., 2006). FXTAS patients may also present psychiatric symptoms, such as anxiety, mood liability and depression (Bacalman et al., 2006; Hessl et al., 2005). FXTAS prevalence is 1 in ~400 males and ~250 females. Penetrance in females is around 8–16% and in males near 40%, but it increases with age to a 60–70% in males of around 80 years (Dombrowski et al., 2002; Jacquemont et al., 2004). These data suggest that FXTAS is one of the most common monogenic forms of gait ataxia and tremor in older males.

Neuroanatomical characterization of FXTAS brain samples shows Purkinje cell loss, Bergmann gliosis and global brain atrophy. Furthermore, the presence of eosinophilic, ubiquitin positive intranuclear inclusions is detected in neuronal and non-neuronal cells throughout the central nervous system (Greco et al., 2002; Iwahashi et al., 2006; Tassone et al., 2004). Inclusions are the pathological hallmark of FXTAS and can be reproduced in cell and mouse models (Arocena et al., 2005; Willemsen, 2003).

Inclusions contain proteins involved in stress responses, including the heat shock proteins HSP27 and HSP70 (Iwahashi et al., 2006) and structural proteins such as lamin A/C and GFAP (Iwahashi et al., 2006). Characteristically, the inclusions contain the *FMR1* mRNA itself (Tassone et al., 2004), which has been shown to sequester some RNA binding proteins through the CGG repeat stretch. These include SAM68, hnRNPA2 and MBNL1, whose loss of function results in alterations of splicing events with consequent detrimental effects in cell function (Iwahashi et al., 2006; Jin et al., 2007; Sellier et al., 2010; Sofola et al., 2007). Despite all the efforts, additional studies are needed to understand the causes of the pathologies associated with the progression of FXTAS.

A common difficulty in neurological disorders is the availability of affected brain tissue to study the pathology. In these cases, non-invasive gene expression profiling in peripheral blood is a useful strategy to define molecular signatures that may provide mechanistic insights into disease pathogenesis and identify biomarkers of disease progression. The use of blood samples to identify potential pathways and genes involved in a neurodegenerative process has been successfully implemented in trinucleotide repeat disorders, including Huntington's disease (Borovecki et al., 2005) and Friedreich's ataxia (Haugen et al., 2010). This strategy identified several genes that distinguished control individuals, pre-symptomatic- and symptomatic patients with Huntington's disease harboring a CAG expansion in the huntingtin gene. Furthermore, the authors showed that blood profiling partially reflected changes occurring in diseased brain (Borovecki et al., 2005). In Friedreich's ataxia, a GAA repeat expansion in the frataxin gene leads to its impaired expression. Sustained frataxin loss in blood results in altered expression of genes involved in immune response and oxidative phosphorylation, thus providing insight into the nature and progression of the disease (Haugen et al., 2010).

Here, we report the first investigation involving transcription profiling of total blood from male *FMR1* premutation carriers. Gene expression deregulation was further analyzed in an in vitro neuronal cell model of FXTAS and in several brain regions of a knock in (KI) mouse model for FXTAS (Bontekoe et al., 2001). Our results provide a comprehensive catalog of the transcriptional changes underlying *FMR1* premutation carriers and provide new insights into the associated pathology.

Material and methods

Human samples

A total of 15 unrelated male individuals were recruited for this study: 9 fragile X premutation carriers and 5 control males (average age of 73) with normal *FMR1* alleles (Table 1). All the carriers were recruited from fragile X families. At the time the samples where collected, a total of 5 carriers where diagnosed with FXTAS. In the course of this study, two additional carriers developed FXTAS. A total of 25 female carriers were recruited, with 12 presenting POF1 (Supplementary Table S1). In addition, 8 control females were included, with 4 presenting POF1. The CGG repeat number and clinical/neurological findings of these patients are summarized in Table 1 and S3. All patients provided written informed consent for testing and for the use of their phenotypic and genetic data. (Local Ethical Committee registration number 2008/4595).

Mouse samples

We used three male CGG-KI mice for the fragile X premutation (range 150–178 CGG repeats) (Bontekoe et al., 2001) at 70 weeks of age and four male wild type (wt) mice of the same age range were included in this study. Mice were sacrificed, and brain tissue was removed from the skull and cut sagittally at the midline into two equal pieces before being frozen and preserved in liquid nitrogen. Subsequent dissection of the different brain areas was performed in transgenic and control mice: prefrontal cortex, striatum, brainstem, motor cortex and hippocampus. Animals were reared and sacrificed in accordance with the recommendations and protocols approved by the local ethics committee.

Total RNA isolation

For each individual, 2.5 mL of peripheral venous blood was collected in 5 mL PAXgene tubes. Whole blood RNA was isolated and purified with the PAXgene Blood RNA Kit according to the manufacturer's instructions. The mouse dissected brain areas were placed immediately in Qiazol solution from Qiagen, followed by RNA extraction with the miRNeasy kit (Qiagen) as indicated by the manufacturer. RNA extraction from our neuronal cell model was also performed with miRNeasy (Qiagen) following manufacturer's instructions. The RNA quality and quantity measures were done with a 2100 Bioanalyzer (Agilent Technologies) and an ND-1000 spectrophotometer (Thermo Scientific), respectively. All RNA samples showed an RNA integration number of seven or more.

Table 1
Clinical and molecular characteristics from male carriers. AS, asymptomatic patients; SP, symptomatic patients. DEF: definite, PROB: Probable, POS: Possible.

Subject	Age	(CGG)n	mRNA *FMR1*[a]	% FMRP[b]	Clinical signs	Radiological signs	Diagnostic[c]	FXTAS onset
SP 1	63	106	1.56	67	2 major	1 major + 1 minor	DEF	62
SP 2	65	113	3.2	88	1 major + 1 minor	1 major	DEF	64
SP 3	62	60	1.72	100	2 major	1 major + 1 minor	DEF	60
SP 4	43	60	1.22	94	1 minor	1 major	PROB	64
SP 5	69	75	2.06	83	2 minor + 1 major	1 minor	POS	62
SP 6	65	83	1.93	78	1 major	2 major	DEF	65
SP 7	55	73	3.82	95	1 major + 2 minor	1 minor	POS	57
AP 1	65	80	1.04	78	None	1 minor	NO	–
AP 2	42	73	0.95	86	None	None	NO	–
Control 1	72	28	1.09	–	None	None	–	–
Control 2	77	29	1.17	–	None	None	–	–
Control 3	69	23	1	–	None	None	–	–
Control 4	71	29	1.45	–	None	None	–	–
Control 5	77	23	0.92	–	None	None	–	–

[a] RNA levels are reported as fold change over those in normal sex-matched control individuals.
[b] Percentage of hair root positive for FMRP by immunohistochemical staining.
[c] Diagnostic criteria for minor and major signs described by Jacquemont et al. (2007).

Microarray hybridization and analysis

100 ng of total RNA was labeled using the LowInputQuick Amp Labeling kit (Agilent) following the manufacturer's instructions. Briefly: mRNA was reverse transcribed in the presence of T7-oligo-dT primer to produce cDNA. cDNA was then in vitro transcribed with T7 RNA polymerase in the presence of Cy3-CTP to produce labeled cRNA. The labeled cRNA was hybridized to the Agilent SurePrint G3 Human gene expression 8 × 60K Microarray according to the manufacturer's protocol. The arrays were washed and scanned on an Agilent G2565CA microarray scanner at 100% PMT and 3 μm resolution. Intensity data was extracted using the Feature Extraction software.

Raw data were taken from the Feature Extraction output files and were corrected for background noise using the normexp method (Ritchie et al., 2007). To assure comparability across samples we used quantile normalization (Bolstad, 2001). Differential expression analysis was carried out on non-control probes with an empirical Bayesian approach on linear models (limma) (Smyth, 2004). Results were corrected for multiple testing according to the False Discovery Rate (FDR) method (Benjamini and Hochberg, 1995). All statistical analyses were performed with the Bioconductor project (http://www.bioconductor.org/) in the R statistical environment (http://cran.r-project.org/) (Gentleman et al., 2004). We used the SurePrint G3 Human GE 8 × 60K Microarray for peripheral blood samples of Fragile X premutation carriers' expression profile, the SurePrint G3 Human Gene Expression 8 × 60K v2 Microarray for our in vitro cellular system and the SurePrint G3 Mouse GE 8 × 60K Microarray for the mouse brain regions analyzed (Agilent).

Pathway enrichment analysis

We used the Ingenuity Pathway Analysis (IPA) online tool (Ingenuity System Inc., www.ingenuity.com) to interpret data in the context of biological processes, pathways, and networks.

qPCR validations

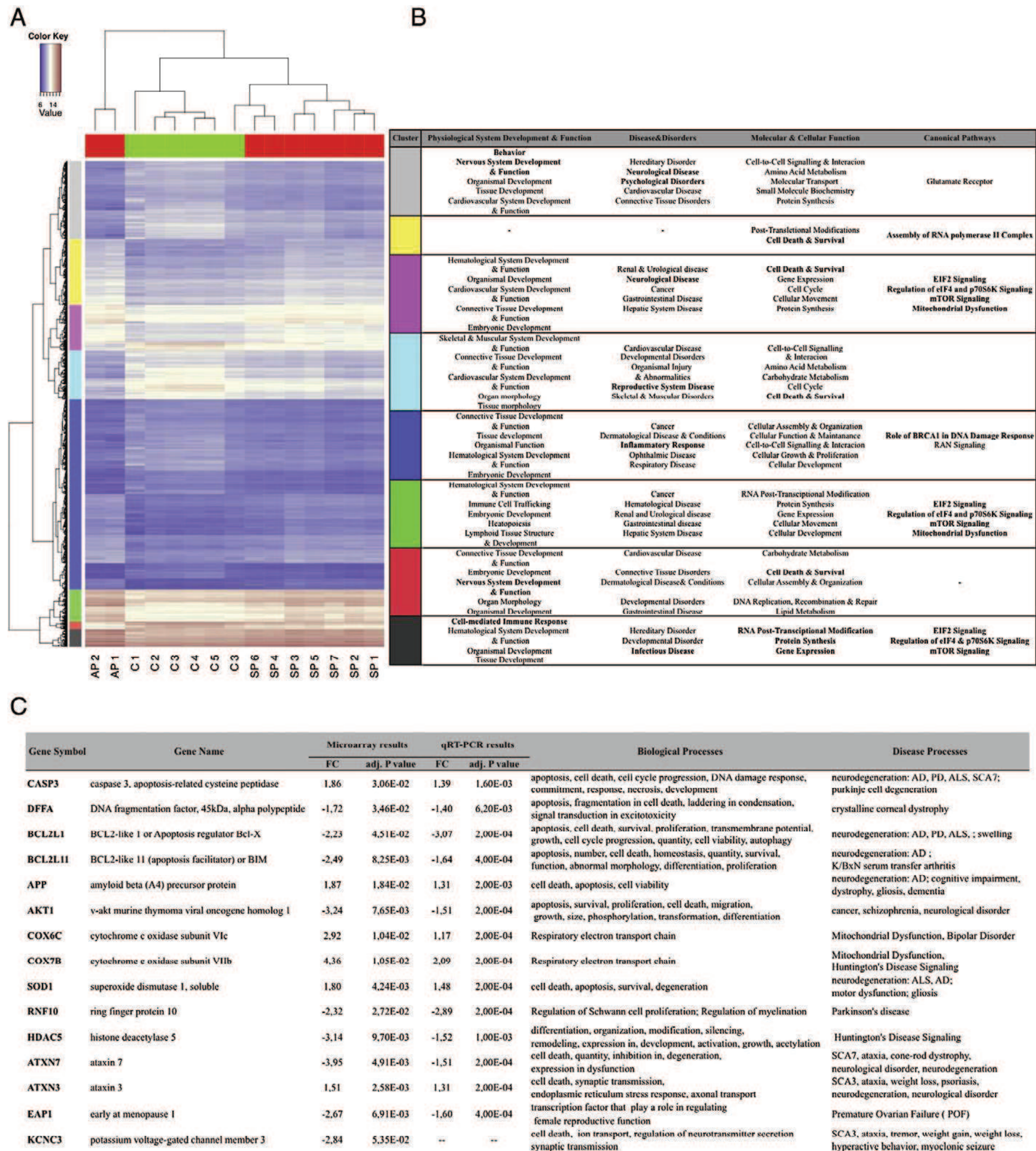
Gene expression analysis was performed using 0.15 μg total RNA from human peripheral blood and 0.3 μg total RNA from CGG KI mice model or SH-SY5Y cell line. cDNA synthesis was performed using the SuperScript III First Strand Synthesis System for RT-PCR (Life Technologies) following the manufacturer's instructions. The cDNA product was diluted to 1/5 with sterile water. Real time PCR (rRT-PCR) was performed using TaqMan gene expression assays, following the manufacturer's instructions in an AB 7900HT Fast Real Time PCR System.

For each experiment, all cases and controls were analyzed in the same rRT-PCR experiment, each sample was run in quadruplicates and the cDNA synthesis was repeated at least twice. Relative quantification (RQ) as shown in graphs was calculated with the 2ΔΔCt method (Livak and Schmittgen, 2001) using ACTB and MRIP as reference genes. RQ was calculated to compare all expression values normalized to the reference genes among carrier and control samples. These RQs and their statistical significance were obtained from a linear mixed effects model (Steibel et al., 2009), which accounted for the different sources of variation derived from the experimental design (Miñones-Moyano et al., 2011). TaqMan Expression Assays are found in Supplementary Table 2.

Generation of FMR1 5′-UTR expanded vectors

Lymphocyte cell lines (LCLs) from two different carriers had been established. One LCL is from a female carrier, which permits the cloning of a normal allele. B lymphocytes were isolated from peripheral blood samples by gradient centrifugation with Ficoll using Leucocep tubes (Greiner Bio-One). Peripheral Blood Mononuclear Cell (PBNC) interphase was collected and washed twice with phosphate buffered solution (PBS), and finally resuspended in 5 mL PBS. PBNCs were incubated with Epstein Barr virus supernatant at room temperature. After the 3 hour incubation 7 mL of incubation medium was added (RPMI 1640 + 20% FBS + 200 ng/mL cyclosporine A). After 5–7 days, 5 mL of medium were replaced with fresh medium. Polymerase chain reaction (PCR) amplification was carried out on genomic DNA (gDNA) isolated from the established LCL. Amplification conditions were as follows: an initial denaturation at 96 °C for 30 s was followed by 25 cycles of 96 °C for 30 s, 64 °C for 30 s and 68 °C for 5 min and a final elongation of 30 min at 68 °C. Each 12.5 μL reaction contained: 3 μL GC-RICH PCR reaction buffer 5x, 3 μL GC-RICH resolution solution 5 M, 0.08 μM of each oligonucleotide (FMR1 F and FMR1 R), 150 μM of each dNTP, 200 ng of gDNA and 1 μL GC-RICH Enzyme Mix (Roche). Products were purified through a 2% agarose gel and gel extracted using a gel extraction kit (Qiagen). Primers used were FMR1 F (5′-AGCCCCGCACTTCCACCACCAGCTCCTCCA) and FMR1 R (5′-TTCACTTCC GGTGGAGGGCCGCCTCTGAGC). Gel purified PCR products were cloned using the pGEM-T Easy Vector Systems (Promega). The recombinant plasmids were transformed into SURE2 supercompetent cells (Stratagene) according to the manufacturer's instructions. The recombinant pGEM-T Easy Vector cloned in a 5′ to 3′ orientation was digested with *Sac*II (New England BioLabs) followed by a 3′ polishing with X-Pfu DNA Polymerase (Kyratec) for 30 min at 37 °C to achieve blunt ends. The insert was then released using *Not*I (New England BioLabs) and gel-purified.

The DNA fragment of interest was subcloned into the pCAGIG (Addgene plasmid 11159) (Matsuda and Cepko, 2004) and transformed into SURE2 supercompetent cells. Positive plasmids were sequenced using the Big Dye 3.1 Termination Cycle Sequencing Kit and DNA Sequencer (ABI3100) from Applied Biosystems. PCR conditions where optimized with 7% DMSO in the final volume.



Cluster	Physiological System Development & Function	Disease&Disorders	Molecular & Cellular Function	Canonical Pathways
	Behavior **Nervous System Development & Function** Organismal Development Tissue Development Cardiovascular System Development & Function	Hereditary Disorder **Neurological Disease** **Psychological Disorders** Cardiovascular Disease Connective Tissue Disorders	Cell-to-Cell Signalling & Interacion Amino Acid Metabolism Molecular Transport Small Molecule Biochemistry Protein Synthesis	Glutamate Receptor
	-	-	Post-Transletional Modifications **Cell Death & Survival**	**Assembly of RNA polymerase II Complex**
	Hematological System Development & Function Organismal Development Cardiovascular System Development & Function Connective Tissue Development & Function Embryonic Development	Renal & Urological disease **Neurological Disease** Cancer Gastrointestinal Disease Hepatic System Disease	**Cell Death & Survival** Gene Expression Cell Cycle Cellular Movement Protein Synthesis	**EIF2 Signaling** **Regulation of eIF4 and p70S6K Signaling** **mTOR Signaling** **Mitochondrial Dysfunction**
	Skeletal & Muscular System Development & Function Connective Tissue Development & Function Cardiovascular System Development & Function Organ morphology Tissue morphology	Cardiovascular Disease Developmental Disorders Organismal Injury & Abnormalities **Reproductive System Disease** Skeletal & Muscular Disorders	Cell-to-Cell Signalling & Interacion Amino Acid Metabolism Carbohydrate Metabolism Cell Cycle **Cell Death & Survival**	
	Connective Tissue Development & Function Tissue development Organismal Function Hematological System Development & Function Embryonic Development	Cancer Dermatological Disease & Conditions **Inflammatory Response** Ophthalmic Disease Respiratory Disease	Cellular Assembly & Organization Cellular Function & Maintanance Cell-to-Cell Signalling & Interacion Cellular Growth & Proliferation Cellular Development	**Role of BRCA1 in DNA Damage Response** RAN Signaling
	Hematological System Development & Function Immune Cell Trafficking Embryonic Development Heatopoiesis Lymphoid Tissue Structure & Development	Cancer Hematological Disease Renal and Urological disease Gastrointestinal disease Hepatic System Disease	RNA Post-Transciptional Modification Protein Synthesis Gene Expression Cellular Movement Cellular Development	**EIF2 Signaling** **Regulation of eIF4 and p70S6K Signaling** **mTOR Signaling** **Mitochondrial Dysfunction**
	Connective Tissue Development & Function Embryonic Development **Nervous System Development & Function** Organ Morphology Organismal Development	Cardiovascular Disease Connective Tissue Disorders Dermatological Disease& Conditions Developmental Disorders Gastrointestinal Disease	Carbohydrate Metabolism **Cell Death & Survival** Cellular Assembly & Organization DNA Replication, Recombination & Repair Lipid Metabolism	-
	Cell-mediated Immune Response Hematological System Development & Function Organismal Development Tissue Development	Hereditary Disorder Developmental Disorder **Infectious Disease**	**RNA Post-Transciptional Modification** **Protein Synthesis** **Gene Expression**	**EIF2 Signaling** **Regulation of eIF4 & p70S6K Signaling** **mTOR Signaling**

Gene Symbol	Gene Name	Microarray results		qRT-PCR results		Biological Processes	Disease Processes
		FC	adj. P value	FC	adj. P value		
CASP3	caspase 3, apoptosis-related cysteine peptidase	1,86	3,06E-02	1,39	1,60E-03	apoptosis, cell death, cell cycle progression, DNA damage response, commitment, response, necrosis, development	neurodegeneration: AD, PD, ALS, SCA7; purkinje cell degeneration
DFFA	DNA fragmentation factor, 45kDa, alpha polypeptide	-1,72	3,46E-02	-1,40	6,20E-03	apoptosis, fragmentation in cell death, laddering in condensation, signal transduction in excitotoxicity	crystalline corneal dystrophy
BCL2L1	BCL2-like 1 or Apoptosis regulator Bcl-X	-2,23	4,51E-02	-3,07	2,00E-04	apoptosis, cell death, survival, proliferation, transmembrane potential, growth, cell cycle progression, quantity, cell viability, autophagy	neurodegeneration: AD, PD, ALS, ; swelling
BCL2L11	BCL2-like 11 (apoptosis facilitator) or BIM	-2,49	8,25E-03	-1,64	4,00E-04	apoptosis, number, cell death, homeostasis, quantity, survival, function, abnormal morphology, differentiation, proliferation	neurodegeneration: AD ; K/BxN serum transfer arthritis
APP	amyloid beta (A4) precursor protein	1,87	1,84E-02	1,31	2,00E-03	cell death, apoptosis, cell viability	neurodegeneration: AD; cognitive impairment, dystrophy, gliosis, dementia
AKT1	v-akt murine thymoma viral oncogene homolog 1	-3,24	7,65E-03	-1,51	2,00E-04	apoptosis, survival, proliferation, cell death, migration, growth, size, phosphorylation, transformation, differentiation	cancer, schizophrenia, neurological disorder
COX6C	cytochrome c oxidase subunit VIc	2,92	1,04E-02	1,17	2,00E-04	Respiratory electron transport chain	Mitochondrial Dysfunction, Bipolar Disorder
COX7B	cytochrome c oxidase subunit VIIb	4,36	1,05E-02	2,09	2,00E-04	Respiratory electron transport chain	Mitochondrial Dysfunction, Huntington's Disease Signaling
SOD1	superoxide dismutase 1, soluble	1,80	4,24E-03	1,48	2,00E-04	cell death, apoptosis, survival, degeneration	neurodegeneration: ALS, AD; motor dysfunction; gliosis
RNF10	ring finger protein 10	-2,32	2,72E-02	-2,89	2,00E-04	Regulation of Schwann cell proliferation; Regulation of myelination	Parkinson's disease
HDAC5	histone deacetylase 5	-3,14	9,70E-03	-1,52	1,00E-03	differentiation, organization, modification, silencing, remodeling, expression in, development, activation, growth, acetylation	Huntington's Disease Signaling
ATXN7	ataxin 7	-3,95	4,91E-03	-1,51	2,00E-04	cell death, quantity, inhibition in, degeneration, expression in dysfunction	SCA7, ataxia, cone-rod dystrophy, neurological disorder, neurodegeneration
ATXN3	ataxin 3	1,51	2,58E-03	1,31	2,00E-04	cell death, synaptic transmission, endoplasmic reticulum stress response, axonal transport	SCA3, ataxia, weight loss, psoriasis, neurodegeneration, neurological disorder
EAP1	early at menopause 1	-2,67	6,91E-03	-1,60	4,00E-04	transcription factor that play a role in regulating female reproductive function	Premature Ovarian Failure (POF)
KCNC3	potassium voltage-gated channel member 3	-2,84	5,35E-02	--	--	cell death, ion transport, regulation of neurotransmitter secretion synaptic transmission	SCA3, ataxia, tremor, weight gain, weight loss, hyperactive behavior, myoclonic seizure

Fig. 1. Gene expression analysis in peripheral blood samples of fragile X premutation carriers. (A) Heatmap of 1660 differentially expressed RNAs (Fold Change ≥ |1.5|and false discovery rate ≤ 1%). Genes with similar expression profiles are grouped in eight different clusters and labeled with different colors. Columns represent samples and rows show the genes. For each gene, red indicates positive log-ratios and blue negative log-ratios. C; Controls, AP; asymptomatic patients, SP; asymptomatic patients. (B) List of the significantly enriched canonical pathways, molecular and cellular functions, diseases and physiological system development and functions altered for each of the eight gene clusters according to IPA. (C) Validation of expression changes by qPCR. Comparison between qPCR and microarray results for selected genes is shown.

Cell lines and transfections

SH-SY5Y neuroblastoma cells were grown in Dulbecco's modified essential medium (DMEM, Invitrogen) supplemented with 10% of inactivated fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/mL Streptomycin (GIBCO, Invitrogen). The differentiation protocol for SH-SY5Y consisted of growing media with 10 mM retinoic acid (RA) (SIGMA). After a four day exposure, the media were removed and replaced by normal growth media plus 80 nM of 12-O-tetradecanoylphorbol-13-acetate (TPA) (SIGMA) during five additional days (Presgraves et al., 2004). All the transfection experiments were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instruction and at a cell confluence of 60%. Vectors were transfected at a concentration that permits an equivalent expression of the plasmids (between 0.6 ng/μL and 0.3 ng/μL). Twenty-four hours after transfection cells were processed, RNA isolated as previously indicated and rRT-PCR determinations of the expression levels of the 5′-UTR transgene were performed, using custom TaqMan assays: EGFP (AGTTCGAGGGCGACACCCTGGTGAA) and FMR1-IRES (GAAGCAGTTCCTCTGGAAGCTTCTT).

Results

Abundant blood gene expression deregulation in FMR1 premutation carriers

To identify mechanisms with possible relevance in the nature and progression of the FXTAS phenotype, we have examined changes in the global gene expression profile in blood samples of male fragile X premutation carriers. We included nine premutation carriers: five FXTAS symptomatic patients, four FXTAS asymptomatic patients and five age-matched controls (Table 1). During the course of the study, two of the asymptomatic patients initiated FXTAS symptomatology and therefore became symptomatic patients. *FMR1* mRNA expression levels in the two other asymptomatic patients were similar to control individuals, while symptomatic subjects presented a clear upregulation of *FMR1* mRNA when compared to controls (~2 fold change, $p < 0.01$) (Table 1). Accordingly, clinical characteristics of individuals used in another study reported asymptomatic patients with no increase in the expression of the *FMR1* gene (Napoli et al., 2011).

We performed gene expression profiling using Agilent based microarrays (SurePrint G3 Human GE 8 × 60K Microarray) (GSE48873). Transcripts that passed stringent filtering criteria (Fold Change ≥ |1.5|and FDR ≤ 1%) were considered as differentially expressed genes. According to these criteria, a total of 1660 RNAs were differentially expressed when comparing symptomatic patients with control individuals. 1003 genes were downregulated and 657 genes were upregulated. Interestingly, more than 30% of the differentially expressed genes were long non-coding RNAs (lncRNAs), most of them (93%) being downregulated. Heatmap analysis of the deregulation across different samples showed three main clusters consisting in controls, asymptomatic patients and symptomatic patients (Fig. 1A). Because *FMR1* mRNA levels are different between symptomatic patients and asymptomatic patients, both the CGG expansion per se and the expression levels of the premutated *FMR1* gene may account for the gene deregulation observed in premutation carriers.

In the heatmap analysis, we identified eight clusters of differentially expressed genes, containing between 1260 (blue) and 51 (red) RNAs (Figs. 1A and B). Ingenuity Pathway Analysis (IPA) in each cluster revealed enrichment in several biological pathways which may underlie FXTAS pathogenic aspects (Fig. 1B), including mitochondrial dysfunction, cell death and survival, inflammatory response, reproductive system disease, and neurological disorders. Thus, differentially expressed genes in blood identify pathways related to different neuropathological aspects of the FXTAS phenotypes. Heatmap analysis showed that 45% of the deregulated lncRNA clustered with protein coding genes involved in the inflammatory response, and another 28% with genes involved in nervous system development and function (Supplementary Table S3), suggesting a participation in analogous functions.

To identify possible modulators explaining the deregulation, IPA uses two statistical tools to determine the activation state of a given molecule: the Z-score, calculated within our data set, and the overlap p-value, which also takes into account already known regulators for a given gene. With this approach, IPA identified several cytokines, transcription factors and a number of miRNAs that could contribute to deregulation of gene expression in FXTAS (Supplementary Table S4).

Microarray expression data were validated by TaqMan based qPCR for 15 genes selected for their probable functional link to FXTAS (Fig. 1C). These genes are involved in nervous system function, inflammatory response, cell death, mitochondrial function, and oxidative stress. We validated 14 out of 15 genes, using two different reference genes: *ACTB* and *TBP*. The only gene that we could not replicate was the potassium voltage gated channel member 3 (*KCNC3*), which was not consistently expressed in our samples (Ct values: 38–40). The validated early at menopause 1 (*EAP1*) gene is an important component in the hypothalamic control for the initiation of puberty and maintenance of the female reproductive cycle in rodents and in non-human primates (Lomniczi et al., 2012). Since POF1 is characteristic of female carriers, we determined the expression of *EAP1* in peripheral blood samples of 25 female carriers, 12 of them presenting POF1. We included eight females without the permutation and four presenting premature menopause (onset before the age of 40 years) (Supplementary Table S1) as controls. RT-PCR analysis showed a significant downregulation of *EAP1* in carrier patients when compared with control individuals without POF1, thus confirming *EAP1* deregulation both in male and female carriers. Downregulation was stronger (24%, $p < 0{,}01$) in female carriers with POF1 compared with female carriers without POF1 (Fig. 2). However, a significant *EAP1* decrease was also detected in controls with POF1 compared with controls without POF1, thus suggesting that *EAP1* low levels may be associated both with the *FMR1* premutation and with POF1. These findings are in agreement with a common involvement of *EAP1* decline in loss/disruption of the menstrual cycle in different genetic conditions. Thus, a possibility exists that decreased *EAP1* levels in female carriers contribute to the high prevalence of POF1 in female carriers.

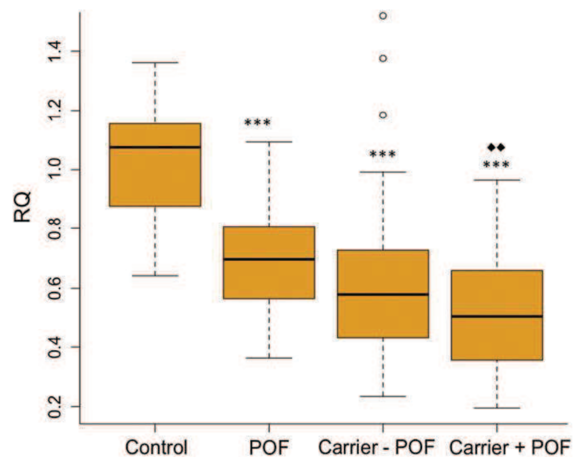


Fig. 2. *EAP1* expression in female fragile X premutation carriers. *EAP1* mRNA expression levels in POF1 control and carrier females. Expression levels are compared to a control sample for RQ. Determinations were performed using two independent reference genes. Boxplot representing the distribution (median and interquartile range). Statistical significance is shown by (***$P < 0.001$; ◆◆$P < 0.01$). Bonferroni correction was applied for multiple comparisons. Significant differences with respect to control individuals without POF1 are indicated with "*" and significant differences between carriers with POF1 and carriers without POF1 are indicated with "◆".

Table 2
qPCR relative quantification of specific FXTAS-blood-DEG in different brain areas of the KI FXTAS mouse model. Determinations were performed in the prefrontal cortex, motor cortex, striatum, hippocampus, cerebellum and brain stem. The deregulation pattern and the corresponding fold change are indicated for each gene and brain area. Down or up indicate a statistically significant deregulation in KI vs. control mice, using a linear mixed effects model, and "–" indicates no deregulation of transcript levels. Deregulation pattern observed with 2 independent endogenous reference genes is indicated in black labeling and deregulation detected with one of the two reference genes is indicated in gray. The last column indicates the deregulation pattern observed in blood of FXTAS patients according to the array and qPCR validations.

Gene ID	Prefrontal cortex	Motor cortex	Striatum	Hippocampus	Cerebellum	Brain Stem	Human Array
Dffa	–	–	FC = −1.28 p−val = 6E−04	–	–	–	Down
Bcl2l1	–	FC = −1.22 p−val = 7E−03	FC = 1.37 p−val = 6E−03	–	–	FC = −1.32 p−val = 2E−04	Down
Bcl2l11	FC = −1.36 p−val = 2E−04	FC = −1.44 p−val = 2E−04	FC = −1.33 p−val = 1E−02	–	–	FC = −1.4 p−val = 2E−04	Down
App	FC = 1.38 p−val = 2E−04	–	–	–	–	FC = 1.18 p−val = 2E−04	Up
Sod1	FC = 1.33 p−val = 5E−03	–	–	–	–	FC = 1.32 p−val = 2E−04	Up
Rnf10	–	FC = −1.14 p−val = 3E−03	FC = −1.38 p−val = 9E−03	–	–	FC = −1.87 p−val = 2E−04	Down
Hdac5	–	FC = −1.12 p−val = 2E−03	FC = −1.35 p−val = 2E−04	FC = −1.22 p−val = 1E−02	–	–	Down
Atxn7	–	FC = −1.2 p−val = 2E−04	FC = −1.21 p−val = 6E−03	–	FC = −1.16 p−val = 2E−03	–	Down
Atxn3	FC = 1.22 p−val = 3E−02	–	–	–	–	FC = 1.34 p−val = 2E−04	Up
Eap1	–	–	–	–	FC = −1.19 p−val = 2E−04	FC = −1.85 p−val = 2E−04	Down
Kcnc3	–	FC = −1.42 p−val = 2E−04	FC = −1.98 p−val = 2E−04	–	FC = −1.24 p−val = 8E−04	FC = −1.33 p−val = 6E−04	Down
Fmr1	FC = 2.59 p−val = 2E−04	FC = 1.99 p−val = 2E−04	FC = 2.17 p−val = 2E−04	FC = 2.67 p−val = 2E−04	FC = 2.28 p−val = 2E−04	FC = 2.86 p−val = 2E−04	Up

Black keys: validated using two independent reference genes.
Gray keys: validation with one independent reference gene.

Expression of selected blood deregulated genes in several brain areas of the FXTAS mouse model

The altered expression of a large amount of transcripts in peripheral blood samples of fragile X premutation carriers raises the question on whether similar changes occur in other tissues affected by the CGG repeat expansion, especially in brain samples for the FXTAS phenotype. We therefore analyzed the expression of selected genes in brain samples of the CGG KI mouse model, in which the endogenous mouse CGG repeats in the murine *Fmr1* gene have been replaced by the human *FMR1* gene carrying 150 CGG repeats (Bontekoe et al., 2001). We analyzed the expression of 12 genes differentially expressed in blood with relevant functions in neuronal homeostasis (Fig. 1C) and in brain samples of three CGG KI and four control mice. We performed TaqMan based qPCR using two different reference genes in six brain areas: prefrontal cortex, motor cortex striatum, hippocampus, cerebellum and brainstem. These areas were selected according to their association with the symptomatology in FXTAS patients and their affectation/involvement in human tissue and mouse samples. The motor cortex, prefrontal cortex and hippocampus show high content of intranuclear inclusions in the human brain as well as in the CGG KI mouse model (Wenzel et al., 2010; Willemsen, 2003). In addition, working memory is impaired in the CGG KI mouse model, suggesting that hippocampal dependent impairments in spatial processing may occur (Hunsaker et al., 2012). Intranuclear inclusions are also present in specific brainstem nuclei and cerebellum layers of FXTAS CGG KI mice (Wenzel et al., 2010; Willemsen, 2003). Moreover, neuroimaging studies in human carriers also revealed volume loss in the cerebral cortex, hippocampus, and cerebellum as well as brainstem and white matter disease in the brainstem and cerebellum (Cohen et al., 2006; Moore et al., 2004). Though intranuclear inclusions are rare in the striatum of CGG KI mice (Wenzel et al., 2010; Willemsen, 2003), other types of alterations in these areas may explain some of the motor deficits in FXTAS patients, including resting tremor and rigidity (D'Hulst et al., 2009; Lee et al., 2007).

Increased levels of *Fmr1* mRNA (2–3 fold change) were detected in all brain areas of the CGG KI mouse (Table 2), which agrees with previous results (D'Hulst et al., 2009; Lee et al., 2007). Of the remaining 11 genes, we validated nine in the brainstem, eight in the striatum and seven in the motor cortex (Table 2) using two independent reference genes (*Hprt1* and *Tbp*). Moreover, half of the genes studied showed the same expression direction in at least three of the six brain regions. Specifically, downregulation of potassium voltage gated channel member 3 (*Kvnc3*) and Bcl-2 interacting mediator of cell death (*Bim*) could be validated in four out of six different brain regions. Also, the ring finger protein 10 (*Rnf10*), the histone deacetylase 5 (*Hdac5*), the Bcl-2-like protein 1 (*Bcl-X*), the spinocerebellar ataxia type 7 protein (*Atxn7*) and *Eap1* showed downregulation in half of the tested brain regions. Interestingly, we found that *Eap1* was significantly downregulated in the brainstem and cerebellum of CGGKI mouse, suggesting that *Eap1* levels in certain brain areas could contribute to POF1 in this model (Lu et al., 2012). All together, these results suggest that gene expression profiling in the blood of fragile X premutation carriers partially reflects changes in the brain transcriptome which may underlie neuropathological aspects in FXTAS.

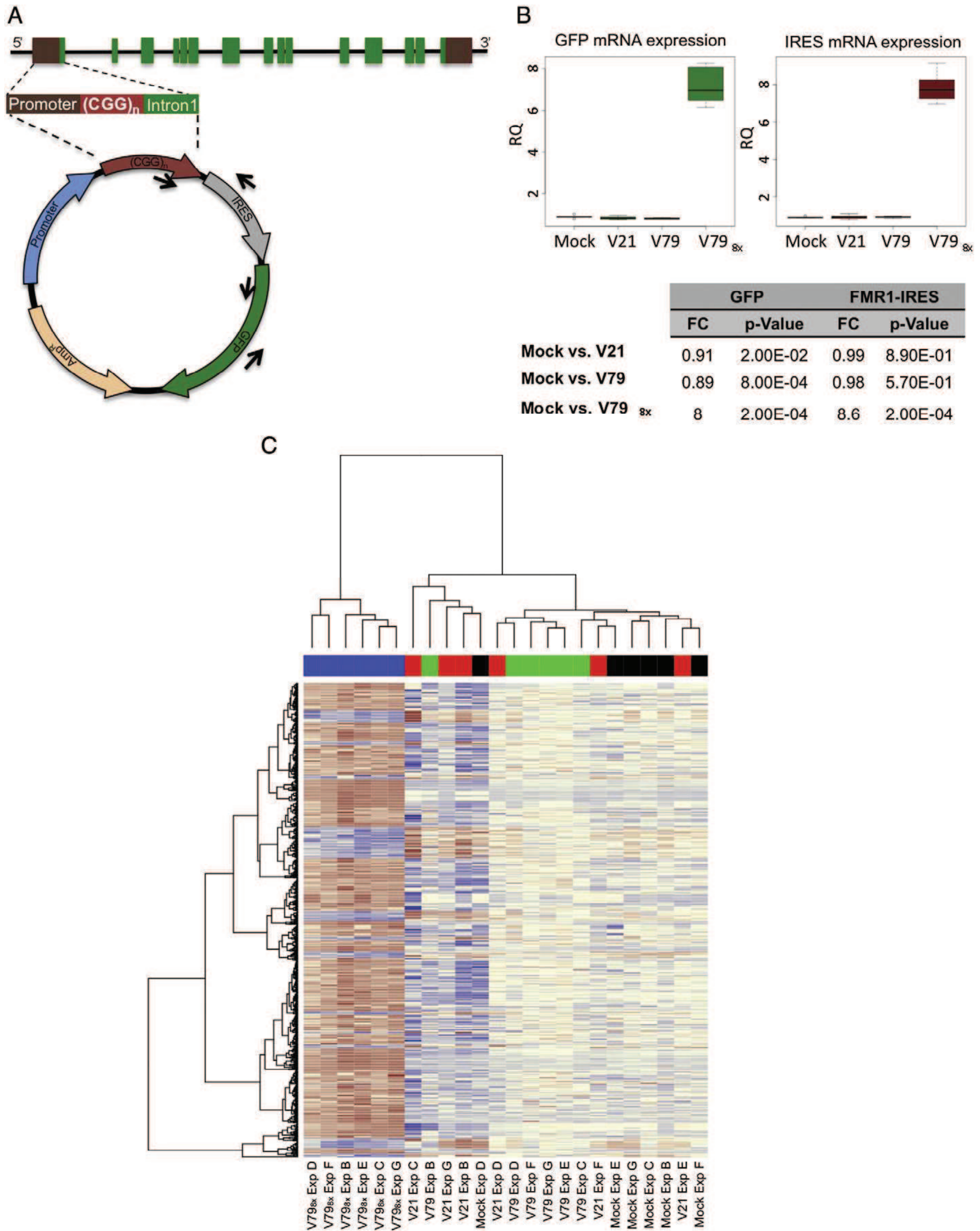
The levels of premutated 5′-UTR FMR1 mRNA influence gene expression

According to patient clustering analysis (Fig. 1A) our data suggest that symptomatic patients and asymptomatic patients present differential gene expression profiles which may be explained by the levels of expression of *FMR1* 5′-UTR. To determine whether premutated 5′-UTR *FMR1* expression levels could contribute to neuronal gene expression deregulation, we used differentiated postmitotic SH-SY5Y neuronal cells transfected with expanded and unexpanded 5′-UTR *FMR1* expression vectors. We used an unexpanded vector with a 21 CGG repeats FMR1 allele (V21) and a premutated/expanded vector with 79 CGG repeats FMR1 allele (V79) to perform the experiments (Fig. 3A). The expression levels of the vectors were determined 24 h after transfection using two different custom TaqMan assays designed to amplify GFP or pIRES (Fig. 3B). Quantification of the expression levels of the transfected vectors showed a group of cells expressing V79 at similar levels as V21 and another group of cells transfected with higher amounts of V79 showing 8-fold overexpression of the vector compared with V21 (V79_8x). These overexpression levels of premutated *FMR1* mRNA have been also reported in FXTAS patients (Kenneson et al., 2001; Tassone et al., 2000). Gene expression was then profiled in the six independent biological replicas, using the Agilent SurePrint G3 Human GE 8 × 60K Microarray (GSE48903).

Heatmap analysis of the probe sets differently expressed in V79_8x vs. V21 (Fold Change ≥ |1.2| and FDR ≤ 5%) showed strong clustering of cells expressing V79_8x with respect to the rest of the samples. The majority of the mock-, V21- and V79-transfected cells were clustered together, in the corresponding groups (Fig. 3C). We found 427 differentially expressed genes (Fold Change ≥ |1.2| and FDR rate ≤ 5%) when comparing cells transfected with V21 with those overexpressing V79

Fig. 3. Gene expression profiling linked to the overexpression of permutated 5′-UTR-*FMR1* in differentiated SH-SY5Y neuronal cells. (A) CGG expanded (V79) and unexpanded (V21) *FMR1* 5′-UTR were cloned into the bicistronic pCAGIG vector and transfected into differentiated SH-SY5Y cells. Two sets of primers (indicated by arrows) were used to quantify the expression of the vectors. (B) Quantification of the expression of the different vectors using two independent sets of primers, covering GFP (right panel) or FMR1-IRES (left panel) fragments. Quantification was normalized using two different reference genes. Expression levels are referred to a control mock-transfected sample for RQ. (C) Heatmap plot of 427 differentially expressed genes comparing V79_8x vs. V21 expressing cells (Fold Change ≥ |1.2|and false discovery rate ≤ 5%) 24 h after transfection of the vectors. Columns represent samples and rows represent genes. For each gene, red indicates up-regulation and blue indicates down-regulation of expression relative to the mean.

(8-fold). A total of 16 of these genes presented a similar deregulation pattern in blood when comparing control individuals with symptomatic patients (Fold Change ≥ |1.2| and FDR ≤ 5%) (Supplementary Table S5). We also detected 197 differentially expressed genes in cells expressing the V79 vector vs. cells overexpressing the same vector (V79_8x). From those, 21 genes presented a similar deregulation



pattern in blood when comparing asymptomatic patients with symptomatic patients (Fold Change $\geq |1.5|$, Supplementary Table S6). Although a little overlap was observed between differentially expressed genes in patient's blood and the cellular model, IPA suggests that similar biological pathways are perturbed. Thus, overexpression of expanded 5′-UTR *FMR1* might be sufficient to deregulate a subset of genes involved in inflammation and nervous system development and function. Moreover, 93% (178 out of 192) of differentially expressed genes in cells expressing the V79 construct vs. cells overexpressing the same vector (V79_8x) were also differentially expressed in cells expressing V21 vs. V79_8x (Supplementary Table S7). This suggests that overexpression of *FMR1* 5′-UTR leads to quantitative rather than qualitative changes in gene expression. In line with this, the levels and pattern of deregulation of 68% of the differentially expressed genes were more substantial in V21 vs. V79_8x than in V79 vs. V79_8x. A similar phenomenon was observed for 41 genes that showed a tendency for deregulation (not reaching statistical significance) when comparing V21 vs. V79 transfected cells (Supplementary Table S8). Again, the deregulation levels of those genes were increased in V21 vs. V79_8x expressing cells.

To study if the sensitivity of differentially expressed genes to V79 dosage was specific to the premutation/expansion, we evaluated whether a similar phenomenon was detected upon overexpression of the V21 control vector. We validated the V79 dosage sensitivity for four out of five examined genes by TaqMan qPCR (Fig. 4A). For these genes we did not observe significant changes when overexpressing V21 (Fig. 4C) suggesting selective sensitivity for overexpression of the expanded *FMR1* 5′-UTR.

We only found ten differentially expressed lncRNA (Fold Change $\geq |1.2|$ and FDR $\leq$ 5%) in the in vitro cellular system, which contrasts with the large number of lncRNA deregulated in the blood of fragile X premutation carriers. Nevertheless, it is worth mentioning that only 15% (919 probes) of the lncRNA probes in the V1 version of the SurePrint G3 Human Gene Expression 8 × 60K Microarray are contained in the newer V2 version. Using the 919 common lncRNA probes for both arrays we found 383 to be differentially expressed in blood samples of patients with FXTAS, with only one being also deregulated in our in vitro cell system: the zinc finger protein 815 (ZNF815) non-coding RNA.

Discussion

Population based studies in newborn for the *FMR1* premutation allele have estimated prevalences ranging from 1/209 in females to 1/430 in males (Tassone et al., 2012). Fragile X premutation carriers are at risk of developing different disorders, including tremor and ataxia (FXTAS), early menopause (POF1), thyroid dysfunction, hypertension, fibromyalgia, and chronic muscle pain (Bacalman et al., 2006; Coffey et al., 2008; Grigsby et al., 2006; Hagerman et al., 2001, 2004; Sullivan et al., 2005). To evaluate the constellation of gene expression consequences of the *FMR1* CGG repeat premutation we have performed comprehensive gene expression profiling in peripheral blood samples of male carriers, including FXTAS patients. The pattern of differentially expressed genes allowed the identification of eight premutation associated clusters, each of them enriched in genes key in physiological processes, whose disruption is likely relevant in several fragile X premutation phenotypes.

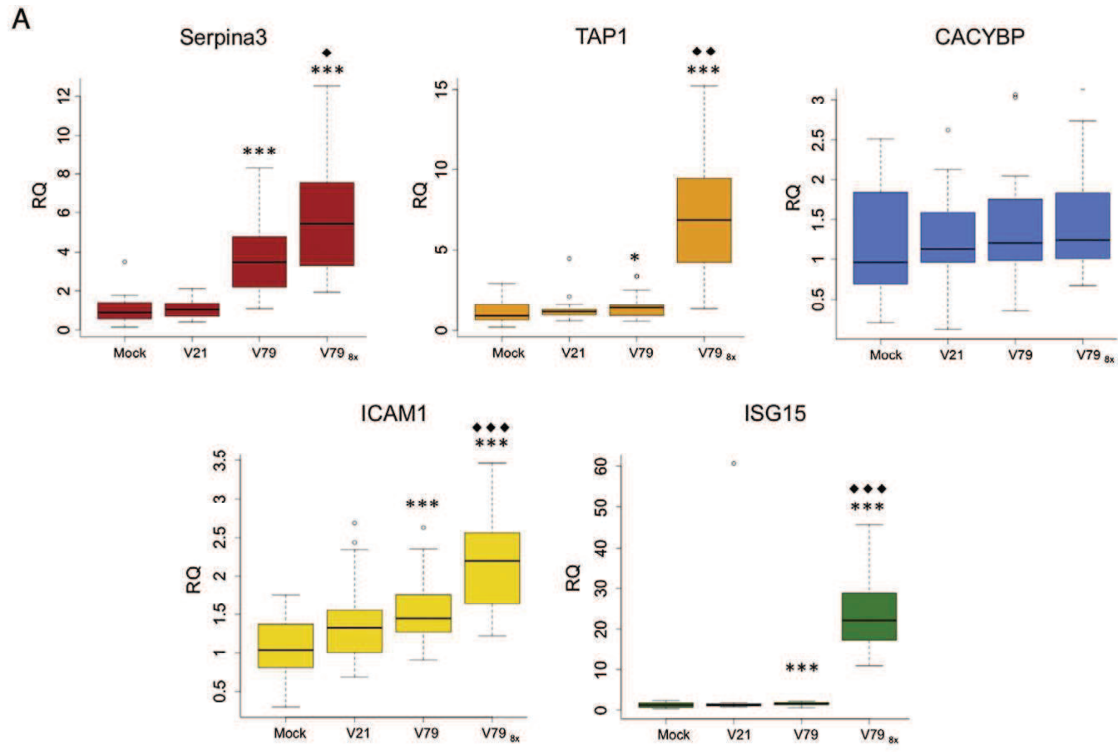
A large number of differentially expressed genes encode for respiratory chain subunits, which are essential for ATP production. We validated the upregulation of cytochrome c oxidase subunit VIc and subunit VIIb (*COX6C* and *COX7B*). The changes we observed in blood RNA of FXTAS patients was also detected in fibroblasts and in brain samples from FXTAS patients, suggesting a shared disease response between different tissues (Napoli et al., 2011; Ross-Inta et al., 2010). It has been proposed that the increase in the expression of mitochondrial function related transcripts in FXTAS patients is a compensatory response to overcome the diminished oxidative phosphorylation capacity (Napoli et al., 2011; Ross-Inta et al., 2010). Compromised oxidative phosphorylation affects ATP production and increases cellular stress by generating mitochondrial reactive oxygen species, which can damage cell components and lead to cell death (Lin and Beal, 2006). In line with this, we detected an upregulation of the superoxide dismutase 1 (*SOD1*), involved in neutralizing free superoxide radicals. Importantly, our data revealed increased expression of genes implicated in other stress responses, including spinocerebellar ataxia type 3 protein (*ATXN3*), involved in the degradation of misfolded chaperone substrates (48), and the amyloid beta (A4) precursor protein (*APP*), participating in endoplasmic reticulum stress induced apoptosis (Scaglione et al., 2011; Takahashi et al., 2009). Among the differentially expressed genes we also detected an enrichment in the oxidative stress response and in the oxidative phosphorylation pathways, which are both largely associated with neurodegenerative processes (Lin and Beal, 2006).

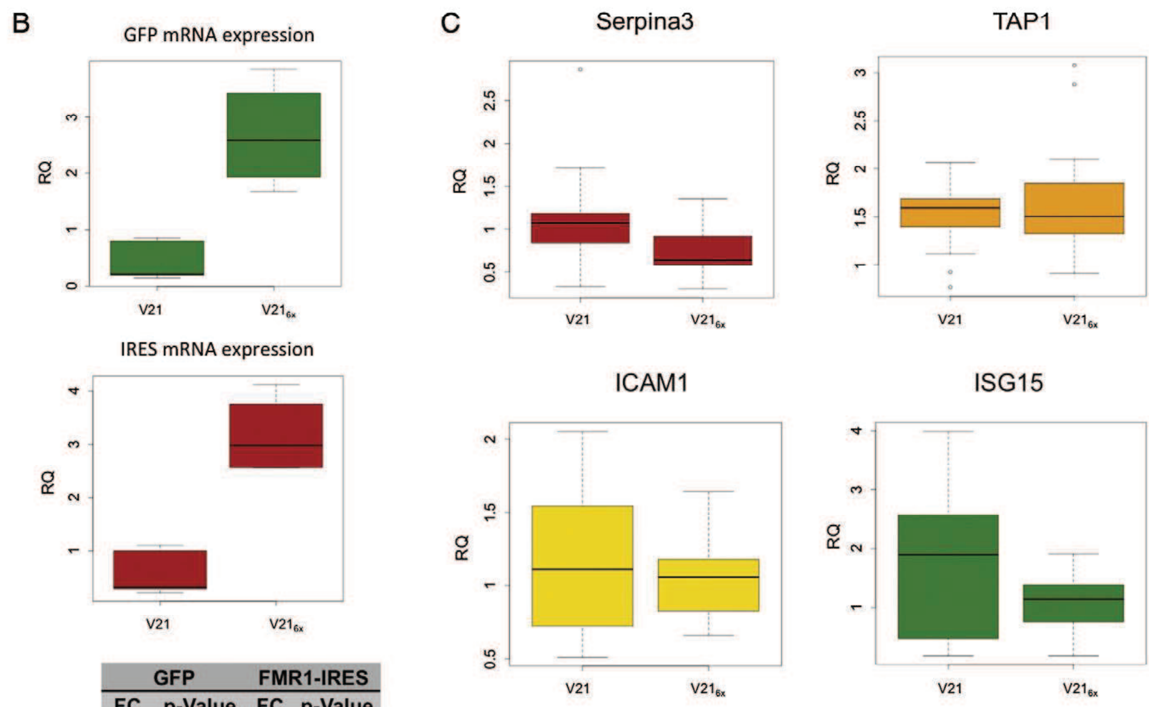
Other gene clusters showed enrichment genes involved in the mTOR signaling pathway and its downstream effectors: eIF2 signaling, eIF4 signaling, and p70S6K signaling regulation. mTOR integrates cellular signals to regulate organismal growth and homeostasis. In the nervous system, mTOR is critical for long term memory formation, axon regeneration, dendrite arborization and spine morphology (Hoeffer and Klann, 2010). The relevance of mTOR in cellular and organismal homeostasis is further supported by its deregulation in aging and neurodegenerative disorders (Laplante and Sabatini, 2012). An upstream regulator of mTOR is the v-akt murine thymoma viral oncogene homolog 1 (*AKT1*), for which we detected decreased expression levels in blood samples of symptomatic patients. It has been shown that knock down of *AKT1* in the fly model of FXTAS enhances the neurodegenerative phenotype in the eye (Lin et al., 2013), indicating its importance FXTAS neuropathology.

Blood profiling identified differentially expressed genes involved in gene expression regulation which are important for normal neuronal homeostasis. *ATXN7* is involved in chromatin remodeling and showed decreased expression in symptomatic patients. Mutations in *ATXN7* leads to spinocerebellar ataxia type 7, a neurodegenerative disorder closely related to FXTAS (David et al., 1997). We also detected decreased expression of *RNF10*, a transcription factor involved in expression regulation of myelin associated glycoprotein (MAG) (Hoshikawa et al., 2008). Studies with cerebral and cerebellar tissues from FXTAS patients exhibit white matter disease, which is characterized among other factors by neuron demyelination (Greco et al., 2002).

We also found decreased expression of *HDAC5* in symptomatic patients. Interestingly, the activity of histone deacetylases (HDACs) is critical to suppress the expanded CGG repeat dependent phenotype in a fly model of FXTAS (Todd et al., 2010). HDAC activation has been shown to rescue the rough eye phenotype in a FXTAS *Drosophila* model. Therefore, decreased expression of *HDAC5* may be involved in the FXTAS phenotype. In addition, abnormal levels of genes involved in cell death and survival pathways were also enriched in symptomatic patients. Decreased expression of *Bcl-X*, a potent inhibitor of cell death, and DNA fragmentation factor subunit alpha (*DFFA*), an inhibitor of DNA fragmentation, together with the upregulation of apoptosis related

Fig. 4. Expression of selective genes linked to *FMR1* 5′-UTR CGG-expansion and expression levels in differentiated SH-SY5Y neuronal cells. (A) qPCR measurements of the expression pattern of SERPINA3, *TAP1*, *CACYBP*, *ICAM1* and *ISG15* for which the array showed significant deregulation when comparing V79 or V79_8x vs. V21 expressing cells. (B) Relative quantification of V21 and V21_6x expression using two independent sets of primers, covering GFP (upper panel) or FMR1-IRES (lower panel) fragments. (C) qPCR analysis of SERPINA3, *TAP1*, *ICAM1* and *ISG15* in SH5Y-SY cells expressing theV21_6x. Plots show distribution and mean RQ ± SE. Expression levels are referred to a control mock transfected cell (A) or V21 transfected cell (B–C) for RQ and in all cases quantification was normalized using two different reference genes. by (**$P < 0.01$; ***$P < 0.001$; ◆◆$P < 0.01$; ◆◆◆$P < 0.001$; using a linear mixed effects model). Bonferroni correction was applied for multiple comparisons. Significant differences with respect to control individuals are indicated with "*" and significant differences between cells expressing V79 and V79_8x are indicated with "◆".





	GFP		FMR1-IRES	
	FC	p-Value	FC	p-Value
V21 vs. V21 6x	6.4	2.00E-04	5.9	2.00E-04

cysteine peptidase 3 (*CASP3*), responsible for apoptosis execution, indicates a higher predisposition in symptomatic patients for apoptosis activation.

Finally, we identified *EAP1* as a gene deregulated in male and female blood samples of fragile X premutation carriers. *EAP1* is an important component in the hypothalamic control of the initiation of puberty and maintenance of female reproductive cycles in rodents and in non-human primates (Dissen et al., 2012; Heger et al., 2007). Female carriers are at a risk for fragile X associated premature ovarian failure (POF1) (Allingham-Hawkins et al., 1999), which is characterized by menopause symptomatology. Studies show that 20% of female carriers will present FXPOI before 40 years of age and 30% before 45 years of age (Jacquemont et al., 2007). Thus, decreased expression of *EAP1* in female carriers could contribute to the appearance of POF1.

In summary, the present study elucidates gene expression profiles in blood samples of symptomatic patients that, at least partially, overlap with differentially expressed genes in brain and fibroblast samples of FXTAS patients.

The study of differentially expressed genes in human blood samples showed a highly overlapping deregulation pattern in the brain stem, motor cortex and striatum of a FXTAS mouse model. The prefrontal cortex and the brain stem displayed upregulation of genes involved in the response to cellular stress, including *APP*, *ATXN3* and *SOD1*. Moreover, four different brain regions showed decreased expression of the histone modifiers *ATXN7* and *HDAC5*, coinciding both in motor cortex and striatum as well as *RNF10* in the motor cortex, striatum and brain stem. We also detected decreased expression of *KCNC3* in four out of six brain areas. Knock-out mice for *KCNC3* display motor deficits such as tremor and severe ataxia (Espinosa et al., 2001), similar to what is observed in FXTAS patients. All together, these results suggest that gene expression profiling in blood partially reflects changes in the brain transcriptome which underlie neuropathological aspects in FXTAS.

We identified characteristic gene expression profiles for asymptomatic patients and symptomatic patients in peripheral blood of fragile X premutation carriers. A previous report (Napoli et al., 2011) and our own data suggest that *FMR1* expression levels contribute to the clinical manifestation of FXTAS. The expression profiles in a neuronal cell model indicated that mutant *FMR1* levels are relevant for the deregulation of genes involved in cell death and survival pathways, the inflammatory response as well as nervous system development and function. We therefore propose that transcriptional changes driven by different levels of *FMR1* premutation may contribute to clinical/phenotypic differences between FXTAS patients.

Changes in gene expression can also be the consequence of variations in miRNA profiles, activity or concentration. Alteration in the miRNA processing machinery has been reported recently in FXTAS (Sellier et al., 2013), leading to a decreased expression of many miRNAs. In agreement with this, IPA upstream regulator analysis predicts a decreased activity of 31 of 32 miRNAs. Of these, four are downregulated in the blood of FXTAS patients (Alvarez-Mora et al., 2013). One of the interesting candidates is miR-221-3p/miR-221 (Z score $= -2.56$ and overlap p-value $= 4.53e-2$), which has an important role in neuronal differentiation and participates in neuronal apoptosis and the inflammation processes (Harder and Libby, 2011; Kim and Hwang, 2011). In our data set we found 49 up-regulated putative miR-221-3p targets, according to Targetscan predictions and increased levels of two validated miR-221-3p targets: cyclin-dependent kinase inhibitor 1B (CDKN1B or $p27^{Kip1}$) and Bcl-2-modifying factor (BMF). Interestingly, miR-221-3p targets the 3′-UTR of *FMR1* mRNA modulating its expression (Zongaro et al., 2013). Thus, decreased levels of miR-221-3p might contribute to *FMR1* upregulation in FXTAS patients.

Increasing evidence suggests that, by perturbing gene expression, lncRNAs play an important role in human disease, including neurodegenerative diseases. We report a significant FXTAS-associated deregulation of lncRNAs in blood samples, which may trigger downstream deregulation of protein coding genes in fragile X premutation carriers. We found that maternally expressed 3 (*MEG3*) and taurine upregulated gene 1 (*TUG1*) are deregulated in our data-set. Both lncRNA are known to associate with polycomb repressive complex 2 (*PRC2*), acting as epigenetic enhancers or repressors in cis- or in trans-, respectively (Khalil et al., 2009; Mondal et al., 2010). Another lncRNA deregulated in our data-set is Nuclear Enriched Abundant Transcript 1 (*NEAT1*), which is essential for the formation and maintenance of paraspeckles (Clemson et al., 2009), an irregular shaped compartment found in the nucleus' interchromatin space (Fox et al., 2002) and which is involved in the regulation of gene expression (Prasanth et al., 2005). Interestingly, *TUG1*, *MEG3* and *NEAT1* are similarly deregulated in Huntington's disease (Johnson, 2012; Khalil et al., 2009), which suggests a common involvement in CNS dysfunction.

Conclusions

In summary, we have shown here a large number of genes with abnormal expression in fragile X premutation carriers. In blood, the main affected functional pathways overlap with previous findings in the brain and primary fibroblasts of carriers, suggesting that blood RNA profiling reflects FXTAS pathogenic alterations. Specifically, we have identified several genes whose expression deregulation likely contributes to FXTAS and/or POF1, including *EAP1*, *HDAC5* and *KCNC3*. Moreover, we provide new evidences for a role of expanded 5′-UTR *FMR1* expression levels in transcriptome modulation. Longitudinal studies in fragile X premutation carriers addressing the relationship between the dynamic changes in the expression of selective genes (specially *FMR1*) and the clinical evolution may shed light onto the pathogenic mechanisms and further target molecules with therapeutic potential.

Conflict of interest statement

The authors declare not to have any conflict of interest.

Acknowledgments

This work was supported by the Spanish Government and FEDER (Fondo Europeo de Desarrollo Regional): PN de I + D + I 2008–2011 PI081367 and PN de I + D + I 2012–2015 PI11/02036, Instituto Carlos III — ISCIII, Subdirección General de Evaluación y Fomento de la Investigación (to E.M), SAF2008-00357 Ministerio de Economia y Competitividad, ISCIII(to X.E), PN de I + D + I 2009–2012 PS09/00413 and PN de I + D + I 2013–2015 PI12/00879 Carlos III — ISCIII, Subdirección General de Evaluación y Fomento de la Investigación (to M.M); Centro de Investigación Biomédica en Red en Epidemiología y Salud Pública (CIBERESP) through the ISCIII (E.M-H., E.M., X.E); and Centro de Investigación Biomédica en Red sobre Enfermedades Neurodegenerativas (CIBERNED) through the ISCIII (L.R-R, MI.A-M, M-M). The Spanish Government supports E.M. (Programa Miguel Servet). Support for E.M-H. was provided by a "La Caixa" fellowship. The Netherlands Brain Foundation supports R.W. We thank the staff of the Genomics Unit and Bioinformatics Unit of the CRG for the analysis of the Agilent arrays.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nbd.2013.12.020.

References

Allingham-Hawkins, D.J., Babul-Hirji, R., Chitayat, D., Holden, J.J., Yang, K.T., Lee, C., …, Vieri, F., 1999. Fragile X premutation is a significant risk factor for premature ovarian failure: the International Collaborative POF in Fragile X study — preliminary data. Am. J. Med. Genet. 83 (4), 322–325.

Alvarez-Mora, M.I., Rodriguez-Revenga, L., Madrigal, I., Torres-Silva, F., Mateu-Huertas, E., Lizano, E., …, Milà, M., 2013. MicroRNA expression profiling in blood from fragile

X-associated tremor/ataxia syndrome patients. Genes Brain Behav. http://dx.doi.org/10.1111/gbb.12061.

Arocena, D.G., Iwahashi, C.K., Won, N., Beilina, A., Ludwig, A.L., Tassone, F., …, Hagerman, P.J., 2005. Induction of inclusion formation and disruption of lamin A/C structure by premutation CGG-repeat RNA in human cultured neural cells. Hum. Mol. Genet. 14 (23), 3661–3671. http://dx.doi.org/10.1093/hmg/ddi394.

Bacalman, S., Farzin, F., Bourgeois, J.A., Cogswell, J., Goodlin-Jones, B.L., Gane, L.W., …, Hagerman, R.J., 2006. Psychiatric phenotype of the fragile X-associated tremor/ataxia syndrome (FXTAS) in males: newly described fronto-subcortical dementia. J. Clin. Psychiatry 67 (1), 87–94.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing author. J. R. Stat. Soc. Ser. B 57 (1), 289–300.

Bolstad, B., 2001. Probe Level Quantile Normalization of High Density Oligonucleotide Array Data. 1–8.

Bontekoe, C.J., Bakker, C.E., Nieuwenhuizen, I.M., van der Linde, H., Lans, H., de Lange, D., …, Oostra, B.A., 2001. Instability of a (CGG)98 repeat in the Fmr1 promoter. Hum. Mol. Genet. 10 (16), 1693–1699.

Borovecki, F., Lovrecic, L., Zhou, J., Jeong, H., Then, F., Rosas, H.D., …, Krainc, D., 2005. Genome-wide expression profiling of human blood reveals biomarkers for Huntington's disease. Proc. Natl. Acad. Sci. U. S. A. 102 (31), 11023–11028. http://dx.doi.org/10.1073/pnas.0504921102.

Clemson, C.M., Hutchinson, J.N., Sara, S.A., Ensminger, A.W., Fox, A.H., Chess, A., Lawrence, J.B., 2009. An architectural role for a nuclear noncoding RNA: NEAT1 RNA is essential for the structure of paraspeckles. Mol. Cell 33 (6), 717–726. http://dx.doi.org/10.1016/j.molcel.2009.01.026.

Coffey, S.M.S., Cook, K., Tartaglia, N., Tassone, F., Nguyen, D.V., Pan, R., …, Hagerman, R.J., 2008. Expanded clinical phenotype of women with the FMR1 premutation. Am. J. Med. Genet. A 146A (8), 1009–1016. http://dx.doi.org/10.1002/ajmg.a.32060.

Cohen, S., Masyn, K., Adams, J., Hessl, D., Rivera, S., Tassone, F., …, Hagerman, R., 2006. Molecular and imaging correlates of the fragile X-associated tremor/ataxia syndrome. Neurology 67 (8), 1426–1431. http://dx.doi.org/10.1212/01.wnl.0000239837.57475.3a.

D'Hulst, C., Heulens, I., Brouwer, J.R., Willemsen, R., De Geest, N., Reeve, S.P., …, Kooy, R.F., 2009. Expression of the GABAergic system in animal models for fragile X syndrome and fragile X associated tremor/ataxia syndrome (FXTAS). Brain Res. 1253, 176–183. http://dx.doi.org/10.1016/j.brainres.2008.11.075.

David, G., Abbas, N., Stevanin, G., Dürr, A., Yvert, G., Cancel, G., …, Brice, A., 1997. Cloning of the SCA7 gene reveals a highly unstable CAG repeat expansion. Nat. Genet. 17 (1), 65–70. http://dx.doi.org/10.1038/ng0997-65.

Dissen, G.A., Lomniczi, A., Heger, S., Neff, T.L., Ojeda, S.R., 2012. Hypothalamic EAP1 (enhanced at puberty 1) is required for menstrual cyclicity in nonhuman primates. Endocrinology 153 (1), 350–361. http://dx.doi.org/10.1210/en.2011-1541.

Dombrowski, C., Lévesque, S., Morel, M.L., Rouillard, P., Morgan, K., Rousseau, F., 2002. Premutation and intermediate-size FMR1 alleles in 10572 males from the general population: loss of an AGG interruption is a late event in the generation of fragile X syndrome alleles. Hum. Mol. Genet. 11 (4), 371–378.

Espinosa, F., McMahon, A., Chan, E., Wang, S., Ho, C.S., Heintz, N., Joho, R.H., 2001. Alcohol hypersensitivity, increased locomotion, and spontaneous myoclonus in mice lacking the potassium channels Kv3.1 and Kv3.3. J. Neurosci. 21 (17), 6657–6665.

Fox, A.H., Lam, Y.W., Leung, A.K.L., Lyon, C.E., Andersen, J., Mann, M., Lamond, A.I., 2002. Paraspeckles: a novel nuclear domain. Curr. Biol. 12 (1), 13–25.

Fu, Y.H., Kuhl, D.P., Pizzuti, A., Pieretti, M., Sutcliffe, J.S., Richards, S., …, Warren, S.T., 1991. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. Cell 67 (6), 1047–1058.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., …, Zhang, J., 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 5 (10), R80. http://dx.doi.org/10.1186/gb-2004-5-10-r80.

Greco, C.M., Hagerman, R.J., Tassone, F., Chudley, A.E., Del Bigio, M.R., Jacquemont, S., …, Hagerman, P.J., 2002. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. Brain 125 (Pt 8), 1760–1771.

Grigsby, J., Brega, A.G., Jacquemont, S., Loesch, D.Z., Leehey, M.A., Goodrich, G.K., …, Hagerman, P.J., 2006. Impairment in the cognitive functioning of men with fragile X-associated tremor/ataxia syndrome (FXTAS). J. Neurol. Sci. 248 (1–2), 227–233. http://dx.doi.org/10.1016/j.jns.2006.05.016.

Hagerman, P.J., Hagerman, R.J., 2004. Fragile X-associated tremor/ataxia syndrome (FXTAS). Ment. Retard. Dev. Disabil. Res. Rev. 10 (1), 25–30. http://dx.doi.org/10.1002/mrdd.20005.

Hagerman, R.J., Leehey, M., Heinrichs, W., Tassone, F., Wilson, R., Hills, J., …, Hagerman, P.J., 2001. Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. Neurology 57 (1), 127–130.

Hagerman, R.J., Leavitt, B.R., Farzin, F., Jacquemont, S., Greco, C.M., Brunberg, J.A., …, Hagerman, P.J., 2004. Fragile-X-associated tremor/ataxia syndrome (FXTAS) in females with the FMR1 premutation. Am. J. Hum. Genet. 74 (5), 1051–1056. http://dx.doi.org/10.1086/420700.

Harder, J.M., Libby, R.T., 2011. BBC3 (PUMA) regulates developmental apoptosis but not axonal injury induced death in the retina. Mol. Neurodegener. 6 (1), 50. http://dx.doi.org/10.1186/1750-1326-6-50.

Haugen, A.C., Di Prospero, N.A., Parker, J.S., Fannin, R.D., Chou, J., Meyer, J.N., …, Van Houten, B., 2010. Altered gene expression and DNA damage in peripheral blood cells from Friedreich's ataxia patients: cellular model of pathology. PLoS Genet. 6 (1), e1000812. http://dx.doi.org/10.1371/journal.pgen.1000812.

Heger, S., Mastronard, C., Dissen, G.G.A., Mastronardi, C., Lomniczi, A., Cabrera, R., …, Ojeda, S.R., 2007. Enhanced at puberty 1 (EAP1) is a new transcriptional regulator of the female neuroendocrine reproductive axis. J. Clin. Invest. 117 (8), 2145–2154. http://dx.doi.org/10.1172/JCI31752.

Hessl, D., Tassone, F., Loesch, D.Z., Berry-Kravis, E., Leehey, M.A., Gane, L.W., …, Hagerman, R.J., 2005. Abnormal elevation of FMR1 mRNA is associated with psychological symptoms in individuals with the fragile X premutation. Am. J. Med. Genet. B Neuropsychiatr. Genet. 139B (1), 115–121. http://dx.doi.org/10.1002/ajmg.b.30241.

Hoeffer, C.A.C., Klann, E., 2010. mTOR signaling: at the crossroads of plasticity, memory and disease. Trends Neurosci. 33 (2), 67–75. http://dx.doi.org/10.1016/j.tins.2009.11.003.

Hoshikawa, S., Ogata, T., Fujiwara, S., Nakamura, K., Tanaka, S., 2008. A novel function of RING finger protein 10 in transcriptional regulation of the myelin-associated glycoprotein gene and myelin formation in Schwann cells. PLoS One 3 (10), e3464. http://dx.doi.org/10.1371/journal.pone.0003464.

Hunsaker, M.R., Wenzel, H.J., Willemsen, Rob, Berman, R.F., 2012. Progressive spatial processing deficits in a mouse model of the fragile X premutation. Behav. Neurosci. 123 (6), 1315–1324. http://dx.doi.org/10.1037/a0017616 (Progressive).

Iwahashi, C.K., Yasui, D.H., An, H.-J., Greco, C.M., Tassone, F., Nannen, K., …, Hagerman, P.J., 2006. Protein composition of the intranuclear inclusions of FXTAS. Brain 129 (Pt 1), 256–271. http://dx.doi.org/10.1093/brain/awh650.

Jacquemont, S., Hagerman, R.J., Leehey, M.A., Hall, D.A., Levine, R.A., Brunberg, J.A., …, Hagerman, P.J., 2004. Penetrance of the fragile X-associated tremor/ataxia syndrome in a premutation carrier population. JAMA 291 (4), 460–469. http://dx.doi.org/10.1001/jama.291.4.460.

Jacquemont, S., Hagerman, R.J., Hagerman, P.J., Leehey, M.A., 2007. Fragile-X syndrome and fragile X-associated tremor/ataxia syndrome: two faces of FMR1. Lancet Neurol. 6 (1), 45–55. http://dx.doi.org/10.1016/S1474-4422(06)70676-7.

Jin, P., Duan, R., Qurashi, A., Qin, Y., Tian, D., Rosser, T.C., …, Warren, S.T., 2007. Pur alpha binds to rCGG repeats and modulates repeat-mediated neurodegeneration in a Drosophila model of fragile X tremor/ataxia syndrome. Neuron 55 (4), 556–564. http://dx.doi.org/10.1016/j.neuron.2007.07.020.

Johnson, R., 2012. Long non-coding RNAs in Huntington's disease neurodegeneration. Neurobiol. Dis. 46 (2), 245–254. http://dx.doi.org/10.1016/j.nbd.2011.12.006.

Kenneson, A., Zhang, F., Hagedorn, C.H., Warren, S.T., 2001. Reduced FMRP and increased FMR1 transcription is proportionally associated with CGG repeat number in intermediate-length and premutation carriers. Hum. Mol. Genet. 10 (14), 1449–1454.

Khalil, A.M., Guttman, M., Huarte, M., Garber, M., Raj, A., Rivea Morales, D., …, Rinn, J.L., 2009. Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression. Proc. Natl. Acad. Sci. U. S. A. 106 (28), 11667–11672. http://dx.doi.org/10.1073/pnas.0904715106.

Kim, E.-M., Hwang, O., 2011. Role of matrix metalloproteinase-3 in neurodegeneration. J. Neurochem. 116 (1), 22–32. http://dx.doi.org/10.1111/j.1471-4159.2010.07082.x.

Laplante, M., Sabatini, D.M., 2012. mTOR signaling in growth control and disease. Cell 149 (2), 274–293. http://dx.doi.org/10.1016/j.cell.2012.03.017.

Lee, J., Li, Z., Brower-Sinning, R., John, B., 2007. Regulatory circuit of human microRNA biogenesis. PLoS Comput. Biol. 3 (4), e67. http://dx.doi.org/10.1371/journal.pcbi.0030067.

Lin, M.T., Beal, M.F., 2006. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature 443 (7113), 787–795. http://dx.doi.org/10.1038/nature05292.

Lin, Y., Tang, C., He, H., Duan, R., 2013. Activation of mTOR ameliorates fragile X premutation rCGG repeat-mediated neurodegeneration. PLoS One 8 (4), e62572. http://dx.doi.org/10.1371/journal.pone.0062572.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25 (4), 402–408. http://dx.doi.org/10.1006/meth.2001.1262.

Lomniczi, A., Garcia-Rudaz, C., Ramakrishnan, R., Wilmot, B., Khouangsathiene, S., Ferguson, B., …, Ojeda, S.R., 2012. A single-nucleotide polymorphism in the EAP1 gene is associated with amenorrhea/oligomenorrhea in nonhuman primates. Endocrinology 153 (1), 339–349. http://dx.doi.org/10.1210/en.2011-1540.

Lu, C., Lin, L., Tan, H., Wu, H., Sherman, S.L., Gao, F., …, Chen, D., 2012. Fragile X premutation RNA is sufficient to cause primary ovarian insufficiency in mice. Hum. Mol. Genet. 21 (23), 5039–5047. http://dx.doi.org/10.1093/hmg/dds348.

Matsuda, T., Cepko, C.L., 2004. Electroporation and RNA interference in the rodent retina in vivo and in vitro. Proc. Natl. Acad. Sci. U. S. A. 101 (1), 16–22. http://dx.doi.org/10.1073/pnas.2235688100.

Miñones-Moyano, E., Porta, S., Escaramís, G., Rabionet, R., Iraola, S., Kagerbauer, B., …, Martí, E., 2011. MicroRNA profiling of Parkinson's disease brains identifies early downregulation of miR-34b/c which modulate mitochondrial function. Hum. Mol. Genet. 20 (15), 3067–3078. http://dx.doi.org/10.1093/hmg/ddr210.

Mondal, T., Rasmussen, M., Pandey, G.K., Isaksson, A., Kanduri, C., 2010. Characterization of the RNA content of chromatin. Genome Res. 20 (7), 899–907. http://dx.doi.org/10.1101/gr.103473.109.

Moore, C.J., Daly, E.M., Tassone, F., Tysoe, C., Schmitz, N., Ng, V., …, Murphy, D.G.M., 2004. The effect of pre-mutation of X chromosome CGG trinucleotide repeats on brain anatomy. Brain 127 (Pt 12), 2672–2681. http://dx.doi.org/10.1093/brain/awh256.

Napoli, E., Ross-Inta, C., Wong, S., Omanska-Klusek, A., Barrow, C., Iwahashi, C., …, Giulivi, C., 2011. Altered zinc transport disrupts mitochondrial protein processing/import in fragile X-associated tremor/ataxia syndrome. Hum. Mol. Genet. 20 (15), 3079–3092. http://dx.doi.org/10.1093/hmg/ddr211.

Orr, H.T., Zoghbi, H.Y., 2007. Trinucleotide repeat disorders. Annu. Rev. Neurosci. 30, 575–621. http://dx.doi.org/10.1146/annurev.neuro.29.051605.113042.

Peprah, E., 2012. Fragile X syndrome: the FMR1 CGG repeat distribution among world populations. Ann. Hum. Genet. 76 (2), 178–191. http://dx.doi.org/10.1111/j.1469-1809.2011.00694.x.

Prasanth, K.V., Prasanth, S.G., Xuan, Z., Hearn, S., Freier, S.M., Bennett, C.F., …, Spector, D.L., 2005. Regulating gene expression through RNA nuclear retention. Cell 123 (2), 249–263. http://dx.doi.org/10.1016/j.cell.2005.08.033.

Presgraves, S.P., Ahmed, T., Borwege, S., Joyce, J.N., 2004. Terminally differentiated SH-SY5Y cells provide a model system for studying neuroprotective effects of dopamine agonists. Neurotox. Res. 5 (8), 579–598.

Ritchie, M.E., Silver, J., Oshlack, A., Holmes, M., Diyagama, D., Holloway, A., Smyth, G.K., 2007. A comparison of background correction methods for two-colour microarrays. Bioinformatics 23 (20), 2700–2707. http://dx.doi.org/10.1093/bioinformatics/btm412.
Ross-Inta, C., Omanska-Klusek, A., Wong, S., 2010. Evidence of mitochondrial dysfunction in fragile X-associated tremor/ataxia syndrome. Biochem. J. 552, 545–552. http://dx.doi.org/10.1042/BJ20091960.
Scaglione, K.M., Zavodszky, E., Todi, S.V., Patury, S., Xu, P., Rodríguez-Lebrón, E., …, Paulson, H.L., 2011. Ube2w and ataxin-3 coordinately regulate the ubiquitin ligase CHIP. Mol. Cell 43 (4), 599–612. http://dx.doi.org/10.1016/j.molcel.2011.05.036.
Sellier, C., Rau, F., Liu, Y., Tassone, F., Hukema, R.K., Gattoni, R., …, Charlet-Berguerand, N., 2010. Sam68 sequestration and partial loss of function are associated with splicing alterations in FXTAS patients. EMBO J. 29 (7), 1248–1261. http://dx.doi.org/10.1038/emboj.2010.21.
Sellier, C., Freyermuth, F., Tabet, R., Tran, T., He, F., Ruffenach, F., …, Charlet-Berguerand, N., 2013. Sequestration of DROSHA and DGCR8 by expanded CGG RNA repeats alters microRNA processing in fragile X-associated tremor/ataxia syndrome. Cell Rep. 3 (3), 1–12. http://dx.doi.org/10.1016/j.celrep.2013.02.004.
Smyth, G.K., 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Stat. Appl. Genet. Mol. Biol. 3. http://dx.doi.org/10.2202/1544-6115.1027 (Article3).
Sofola, O.A., Jin, P., Qin, Y., Duan, R., Liu, H., de Haro, M., …, De, M., 2007. RNA-binding proteins hnRNP A2/B1 and CUGBP1 suppress fragile X CGG premutation repeat-induced neurodegeneration in a *Drosophila* model of FXTAS. Neuron 55 (4), 565–571. http://dx.doi.org/10.1016/j.neuron.2007.07.021.
Steibel, J.P., Poletto, R., Coussens, P.M., Rosa, G.J.M., 2009. A powerful and flexible linear mixed model framework for the analysis of relative quantification RT-PCR data. Genomics 94 (2), 146–152. http://dx.doi.org/10.1016/j.ygeno.2009.04.008.
Sullivan, A.K., Marcus, M., Epstein, M.P., Allen, E.G., Anido, A.E., Paquin, J.J., …, Sherman, S.L., 2005. Association of FMR1 repeat size with ovarian dysfunction. Hum. Reprod. 20 (2), 402–412. http://dx.doi.org/10.1093/humrep/deh635.
Takahashi, K., Niidome, T., Akaike, A., Kihara, T., Sugimoto, H., 2009. Amyloid precursor protein promotes endoplasmic reticulum stress-induced cell death via C/EBP homologous protein-mediated pathway. J. Neurochem. 109 (5), 1324–1337. http://dx.doi.org/10.1111/j.1471-4159.2009.06067.x.
Tassone, F., Hagerman, R.J., Iklé, D.N., Dyer, P.N., Lampe, M., Willemsen, R., …, Taylor, A.K., 1999. FMRP expression as a potential prognostic indicator in fragile X syndrome. Am. J. Med. Genet. 84 (3), 250–261.
Tassone, F., Hagerman, R.J., Taylor, A.K., Gane, L.W., Godfrey, T.E., Hagerman, P.J., 2000. Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. Am. J. Hum. Genet. 66 (1), 6–15. http://dx.doi.org/10.1086/302720.
Tassone, F., Hagerman, R.J., Garcia-Arocena, D., Khandjian, E.W., Greco, C.M., Hagerman, P.J., 2004. Intranuclear inclusions in neural cells with premutation alleles in fragile X associated tremor/ataxia syndrome. J. Med. Genet. 41 (4), e43-e43. http://dx.doi.org/10.1136/jmg.2003.012518.
Tassone, F., Long, K.P., Tong, T.-H., Lo, J., Gane, L.W., Berry-Kravis, E., …, Hagerman, R.J., 2012. FMR1 CGG allele size and prevalence ascertained through newborn screening in the United States. Genome Med. 4 (12), 100. http://dx.doi.org/10.1186/gm401.
Todd, P.K., Oh, S.Y., Krans, A., Pandey, U.B., Di Prospero, N.A., Min, K.-T., …, Paulson, H.L., 2010. Histone deacetylases suppress CGG repeat-induced neurodegeneration via transcriptional silencing in models of fragile X tremor ataxia syndrome. PLoS Genet. 6 (12), e1001240. http://dx.doi.org/10.1371/journal.pgen.1001240.
Verkerk, A.J., Pieretti, M., Sutcliffe, J.S., Fu, Y.H., Kuhl, D.P., Pizzuti, A., …, Warren, S.T., 1991. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65 (5), 905–914.
Wenzel, H.J., Hunsaker, M.R., Greco, C.M., Willemsen, R., Berman, R.F., 2010. Ubiquitin-positive intranuclear inclusions in neuronal and glial cells in a mouse model of the fragile X premutation. Brain Res. 1318, 155–166. http://dx.doi.org/10.1016/j.brainres.2009.12.077.
Willemsen, R., 2003. The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. Hum. Mol. Genet. 12 (9), 949–959. http://dx.doi.org/10.1093/hmg/ddg114.
Zongaro, S., Hukema, R., D'antoni, S., Davidovic, L., Barbry, P., Catania, M.V., …, Simona, D., 2013. The 3′UTR of FMR1 mRNA is a target of mi, R-101, miR129-5p and miR-221: implications for the molecular pathology of FXTAS at the synapse. Hum. Mol. Genet. 1–41. http://dx.doi.org/10.1093/hmg/ddt044.