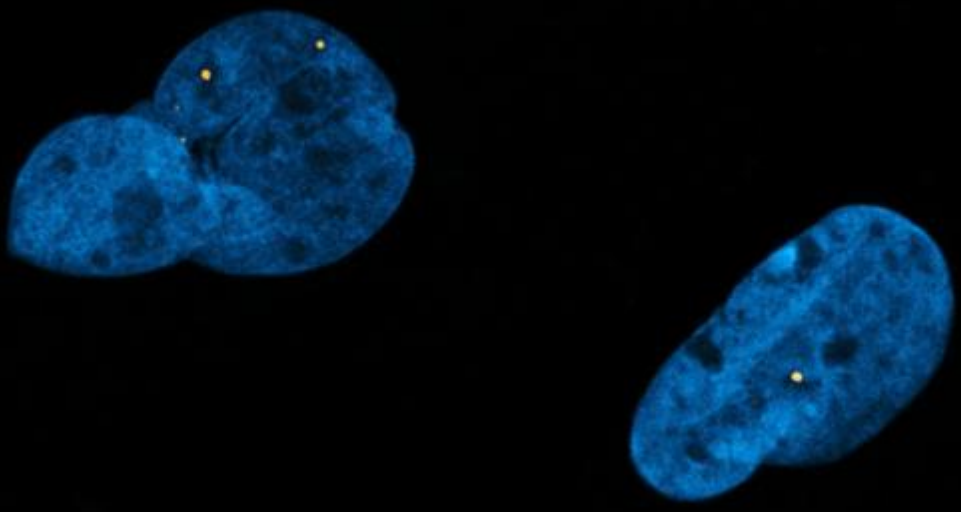
Nuevas estrategias terapéuticas en la Distrofia Miotónica tipo 1 basadas en los mecanismos moleculares del envejecimiento y del cáncer



Mikel García Puga

Tesis Doctoral

2020

eman ta zabal zazu

Universidad del País Vasco

Euskal Herriko Unibertsitatea

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Directores: Dr. Ander Matheu y Dr. Adolfo López de Munain

Memoria presentada para optar al título de Doctor por la Universidad del País Vasco / Euskal Herriko Unibertsitatea

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Universidad del País Vasco | Euskal Herriko Unibertsitatea

NAZIOARTEKO BIKAINTASUN CAMPUSA
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TESI ZUZENDARIAREN BAIMENA TESIA AURKEZTEKO	AUTORIZACIÓN DEL/LA DIRECTORA/A DE TESIS PARA SU PRESENTACIÓN

Zuzendariaren izen-abizenak /Nombre y apellidos del/la director/a:

Adolfo López de Munain con NIF 16246491G
Ander Matheu Fernández con NIF 44144685A

Tesiaren izenburua / Título de la tesis: Nuevas estrategias terapéuticas en la Distrofia Miotónica tipo 1 basadas en los mecanismos moleculares del envejecimiento y del cáncer

Doktorego programa / Programa de doctorado: Biología Molecular y Biomedicina

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Tokia eta data / Lugar y fecha: San Sebastián 20 de Julio de 2020

Ander Matheu Fernández

Adolfo López de Munain

Sin. / Fdo.: Tesiaren zuzendaria / El/La director/a de la tesis

AUTORIZACIÓN DE LA COMISIÓN ACADÉMICA DEL PROGRAMA DE DOCTORADO

La Comisión Académica del Programa de Doctorado en Biología Molecular y Biomedicina en reunión On Line celebrada el día **17** de Julio de 2020, ha acordado dar la conformidad a la presentación de la Tesis Doctoral titulada: *Nuevas estrategias terapéuticas en la Distrofia Miotónica tipo 1 basadas en los mecanismos moleculares del envejecimiento y del cáncer* dirigida por el Dr. Ander Matheu Fernández y Adolfo López de Munain y presentada por Don. Mikel García Puga adscrito al Departamento de Neurociencias.

En Leioa (Vizcaya), a 17 de Julio de 2020.

EL/LA RESPONSABLE DEL PROGRAMA DE DOCTORADO

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Fdo.: Prof. Miguel A. Trueba

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El Consejo del Departamento de Neurociencias

en reunión celebrada el día 23 de julio de 2020 ha acordado dar la conformidad a la

admisión a trámite de presentación de la Tesis Doctoral titulada:

Nuevas estrategias terapéuticas en la Distrofia Miotónica tipo 1 basadas en los mecanismos moleculares del envejecimiento y del cáncer

dirigida por los Dres. ADOLFO LÓPEZ DE MUNAIN ARREGUI y ANDER MATHEU FERNÁNDEZ

y presentada por Don MIKEL GARCÍA PUGA ante este Departamento.

En LEIOA, a 23 de julio de 2020

VºBº DIRECTOR DEL DEPARTAMENTO

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ACTA DE GRADO DE DOCTOR O DOCTORA
ACTA DE DEFENSA DE TESIS DOCTORAL

DOCTORANDO **DON MIKEL GARCÍA PUGA**

TITULO DE LA TESIS: ***Nuevas estrategias terapéuticas en la Distrofia Miotónica tipo 1 basadas en los mecanismos moleculares del envejecimiento y del cáncer***

El Tribunal designado por la Comisión de Postgrado de la UPV/EHU para calificar la Tesis Doctoral arriba indicada y reunido en el día de la fecha, una vez efectuada la defensa por el/la doctorando/a y contestadas las objeciones y/o sugerencias que se le han formulado, ha otorgado por_____la calificación de:

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Fdo.: Fdo.: Fdo.:

Dr/a: _____Dr/a:_____Dr/a:_____

EL DOCTORANDO, Fdo.: Mikel García Puga

Agradecimientos

La verdad es que cuando empiezas el doctorado piensas que este día queda muy lejano y, sin embargo, aquí estoy escribiendo los agradecimientos de mi tesis, cerrando una etapa que no habría sido posible sin el apoyo de muchísima gente. La andadura por la tesis no ha sido precisamente fácil y por ello quiero dar las gracias a todos lo que me han ayudado en este camino. Ha sido un periodo de aprendizaje continuo, no solo en el campo científico, sino también a nivel personal.

En primer lugar, esta tesis no sería posible sin el apoyo que he recibido por parte de toda mi familia. Por inculcarme que todo es posible con esfuerzo y por apoyarme en las elecciones que he tomado.

Agradecer a mis directores de tesis Ander Matheu y Adolfo López de Munain, por haberme apoyado desde el primer momento y haberme acogido en vuestros laboratorios a pesar de no conocerme previamente. Durante estos años habéis sido clave en mi desarrollo como investigador, empezando con los pequeños consejos del día a día y terminando en cómo estructurar la escritura de una tesis. Pero también a nivel personal, celebrando los pequeños logros que hemos ido superando durante estos años. **Ander**, me has transmitido la confianza para poder celebrar los pequeños avances con unas cañas. Además, creo que ninguno de tus becarios olvidará cuál es el mejor ron del mundo. **Adolfo**, quería decirte que has aportado la parte humana a esta tesis, siempre me has apoyado y ayudado, indicándome cómo mejorar. Además, a pesar de estar siempre tan ocupado, has encontrado un momento para escucharme y guiarme. Nunca olvidaré tus correcciones como María Moliner.

A continuación, me gustaría agradecer a mis compañeros de laboratorio por el enorme apoyo que he recibido y también el que hayáis estado siempre ahí para ayudarme en mis malos momentos. Han sido muchos años y no quiero olvidarme de la gente que ya no está en el laboratorio. **Olatz**, los cafés perdieron mucho tras tu marcha, siempre con una historia graciosa que contar. **Pauli**, compañera de pecera, *Mrs. Firefly*, tengo algo que confesar… En realidad, nunca se equivocaron en la fábrica al repartir las tallas de los guantes, era muy gracioso verte no comprender cómo podía haber guantes diminutos en tu caja jajaj. Siempre me acordaré de esos agostos solos en Biodonostia, los dos mano a mano en el lab con nuestra amiga la Firefly. ¡¡Ya nos veremos por Iruña!! **Estitxu**, empecé contigo, tú me enseñaste todo lo que sé en el laboratorio, y a día de hoy me siento muy orgulloso de que me hayas enseñado. Me has transmitido la idea de ser metódico y correcto en mis experimentos, con la confianza de echarnos unas cañas. **Idoia**, ahora entiendo muchas cosas que no entendía cuando llegué al laboratorio, y muchos de los consejos que en aquella época nos dabas a los txikis y que nos parecían una locura. María, más conocida como **Sa-**

tti, junto con Estitxu y Adolfo habéis sido mis correctores oficiales de esta tesis. Has aportado el aire gallego que tanta falta le hacía a este laboratorio. Espero y deseo que montes un laboratorio en tu aldea y en ese momento te acuerdes de nosotros. Además, las txarletas en la recta final de esta tesis intentando arreglar el mundo y la ciencia me han ayudado a definir mi futuro.

Tampoco quiero olvidarme de las nuevas incorporaciones o no tan nuevas como **María García**, si algún día decides apartarte de la ciencia ya sabes que tendrás un gran futuro como baserritarra de aguacates, y **Sabrina**, espero y deseo que encuentres tu camino. Mi último pollito **Alex**, que soñaba con células azules. Y por God no puedo olvidarme de mi **Aneeeeeeeeeeeeee**, junto con Ander Thor, habéis sido los mejores pollitos que he podido tener. Sin duda, Aneeee eres de las personas que más huella han dejado entre nosotros. No me puedo olvidar de ese viaje celular que hice a tu labo en Leioa y de los arcos y la ropa que me dejaste para bailar el aurresku de Vir. Sigue siendo tú misma y transmitiendo la alegría que siempre transmites.

Los **tweens** habéis sido clave para el desarrollo de esta tesis, sobre todo para mantener la cordura durante todos estos años y para desconectar de este mundo tan extenuante. Sin tener un grupo de gente así hubiera sido imposible que hubiera llegado a escribir estos agradecimientos. Y cuánto hemos hablado de su escritura. Jaione, Juncal, Leire, Ander, Vir, Carla, Araika, Laura, Miiiiren, Maddalen. Cuántas aventuras hemos vivido: Santo Tomás, San Sebastián, San Fermín, Oiartzun jaiak, Logroño, una tesis, dos despedidas, dos bodas, una hija, una bodega en Logroño, unas salinas, múltiples comidas y cenas, un aurresku, varias Korrikas y seguro que me olvido de muchas más. Con vosotros me he sumergido de lleno en la kultura baska.

Mi **pequeña Leeeeeire**, ¿qué voy a hacer sin ti? O mejor ¿qué estoy haciendo sin ti? Fíjate que empezamos compartiendo mesa y hasta silla, y acabamos compartiendo piso junto con el Señor Francisco. Y bueno, antes de eso tomábamos café juntos sin saber quiénes éramos... Desde el principio conectamos y las primeras frases que aprendí nada más llegar a Donosti fueron gracias a ti: Non da kotxea? Urrun edo gertu? Etorri pa'ká beltzarana, itzali nire sua zure mangerarekin, pero sobre todo "eso en euskera no se dice", jajajajajaj. Hemos compartido grandes momentos y atakitos, hemos solucionado el mundo (también científico) mil millones de veces y hemos viajado por un montón de sitios: Ámsterdam, París, Berlín, Tulú. Hemos aprendido a llevar una dieta sana y equilibrada todos los lunes y todas las noches nos hemos visto medio netflix junto con el sakito, hahahaha. Además, siempre has estado ahí en los momentos complicados. Seguro que todavía nos queda mucho que vivir juntos allá donde nos lleve!!!!!

Ay hijas mías, qué orgulloso estoy de vosotras: Jaione, Juncal y Leire; cómo habéis mejorado vuestras habilidades lingüísticas desde que llegasteis aquí.

Jaione, hija de mi vida y de mi corazón, qué decirte a estas alturas maju. Me has hecho reírme en multitud de ocasiones, nunca pierdas esa espontaneidad que te caracteriza. Has sufrido gran cantidad de mis sustitos, pero siempre seguirás siendo nuestra ama rata particular. Te recordaré por haber puesto Hernialde en el centro de Euskadi, pero sobre todo por tu jersey de farra, has creado estilo. Y recuerda, aún tenemos unos cuantos akelarres pendientes. ¡¡¡Eeeeellll fiiiiiiinaaaaaallll iss cooooming!!!. **Juncalita de mis amores**, nunca olvidaré tus habilidades para solucionar los problemas, incluido el faro del coche con cinta de autoclave en mitad de la nada jajajaja y por haber sido nuestra txofer particular en todas las salidas que hemos hecho, acabando siempre en el polígono de Eibar. **Miiiiirenn**, has pasado a la posteridad con esta gran frase "¿Algo bueno tiene que tener no?" jajajaj. Fuiste muy buena anfitriona en Berlín, nos llevaste a muy buenos sitios con una cerveza muy rica, pero todavía no me ha quedado claro si ir al Bérgamo, los bunkers o a Sachsenhausen!!! Hahahah

Maddalen, compañera de pecera, nunca olvidaré tus múltiples frases: ay pollito, cuanto bonsái hay en virus, vamos xikito, ay josetxomaria jajajjaja. Fuiste otro pollito mío veraniego, te volví crazy con los fibros, que si se pegan no se pegan, que si tratamiento por aquí, baina benetan? Eso en un ti tá. Nos trajiste bonsáis y ese arroz con leche caxero, Dios qué rico estaba. **Araika**, nunca olvidaré tus cafés y tus burpies en pleno Buen Pastor. Cada vez que me preparo un café vivo con miedo de que aparezcan grumos hahah. **Carla**, llegaste a nuestras vidas tras un monólogo y fíjate que acabamos hasta organizando tu despedida y yendo a tu boda jajaja

Mi querida **Antonia**, ay Antonia qué momentos hemos vivido. Rápidamente conectamos en los cafés desde la primera semana y fíjate que hasta aprendí a bailar un aurresku sudando la gota gorda en pleno junio. Los señores del museo de Zarautz fliparon cuando nos vieron entrar corriendo vestidos de boda para vestirnos para bailar. Siempre me has escuchado y me has ayudado a tomar las decisiones correctas, aunque muchas veces no te he hecho mucho caso y luego me he arrepentido de ello jajaj. Sin lugar a dudas no habría llegado a este momento sin esas conversaciones, he aprendido lo complicadas que son las relaciones personales jajajaj, espero que no llores en mi presentación que te estaré vigilando hahahah.

Quiero mencionar también al grupo de Oncología Molecular, con los que he compartido más que laboratorio. Nunca olvidaré la risa de **Esther,** JA JA ó sisisisi sí sí, los bailes de **Erika**, las habilidades informáticas de **Giovanni**, los audios de **Andrea** y el perro que se comió el paipo, **Lorea**, siempre disponible a ayudarte. También al resto de labo por haberme ayudado cuando lo he necesitado: Caffarel, Armesto, Arestin.

Tampoco quiero olvidar al grupo de Bioingeniería: **Yndri**, nunca olvidaré nuestra primera conversación y esos centímetros de menos que no te resultaron útiles para

alcanzar esa placa de cultivos hahahahah, sin olvidarme de ese maravilloso viaje a las Azores. También en especial también al resto del grupo: Haizea, la divina Ainhoa, Paula Vázquez y sus concursos televisivos, Leire y su hipito, Sandri, Héctor e Izeti.

Neurociencias, tampoco me podría olvidar de ellos; son la otra mitad. Miren y Karmele, siempre me habéis considerado uno más de neuro y eso es de agradecer. **Karmele**, tan resolutiva y rápida, y siempre con una sonrisa, Adolfo no será el mismo sin ti. **Miiiren**, qué momentos hemos vivido, gracias por escucharme en todos ellos y por esas terapias que hacíamos y seguiremos haciendo, gracias por abrirme las puertas del labo de neuro y de Adolfo, por aconsejarme, guiarme y apoyarme en todos los momentos. Seguimos teniendo pendientes unas cañas, pero las echaremos por Easo cualquier día. No me puedo olvidar de **Jaioneitor**, *I want my money back*, por todos los txismorreos vividos. Espero que sigas siendo siempre feliz y gracias por haber sido siempre fiel a la matutina de Ordizi!!! Junto con Iván, Neia, Haizpea, el tractor, la paja y el AHK. Y aquí aparece nuestro queridísimo equipo AHK y las nuevas generaciones con Laura!!! No me puedo olvidar de **Alba** y **María Goicoechea**, que siempre me recibían con una sonrisa, ni de **Aias**, todo comenzó con ella, bajando a neuro todos los días a buscarte para aprender a cultivar fibroblastos, junto con esas tardes descifrando el seahorse. **Gaaaara** zeeer mooduzz, por esos fantásticos medios para cultivar mioblastos; **Moni**, por esas conversaciones sobre metabolismo y mitocondrias y esa energía positiva y motivadora que siempre desprendes y **Gorka** con esas moskis tan monas.

Pero a lo largo de estos años no todo ha sido trabajo y quiero mencionar a muchísima gente que he conocido durante estos años y que han ayudado a que mi estancia en Donosti fuera mucho más agradable. En el Gorbea empezaron los pintxopotes de Amara y con su cierre ganamos en salud, pero esas quedadas no acabaron y salvo causa mayor, es decir, que estuviésemos fuera del país, cada semana nos reuníamos, haciendo que el fin de semana empezara los miércoles. Pero una pandemia hizo que los pintxopotes de los miércoles dejaran de tener éxito. ¡¡Supimos reponernos a eso y ahora los findes empiezan los txamartes!! El **señor Aner**, tan diligente con ese saber estar típico de Bermeo; **Peeedro y Nuno**, la aportación internacional al pinxopote del Haritza, **Lander**, nuestro *God* particular; **Mikel** "el realmente malvado" y Jon, ese matrimonio tan particular. **Jon,** he aprendido muchas maldades contigo, las mejores dar la vuelta a la pantalla del ordenador pero la mejor fue poner el fondo de pantalla con iconos falsos, seguro que alguien ahora mismo se sentirá aludid@ jajajajaj. **Alazne,** esa party hard en tu casa después de una pandemia y esa fuente perdida en mitad de una carretera, cuantas vidas habrá salvado incluidas las nuestras, pero qué caminata tan bonita, ay po dio; y **Xabi**, el único realmente de ciencias.

¿Quién diría que correría varias carreras? Pues sí y han sido unos cuantas, que no hubiera sido posible sin el equipo runner EHU: **Leire Merino, Ibone y Aitor**. Creía que no iba a acabar esa carrera y al final la acabamos todos juntos. **Aitor**, ¿quién iba a pensar que acabaría echando siestitas con Leire y Xabi en tu sofá rojo? Empezamos como *best friends forever* a altas horas de la noche y al final acabamos yendo de vacaciones juntos. **Ibone** más conocida como *Hi bon*, siempre con una sonrisa. Sin duda, la carrera más divertida ha sido la carrera nocturna en semana grande con hepato and the sisters! Hepato, siempre salvándome la vida en el último momento cuando se nos acababa algo. Pero sin duda, hay que agradecer la gran aportación de nuevos pisukides, piso ahora rebautizado como **HepatoTalent**.

Dra. Taati, taati, taati ¿quién diría que acabaríamos viviendo juntos? Pues sí, empezamos como con Aitor, siendo los mejores amigos de madrugada y mira cómo acabamos luego. Siempre estaréis en mi memoria Leire y tú siendo arrastradas por el coche escoba en la korrika jijijijiji, pero sobre todo por aquel viaje a Tulú y aquella cena, nunca he comido como aquel día jajajaj.

Pero todos estos años no hubieran sido lo mismo si no hubiera conocido a "La Cuadrilla": Angela, Nerea, Iñigo, Marie, Xabi, Irene, Ainize, David, Joana, Javi. Con vosotros he hecho multitud de planes, sagardotegis, un apocalipsis zombi, Herri Urrats, un finde en Logroño y todo lo que aún queda. Pero sobre todo, por haber hecho más amena la cuarentena con los skypes de los viernes y el club del cine que montamos. **Angela**, conectamos desde el primer momento, nos hicimos muy amigos en muy poco tiempo y hemos vivido un montón de cosas juntos. Sin lugar a dudas, no hubiera llegado a este punto sin tu ayuda. Gracias a ti conocí a este grupo de gente con los que tantas cosas hemos vivido y que considero muy buenos amigos, y también gracias a ti ahora soy una persona mucho más abierta. **Iñigo**, nunca me he reído tanto contigo y créeme que vivo con miedo a coger un avión y que el piloto seas tú, de Tolosa al cielo jajajajajaj. **Nere**, nos hicimos amigos desde el principio y sé que siempre estarás ahí, ahora junto a Odei. Sin lugar a dudas eres una de las personas especiales que esta tesis ha traído a mi vida. Nere, ¿qué haces hoy? ¿en media hora en el puente? Hahahahah. **Xabi**, tan buena persona, siempre aportas la sensatez en este grupo e **Irene**, he aprendido de ti la riqueza de Euskadi y el amor por tu tierra. **David**, al final siempre quedamos nosotros jajaja ¿bueno qué, una copa no?. La de cenas y sidrerías en las que hemos cerrado todo mano a mano jajaj y seguiremos cerrando. **Joana**, aportas este punto alocado y despistado tan necesario, tan pronto aparece una hora antes y te la encuentras bebiendo sola o una hora más tarde en otro barrio. **Ainize**, nuestra óptica particular y **Javi**, nuestro niño pequeño tan especial.

Bueno creo que llevas todo el rato esperando en que parte aparecerás en estos agradecimientos. **Ander**, fuiste mi primer pollito, la primera persona a mi cargo y la primera persona a la que tuve que enseñar, me moría de vergüenza los primeros

meses. Casi sin conocerme estuviste ahí apoyándome en ese profundo punto por el que pasamos todos en mitad de una tesis y fíjate que ahora somos casi, bueno "casi" por poner algo, inseparables. Has aguantado muchas veces mis momentos "vagüina by Jaione", pero siempre con la sonrisa que te caracteriza. Creo que en estos años hemos hecho más kilómetros por Guipúzcoa que en toda la vida juntos y ahora puedo decir que soy un experto en Fender (impresionante su sonido), Gretsch (menuda compraza), Zildjian, Mooer, Yamaha, Pearl, Tama y los múltiples y variados Emauses recorridos gracias a ti. Ya sabes que nos queda pendiente ese viaje a Thomann.

Deeeesi, esta tesis también te pertenece a ti, por esas conversaciones sobre la vida, la tesis, el futuro jajaja. Casi 10 años desde que empezamos a vivir juntos en Bilbao, cómo nos ha cambiado la vida desde ese primer día en el que empezamos la carrera, con Laura y Leire. Juanri, Iraia y el futuro!!! ¡¡La siguiente tesis será la tuya!!

Índice de contenidos

Abreviaturas, acrónimos y símbolos

	3T3	*3-day transfer inoculum 3 × 10^5 cells* (Transferencia de 3 días, inóculo de 3 × 10^5 células)
A	ADN	Ácido desoxirribonucleico
	ADNc	Ácido desoxirribonucleico complementario
	AHK	Ahulken
	AHK6	Ahulken 6
	AHK7	Ahulken 7
	AMPK	*AMP-activated protein kinase* (Proteína quinasa activada por AMP)
	ARN	Ácido ribonucleico
	ARNm	Ácido ribonucleico mensajero
	ASO	*Antisense oligonucleotides* (Oligonucleótido antisentido)
	ATG	Adenina-Tirosina-Guanina
	ATP	Adenosina trifosfato
B	BCA	Ácido bicinconínico
	bp	*Base pair* (Pares de bases)
C	CCG	Citosina-Citosina-Guanina
	CCTG	Citosina-Citosina-Timina-Guanina
	CM	*Conditioned medium* (Medio condicionado)
	CO_2	Dióxido de carbono
	CTC	Citosina-Timina-Citosina
	CTG	Citosina-Timina-Guanina
	$CuSO_4$	Sulfato de cobre II
D	D	Dasatinib
	DDR	*DNA Damage Response* (Respuesta de Daño en el ADN)
	DEPC	Dietil pirocarbonato
	DM	Distrofia Miotónica
	DM1	Distrofia Miotónica tipo 1
	DM2	Distrofia Miotónica tipo 2
	DMEM	Medio de cultivo Eagle modificado por Dulbecco
	DMSO	Dimetilsulfóxido
	DOX	Doxorrubicina
E	ECAR	*Extracellular Acidification Rate* (Ratio de acidificación extracelular)
	EDTA	Ethylenediaminetetraacetil acid (Ácido Etileno Diamino Tetracético)
	EdU	*5-ethynyl-2'-deoxyuridine* (5-etinilo 2'-deoxiuridina)
	EGF	*Epidermal Growth Factor* (Factor de crecimiento epidérmico)

F	FBS	*Fetal Bovine Serum* (Suero Bovino Fetal)
	FCCP	*Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone* (Carbonilcianuro-p-trifluorometoxifenilhidrazona)
	FGF	*Fibroblast Growth Factor* (factor de crecimiento de fibroblastos)
	FSC	*Forware Scatter* (Detector de dispersión frontal)
G	GFP	*Green Fluorescent Protein* (Proteína verde fluorescente)
	GGC	Guanina-Guanina-Citosina
H	H_2O_2	Peróxido de hidrógeno
	HBSS	*Hank's Balanced Salt Solution* (Solución salina de Hank)
	HCl	Ácido clorhídrico
	IgG	*Immunoglobulin G* (Inmunoglobulina G)
	HRP	*Horseradish peroxidase* (Peroxidasa de rábano)
	HSA	*Human Skeletal α-Actin* (Actina esquelética humana alfa)
I	IF	Inmunofluorescencia
K	KO	*Knock-Out*
M	M-199	*Medio de cultivo 199 suplementado con las sales de Earle*
	MGB	*Minor groove binder* (Sonda de unión al surco menor del ADN)
	MIRS	Muscular Impairment Rating Scale (Escala de discapacidad muscular)
	MOI	*Multiplicity Of Infection* (Multiplicidad de infección)
	MTT	Bromuro 3-(4,5 dimetil 2-tiazolil)-2,5-difeniltetrazolio
N	N	Navitoclax
	NaCl	Cloruro de sodio
	NADH	*Nicotinamide Adenine Dinucleotide* (Nicotinamida adenina dinucleótido)
	NaF	Fluoruro de sodio
	NP-40	Nonyl phenoxypolyethoxylethanol
	Na_4P2O_7	Pirofosfato de sodio
	Na_3VO_4	Ortovanadato de sodio
O	OCR	*Oxygen Consumption Rate* (Ratio de consumo de oxígeno)
	OMIM	*Online Mendelian Inheritance in Man* (Base de datos Online de Herencia Mendeliana en Humanos)
	OXPHOS	*Mitochondrial Oxidative Phosphorylation System* (Fosforilación Oxidativa Mitocondrial)
P	PBMC	*Peripheral Blood Mononuclear Cells* (Células mononucleares de sangre periférica)
	PBS	*Phosphate buffered saline* (Tampón fosfato salino)
	PCR	*Polymerase Chain Reaction* (Reacción en cadena de la polimerasa)
	PDL	*Population Doubling Level* (Nivel de duplicación de la población)
	pH	Potencial de hidrógeno

	PH3	*Phospho Histone H3* (Fosfo histona H3)
	PMSF	*Phenyl Methyl Sulfonyl Fluoride* (Fluoruro de fenilmetilsulfonilo)
Q	Q	Quercetina
	qRT-PCR	*Reverse Transcription Quantitative Polymerase Chain Reaction* (Transcripción reversa - Reacción en cadena de la polimerasa cuantitativa)
R	RAN	*Non-conventional repeat-associated non-ATG translation* (Traducción no convencional asociada a un inicio no ATG)
	RIN	*RNA Integrity Value* (Valor de la Integridad del ARN)
	ROS	*Reactive oxygen species* (Especies reactivas de oxígeno)
	RT	*Reverse Transcription* (Transcripción Reversa)
	RTL	*Relative Telomere Length* (Longitud Telomérica Relativa)
S	SASP	*Senescence Associated Secretory Phenotype (Fenotipo secretor asociado a la senescencia)*
	SDS-PAGE	*Sodium dodecyl sulfate polyacrylamide gel electrophoresis* (Electroforesis en gel de poliacrilamida con dodecilsulfato sódico)
	SEM	*Standard Error of the Mean* (Error estándar de la media)
	SIR	*Standardized Incidence Ratio* (Ratio Estandarizado de Incidencia)
	SNC	Sistema Nervioso Central
	SSC	*Side Scattered* (Detector de dispersión lateral)
T	TBS-T	*Tris Buffered Saline with Tween 20* (Solución salina tamponada con Tris y con Tween 20)
	Tris	Trisaminometano
	TTAGGG	Timina-Timina-Adenica-Guanina-Guanina-Guanina
U	UTR	*Untranslated region* (Región no traducida)
W	WB	*Western-Blot*
X	X-Gal	5-bromo-4-cloro-3-indolil-β-D-galactopiranósido

Nomenclatura de los genes más importantes

	53BP1	*Tumor protein p53 binding protein 1* (Proteína tumoral 1 de union a p53)
B	BMI1	*Proto-oncogene, Polycomb ring finger protein* (Proteína proto-oncogénica del tipo “dedo de anillo” perteneciente al complejo Policomb)
	BRCA1	*BRCA1 DNA repair associated* (Proteína asociada a la reparación del ADN)
C	CUGBP	*CUGBP Elav-like family proteins* (Familia de proteínas de tipo Elav)
D	DMPK	*Dystrophia Myotonica-Protein Kinase* (Proteína Quinasa de la Distrofia Miotónica)
	DMWD	*Myotonica-containing WD repeat motif protein* (Proteína de la Distrofia Miotónica que contiene secuencias repetidas del tipo WD)
	DRP1	*Dynamin 1 like protein* (Proteína Dinamina del tipo 1)
G	GPx1	*Glutathione Peroxidase 1* (Glutatión peroxidasa 1)
H	γ-H2AX	*Variante fosforilada de la familia de histonas H2A*
I	IL-6	*Interleukin 6* (Interleucina 6)
	KI-67	*Marker of proliferation Ki-67* (Marcador de proliferación KI-67)
M	MBNL	*Muscleblind like splicing regulator family proteins* (Familia de proteínas de Muscleblind reguladoras del *splicing*)
	MFN	*Mitofusin* (Mitofusina)
O	OPA1	*Mitochondrial dynamin like GTPase* (Dinamina mitocondrial de tipo GTPasa)
P	$p14^{ARF}$	*Alternate Reading Frame protein of Cyclin dependent kinase inhibitor 2A* (Proteína alternativa del inhibidor 2A de quinasa dependiente de ciclina)
	$p16^{INK4a}$	*Cyclin dependent kinase inhibitor 2A* (Inhibidor 2A de quinasa dependiente de ciclina)
	$p21^{CIP1}$	*Cyclin dependent kinase inhibitor 1A* (Inhibidor 1A de quinasa dependiente de ciclina)
	$p27^{KIP1}$	*Cyclin dependent kinase inhibitor 1B* (Inhibidor 1B de quinasa dependiente de ciclina)
	p38 MAPK	*p38 Mitogen-Activated Protein Kinase* (Proteína quinasa p38 activada por mitógenos)
	PGC1-α	*Peroxisome proliferator-activated receptor gamma coactivator 1 alpha* (Receptor gamma y co-activador 1 alfa de peroxisoma que se activa por proliferación)
S	SIX5	*SIX homeobox 5 transcription factor* (Factor de transcripción homeobox 5)
T	TFAM	*Mitochondrial transcription factor A* (Factor de transcripción mitocondrial A)
	TOMM20	*Translocase of outer mitochondrial membrane 20* (Translocador 20 de la membrana mitocondrial externa)
Z	ZEB	*Zinc finger E-box binding homeobox proteins* (Proteínas homeobox con dedo de zinc de unión a E-box)

Anglicismos

Se ha procurado evitar aquellos términos que tienen un equivalente adecuado en castellano y permanecen aquellos que por su amplia difusión en la comunidad científica resultan más adecuados para la correcta comprensión del texto.

Splicing: Con este término nos referimos al proceso mediante el cual los intrones son escindidos del transcrito de ARN mensajero primario y los exones se unen para generar un ARN mensajero maduro.

Knock-out: Con este término hacemos referencia a un organismo, un ratón o una mosca, modificado por ingeniería genética para que uno o más de sus genes estén inactivados mediante la técnica de bloqueo de genes.

Resumen

La distrofia miotónica (DM) es un desorden neuromuscular multisistémico transmitido con herencia autosómica dominante, del que se distinguen dos tipos en base a su diferente origen molecular. La DM1 está causada por la expansión del trinucléotido *CTG* en la región 3' no traducida del gen *DMPK* localizado en el cromosoma 9, y la DM2 está causada por la expansión del tretranucleótido *CCTG* en el gen *CNBP* localizado en el cromosoma 3. En términos generales, aunque ambas distrofias presentan un catálogo de síntomas y signos superponibles, la DM1 presenta un fenotipo más grave que la DM2. Además, en la DM1 el tamaño de la expansión CTG se asocia con la gravedad de la enfermedad, de manera que un individuo sano posee hasta 35-50 repeticiones, mientras que los individuos gravemente afectos presentan entre 100 y 3.000 repeticiones CTG. A pesar de que la DM1 es una enfermedad neuromuscular rara, en la región de Guipúzcoa la prevalencia es superior a 26 casos cada 100.000 habitantes.

Desde un punto de vista clínico, los pacientes con DM1 experimentan un proceso degenerativo multisistémico que cursa con atrofia y debilidad muscular, miotonía, cardiomiopatía, cataratas, alopecia, síndrome metabólico con resistencia a la insulina, mayor riesgo a desarrollar neoplasias y una afectación del sistema nervioso central, entre otros. Todo ello conlleva una reducción en su esperanza de vida y una importante pérdida en su calidad de vida. Desafortunadamente, a día de hoy, no existe ningún tratamiento eficaz contra la DM1.

La mayoría de estas manifestaciones clínicas se podrían considerar manifestaciones de un envejecimiento fisiológico precoz, por lo que las alteraciones presentes en el envejecimiento podrían estar jugando un papel importante en el desarrollo de la DM1. En este contexto, los objetivos de esta tesis son el análisis de los procesos moleculares propios del envejecimiento celular en los pacientes con DM1, el estudio del riesgo a desarrollar cáncer en la cohorte de pacientes con DM1 de Guipúzcoa y el testado de diferentes compuestos con función anti envejecimiento como posible nueva estrategia terapéutica frente a la DM1.

En esta tesis describimos, mediante un análisis transcriptómico en fibroblastos primarios derivados de pacientes con DM1, que los procesos de regulación del ciclo celular, de división celular y de respuesta al daño en el ADN están disminuidos en fibroblastos, además de en células sanguíneas de PBMC de dichos pacientes, y que estas alteraciones serían un requisito necesario para el desarrollo de una senescencia prematura, donde la vía de BMI1 – $p16^{INK4a}$ ejerce un papel importante. Por otra parte, demostramos una disminución en el metabolismo y actividad mitocondrial en los fibroblastos derivados de pacientes con DM1. Adicionalmente, estos resultados han sido validados en muestras sanguíneas confirmando que las alteraciones

en procesos clave que ocurren durante el envejecimiento fisiológico se dan de una manera prematura y exacerbada en la DM1.

En línea con esta idea, observamos, mediante un estudio longitudinal, que las PBMC derivadas de pacientes con DM1 sufren un acortamiento telomérico más acentuado que las de los controles con el paso del tiempo. Dado que el cáncer y el envejecimiento están estrechamente relacionados, la alteración de estos mismos procesos moleculares podría explicar la observación clínica de que los pacientes con DM1 presentan un mayor riesgo para desarrollar neoplasias, siendo este riesgo mayor en mujeres que en hombres en la cohorte de pacientes establecida en el Hospital Universitario Donostia.

En resumen, los pacientes con DM1 presentan una alteración en la regulación de la proliferación celular, en la respuesta al daño en el ADN, en el desarrollo de una senescencia prematura y en el metabolismo mitocondrial, junto con un acortamiento telomérico exacerbado, todos ellos procesos moleculares y patologías vinculadas al envejecimiento y al riesgo oncogénico, lo que refuerzan la hipótesis de considerar a la DM1 como una enfermedad caracterizada por un envejecimiento prematuro de los sistemas biológicos.

Por último, esta tesis ha querido investigar de manera preclínica el potencial efecto terapéutico tanto de compuestos nuevos y de fármacos reposicionados como terapias antienvejecimiento. Se han testado los compuestos senolíticos (quercetina, dasatinib y navitoclax) y la metformina, así como una nueva serie de compuestos de desarrollo propio, denominados ahulkenoides (AHK). Todas estas estrategias tuvieron un efecto beneficioso muy potente en cultivos celulares *in vitro* y también *in vivo* en el modelo de DM1 en *Drosophila melanogaster.*

En definitiva, en esta tesis aportamos datos experimentales robustos que avalan que los pacientes con DM1 poseen alteraciones en varios procesos clave del envejecimiento fisiológico y que dichas alteraciones se pueden revertir mediante distintas aproximaciones antienvejecimiento, constituyendo éstas una nueva aproximación terapéutica para los pacientes con DM1.

Introducción

1. Distrofia Miotónica

1.1. Concepto. Historia de la entidad. Nosología. Distrofia miotónica tipo 1 y tipo 2

La Distrofia Miotónica (DM) fue descrita por primera vez por el médico alemán Hans Steinert en 1909 (Mishra *et al.*, 2018). Sin embargo, ya había sido documentado algún caso aislado por Frederick Eustace Batten y Heinrich Curschmann, su mentor, pero ninguno de ellos había asociado los síntomas a una enfermedad concreta. Es por ello por lo que también se le denomina enfermedad de Curschmann-Steinert-Batten (Olbrych-Karpinska and Tutaj, 1981; Wagner and Steinberg, 2008). En su artículo original, Steinert describió 6 casos de la enfermedad, incluyendo desde pacientes con inicio de la enfermedad en la infancia hasta pacientes con debut en la edad adulta, tanto con antecedentes familiares como sin ellos. Muchos de estos pacientes recapitulaban de manera bastante fiel lo que hoy en día entendemos como DM. Fue el fallecimiento de uno de sus pacientes lo que le dio a Steinert la oportunidad de estudiar en detalle gran parte de la musculatura que se encuentra afectada en la enfermedad. Sin embargo, no fue hasta 83 años después, en 1992, cuando se identificó la causa molecular de la enfermedad (Brook *et al.*, 1992; Wagner and Steinberg, 2008).

A día de hoy se ha acumulado un gran conocimiento sobre la fisiopatología de la enfermedad, de la que se conoce que es multisistémica, transmitida por herencia autosómica dominante, y con un fenotipo clínico variable y complejo. Entre los síntomas más característicos de la enfermedad se encuentran la miotonía progresiva, la degeneración y debilidad muscular, la pérdida de masa muscular, los defectos en la conducción y el ritmo cardiaco, las cataratas iridiscentes subcapsulares posteriores y los trastornos endocrinos, entre otros. Por tanto, aunque la DM se clasifica dentro de las enfermedades neuromusculares, afecta a tantos sistemas que debe visualizarse realmente como una condición multisistémica (Harper, 2002).

Se distinguen dos tipos de distrofia miotónica con bases moleculares diferentes: la DM tipo 1 (DM1, OMIM #160900) y la DM tipo 2 (DM2, OMIM #602668) **(Tabla I1)**. Ambos tipos presentan un conjunto de rasgos clínicos semejantes, pero tienen una causa genética diferente y, aunque ambas enfermedades se consideran afecciones degenerativas de progresión lenta, generalmente la DM1 es clínicamente más grave que la DM2 (Thornton, 2014).

La DM1 está causada por la expansión de un trinucleótido “CTG” en la región 3’ UTR del gen de la proteína quinasa de la distrofia miotónica, denominado *DMPK*. En cambio, la DM2 está causada por la expansión del tetranucleótido “CCTG” en el intrón 1 del gen *CNBP*, que codifica una proteína de unión a ADN y a ARN de tipo “dedos de

zinc". El tamaño de la expansión del nucleótido también difiere entre las dos distrofias, siendo mucho mayor en el caso de la DM1. Otra diferencia relevante es el hecho de que en la DM1 la longitud de la expansión correlaciona de manera negativa con la edad de inicio y con la gravedad de la enfermedad, lo que no se observa en la DM2 (Meola *et al.,* 2015; De Antonio *et al.,* 2016).

Por otra parte, ambas enfermedades también difieren en cuanto a su distribución geográfica. Así, mientras la DM1 se encontra distribuida ampliamente por todo el mundo, aunque es más frecuente en poblaciones caucásicas y muy rara en África subsahariana, la DM2 tiene una distribución geográfica mucho más restringida, siendo más frecuente en poblaciones originarias del norte de Europa (Meola and Cardani, 2017).

Tabla I1. Semejanzas y diferencias entre DM1 y DM2. Modificado de Meola *et al.* 2015.

	DM1	DM2
Aspectos moleculares		
Gen afectado	*DMPK*; Cromosoma 19	*CNBP*; Cromosoma 3
Microsatélite repetido	CTG	CCTG
Localización de la expansión	3' UTR	Intrón 1
Tamaño de la expansión	100-3.000	100-10.000
Penetrancia	100%	100%
Inestabilidad somática	Presente	Presente
Anticipación	Presente	En algunos casos
Inestabilidad intergeneracional	Sí	Sí
Aspectos generales		
Distribución	Mundial, a excepción de África	Preferentemente en poblaciones de origen europeo
Prevalencia	12.5-189/100.000	1-9/100.000
Edad de debut	A cualquier edad	En edad adulta
Forma congénita	Presente	Ausente
Esperanza de vida	Reducida	Normal
Músculo afecto	Distal/Proximal	Proximal
Fibras afectas	Tipo 1	Tipo 2

1.2. Epidemiología

La DM1 es la enfermedad neuromuscular hereditaria más frecuente en la edad adulta. Las tasas de prevalencia detectadas en varios estudios epidemiológicos oscilan entre 2.1 y 189 casos por cada 100.000 habitantes, siendo la prevalencia media mundial estimada de 12.5/100.000 (Harper, 2002; Musova *et al.*, 2009).

Aunque se observa una amplia distribución de la enfermedad en todo el mundo, algunas regiones presentan unas prevalencias especialmente elevadas. La prevalencia más elevada se da en la población francocanadiense de la región del Saguenay-Lac-St-Jean en Quebec (Canadá), con 189 casos por cada 100.000 habitantes (De Braekeleer, 1991; Mathieu *et al.*, 1990). En dicha región se identificó a 746 pacientes en una población de 285.000 habitantes distribuidos en 88 familias de esta región. En otras regiones del mundo también se presentan prevalencias superiores a la media, como es el caso de la región de Guipúzcoa, con 26.5 casos cada 100.000 habitantes (Lopez de Munain *et al.*, 1993); Finlandia, con 44/100.000 (Suominen *et al.*, 2011) 2011; la región de Norrbottens en Suecia, con 36.50/100.000 y la isla de Guam (Micronesia, Pacífico Occidental), con 76.30/100.000, entre otros. Como causa de la elevada prevalencia en Canadá se argumenta la presencia de un efecto fundador con un fuerte aislamiento tanto genético como geográfico de la población, que, a su vez, podría explicar la elevada prevalencia hallada en Guipúzcoa y en Finlandia, debido a que sus poblaciones también presentan un fuerte aislamiento genético y geográfico (Behar *et al.*, 2012; Kaariainen *et al.*, 2017). Se ha determinado que la introducción de la mutación en Quebec se dio en 1657 cuando Noël Simard emigró de Francia a Canadá y allí se casó con Marie-Madeleine Racine, también de origen francés, siendo uno de los dos el que transmitió la enfermedad a su descendencia, documentándose muy rigurosamente la segregación de la mutación durante 14 generaciones (Mathieu *et al.*, 1990).

En otras regiones del mundo se han encontrado prevalencias en torno a la media mundial como es el caso del norte de Italia, con 9.31/100.000 (Siciliano *et al.*, 2001a); Japón con 9.40/100.000 (Nakagawa *et al.,* 1991), Irlanda del Norte, con 11.95/100.000 (Magee and Nevin, 1999) y 17.8/100.000 en la región de Gotemburgo, en Suecia (Lindberg and Bjerkne, 2017). En Israel se han observado prevalencias inferiores a las de Europa, con 2 casos cada 100.000 habitantes (Ashizawa and Epstein, 1991). No obstante, en estudios más recientes en población la judía de Israel se han documentado unas prevalencias de 15.7/100.000, con marcadas diferencias entre comunidades. Así, los judíos Askhenazis poseen la prevalencia más baja, con 5.7/100.000 mientras que los sefardíes/judíos orientales y los judíos yemeníes presentan una prevalencia destacada, con 20 y 47.3 casos cada 100.000 habitantes, respectivamente (Segel *et al.*, 2003).

En otras regiones del mundo se han hallado las prevalencias más bajas de la enfermedad, como en Hong Kong y Taiwán (con 0.37 y 0.46 casos cada 100.000, respectivamente) (Pan *et al.*, 2001). Además, la presencia de la enfermedad en poblaciones autóctonas tanto de África como de Australia y América, es prácticamente nula, con algunos casos aislados detectados (Ashizawa and Epstein, 1991; Goldman *et al.*, 1996a; Goldman *et al.*, 1994, 1996b).

Es importante mencionar que es probable que la tasa de prevalencia en casi todas las poblaciones esté infraestimada, tanto en las regiones donde encontramos prevalencias bajas, como en las regiones con una alta prevalencia, debido, entre otras causas, a la existencia de individuos presintomáticos o con una sintomatología muy leve, los cuales no se contabilizan en los estudios epidemiológicos. Cabe destacar que en las regiones donde se ha estudiado la evolución de la prevalencia a lo largo del tiempo, como es el caso de Japón, Croacia o Inglaterra, se ha producido un aumento en el diagnóstico de la misma, probablemente debido al mayor conocimiento que se tiene hoy en día de la enfermedad y a las mejoras diagnósticas (Harper, 2002).

1.3. Bases genéticas de la enfermedad

Como se ha comentado anteriormente, la DM1 está causada por la expansión del microsatélite CTG en el gen de la proteína quinasa de la distrofia miotónica (*DMPK,* del inglés,*"Dystrophia Myotonica Protein Kinase"*) (OMIM #605377). Este gen localizado en el cromosoma 19, tiene un tamaño de 3.243 bp y contiene 15 exones, habiéndose identificado hasta 7 variantes de *splicing* que difieren en su extremo 5'. La inserción de este microsatélite se produce en la región 3' no traducida de dicho gen, con lo que no se altera la estructura codificante de la proteína. La inserción del microsatélite va a causar la afectación de los genes adyacentes *SIX5* y *DMWD* **(Figura I1)**. El patrón de herencia es autosómico dominante, por lo que los individuos afectos o los portadores, aunque sean oligosintomáticos o asintomáticos, pueden trasmitir la enfermedad, sin diferencias en la incidencia ni en el riesgo de transmisión entre ambos sexos.

La DM1 es uno de los aproximadamente 30 desórdenes neurológicos causados por expansiones inestables de repeticiones en tándem, junto con la ataxia de Friedreich o la enfermedad de Huntington, (Jones *et al.*, 2017; McMurray, 2010; Monckton and Caskey, 1995).

En la población sana el número de tripletes varía entre 5 y 37 repeticiones, siendo la transmisión intergeneracional estable, mientras que en los pacientes con DM1 oscila entre 100 y 3.000 repeticiones, siendo la transmisión intergeneracional inestable. El rango de repeticiones tiene un importante impacto en la enfermedad. En este sentido, en un rango de tripletes que oscila entre 35 y 100 repeticiones, la expansión

se corresponde a una fase de la enfermedad que se denomina de premutación. En esta situación, los individuos son prácticamente asintomáticos sin una evidencia clínica de enfermedad neuromuscular, pero pueden trasmitir el alelo afecto a la descendencia. Este alelo inestable puede expandirse, es decir, aumentar el número de tripletes, de generación en generación, presentándose la DM1 con una gravedad mayor en las generaciones futuras. Este fenómeno se conoce como anticipación genética (De Temmerman *et al.*, 2004). Se ha demostrado que la presencia de estos alelos inestables se produce tanto en células germinales como en las somáticas, de forma que a mayor número de repeticiones encontramos una mayor inestabilidad del triplete y una mayor probabilidad de una expansión de la mutación (Martorell *et al.*, 1998; Meola and Cardani, 2015; Monckton and Caskey, 1995; Pratte *et al.*, 2015).

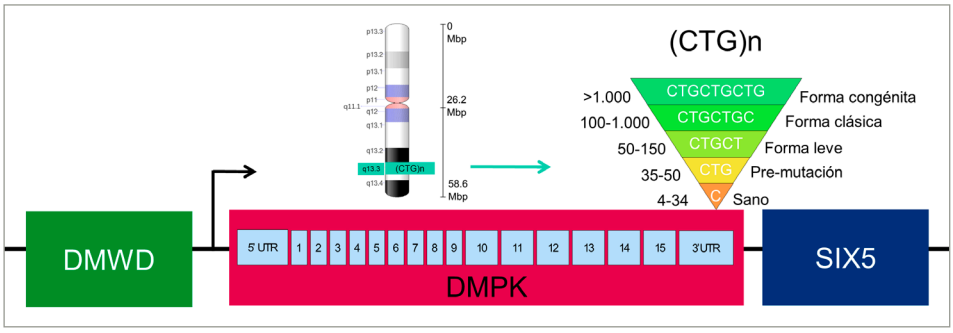

Figura I1. Representación gráfica del *locus DMPK,* junto con los genes adyacentes *SIX5* y *DMWD*. En la imagen se representa la localización citogenética del gen, así como la localización y tamaño de la expansión CTG, la cual se inserta en la región 3' UTR de *DMPK,* pero también afecta a la región promotora de SIX5. En función del tamaño de la expansión se distinguen 4 formas de la enfermedad: premutación, leve, clásica y congénita. Modificado de Turner *et al.* 2014.

Numerosos estudios han puesto de manifiesto que la gravedad de la enfermedad y la edad de debut se asocian con el tamaño de la expansión (Aldenbratt *et al.*, 2017; De Antonio *et al.*, 2016; Landfeldt *et al.*, 2019; Park *et al.*, 2018; Tsilfidis *et al.*, 1992), lo que se traduce en que a una mayor longitud del triplete los pacientes presenten una sintomatología precoz y más grave (Cumming *et al.*, 2019; De Antonio *et al.*, 2016; Hunter *et al.*, 1992).

Por otra parte, se ha visto que el sexo del progenitor que transmite la expansión del microsatélite influye en la magnitud de la expansión intergeneracional y también en la probabilidad de que se produzca una contracción.

En el caso de individuos con premutación –es decir con un tamaño de repeticiones CTG de hasta 100 repeticiones–, se ha observado que las expansiones ocurren en un 92% si la han heredado por vía paterna y en un 44% si la han heredado por vía materna. Además, el aumento relativo de la expansión es mayor cuando se hereda

por vía paterna, (Brunner *et al.*, 1993; Martorell *et al.*, 2001; Pratte *et al.*, 2015). En cambio, en el caso de pacientes con más de 100 repeticiones es más probable que el alelo se expanda si se hereda por vía materna Las mayores expansiones intergeneracionales se dan cuando el alelo transmitido viene de la madre, lo que se conoce como impronta genómica (Tomé and Gourdon, 2020; Lavedan et al., 1993; Salehi *et al.,* 2007). Sin embargo, también hay casos descritos en los que en vez de expansiones se producen contracciones en la longitud del triplete (3-10%, según la vía de herencia materna o paterna). Estos fenómenos son menos frecuentes que las expansiones y se dan preferentemente cuando el alelo patogénico es heredado por vía paterna (Ashizawa *et al.*, 1994).

Más recientemente se han identificado variantes en la secuencia del alelo expandido. Se ha observado que una proporción escasa de pacientes (3-10%) tiene interrumpido el triplete CTG por secuencias CCG, CTC y/o GGC. Estas interrupciones se han asociado a contracciones en la longitud del triplete. Además, los pacientes con DM1 que presentan estas interrupciones tienen rasgos clínicos más leves o manifestaciones clínicas atípicas de la enfermedad, lo que lleva a pensar que la presencia de estas interrupciones podría estar explicando por un lado el fenómeno de contracción, pero también los casos en los que la longitud del triplete no correlaciona con las manifestaciones clínicas de la enfermedad (Cumming *et al.*, 2018; Pešović *et al.*, 2017; Santoro *et al.*, 2017; Tome *et al.*, 2018).

Finalmente, se ha descrito que un mismo paciente puede tener un número diferente de repeticiones en diferentes tejidos, lo que se conoce como mosaicismo somático (Ashizawa *et al.*, 1993; Lavedan *et al.*, 1993), y que las repeticiones pueden aumentar con el tiempo en un mismo paciente debido a la inestabilidad del triplete (Martorell *et al.*, 1998).

1.4. Manifestaciones clínicas

La DM1 es una enfermedad multisistémica de expresión variable en su presentación clínica y gravedad. Es una enfermedad neuromuscular que se caracteriza principalmente por la presencia de miotonía además de atrofia y debilidad muscular, pero también por la afectación de prácticamente todos los órganos en el cuerpo **(Tabla I2** y **Figura I2)**, lo que conlleva a una pérdida importante en la calidad de vida de estos pacientes (Heatwole *et al.*, 2012; Laberge *et al.*, 2009).

Las principales características clínicas han sido agrupadas en afectación del sistema muscular, del sistema cardíaco, del sistema respiratorio, del sistema nervioso central y, finalmente, del sistema endocrino, además de las afectaciones provocadas en otros órganos.

a) Afectación del sistema muscular

En la DM1, a diferencia de otras distrofias musculares, la debilidad muscular comienza afectando primeramente a la musculatura distal. Dicha debilidad muscular se acompaña de atrofia y de una degeneración muscular progresiva que se puede evaluar mediante la escala MIRS (del inglés, *"Muscular Impairment Rating Scale",* ver **Anexo 4**) (Mathieu *et al.*, 2001).

Además de la debilidad muscular, la DM1 se caracteriza por la presencia de miotonía, definida como la relajación lenta de los músculos después de la contracción voluntaria o provocada. Esta miotonía es espontánea tras la contracción y se puede provocar por percusión en la lengua (presencia de rodete miotónico que es una contracción transversal) y en la eminencia tenar que es un conjunto de músculos presentes en la mano.

La debilidad se caracteriza por la afectación de la musculatura facial y temporal y de los músculos extensores y flexores del cuello, siendo una de las características más tempranas y constantes de la enfermedad. La ptosis es un descenso permanente del párpado superior que en estos pacientes es generalmente simétrica, siendo uno de los rasgos faciales más llamativos. Por otra parte, la parálisis de las cuerdas vocales es un síntoma de aparición tardía y rara vez es completa. La alta frecuencia de la debilidad facial, maxilar, palatal y de la lengua, junto con la miotonía, provoca problemas en el habla. A todo ello se añade la debilidad en extremidades, de predominio distal, que se puede hacer proximal a lo largo del curso evolutivo de la enfermedad.

Desde el punto de vista histológico, los pacientes con DM1 muestran una atrofia muscular selectiva de las fibras musculares de tipo 1 (Thornton, 2014). También es frecuente la presencia de núcleos en posición central en fibras musculares individuales, un aumento de su diámetro, la presencia de fibras ramificadas e inmaduras y de tejido adiposo (Vihola *et al.*, 2010).

Desde un punto de vista molecular, la miotonía se ha asociado con un *splicing* incorrecto del mensajero del canal de cloro CLCN1, debido a una función anormal tanto de MBNL1 como de CUGBP1 (Cardani *et al.*, 2013), y la debilidad muscular se ha asociado a un *splicing* anormal tanto de *BIN1* (isoforma Ex11-) (Fugier *et al.*, 2011), una proteína implicada en la organización y la biogénesis muscular pero también al canal de calcio *CACNA1S* (isoforma Ex29-) (Tang *et al.*, 2012). En concordancia con las observaciones en los pacientes con DM1, los modelos de ratón que mejor recapitulan la afectación muscular son los modelos donde se han inactivado diferentes miembros de proteínas *MBNL* (Kanadia *et al.*, 2003; Thomas *et al.*, 2017), pero también los modelos de mosca donde se han insertado las repeticiones CTG, como es el caso del modelo 480i que presenta una degeneración muscular, además de una menor longevidad (García-López *et al.*, 2008).

Tabla I2. Principales síntomas de la Distrofia Miotónica 1. Tomado de Meola *et al.,* 2015.

Tejido afectado	Síntomas	Referencia
Muscular	Debilidad de la musculatura distal	Harper, 2002
	Pérdida de fibras musculares tipo 1	Vihola *et al.*, 2003
	Miotonía	Harper, 2002
	Ptosis facial	Jameson, 2018
	Fatiga	Kalkman *et al.*, 2005
	Insuficiencia respiratoria	Jameson, 2018
	Sarcopenia	Malatesta, 2012
Cardíaco	Defectos en la conducción cardíaca	Jameson, 2018
	Arritmias	Pelargonio *et al.*, 2002
	Fallo cardíaco	Harper, 2002
Respiratorio	Insuficiencia respiratoria	Jameson, 2018
	Infección respiratoria	Harper, 2002
SNC y sistema cognitivo	Trastornos cognitivos	Sistiaga *et al.*, 2010
	Trastornos de la personalidad	Bungener *et al.*, 1998
	Ansiedad y depresión	Antonini *et al.*, 2006
	Somnolencia diurna	Dauvilliers and Laberge, 2012
	Apnea del sueño	Pincherle *et al.*, 2012
	Leve atrofia cortical	Giorgio *et al.*, 2006
	Cambios en la materia blanca	Wozniak *et al.*, 2013
Endocrino	Hiperinsulinemia	Orngreen *et al.*, 2012
	Disfunción tiroidea	Orngreen *et al.*, 2012
	Diabetes mellitus	Orngreen *et al.*, 2012
	Atrofia testicular	Harper, 2002
	Alteraciones en la homeostasis del calcio	Santoro *et al.*, 2014
	Infertilidad	Matsumura *et al.*, 2009
Digestivo	Disfagia	Bellini *et al.*, 2006
	Estreñimiento o diarrea	Bellini *et al.*, 2006
	Cálculos biliares	Cardani *et al.*, 2008
	Transaminasas elevadas	Heatwole *et al.*, 2006
	Esteatosis no alcohólica	Achiron *et al.*, 1998
	Elevación de enzimas hepáticas	Achiron *et al.*, 1998
Ocular	Cataratas iridiscentes	Jameson, 2018
Piel	Pilomatricomas	Zampetti *et al.*, 2015
Todos	Mayor riesgo oncogénico	Emparanza *et al.*, 2018

b) Afectación del sistema cardíaco

La afectación cardíaca es un hecho frecuente e importante en la DM1 y constituye la segunda causa de muerte (Ashizawa *et al.*, 2018; Groh *et al.*, 2008; Mathieu *et al.*, 1999; Rajdev and Groh, 2015). Se estima que entre el 75-80% de los pacientes presenta algún grado de afectación cardíaca, desde alteraciones leves como bloqueos de la conducción hasta arritmias ventriculares graves que pueden ocasionar muerte súbita (Finsterer *et al.*, 2012; Petri *et al.*, 2012). Se ha demostrado que la edad de inicio del daño cardíaco correlaciona inversamente con el tamaño de la expansión y el grado de debilidad muscular (Antonini *et al.*, 2000). También se ha determinado que la sustitución del tejido cardiaco por tejido fibroadiposo juega un papel importante en la afectación de este sistema (Pelargonio *et al.*, 2002).

Desde un punto de vista molecular, este fenotipo cardiaco se ha asociado con un *splicing* anormal del canal de sodio *SCN5A* (isoforma Ex6A+) (Freyermuth *et al.*, 2016), predominando la isoforma fetal de este gen. Los modelos en ratón que mejor recapitulan los defectos en el sistema cardíaco son aquellos en los que se ha eliminado genéticamente DMPK y en consecuencia su función (Reddy *et al.*, 1996) y aquellos en los que se han insertado 960 repeticiones CTG específicamente en cardiomiocitos (Orengo *et al.,* 2008; Wang *et al.,* 2007).

c) Afectación del sistema respiratorio

La afectación respiratoria en la DM1 es frecuente y constituye la primera causa de muerte prematura en estos pacientes (Ashizawa *et al.*, 2018; Hawkins *et al.*, 2019; Mathieu *et al.*, 1999). Es, junto con los déficits del SNC y la debilidad muscular, uno de los factores que más influyen en el deterioro de la calidad de vida de los pacientes con DM1 (Thornton, 2014). En la mayoría de los casos esta afectación es de aparición progresiva y ocurre tardíamente, por lo que muchas veces se diagnostica con retraso.

La afección del sistema respiratorio puede venir acompañada de infecciones respiratorias recurrentes y un mayor riesgo de sufrir neumonía, derivado por las dificultades en la deglución.

En cuanto a las causas que estarían detrás de este fenotipo, por un lado, se atribuye a la afectación muscular y a la miotonía de la musculatura torácica, y, por otro, a la afectación del SNC que conduce a una alteración en el control de la respiración (Poussel *et al.*, 2015; Sansone *et al.*, 2015).

d) Afectación del sistema nervioso central

Además del músculo, del sistema cardíaco y del respiratorio, el SNC también se ve comprometido por la enfermedad, llegando a ser esta afectación uno de los mayores problemas para la vida diaria de los pacientes.

Las manifestaciones son sumamente variables e incluyen el déficit cognitivo (Labayru *et al.*, 2019), la apatía, la fatiga y las alteraciones del sueño. Las alteraciones en el sueño son una de las manifestaciones más frecuentes de la enfermedad, presentándose en hasta un tercio de los pacientes. Se caracteriza, fundamentalmente, por una dificultad para mantenerse despiertos, sobre todo tras las comidas (Laberge *et al.*, 2004), teniendo un origen multifactorial.

Las formas más graves de la enfermedad se presentan con discapacidad intelectual, déficit de atención, trastornos del lenguaje y trastornos del espectro autista (Labayru *et al.*, 2018; Okkersen *et al.*, 2017; Sistiaga *et al.*, 2010), que correlacionan inversamente con la longitud del triplete en algunos aspectos, como es el caso del cociente intelectual (Sistiaga *et al.*, 2010; Winblad *et al.*, 2006).

Aunque los problemas psiquiátricos no suelen ser graves, la depresión mayor y las alteraciones de la personalidad confieren a estos pacientes unas características especiales. Es frecuente que muestren rasgos de trastorno esquizoide, ansiedad, histeria, compulsión, neurosis depresiva y falta de iniciativa (Gallais *et al.*, 2015; Seijas-Gomez *et al.*, 2015; Sistiaga *et al.*, 2010).

Desde un punto de vista biológico, se ha determinado que los pacientes con DM1 presentan una reducción en el volumen del cerebro, tanto de la sustancia blanca como de la gris, atrofia cortical y lesiones en la sustancia blanca. Se ha determinado que la reducción en el volumen de la sustancia blanca correlaciona con la longitud del triplete y con el grado de debilidad muscular (Labayru *et al.*, 2019).

En cuanto a los modelos de ratón que tendrían afectación del SNC, encontramos el modelo DMSXL (>1.000 CTG), con acumulación de Tau en el cerebro, de *foci* nucleares y defectos en el *splicing* (Gomes-Pereira *et al.*, 2011; Seznec *et al.*, 2001); y el modelo donde se ha eliminado genéticamente *MBNL* que presenta apatía y falta de motivación (Matynia *et al.*, 2010).

e) Afectación endocrina

Los pacientes con DM1 presentan una afectación endocrina que se manifiesta de forma variable. Las disfunciones más comunes son el hipogonadismo, las alteraciones tiroideas y las alteraciones en los niveles de insulina y glucosa.

El hipogonadismo es la manifestación del sistema endocrino más frecuente en la DM1, describiéndose una atrofia testicular en hasta un 80% de los varones. Este hecho, junto con la insuficiencia ovárica presente en las mujeres, condiciona una baja fertilidad y un aumento en las pérdidas gestacionales (Johnson *et al.*, 2015; Rudnik-Schoneborn and Zerres, 2004).

En cuanto al metabolismo de la insulina, generalmente se describe una resistencia a la acción de esta hormona por una menor sensibilidad a la misma a nivel muscular, que puede manifestarse como hiperinsulinismo o como una diabetes mellitus tipo 2. Desde el punto de vista molecular, la resistencia a la insulina se ha asociado con una mayor presencia de la variante de *splicing* IR-A (isoforma embrionaria) del receptor de la insulina INSR (Renna *et al.*, 2019; Santoro *et al.*, 2013). El modelo animal que desarrolla una afección endocrina con una hiperglicemia e intolerancia a la glucosa es el modelo DMSXL (>1.000 CTG) (Huguet *et al.*, 2012).

Con menor frecuencia encontramos alteraciones en el eje corticotropo, dislipemias y alteraciones electrolíticas. Estas alteraciones no son menos importantes, ya que una elevación de triglicéridos predispone a un mayor riesgo de enfermedad vascular.

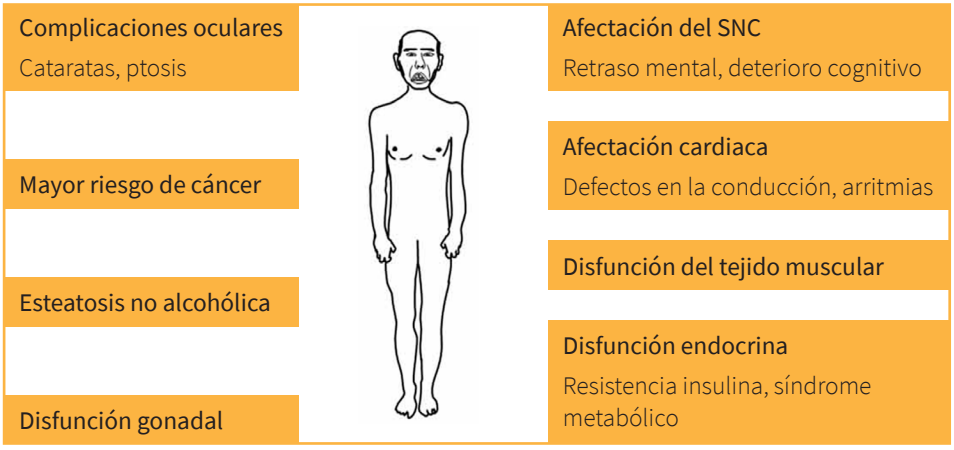

Figura I2. Enumeración de los síntomas clínicos más característicos de la DM1. Modificado de Mateos-Aierdi *et al.,* 2015.

f) Afectación de otros sistemas

Por último, cabe destacar otros sistemas que también se encuentran alterados en grado variable en la DM1.

El sistema digestivo también se encuentra afectado en grado variable en la DM1, incluyendo alteraciones como la hipomotilidad del esófago y colon, la disfagia y la esteatosis hepática (Gourdon and Meola, 2017; Thornton, 2014).

El desarrollo de cataratas precoces, es decir, antes de los 55 años, es una de las características distintiva de los pacientes con DM1, presentándose en casi todos ellos. Las cataratas más comunes que desarrollan estos pacientes son muy específicas y reciben el nombre de cataratas en forma de árbol de navidad, clasificándose como cataratas posteriores subescapulares de apariencia iridiscente (Smith and Gutmann, 2016; Thornton, 2014). Adicionalmente, se ha observado en pacientes con DM1 que presentan un pelo fino y poca densidad de folículos pilosos, teniendo una alta incidencia de alopecia androgénica (Campanati *et al.*, 2015; Campione *et al.*, 2017).

Cabe destacar que los tejidos más afectados en la DM1 son los que presentan un mayor incremento en la expansión del trinucleótido, como son el tejido muscular y el corazón (Ashizawa *et al.*, 1993). Además, con el paso del tiempo, esta expansión puede aumentar en un mismo individuo. Se ha determinado en muestras de sangre de pacientes con DM1, que a partir de 200 repeticiones CTG la expansión aumenta cada año. En cambio, esto no sucede en expansiones con menores a 200 repeticiones CTG (Martorell *et al.*, 1995; Martorell *et al.*, 1998; Wong *et al.*, 1995). Lo mismo ocurre en algunos modelos animales de ratón (Fortune *et al.*, 2000). Por otra parte, también se ha descrito *in vitro* que el mantenimiento de las células en cultivo, modelo que fenocopia las características celulares del envejecimiento fisiológico, produce un aumento en el número de repeticiones CTG (Ueki *et al.*, 2017).

Con todo ello, la esperanza de vida de estos pacientes se encuentra reducida, siendo la principal causa de muerte las complicaciones respiratorias, el fallo cardíaco, la muerte súbita por arritmias y el desarrollo de neoplasias (Ashizawa *et al.*, 2018; Gutiérrez Gutiérrez *et al.*, 2019; Turner and Hilton-Jones, 2014).

1.5. Subtipos clínicos de DM1

Recientemente la DM1 ha sido clasificada en 4 subtipos clínicos en función de la edad de inicio de los síntomas, la severidad y rangos de expansión del trinucleótido, aunque estos se solapan ampliamente entre los diferentes subtipos **(Figura I3 y Tabla I3)**. Estos grupos son los siguientes: la forma congénita, las formas pediátricas, la forma adulta y la forma leve (de Die-Smulders *et al.*, 1998; IDMC-2, 2000; Lanni and Pearson, 2019; Mathieu *et al.*, 1999).

La **forma congénita** (Campbell *et al.*, 2013; Echenne and Bassez, 2013) (tamaño de la expansión en rangos generalmente > 1.000 CTG) es la forma más grave de DM1, pero también la menos frecuente. Se caracteriza por una severa hipotonía al nacer,

frecuentemente acompañada de insuficiencia respiratoria, discapacidad intelectual y de distrofia muscular progresiva. A pesar de ello, al sexto mes de vida se produce una ligera mejoría, aunque hay un retraso evidente en el desarrollo. Hacia los 3-4 años mejora la hipotonía y prácticamente todos los pacientes consiguen una marcha autónoma. Sin embargo, ya en la adolescencia aparece la miotonía y con el paso de los años surge la característica debilidad muscular en las extremidades, generalmente de predominio distal (a diferencia de la DM2, en la que es predominantemente proximal), así como el resto de manifestaciones clásicas de la DM1.

El papel de la metilación en el locus de *DMPK* se ha asociado con la vía de herencia predominantemente materna que se observa en la DM1 congénita, estando más frecuentemente metilada la zona en la región 5' de DMPK en las herencias por esta vía materna (Barbé *et al.*, 2017).

Las formas **pediátricas** (con rangos generalmente entre 350-850 CTG), tradicionalmente se habían separado en dos grupos diferenciados denominados formas infantiles y juveniles, pero actualmente se tiende al consenso de incluirlas dentro de un mismo grupo denominado pediátrico (De Antonio *et al.*, 2016). Estas formas en muchas ocasiones no están diagnosticadas debido a la ausencia clara de síntomas neurológicos. Son menos graves que la forma congénita, pero presentan grados variables de deterioro cognitivo, dificultades de aprendizaje, degeneración muscular y somnolencia (Ho *et al.*, 2019; Lagrue *et al.*, 2019).

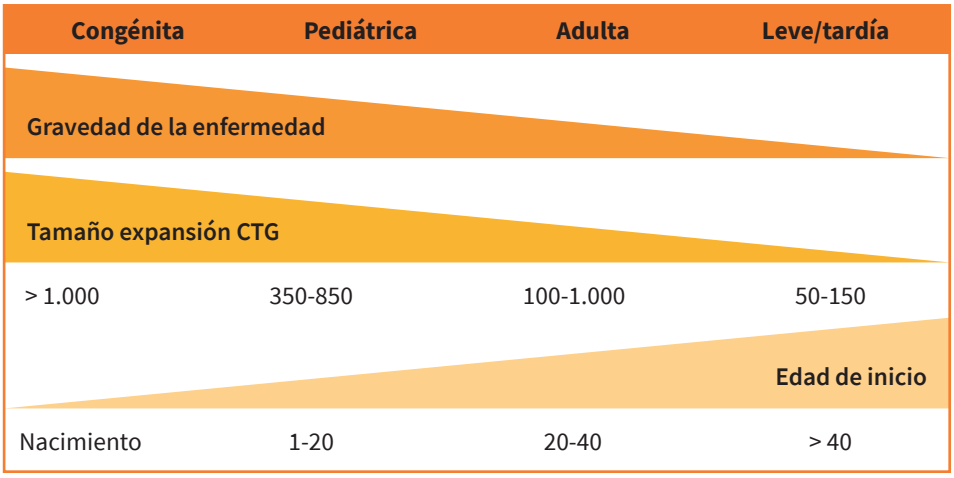

Figura I3. Correlación entre el subtipo clínico y la gravedad de la enfermedad, el tamaño de la expansión CTG y la edad de inicio. A la izquierda el mayor tamaño en la expansión correlaciona con una gravedad mayor y con una edad de inicio de la enfermedad temprana. En cambio, a la derecha, un tamaño de expansión de 50 repeticiones se relaciona con un fenotipo leve y de aparición tardía.

En cuanto a la forma con inicio en la edad **adulta** o forma **clásica** (con un amplio margen de variabilidad en el rango de la expansión, de entre 100-1.000 CTG), se caracteriza por miotonía, desgaste muscular progresivo y atrofia, todo ello acompañado por el res-

to de manifestaciones extramusculares descritas anteriormente en el capítulo de *"Manifestaciones Clínicas"* (Gourdon and Meola, 2017; Thornton, 2014).

Finalmente encontramos la forma **leve** o forma **tardía** (que generalmente se manifiesta en rangos de entre 50-150 CTG), que presenta un fenotipo menos grave, con una menor afectación muscular con leve miotonía (a veces solo eléctrica), escasa o nula debilidad y la presencia de cataratas precoces (Arsenault *et al.*, 2006).

Tabla I3. Correlación del fenotipo con la longitud de la expansión CTG en DM1. Basado en Die-Smulders *et al.,* 1998, Mathieu *et al.,* 1999, International Myotonic Dystrophy Consortium 2000 y 2019, De Antonio 2016 y Lanni *et al.,* 2019.

Subtipo	Síntomas clínicos relevantes	CTG[1,2]	Edad de debut	Esperanza de vida	Herencia materna	Herencia paterna
Pre-mutación	Ninguno	37-50 (riesgo de expansión del alelo)	No procede	Normal		
DM1 leve	Cataratas, leve miotonía	50-150	> 40 años	Normal	30%	70%
DM1 adulta	Síntomas clásicos de la DM1	100-1.000	20-40 años	Reducida	30%	70%
DM1 pediátrica	Deterioro cognitivo, dificultades de aprendizaje, degeneración muscular y somnolencia Menos grave que la congénita pero más grave que la adulta	350-850	1 mes – 20 años	Reducida	45%	55%
DM1 congénita	Defectos en el desarrollo, hipotonía, insuficiencia respiratoria, defectos cardiacos, deterioro cognitivo severo, disfagia	> 1.0003	< 1 mes	Mayor riesgo de muerte en la infancia[4]	91-87.5%	9-12.5%

1. Un mismo tamaño de la expansión CTG puede asociarse a diferentes subtipos clínicos de la enfermedad.
2. El tamaño de la expansión CTG en individuos sanos es de entre 4-37 repeticiones.
3. Se han observado casos con repeticiones menores a 1.000 CTG.
4. No incluye muertes neonatales.

1.6. Diagnóstico

Hasta el descubrimiento de la expansión del microsatélite en 1992 (Brook *et al.*, 1992), el diagnóstico de la enfermedad era fundamentalmente clínico tras detectar en el examen clínico de los rasgos más característicos de la enfermedad como son la miotonía, la debilidad y la atrofia muscular. Ante esta sospecha clínica, se podían realizar pruebas complementarias como la electromiografía muscular y así poner de manifiesto las descargas miotónicas, que en los casos más leves pueden ser subclínicas. La determinación de los niveles de creatina quinasa, así como la biopsia muscular, usadas ampliamente en el diagnóstico de otras miopatías, son de escasa utilidad en la DM1 (Gutiérrez Gutiérrez *et al.*, 2019).

En la actualidad, ante la sospecha clínica de un paciente con DM1, se determina el tamaño de la expansión en una muestra de ADN obtenida de la sangre del paciente. Para ello se han desarrollado diferentes técnicas basadas en la reacción en cadena de la polimerasa (PCR) (IDMC-2, 2000; Prior, 2009). El primer barrido se realiza mediante PCR convencional para la determinación de alelos de hasta 150 repeticiones. La implementación de la *triple-PCR* (Warner *et al.*, 1996) permite detectar si el alelo está expandido, pero sin poder cuantificar el número exacto de repeticiones. Esta técnica arroja hasta un 9% de falsos positivos, debido a que no es capaz de detectar la presencia de interrupciones en la secuencia CTG (Kakourou *et al.*, 2010; Radvansky *et al.*, 2011). Sin embargo, modificaciones posteriores de dicha técnica (Radvansky *et al.*, 2011) son capaces de detectar dichas interrupciones. La cuantificación exacta del número de repeticiones es más precisa mediante la *small-pool* PCR (Dandelot and Gourdon, 2018) seguida de *Southern-Blot* (Gutiérrez Gutiérrez *et al.*, 2019; IDMC-2, 2000). No obstante, ante la dificultad que entraña la determinación exacta del número de repeticiones y la variedad de técnicas disponibles con resultados no homogéneos entre ellas (Ballester-López *et al.,* 2020), es habitual que en la práctica clínica asistencial sólo se determina si uno de los alelos está expandido, sin abordar la medición del número exacto de repeticiones.

1.7. Mecanismos moleculares de la enfermedad

A día de hoy se postulan tres mecanismos moleculares diferentes y no excluyentes, que estarían detrás de la patogénesis de la enfermedad **(Figura I4)**: a) la haploinsuficiencia de *DMPK*, b) el papel de la metilación y la alteración en la expresión de los genes adyacentes *SIX5* y *DMWD;* y c) el efecto tóxico que produce la acumulación del ARN mutado.

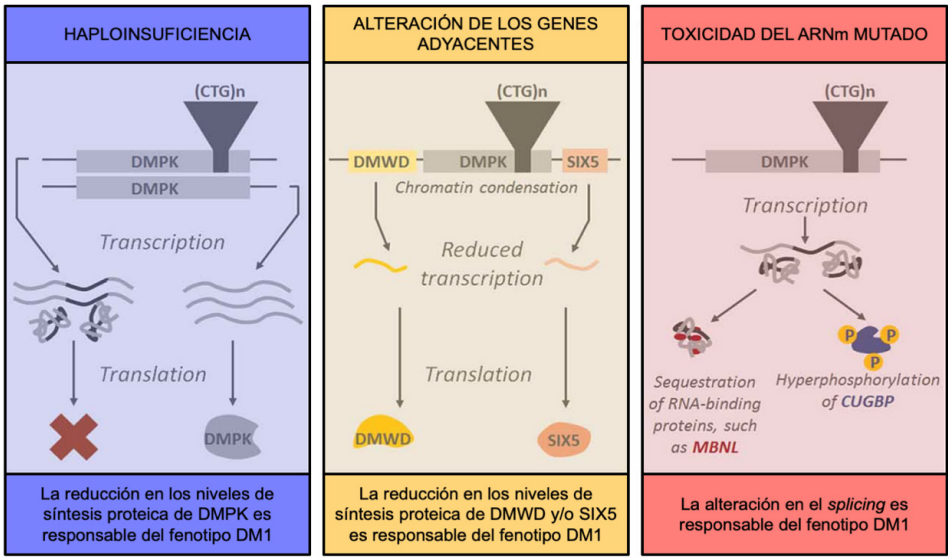

Figura I4. Distintos mecanismos moleculares que contribuyen a la patología de la DM1. 1) La haploinsuficiencia de DMPK, 2) la alteración en la expresión de los genes adyacentes y el papel de la metilación y 3) la toxicidad del ARNm mutado que va a provocar el secuestro de MBNL y la sobrexpresión de CUGBP. Modificada de Mateos-Aierdi *et al.,* 2015

a) Haploinsuficiencia de DMPK

La haploinsuficiencia de *DMPK* se basa en el efecto deletéreo de poseer una única copia funcional del gen, disminuyendo la producción de proteína funcional.

DMPK es una quinasa del tipo serina/treonina (Manning *et al.*, 2002) con una alta homología en su dominio quinasa con la quinasa MRCK (del inglés *"myotonic dystrophy kinase-related Cdc42-binding kinase"*) (Leung *et al.*, 1998), cuya función es la organización del citoesqueleto y a la quinasa ROCK1 de la familia de quinasas de tipo Rho (Wansink *et al.*, 2003), cuya función es la formación de las adhesiones focales y las fibras de estrés. DMPK pertenece a la familia de quinasas AGC, como es el caso de NDR1, cuya función está implicada en la regulación del ciclo celular y la apoptosis, o de AKT1, cuya función está relacionada con la proliferación celular AKT (Wansink *et al.*, 2003).

En relación a su función, en el músculo esquelético DMPK regula la miogénesis y su ausencia provoca atrofia muscular, miopatía, alteraciones en la homeostasis del calcio, defectos cardiacos y una disminución en la sensibilidad por la insulina en modelos murinos (Berul *et al.*, 1999; Jansen *et al.*, 1996; Llagostera *et al.*, 2007; Reddy *et al.*, 1996). Recientemente, se ha observado que la expresión de DMPK se activa en respuesta a un daño en el ADN a través de p53 (Itoh *et al.*, 2019).

Se han identificado 7 isoformas de DMPK (DMPK A-G) producidas mediante tres combinaciones diferentes en su *splicing* alternativo, definido por el tipo celular y su localización subcelular, preferentemente en el citoplasma (Groenen *et al.*, 2000). Todas las isoformas comparten un dominio N-terminal, un dominio quinasa y una región con una cola en forma de espiral. Las diferencias entre las diferentes isoformas difieren fundamentalmente en la presencia o ausencia de un motivo VSGG y de la naturaleza de su dominio C-terminal. La última isoforma identificada, la DMPK-G carece del dominio donde se introduce la expansión CTG por lo que, a diferencia del resto de isoformas, no queda retenida en el núcleo (Wansink *et al.*, 2003).

En cuanto a su patrón de expresión tiene una preferencia por el tejido muscular esquelético y por las fibras musculares de tipo 1, las más afectadas en DM1, donde co-localiza con el transportador de calcio SERCA2a. Dentro del músculo también se ha visto expresión en el retículo sarcoplásmico y en las uniones neuromusculares (revisado por Ueda *et al.,* 2000). Por otra parte, también se ha demostrado que se localiza en la membrana nuclear donde interacciona con la proteína lámina A/C. A su vez, también se ha observado expresión de DMPK en el músculo cardiaco y en el SNC.

En relación a las dianas de DMPK, se ha determinado una preferencia por los sustratos con el siguiente dominio, "RXXS/TL/VR", muy similar al dominio de PKC o CaMK II (Wansink *et al.*, 2003). Se ha observado que DMPK modula la actividad de los canales de sodio en el músculo esquelético (Mounsey *et al.*, 1995) y que sus sustratos son el receptor de dihidropiridina, CUGBP (Roberts *et al.*, 1997), DMPA, MKBP, PLM (Mounsey *et al.*, 2000) y la miosina fosfatasa. Se ha determinado que DMPK fosforila a la miosina fosfatasa en su región C-terminal y con ello inhibe su función fosfatasa (Muranyi *et al.*, 2001). La inhibición de la miosina fosfatasa provoca un aumento en la fosforilación de la miosina de cadena ligera que provoca una sensibilización por el Ca^{2+} en el músculo liso y cambios en la estructura del citoesqueleto en células no musculares (Kaibuchi *et al.*, 1999; Muranyi *et al.*, 2001).

De acuerdo a esta teoría, en la DM1 los transcritos de *DMPK* que contienen las repeticiones expandidas quedan retenidos en el núcleo, bloqueando su transporte al citoplasma donde deberían traducirse para dar lugar a la proteína funcional. La acumulación de estos transcritos mutados en los *foci* nucleares es una característica esencial de la enfermedad (Miller *et al.*, 2000; Sznajder and Swanson, 2019).

Este mecanismo postula que la expresión reducida de *DMPK* es la causante del desarrollo de la enfermedad, lo que correlaciona con niveles reducidos de *DMPK* en pacientes con DM1, que a su vez correlaciona inversamente con el tamaño de la expansión del microsatélite (Fu *et al.*, 1993).

b) Papel de la metilación y la alteración en la expresión en los genes adyacentes

El segundo de los mecanismos propuestos se basa en la alteración de los dos genes que flanquean a *DMPK*: *SIX5* y *DMWD*.

SIX5 (del inglés, *"SIX homeobox 5"*) codifica un factor de transcripción de la familia SIX y se localiza en la región 3' inmediatamente posterior a DMPK **(Figura I1)**. SIX5 se expresa durante el desarrollo embrionario y tiene un papel preferente en la córnea, el epitelio de la lente y los fotorreceptores de la retina (Sato *et al.*, 2002). También se ha descrito que tiene un papel en la espermatogénesis (Sarkar *et al.*, 2004). La expansión CTG va a afectar a su secuencia promotora, provocando un cambio en la metilación del *locus* y con ello una reducción en su expresión (Boucher *et al.*, 1995).

DMWD (del inglés, *"Myotonica-containing WD repeat motif")* es un gen que se localiza en la región 5' inmediatamente anterior a DMPK **(Figura I1)** (Jansen *et al.*, 1995). Al contrario que SIX5, se ha observado que se expresa en casi todos los tejidos adultos (Westerlaken *et al.*, 2003).

Al igual que *DMPK*, se ha demostrado que existe una reducción de *SIX5* que correlaciona inversamente con el tamaño de la expansión (Korade-Mirnics *et al.*, 1999). Sin embargo, se desconoce si esta correlación también se da con *DMWD*.

Recientemente, se ha descrito un cambio importante en los niveles de metilación en las regiones flanqueantes a las repeticiones CTG. Se ha determinado que la región anterior a DMPK presenta unos niveles superiores de metilación, mientras que las regiones donde se introduce el triplete y la región posterior a DMPK no están metiladas (Ghorbani *et al.*, 2013; López Castel *et al.*, 2011), proponiéndose este patrón de metilación aberrante como un marcador diagnóstico de la enfermedad (Barbé *et al.*, 2017).

Todo ello hace que se produzcan cambios importantes en la estructura de la cromatina, lo que induce una condensación del ADN en esta región (Otten and Tapscott, 1995) y con ello la reducción de la expresión tanto de *SIX5* (Klesert *et al.*, 1997; Thornton *et al.*, 1997; Yanovsky-Dagan *et al.*, 2015) como de *DMWD* (Alwazzan *et al.*, 1999; Frisch *et al.*, 2001; Thornton *et al.*, 1997).

c) La toxicidad del ARN mutado

El tercer mecanismo propone la toxicidad del ARN portador de las repeticiones expandidas como el principal factor patogénico de la enfermedad. Este ARN se pliega sobre sí mismo a nivel de las repeticiones, formando una horquilla de doble cadena (Michalowski *et al.*, 1999) que interfiere con su propio procesamiento y con el de otros ARNs, lo que se conoce como ganancia de función tóxica de ARN. Este ARN tóxico interfiere con proteínas que participan en la regulación del *splicing* alternativo

de otros muchos transcritos, entre los que destacan la familia de *MBNL* (Miller *et al.*, 2000) y la familia de *CUGBP*/*CELF* (Kuyumcu-Martinez *et al.*, 2007).

El resultado de esta acumulación de ARN tóxico va a provocar una inmovilización de MBNL en agregados nucleares denominados *foci*, que se visualizan usando la microscopia de fluorescencia. Esto es debido a la alta afinidad de MBNL por las expansiones CTG, lo que finalmente va a provocar una pérdida de su función (Fardaei *et al.*, 2002; Pettersson *et al.*, 2015; Wheeler *et al.*, 2007). Por otro lado, se va a producir una unión de *CUGBP1*/*CELF1* a los agregados CTG que va a provocar su acumulación debido a una híperfosforilación por parte de la proteína quinasa C que induce su estabilización (Cardani *et al.*, 2013; Kuyumcu-Martinez *et al.*, 2007). Además de MBNL y CUGBP1 se ha demostrado que otras proteínas de unión al ARN se acumulan de manera tóxica en los *foci* CTG, como son las proteínas hnRNP F, hnRNP H, DDX5, DDX56, DDX517 y Staufen (Apponi *et al.*, 2011; Bondy-Chorney *et al.*, 2016; Crawford Parks *et al.,* 2020; Pettersson *et al.*, 2015; Ravel-Chapuis *et al.*, 2012).

Todas estas alteraciones van a provocar disfunciones en los procesos de proteostasis y ribostasis en la célula con una inhibición de la familia de MBNL, un cambio en su localización subcelular y un incremento en la distribución de la familia de CUGBP/CELF. El resultado final de ello es una alteración de todo el transcriptoma de la célula en relación a la expresión patogénica de variantes de *splicing* neonatales en tejidos adultos de pacientes con DM (Bland *et al.*, 2010; Hino *et al.*, 2007; Sicot *et al.*, 2011), además de una poliadenilación alternativa (Batra *et al.*, 2014; Batra *et al.*, 2015) y un transporte de transcritos entre el núcleo y citoplasma alterado (Andre *et al.*, 2018). Este mecanismo explica que las enfermedades DM1 y DM2, causadas por la expansión de repeticiones similares en regiones no codificantes de genes con diferente localización, presenten una sintomatología común.

En la DM1 se han encontrado variantes en el *splicing* de multitud de genes (Ravel-Chapuis *et al.*, 2018), pero hay una serie de variantes que se han asociado a aspectos clínicos de la enfermedad, como son las variantes del receptor de insulina asociadas a la resistencia a la insulina; del canal de cloro, responsables principalmente de la miotonía (Osborne *et al.*, 2009) y de los canales tipo SERCA (Kimura *et al.*, 2005), asociadas a la alteración de la homeostasis del calcio, entre otras muchas variantes. En consecuencia, la DM1 ha llegado a considerarse como una *spliceopatía*.

Por otro lado, se ha descrito que estas repeticiones pueden producir péptidos tóxicos mediante una traducción del ARN que no se inicia mediante la secuencia consenso ATG, lo que se conoce como traducción de tipo RAN (del inglés, *"Repeat-Associated Non-ATG translation"*). La producción de los transcritos antisentido de *DMPK* genera la acumulación de péptidos con cadenas largas de polileucina, policisteína y polialanina que provocan una toxicidad celular (Zu *et al.*, 2011). Sin embargo, el me-

canismo exacto de la traducción tipo RAN, así como su implicación en la patogénesis de la DM1 está por esclarecer.

En definitiva, se han postulado diferentes mecanismos patogénicos que explican la presencia de determinadas manifestaciones que caracterizan la DM1. La presencia de estos procesos y, probablemente, algún otro aún desconocido, lleva a pensar que el complejo fenotipo de la enfermedad es el resultado de la suma de todos ellos (Andre *et al.*, 2018; Kearse and Todd, 2014; Konieczny *et al.*, 2014; Meola and Cardani, 2015; Meola *et al.*, 2013; Rohilla and Gagnon, 2017).

1.8. Modelos animales para el estudio de la DM1

La contribución que tiene cada mecanismo propuesto en la patogénesis de la DM1 se ha estudiado mediante la generación de diferentes modelos animales. Por analogía a los mecanismos moleculares propuestos para la enfermedad, podemos clasificar los modelos animales en 1) modelos *knock-out (KO)* para *DMPK*; 2) modelos *KO* para *SIX5* y *DMWD*; 3) modelos *KO* para *MBNL* y modelos de sobrexpresión de *CUGBP* y, por último, 4) modelos en los que se han insertado las repeticiones CTG.

1) Modelos *KO* para *DMPK*

Los primeros modelos que se desarrollaron fueron los ratones donde se eliminó DMPK (modelos $Dmpk^{+/-}$ y $Dmpk^{-/-}$) de manera multisistémica. Estos modelos desarrollan una miopatía tardía, defectos en la conducción cardiaca e intolerancia a la glucosa, pero no reproduce ni el fenotipo muscular ni el multisistémico de la enfermedad (Berul *et al.*, 1999; Jansen *et al.*, 1996; Reddy *et al.*, 1996). Estos resultados, junto con el hecho de que los síntomas de la DM2 sean tan similares a los de la DM1 a pesar de no implicar a *DMPK*, sugieren que una reducción en la función de esta proteína sería la causa, al menos, de parte de la sintomatología de los pacientes con DM1.

2) Modelos *KO* para SIX5 y *DMWD*

Posteriormente se desarrollaron modelos en los que se eliminó SIX5 en todo el organismos (modelos $Six5^{+/-}$ y $Six^{-/-}$). La eliminación de una sola copia (Sarkar *et al.*, 2000) de este gen ya es suficiente para el desarrollo de cataratas. No obstante, el tipo de cataratas que desarrolla este modelo no son del tipo del que desarrollan los pacientes con DM1. Por otra parte, la eliminación de ambas copias de *SIX5*, además de provocar el desarrollo de cataratas (Sarkar *et al.*, 2000), provoca una menor fertilidad en machos (Sarkar *et al.*, 2004) y una alteración de la función cardíaca (Wakimoto *et al.*, 2002). Sin embargo, ninguno de los modelos presenta el fenotipo multisistémico característico de la DM1.

Recientemente se ha desarrollado un modelo heterocigoto para *DMWD* y un modelo doble transgénico para *DMWD* y *SIX5* (Yin *et al.*, 2020), que desarrollan una leve pérdida muscular, señalando que la reducción a la mitad en la dosis génica de *SIX5* y/o *DMWD* no es suficiente para recapitular los rasgos fenotípicos principales de la enfermedad, como son la debilidad muscular y la miotonía.

3) Modelos *KO* para *MBNL* y modelos de sobrexpresión de *CUGBP*

El impacto de las proteínas MBNL y CUGBP/CELF en la patogénesis de la DM1 se ha estudiado mediante otro grupo importante de modelos de ratón, como son los modelos *knock-out* para *MBNL1*, *MBNL2* y *MBNL3,* y los modelos de sobrexpresión de *CUGBP*/*CELF*. Gracias a ellos se han podido identificar las variantes de *splicing* dependientes de la familia de *MBNL* y de la familia de *CUGBP*.

Los modelos con inactivación de *MBNL1* (*Mbnl1*$^{\Delta 3/\Delta 3}$ y *Mbnl1*$^{-/-}$) de manera ubicua desarrollan una miotonía asociada al defecto en el splicing de *CLCN1*, cataratas, falta de motivación y apatía. Sin embargo, no desarrollan debilidad muscular (Kanadie *et al.,* 2003 and Matynia *et al.,* 2010). En cambio, en los modelos con inactivación de *MBNL2* (*Mbnl2*$^{\Delta 2/\Delta 2}$, *Mbnl2*$^{GT4/GT4}$ y *Mbnl2*$^{GT2/GT2}$) se han descrito resultados contradictorios. El modelo *Mbnl2*$^{GT2/GT2}$ desarrolla una patología muscular leve con miotonía y defectos en el *splicing*. Sin embargo, los modelos *Mbnl2*$^{\Delta 2/\Delta 2}$, *Mbnl2*$^{GT4/GT4}$ no presentan alteraciones musculares reseñables, pero sí un marcado deterioro del SNC en el modelo *Mbnl2*$^{\Delta 2/\Delta 2}$ (Charizanis *et al.,* 2012; Hao *et al.,* 2008; Lin *et al.,* 2006). Por último, el modelo *KO* para *MBNL3* muestra una reducción en la fuerza muscular y una alteración de la regeneración muscular dependiente de la edad (Poulos *et al.*, 2013), además de intolerancia a la glucosa, defectos cardíacos y la presencia de cataratas (Choi *et al.*, 2016). No obstante, ninguno de estos modelos parece recapitular todo el espectro de la enfermedad y se han observado fenómenos compensatorios entre diferentes miembros de la familia MBNL cuando uno de ellos se encuentra inactivado. Por ello, se han desarrollado los modelos doble *KO MBNL1 y* 2 y triple *KO MBNL1*, *2* y *3* que recapitulan en mayor medida el fenotipo de la enfermedad (Choi *et al.*, 2015; Thomas *et al.*, 2017). Este último modelo tiene una alta tasa de mortalidad neonatal, con retraso en el crecimiento y debilidad muscular con profundos cambios en el *splicing*.

Por su parte, los modelos de sobrexpresión de *CUGBP1*/*CELF1* reproduce el desgaste muscular y la miopatía cuando se introduce en el músculo esquelético (Ward *et al.*, 2010) y defectos en la conducción cardiaca y cardiomiopatía cuando se introduce en el tejido cardiaco (Koshelev *et al.*, 2010).

Recientemente se ha desarrollado un modelo de ratón en el que se ha eliminado un alelo de DMPK, SIX5, MBNL1 y DMWD, que presenta una fuerte mortalidad por

complicaciones respiratorias y se asocia a la forma congénita de la DM1. Sin embargo, muchos de los defectos de *splicing* presentes en la enfermedad no son recapitulados en este modelo (Yin *et al.*, 2020). Además, las afectaciones, en conjunto, no son tan graves como cabría esperar en un modelo con 4 de las alteraciones que se dan en la DM1.

4) Modelos con expansiones CTG

Son los modelos en los que se ha insertado el triplete expandido los que parecen recapitular en mayor medida los fenotipos musculares y multisistémicos de la enfermedad (Braz *et al.*, 2018; Gomes-Pereira *et al.*, 2011).

El modelo de ratón en el que se insertaron 250 repeticiones CTG bajo el control del promotor de la actina muscular humana (modelo HSA^{LR}) presenta una significativa degeneración muscular, miotonía, una alta mortalidad y profundas alteraciones en el *splicing* dependiente de MBNL (Mankodi *et al.*, 2000). Sin embargo, la ausencia de debilidad y de pérdida muscular representa una limitación adicional.

Por otro lado, se ha desarrollado otro modelo donde se han insertado 300 repeticiones CTG de manera ubicua y se ha visto que presenta miotonía y cambios histológicos y musculares asociados a una atrofia muscular. Estos síntomas no son tan graves como en la enfermedad humana y normalmente aparecen en los ratones transgénicos envejecidos (Panaite *et al.*, 2008; Seznec *et al.*, 2001; Vignaud *et al.*, 2010). Sin embargo, se ha generado *de novo* una nueva línea transgénica por simple cruzamiento de la línea con 300 repeticiones, que ha dado lugar a una línea con más de 1.000 repeticiones CTG (modelo DMSXL), demostrando con ello la inestabilidad del triplete y su expansión intergeneracional (Gomes-Pereira *et al.*, 2007). Este ratón DMSXL presenta una alta tasa de mortalidad, retraso en el crecimiento e importante afectación muscular (Huguet *et al.*, 2012), además de fallo respiratorio (Panaite *et al.*, 2013) y cambios en el comportamiento y en la cognición, presentando un fenotipo multisistémico y siendo el que mejor recapitula los rasgos de la enfermedad hasta la fecha.

Al margen de los modelos murinos, cabe resaltar la existencia de los modelos en mosca que reproducen los cambios moleculares e histológicos de la enfermedad, como la acumulación de *foci* nucleares, las alteraciones en el *splicing*, la afectación del SNC y los problemas cardiacos, entre otros (Souidi *et al.*, 2018). Entre ellos, cabe destacar el modelo con inserción 480 repeticiones CTG interrumpidas, que presenta alteraciones en el *splicing*, pérdida de función de MBNL y acumulación de transcritos a nivel molecular, además de degeneración muscular y una menor supervivencia global (García-López *et al.*, 2008).

1.9. Tratamiento actual y aproximaciones terapéuticas en curso

En cuanto al tratamiento, en la actualidad no existe ninguno que retrase, detenga o revierta el curso propio de la enfermedad. Los tratamientos que se utilizan simplemente van dirigidos a paliar o tratar los síntomas, la mayor parte de ellos de forma tejido dependiente (Gutiérrez Gutiérrez *et al.*, 2019).

La *debilidad muscular* se puede trabajar mediante ejercicio físico regular para mantener la masa muscular. La *miotonía* se puede tratar con mexiletina, aunque con un control terapéutico estricto debido a que este compuesto bloquea los canales de sodio cardiacos. Las *mialgias* se pueden paliar con analgésicos o gabaérgicos y la *fatiga* con modafinilo. Las *cataratas* se pueden intervenir quirúrgicamente con un buen resultado funcional y los *problemas cardíacos* pueden prevenirse mediante la implantación de un marcapasos o de un desfibrilador automático. La *diabetes mellitus* se trata con fármacos sensibilizadores a la insulina como la metformina. Finalmente, los problemas de *deglución* se pueden mitigar mediante una dieta especial. También se pueden usar fármacos antiespasmódicos para tratar el dolor abdominal y los síntomas del colon irritable. En paralelo puede ofrecerse una terapia psicológica encaminada a mejorar los problemas *conductuales y psicológicos* y una terapia logopédica para tratar los problemas del *habla*.

En los últimos años se ha abordado la investigación en terapias frente a DM1 en 3 ejes principales: 1) estrategias encaminadas a la degradación de los transcritos de *DMPK* mutantes; 2) inhibición del secuestro de MBNL1 por el transcrito mutado y, finalmente, 3) estrategias basadas en reducir los niveles de CUGBP1/CELF1.

Varios grupos han centrado sus esfuerzos en el bloque del ARNm de DMPK como estrategia para reducir la expresión de las repeticiones tóxicas. En este sentido se han desarrollado varios tipos de oligonucleótidos antisentido (ASO, del inglés *"Anti-Sense Oligonucleotides"*), que se van a unir al ARNm y por lo tanto van a inactivar su acción. Los ASO son pequeñas moléculas de ADN monocatenario complementarias a determinadas secuencias específicas de un ARNm diana. Se ha demostrado que los ASO, administrados intramuscularmente al ratón HSA^{LR} provocan una disminución del ARN tóxico en el músculo esquelético, la liberación de MBNL1 y una mejora en la arquitectura del músculo (Wheeler *et al.*, 2012). No obstante, tras el testado de los ASO en ensayos clínicos, se decidió abandonar su desarrollo, ya que los niveles de ASO encontrados en la biopsia muscular de los pacientes tratados, tras administración de forma subcutánea, no eran lo suficientemente altos (NCT02312011). Más recientemente, se ha anunciado el desarrollo de nuevos ASO más potentes para tratar la DM1 mejorando su distribución muscular (Ionis, 2017).

Por otra parte, cabe destacar las estrategias encaminadas a recuperar la actividad de MBNL1 a niveles no patológicos. Para ello, se han realizado cribados (Konieczny *et al.*, 2017; Rzuczek *et al.*, 2015) de miles de moléculas con el objetivo de identificar aquellas que inhiban la interacción MBNL1-CTG. Entre las moléculas identificadas cabe destacar el *Ligando 1* (Arambula *et al.*, 2009) y sus posteriores modificaciones (Jahromi *et al.*, 2013), que son capaces de liberar a MBNL1 de los *foci* celulares; y el *Ligando 3*, que mejora el fenotipo de DM1 en un modelo de *Drosophila* (Nguyen *et al.*, 2015). Sin embargo, la principal desventaja de este tipo de aproximaciones terapéuticas es que, además de inhibir la interacción de MBNL1 con la expansión CTG, también podrían inhibir otras interacciones de MBNL con otros transcritos (Warf *et al.*, 2009).

En relación a los compuestos encaminados a la modulación de CUGBP1/CELF1, podemos destacar los inhibidores de GS3Kβ y los inhibidores de la proteína quinasa C (PKC). Se ha observado una activación de ambas rutas en pacientes con DM1, que conlleva una fosforilación aberrante de CUGBP1/CELF1. Con esta idea han surgido compuestos que inhiben dicha fosforilación como es el caso del litio (Jones *et al.*, 2012), la quinolona 53 (Palomo *et al.*, 2017) o el tideglusib (Wang *et al.*, 2019). Este último ha llegado a ensayos clínicos (NCT03692312) con resultados positivos en varios aspectos como la somnolencia, la cognición, el autismo, la función muscular y la miotonía (AMO-Pharma, 2019). Por otra parte, se encuentra el compuesto Ro-31-8220, un inhibidor de PKC, que se encuentra en estudios preclínicos (Hambleton *et al.*, 2006).

En resumen, en los últimos años se han identificado gran cantidad de moléculas con un potencial terapéutico anti-DM1 (Konieczny *et al.,* 2017; Overby *et al.,* 2018; Thornton *et al.,* 2017). Sin embargo, ninguna de estas estrategias se ha implementado a día de hoy como tratamiento a nivel asistencial y es necesaria la búsqueda de nuevos compuestos.

2. Distrofia miotónica tipo 1 y envejecimiento

2.1. El concepto de envejecimiento

A simple vista es sencillo distinguir un individuo joven de una persona mayor. Sin embargo, definir el proceso de envejecimiento resulta mucho más complejo de lo que pueda parecer en un primer momento. Existen numerosas definiciones, pero todas coinciden en una misma idea: "el envejecimiento comprende cambios que ocurren en nuestras células y tejidos a lo largo del tiempo, que propician la pérdida progresiva de la homeostasis del organismo y que conllevan una mayor vulnerabilidad a morir" (López-Otín *et al.*, 2013).

Por otra parte, se ha llegado al consenso de que el proceso de envejecimiento se caracteriza por ser prácticamente universal ocurriendo en todas las especies a lo largo de la escala evolutiva, con algunas notables excepciones; ha de ser intrínseco, teniendo su origen de manera endógena, pero también afectado por el entorno; ha de ser progresivo y, finalmente, ha de ser deletéreo, ya que tiene un carácter perjudicial para el individuo (Viña *et al.*, 2007).

El aumento en la esperanza de vida que se ha producido en las últimas décadas en los países desarrollados ha provocado un profundo cambio en la pirámide poblacional. Las proyecciones europeas para 2080 arrojan el dato de que casi un 30% de la población tendrá más de 65 años. Cabe señalar que el aumento en la esperanza de vida trae consigo el incremento en el desarrollo de enfermedades crónicas, siendo el envejecimiento el factor de riesgo más importante para el desarrollo de estas enfermedades (Dillin *et al.*, 2014; MacNee *et al.*, 2014). Hoy en día, casi dos tercios de las muertes están relacionadas con el envejecimiento, siendo éste el mayor factor de riesgo para morir (MacNee *et al.*, 2014).

Entre estas enfermedades asociadas al envejecimiento cabe destacar el declive progresivo que se da en la funcionalidad del sistema respiratorio (Angelidis *et al.*, 2019; Lowery *et al.*, 2013) y del sistema cardiovascular, que aumentan considerablemente con la edad, siendo la principal causa de muerte en personas de más de 65 años. A modo de ejemplo, los problemas coronarios y el fallo cardiaco ocurren en más de un 70% en personas con más de 65 años (Rosamond *et al.*, 2008). De la misma manera, la osteoporosis es una condición que se da con el envejecimiento (Papaioannou *et al.*, 2009), al igual que la pérdida de masa muscular (Cruz-Jentoft *et al.*, 2010), el desarrollo de tumores (Finkel *et al.*, 2007; Serrano and Blasco, 2007) y el aumento de las enfermedades neurodegenerativas (Hou *et al.*, 2019; Walker *et al.*, 2019).

A todo ello hay que añadir que todavía se encuentra sin resolver la pregunta de cuál es la esperanza de vida máxima de la especie humana. En 2016, en base a datos de-

mográficos, se determinó que el ser humano posee una esperanza de vida teórica y fija de 115 años (Dong *et al.*, 2016). Sin embargo, otros estudios ponen de manifiesto que no hay un límite teórico para la esperanza de vida, llegando las tasas de mortalidad a una estabilidad a los 105 años, edad a partir de la cual la probabilidad de muerte sería del 50%, con lo que técnicamente no existiría un límite teórico para la longevidad (Barbi *et al.*, 2018).

2.2. Manifestaciones de envejecimiento en la DM1 a nivel sistémico

Como se ha indicado anteriormente, la DM1 es una enfermedad multisistémica caracterizada por deficiencias en múltiples tejidos **(Tabla I2)**, donde encontramos una pérdida de la masa muscular conocida como sarcopenia, problemas cardíacos, una pérdida de la función pulmonar y un deterioro del SNC, entre otras afectaciones, siendo todos ellos procesos que se dan durante el envejecimiento fisiológico de manera natural.

Todas estas alteraciones funcionales en pacientes con DM1 sugieren un declive funcional acorde con un envejecimiento exacerbado. En concordancia, la esperanza de vida de estos pacientes se encuentra reducida (Gutiérrez Gutiérrez *et al.,* 2019), lo que ha llevado a proponerla como un modelo de envejecimiento prematuro (Mateos-Aierdi et al., 2015), e incluso como un síndrome progeroide (Meinke et al., 2018).

Hay otras enfermedades como los síndromes de Hutchinson-Gilford (Gonzalo *et al.*, 2017) o de Werner (Ghosh and Zhou, 2014) que también presentan síntomas de envejecimiento prematuro. Sin embargo, estas enfermedades son modelos extremos del envejecimiento (progeria) que no recapitulan todos los procesos afectos durante el envejecimiento fisiológico, como por ejemplo la afección del SNC o la presencia de neoplasias (Gonzalo *et al.*, 2017; Gordon *et al.*, 2014). En cambio, la DM1, al menos en su etapa adulta, recapitula en mayor medida y de manera mucho más fisiológica, aunque acelerada, los procesos moleculares y las alteraciones clínicas involucradas en el envejecimiento normal o fisiológico. Dentro de estos procesos en esa tesis nos vamos a centrar en los cambios subyacentes al incremento del riesgo oncogénico y en las alteraciones en la proliferación y senescencia celular, así como en el metabolismo mitocondrial.

En los últimos años se ha observado que los pacientes con DM1 tienen un mayor riesgo a desarrollar tumores, tanto benignos como malignos. El primer estudio que lo documentó fue en el año 1965, donde reportaban la presencia de un tumor benigno de la piel denominado pilomatricoma (Cantwell and Reed, 1965). En 1971, el profesor Peter Harper encontró la primera asociación entre la presencia de pilomatricomas y DM1 dentro de la misma familia (Harper, 1971). Posteriormente, la

presencia de este tipo tumoral ha sido ampliamente documentada en pacientes con DM1, siendo una de sus afecciones características (Marcoval *et al.*, 2016; Zampetti *et al.*, 2015). De la misma manera, se ha determinado que los pacientes con DM1 tienen un riesgo 1.5 veces superior a desarrollar tumores benignos, siendo los más frecuentes los endocrinos del tiroides, seguidos por los del sistema reproductor femenino y, finalmente, por los de la piel, donde destaca la presencia de pilomatricomas, que es casi exclusivo de pacientes con DM1 (Alsaggaf *et al.*, 2019b; Alsaggaf *et al.*, 2018; Chan *et al.*, 1999; Gadalla *et al.*, 2017).

Tabla I4. Tipos tumorales identificados con mayor riesgo en pacientes con DM1 según cada estudio.

Tipo Tumoral	SIR[a]	Significación estadística	Cohorte
Melanoma uveal	27.54	p<0.001	Rochester
Ojo	12.00	p=0.01	Nórdica
Otros femeninos	9.60	p=0.02	Nórdica
Endometrio	7.60	p=0.00001	Nórdica
Tiroides	7.10	p=0.01	Nórdica
	5.54	p=0.001	Rochester
Cerebro	5.30	p=0.0005	Nórdica
Ovario	5.20	p=0.0006	Nórdica
Páncreas	3.20	p=0.05	Nórdica
Colon	2.90	p=0.004	Nórdica
Tipo Tumoral	**RR[b]**	**Significación estadística**	**Estudio**
Timoma	100	Sí	Francia
Endometrio	21.7	Sí	Francia
Testículo	10.74	0.01	Utah
Ovario	9.3	Sí	Francia
Endometrio	6.98	0.03	Utah
Linfoma No-Hodgkins	4.25	0.03	Utah

En la tabla se enumeran los tipos tumorales detectados en cada estudio con significación estadística.

[a] SIR del inglés, "Standardized Incidence Ratio".

[b] Riesgo relativo.

Recientemente, mediante el estudio de dos grandes cohortes nórdicas que suman 1600 pacientes con DM, se ha observado una incidencia 2 veces superior a desarrollar tumores malignos en pacientes con DM respecto a la población general, siendo los tipos tumorales con mayor riesgo el cáncer ocular, seguido por el de endometrio, de tiroides, de cerebro, de ovario, de páncreas y de colon (Gadalla *et al.*, 2011). También se ha determinado que estos tumores se presentan más frecuentemente en el género femenino (Alsaggaf *et al.*, 2019b; Alsaggaf *et al.*, 2018; Chan *et al.*, 1999; Gadalla *et al.*, 2017). Estudios posteriores en cohortes adicionales han confirmado dicho aspecto en pacientes DM1 **(Tabla I4)**.

El mecanismo por el cual los pacientes con DM1 tienen un mayor riesgo para desarrollar tumores tanto benignos como malignos se desconoce por completo. Se ha propuesto la ruta de β-catenina como posible causa (Chan *et al.*, 1999; Mueller *et al.*, 2009). Recientemente, se han descrito mutaciones en β-catenina y en ATM en tumores de pilomatricomas de pacientes con DM1 (Rübben *et al.,* 2020)

2.3. Fisiología molecular del envejecimiento en la DM1

En 2013 se definieron las características moleculares distintivas responsables del envejecimiento fisiológico, los denominados "*Hallmarks of Aging*". Estas características se consideraron las responsables, en parte, del envejecimiento porque cumplían con tres características: se manifestan durante el envejecimiento fisiológico, promueven un envejecimiento prematuro si se exacerban y, por último, ralentizan el envejecimiento si se bloquean (López-Otín *et al.*, 2013).

Los autores describieron 9 características distintivas responsables del envejecimiento, divididas en 3 grupos: primarias, antagonistas e integrativas. Entre las primarias se incluyen procesos tales como la inestabilidad genómica, el acortamiento telomérico, las alteraciones epigenéticas y la pérdida de la proteostasis. Todos estos procesos son considerados negativos para el organismo y son los iniciadores del proceso de envejecimiento. En cambio, las antagonistas, cuando tienen lugar de manera moderada y en momentos puntuales, son beneficiosos para el organismo, pero si ocurren a niveles exacerbados o se convierten en crónicos, son perjudiciales. En esta categoría encontramos la falta en la regulación de la sensibilidad nutricional, la senescencia celular y la disfunción mitocondrial. La última categoría, o las integrativas, son los procesos que afectan directamente a la homeostasis y a la función del tejido, dando lugar a un fenotipo envejecido. En esta categoría encontramos al agotamiento de las células madre y la alteración en la comunicación intercelular (López-Otín *et al.*, 2013).

Distintos estudios han detectado alteraciones en estos procesos, en mayor o menor medida, en diferentes modelos de DM1 (**Figura I5**).

Entre los procesos primarios se ha observado que las células satélite, la población de célula madre muscular, tienen un mayor ratio de ***acortamiento telomérico*** *in vitro* (Bigot *et al.*, 2009; Thornell *et al.*, 2009), que se asocia con una diferenciación y maduración anormal.

Otro proceso primario alterado en DM1 es la ***proteostasis***, que es fundamental para preservar un proteoma sano en la célula. En DM1 se ha observado una inducción exacerbada de la autofagia, que es un proceso de autodegradación de proteínas y orgánulos dañados, en los mioblastos que no logran diferenciarse (Beffy *et al.*, 2010), en neuronas derivadas de células madre embrionarias (Denis *et al.*, 2013) y en un modelo de *Drosophila melanogaster* contribuyendo a la atrofia muscular (Bargiela *et al.*, 2015). También, se ha hallado una acumulación de chaperonas, proteínas responsables del plegamiento de las proteínas recién sintetizadas, en el músculo esquelético de pacientes con DM1 (Suzuki *et al.*, 1998). Finalmente, se ha documentado una sobreactivación de la ruta ubiquitina-proteosoma, cuya función es la de marcar proteínas para ser degradadas en el proteasoma, tanto en músculo esquelético como cardíaco, en dos modelos animales murinos de DM1 (Huguet *et al.*, 2012; Vignaud *et al.*, 2010).

Por otra parte, y como se ha comentado anteriormente, se ha observado un cambio en el patrón de ***metilación*** en el locus de DMPK, proponiéndose incluso el patrón de metilación como marcador diagnóstico de la enfermedad (Barbé *et al.*, 2017).

Entre los procesos antagonistas alterados en la DM1 encontramos, en primer lugar, la ***senescencia celular***. Éste es un proceso que se caracteriza por una parada proliferativa irreversible, pero en un estado metabólicamente activo. En concreto, se ha descrito que las células satélite de pacientes con DM1 requieren de un menor número de divisiones celulares para entrar en senescencia (Bigot *et al.*, 2009; Thornell *et al.*, 2009).

La ***disfunción mitocondrial*** es otro proceso antagonista alterado en DM1. Se ha visto que la isoforma A de DMPK co-localiza con la membrana mitocondrial externa, alterando su morfología (van Herpen *et al.*, 2005), y que el músculo de pacientes con DM1 posee una acumulación de mitocondrias (Ueda *et al.*, 1999). Por otra parte, también se ha demostrado, tanto en músculo como en sangre, que pacientes con DM1 presentan niveles reducidos de la coenzima Q10 y que estos niveles correlacionan inversamente con la expansión CTG (Siciliano *et al.*, 2001b). Finalmente, también se ha determinado que el ADN mitocondrial de pacientes con DM1 presenta una mayor tasa de deleciones (Sahashi *et al.*, 1992). No obstante, son necesarias más evidencias que caractericen el déficit de la función mitocondrial en pacientes con DM1.

La ***pérdida de la sensibilidad a los nutrientes*** es otro de los procesos antagonistas alterados en pacientes con DM1. Hay trabajos que han demostrado una alteración de este fenómeno en DM1, como, por ejemplo, la presencia de resistencia a la glucosa, hiperinsulinemia y, en algunos casos, el desarrollo de diabetes mellitus, además de alteraciones en el metabolismo del calcio, insuficiencia tiroidea o hipogonadismo (Orngreen *et al.*, 2012). Algunas de estas alteraciones son debidas, en parte, a una hiperfosforilación de CUGBP1/CELF1. Esta fosforilación lleva a un *splicing* anormal del receptor de insulina y resulta en una resistencia a la insulina en estos pacientes (Savkur *et al.*, 2001).

En definitiva, la DM1 presenta múltiples alteraciones clínicas y biológicas que se asocian al envejecimiento fisiológico, lo que ha llevado a visualizar esta enfermedad como un cuadro progeroide o una enfermedad con un envejecimiento prematuro sistémico (Mateos-Aierdi *et al.*, 2015; Meinke *et al.*, 2018).

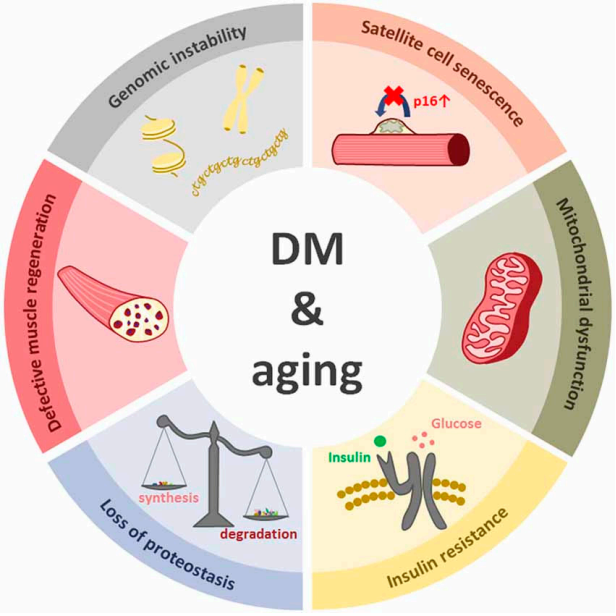

Figura I5. Distintas alteraciones moleculares compartidas entre la DM1 y el envejecimiento celular. Modificado de Mateos-Aierdi *et al.*, 2015

2.4. El calcio durante el envejecimiento

El Ca^{2+} desempeña un papel vital en una amplia gama de procesos celulares tanto en la fibra muscular como en otros tipos celulares, regulando la homeostasis de las membranas celulares, el acoplamiento entre la excitación y la contracción muscular (E-C), la neurotransmisión, la diferenciación, la proliferación o la supervivencia celular (Berridge *et al.*, 2000; Cheng *et al.*, 2015). Además, el Ca^{2+} regula el metabolismo y la biogénesis mitocondrial, así como su motilidad, su distribución y su plasticidad (Celsi *et al.*, 2009; Yi *et al.*, 2004). Por lo tanto, una regulación estricta de los niveles de Ca^{2+} es esencial para la función adecuada del músculo esquelético.

Recientemente, se ha demostrado que la DM1 se asocia con un *splicing* alternativo desregulado del gen CACNA1S que es un sensor de voltaje con un papel central en la E-C (Tang *et al.*, 2012). Además se han observado alteraciones en el *splicing* de otros genes relacionados con la homeostasis del Ca^{2+} como en los canales del cloro tipo 1, el transportador del calcio SERCA que transporta el Ca^{2+} desde el citosol al retículo sarcoplásmico y los receptores de rianodina RyR1 (Kimura *et al.*, 2005; Santoro *et al.*, 2014). Todo ello conduce a un incremento crónico de Ca^{2+} citosólico (Benders *et al.*, 1997; Jacobs *et al.*, 1990; Vihola *et al.*, 2013), que es un escenario común de otras distrofias musculares (Vallejo-Illarramendi *et al.*, 2014).

Todos estos hallazgos sugieren que el efecto combinado de un *splicing* mal regulado de varios genes involucrados en la regulación del Ca^{2+}, junto con la desregulación del acoplamiento de E-C en la DM1, podrían jugar un papel de magnitud desconocida en la degeneración muscular y multisistémica que acontece en la DM1.

2.5. Rasgos moleculares alterados en el envejecimiento y en el cáncer

En 2000 Hanahan y Weinberg describieron los "*Hallmarks of Cancer*" (Hanahan and Weinberg, 2000) entendidos como los principales rasgos moleculares característicos que va adquiriendo una célula durante el desarrollo y la progresión del cáncer. Estos procesos van encaminados a mantener la señalización proliferativa y así evadir la supresión del crecimiento mediante la inmortalidad replicativa, resistir las señales de muerte celular, activar la invasión y el proceso de metástasis, así como de inducir la angiogénesis, lo que permite un suministro incrementado de nutrientes para el crecimiento ilimitado del tejido tumoral. En 2011 se añadieron dos nuevas características, la habilidad para reprogramar su metabolismo energético y la habilidad para evadir al sistema inmune (Hanahan and Weinberg, 2011).

En un primer momento, cáncer y envejecimiento pueden parecer dos procesos contrapuestos. Sin embargo, comparten el mismo origen, la acumulación de daño celular (López-Otín *et al.*, 2013), y los mismos mecanismos biológicos. Si comparamos los procesos implicados en el desarrollo del cáncer y del envejecimiento observamos que hay procesos compartidos, como son la inestabilidad genómica, la alteración en la población de células madre, la senescencia celular, las alteraciones metabólicas o el mantenimiento de los telómeros, pero con diferente intensidad y, en muchos casos con efectos opuestos (Hanahan and Weinberg, 2011; López-Otín *et al.*, 2013). El cáncer sería el resultado de una ganancia aberrante de funcionalidad de estos procesos, mientras que el envejecimiento sería el resultado de una pérdida de funcionalidad de los mismos mecanismos.

2.5.1. Acortamiento telomérico en cáncer y envejecimiento

Los telómeros son unas secuencias de ADN repetitivas TTAGGG que se encuentran al final de cada cromosoma y cuya función es proteger la integridad del ADN en cada proceso de réplica. En este sentido, en cada división celular se produce un acortamiento de dichos telómeros debido a que la maquinaria replicativa del ADN es incapaz de replicar por completo estas secuencias (Blasco, 2005).

Para evitar este fenómeno, la célula posee mecanismos compensatorios como es el enzima telomerasa, encargado de mantener el tamaño de los telómeros (Greider and Blackburn, 1985). Sin embargo, en los tejidos adultos la actividad de este enzima no es suficiente para compensar dicha pérdida telomérica que se produce en cada división celular (Collins and Mitchell, 2002).

Las células cancerosas se caracterizan, entre otras cosas, por una proliferación descontrolada, con lo que en un momento dado, el acortamiento telomérico producido debería detener la proliferación de las células que se están transformando en neoplásicas. Sin embargo, esto no ocurre así, en parte debido a que las células tumorales desarrollan mecanismos para mantener altos niveles de telomerasa y así evitar su acortamiento en cada ronda replicativa (Stewart and Weinberg, 2006).

Numerosos estudios han demostrado una correlación inversa entre la longitud telomérica y la edad, observándose telómeros más cortos en personas de mayor edad (Cawthon *et al.*, 2003; von Zglinicki and Martin-Ruiz, 2005). También se ha documentado una exacerbación del acortamiento telomérico en enfermedades y procesos asociados al envejecimiento como en las alteraciones cardiovasculares, la aterogénesis o en la enfermedad de Alzheimer (Ogami *et al.*, 2004; Panossian *et al.*, 2003). De la misma manera las enfermedades progeroides citadas anteriormente como el síndrome de Hutchinson-Gilford o de Werner presentan un acortamiento telomérico mayor (Chang *et al.*, 2004; Decker *et al.*, 2009; Mason and Bessler, 2004).

La relación entre la longitud de los telómeros con el cáncer y el envejecimiento se ha demostrado generando el modelo de ratón deficiente para el enzima telomerasa. Con el desarrollo de las diferentes generaciones, se produce un mayor acortamiento telomérico, así como un envejecimiento más prematuro de este ratón. Además, este modelo es resistente a la inducción de tumores (Blasco, 2005; Blasco *et al.*, 1997; García-Cao *et al.*, 2006; Lee *et al.*, 1998). Por el contrario, el ratón con sobrexpresión del enzima de la telomerasa es más susceptible al desarrollo de cáncer (Canela *et al.*, 2004; Gónzalez-Suárez *et al.*, 2001).

2.5.2. Senescencia celular en cáncer y envejecimiento

La senescencia celular es un proceso biológico que provoca la incapacidad de las células para proliferar, quedándose retenidas en fase G0 del ciclo celular, aunque éstas siguen siendo activas metabólicamente. La senescencia celular es un mecanismo conservado que juega un papel fundamental en el equilibrio entre el cáncer y el envejecimiento fisiológico, asociándose su presencia a distintos procesos tanto fisiológicos durante el desarrollo embrionario o en la etapa adulta, como en enfermedades asociadas al envejecimiento (**Figura I5**) (Campisi, 2013; Campisi *et al.*, 2011; Gorgoulis *et al.*, 2019; Muñoz-Espín *et al.*, 2013; Storer *et al.*, 2013).

Los principales responsables de desencadenar el proceso de senescencia son el acortamiento telomérico junto con la activación de $p16^{INK4a}$ y p53 en respuesta al daño excesivo en el ADN, siendo el objetivo principal de la senescencia la eliminación de las células dañadas y así mantener la homeostasis tisular (Muñoz-Espín and Serrano, 2014).

Se ha demostrados *in vitro,* pero también *in vivo,* que la senescencia celular juega un papel supresor tumoral ya que está favorecida en procesos precancerosos y tumores benignos y su eliminación o inactivación es necesaria para la transformación neoplásica y para la progresión del tumor maligno (Bartkova *et al.*, 2006; Braig *et al.*, 2005; Chen *et al.*, 2005; Collado *et al.*, 2005; Di Micco *et al.*, 2006; Sarkisian *et al.*, 2007; Xue *et al.*, 2007).

Sin embargo, también se ha observado una acumulación de células senescentes dentro de los tejidos durante el envejecimiento fisiológico, tanto en humanos (Dimri *et al.*, 1995), como en ratones (Wang *et al.*, 2009). Esta acumulación se ha descrito en casi todos los tejidos, pero con una especial acumulación en la piel, el hígado, los pulmones y el bazo (Kurz *et al.*, 2000; Young *et al.*, 2009). Las células senescentes se acumulan debido a su ineficaz eliminación por el sistema inmune y a su propia secreción de factores proinflamatorios, lo que se conoce como el fenotipo secretor asociado a la senescencia (*SASP*, del inglés, *"Senescence-associated secretory phenotype"*) (Freund *et al.*, 2010; López-Otín *et al.*, 2013). Esta acumulación de células

senescentes perjudicial para el organismo se ha observado también en diferentes patologías asociadas a la edad como la fibrosis (Hecker *et al.*, 2014; Krizhanovsky *et al.*, 2008), la diabetes mellitus tipo 2 (Chesnokova *et al.*, 2009; Sone and Kagawa, 2005), la sarcopenia (Cosgrove *et al.*, 2014; Du *et al.*, 2014; Sousa-Victor *et al.*, 2014), la enfermedad de Parkinson y de Alzhéimer (Bhat *et al.*, 2012; Chinta *et al.*, 2013) o el desarrollo de cataratas (Baker *et al.*, 2008). Además, se ha determinado que la eliminación de dichas células senescentes mediante la eliminación genética de su regulador p16INK4a, o a través de la administración de los compuestos denominados senolíticos, retrasa la aparición del tejido dañado, mantiene la homeostasis tisular y del organismo y retrasa el envejecimiento, además de aumentar la longevidad (Baar *et al.*, 2017; Baker *et al.*, 2011; Sousa-Victor *et al.*, 2014).

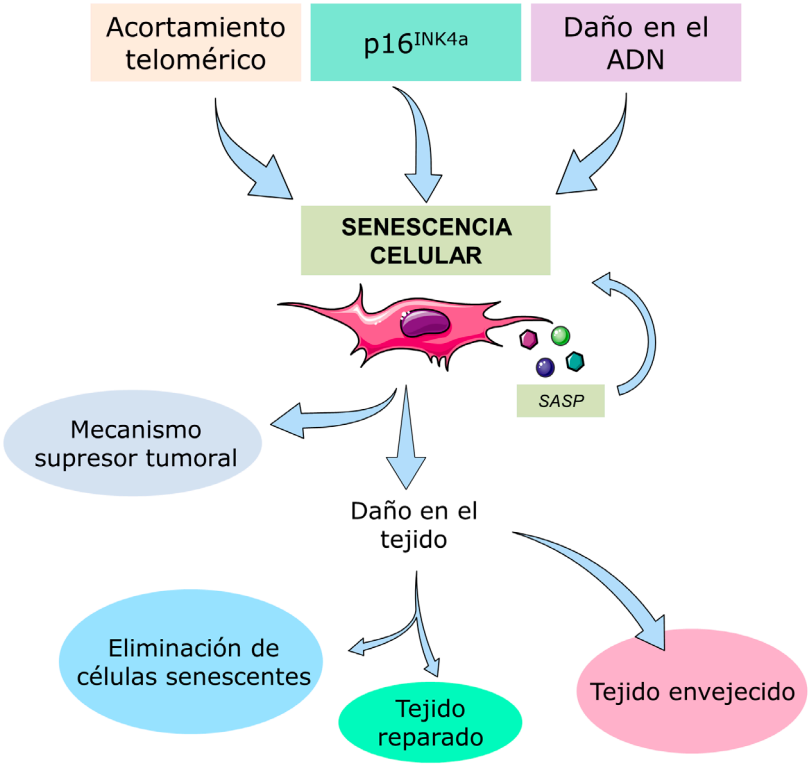

Figura I6. La senescencia celular en los procesos moleculares del cáncer y del envejecimiento. La senescencia celular se puede desencadenar por varios factores, entre los que destacan el acortamiento telomérico, la acumulación de $p16^{INK4a}$ y el daño en el ADN: los denominados factores de Hayflick. En los tejidos adultos, la principal función de la senescencia celular es la de proporcionar protección frente al cáncer. Esta protección se consigue mediante la activación de los mecanismos de reparación y regeneración tisular. En cambio, si este daño no es reparado adecuadamente se van a acumular dichas células senescentes, provocando la disfunción y envejecimiento del tejido. Modificado de Collado *et al.,* 2007.

3. Bases racionales para una terapia anti envejecimiento en la DM1

3.1. Nuevas aproximaciones basadas en compuestos anti envejecimiento

Gran parte de la investigación en torno al envejecimiento se basa en discernir si es posible retrasarlo sin promover las enfermedades asociadas a él (Longo *et al.*, 2015).

Uno de los hallazgos más significativos en esta línea ha demostrado en modelos preclínicos que la restricción calórica es capaz de alargar la supervivencia y retrasar enfermedades o condiciones asociadas al envejecimiento como son la obesidad, la diabetes, la inflamación, la hipertensión o las enfermedades cardiovasculares (Bruens *et al.,* 2020; Fontana *et al.,* 2004; Mercken *et al.,* 2013). Los estudios preliminares en humanos también demuestran que la restricción calórica retrasa la aparición de fenotipos asociados al envejecimiento, aunque la duración y la severidad de este régimen calórico hacen que no sea factible o apetecible para muchas personas (Most *et al.*, 2017).

La rapamicina, la metformina, el resveratrol o los compuestos senolíticos (de Magalhaes *et al.*, 2017; López-Otín *et al.*, 2013), junto con la restricción calórica, son aproximaciones en curso que se están desarrollando como terapia antienvejecimiento. La administración de metformina se ha planteado en un ambicioso ensayo clínico frente al envejecimiento poblacional (Check Hayden, 2015). Desde un punto de vista traslacional y clínico, existe la posibilidad teórica de que uno de estos compuestos antienvejecimiento pueda ser utilizado para el tratamiento de la DM1. De hecho, el resveratrol aumenta el *splicing* del receptor de la insulina en fibroblastos de pacientes de DM1 (Takarada *et al.*, 2015), mientras que hay resultados que avalan la efectividad de los inhibidores mTOR (rapamicina) sobre la función muscular en un modelo de ratón de DM1 (Brockhoff *et al.*, 2017), y también se ha identificado que la metformina revierte los defectos en el *splicing* y los niveles de glucemia en las células de individuos con DM1 (García-López *et al.*, 2011; Laustriat *et al.*, 2015).

3.1.1. Metformina

La metformina es un fármaco que se usa en la actualidad para tratar la diabetes mellitus, y que a su vez también incide sobre varios procesos asociados al envejecimiento (Barzilai *et al.*, 2016). Entre sus efectos, se ha observado que es capaz de regular la inflamación (Saisho, 2015), la autofagia (Song *et al.*, 2015), la senescencia celular (Jadhav *et al.*, 2013), la producción de especies reactivas de oxígeno (Batandier *et al.*, 2006) y de reducir los niveles de daño en el ADN (Algire *et al.*, 2012). Todos

estos procesos están implicados en el envejecimiento fisiológico y también en el desarrollo de tumores.

Recientemente se ha analizado el efecto que tiene la metformina en el aumento de la longevidad en diversos modelos animales. Se ha demostrado que tanto en *Caenorhabditis elegans* (Onken and Driscoll, 2010) como en modelos murinos (Anisimov *et al.*, 2008; De Haes *et al.*, 2014), la metformina incrementa la longevidad, siendo este incremento mayor si la administración comienza a edades tempranas. Además, este†† efecto anti-envejecimiento no se restringe a individuos sanos, observándose que la administración de metformina a un modelo de ratón de enfermedad de Huntington incrementa su esperanza de vida un 20% (Ma *et al.*, 2007), mejorando también su capacidad cognitiva y neuromuscular (Allard *et al.*, 2016). En humanos se ha descrito que puede reducir el riesgo de enfermedades cardiovasculares (Kooy *et al.*, 2009), prevenir la demencia (Cheng *et al.*, 2014) y contribuir a una disminución de la mortalidad (Bannister *et al.*, 2014).

Por otro lado, se ha determinado que los pacientes diabéticos desarrollan cáncer más frecuentemente que la población no diabética (Giovannucci *et al.*, 2010), pero en aquellos pacientes diabéticos tratados con metformina su incidencia se reduce hasta en un 30% (Evans *et al.*, 2005; Gandini *et al.*, 2014), lo que no sucede en pacientes diabéticos tratados con otros medicamentos anti-diabéticos (Bowker *et al.*, 2006).

En la DM1 se ha visto que, *in vitro*, la metformina es capaz de rescatar algunos procesos de *splicing* alternativo (Laustriat *et al.*, 2015), pero sin grandes beneficios cuando se traslada al modelo murino HSA^{LR} (Brockhoff *et al.*, 2017). Sin embargo, recientemente se ha llevado a cabo un ensayo clínico, donde se ha visto que los pacientes con DM1 tratados con metformina mejoran en el test de la marcha (Bassez *et al.*, 2018). Por otra parte, se ha demostrado que los pacientes con DM1 y diabéticos que toman metformina tienen un menor riesgo a desarrollar cáncer (Alsaggaf *et al.*, 2019a).

No obstante, son necesarios nuevos estudios para entender el mecanismo de acción de la metformina en pacientes con DM1 y dilucidar si estos pacientes se podrían beneficiar del tratamiento con este fármaco.

3.1.2. Compuestos senolíticos

Como hemos visto anteriormente, las células senescentes se acumulan en los tejidos tanto durante el envejecimiento fisiológico como durante el desarrollo de enfermedades crónicas. Se ha demostrado que la eliminación de las células senescentes alarga la supervivencia y retrasa el envejecimiento (Baker *et al.*, 2011). Esto ha hecho que, recientemente, se estén descubriendo y caracterizando compuestos capaces de eliminar las células senescentes en los tejidos, los denominados compuestos senolíticos (Zhu *et al.*, 2015) **(Figura I7)**.

Los primeros compuestos senolíticos que se descubrieron fueron el dasatinib, un inhibidor de tirosina quinasas (Zhu *et al.*, 2015) y la quercetina, con numerosas dianas entre las que se encuentra la modulación del estrés oxidativo y la inflamación (Costa *et al.*, 2016). Ambos son capaces de inducir apoptosis selectivamente en las células senescentes (Zhu *et al.*, 2015). Posteriormente, se observó que otro compuesto, el navitoclax, un inhibidor de las proteínas anti-apoptóticas Bcl-2, Bcl-XL y Bcl-W (Chang *et al.*, 2016), también tenía acción senolítica (Chang *et al.*, 2016; Zhu *et al.*, 2016). Actualmente, hay aproximadamente 20 compuestos candidatos que se están testando como potenciales compuestos senolíticos actuando a diferentes niveles (Childs *et al.*, 2017).

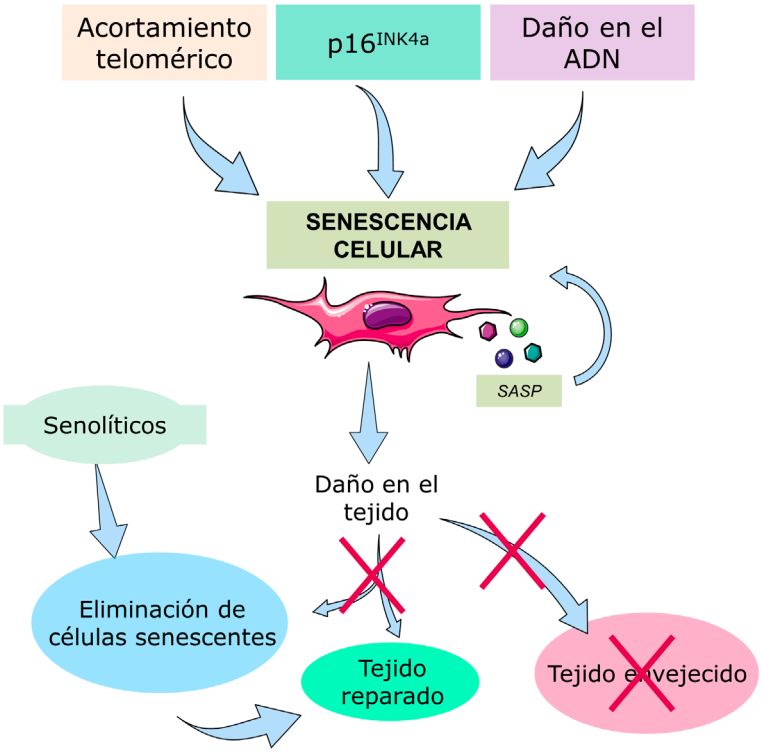

Figura I7. Durante el envejecimiento se produce una acumulación de células senescentes en los tejidos que van a provocar su envejecimiento, debido a que el tejido ha perdido los mecanismos de reparación tisular. Los compuestos senolíticos son compuestos que promueven la eliminación de dichas células senescentes mediante apoptosis, evitando su acumulación en los tejidos y, con ello, evitando la disfunción de los mismos. Modificado de Collado *et al.,* 2007.

Una fortaleza de estos compuestos es que parecen ser selectivos para células senescentes, siendo capaces de mitigar los rasgos asociados al envejecimiento en modelos animales (Chang *et al.*, 2016). Tanto la quercetina como el dasatinib incrementan la supervivencia y reducen la mortalidad en ratones envejecidos, incluso en ratones

jóvenes a los que se les trasplantan células senescentes (Xu *et al.*, 2018). Por otra parte, en humanos, se ha demostrado que la administración de ambos compuestos durante tan solo 11 días, reduce el número de células senescentes en tejido adiposo y en la epidermis (Hickson *et al.*, 2019; Justice *et al.*, 2019).

Actualmente se están realizando nuevos ensayos clínicos para evaluar el impacto de los compuestos senolíticos en enfermedades asociadas al envejecimiento fisiológico, pero se desconoce por completo la función que tienen estos compuestos en la DM1.

3.2. Aproximaciones orientadas a mejorar la homeostasis del calcio

Son numerosas las aproximaciones terapéuticas en curso basadas en corregir una deficiente homeostasis del Ca^{2+}, un hecho que se da tanto en el envejecimiento fisiológico como en las enfermedades neuromusculares (Vallejo-Illarramendi *et al.*, 2014; Weisleder and Ma, 2008). Diferentes estudios han demostrado efectos beneficiosos de estabilizadores de RyR-calstabina, tanto en modelos murinos de distrofia de Duchenne como de distrofia de cinturas tipo 2E. Los complejos RyR y calstabina-2 componen un importante canal de calcio que regula los niveles de calcio intracitoplasmático. El tratamiento con el estabilizador de dicha interacción, S107, resultó en una reducción de la nitrosilación del receptor 1 de RyR, una reversión del agotamiento de calstabina-1 y una mejora general de la función muscular y del rendimiento físico (Andersson *et al.*, 2012; Bellinger *et al.*, 2009; Mackrill, 2010). Por otro lado, se han desarrollado otras estrategias terapéuticas para normalizar la homeostasis del Ca^{2+} basadas en conseguir una sobrexpresión de SERCA1 o SERCA2 mediante el tratamiento con BGP-15 o la transferencia de genes mediada por virus adenoasociados (Goonasekera *et al.*, 2011; Morine *et al.*, 2010), como forma de recuperar la homeostasis del calcio.

En la DM1 se han observado alteraciones en el *splicing* en el canal de Ca^{2+} CACNA1S, el receptor de RyR y la bomba SERCA, que contribuyen a la elevación característica del Ca^{2+} basal intracelular en células musculares, la cual tiene un importante impacto a su vez en el desarrollo de la enfermedad (Vallejo-Illarramendi *et al.*, 2014). Por otra parte, existen evidencias de que agentes como la rapamicina, un inhibidor de mTOR, modula el envejecimiento del organismo retrasándolo en diferentes especies como gusano, mosca y ratón (Blagosklonny, 2017; Ehninger *et al.*, 2014). En DM1 hay resultados que avalan la efectividad sobre la función muscular de los inhibidores mTOR en un modelo de ratón de DM1 (Brockhoff *et al.*, 2017).

Por lo tanto, la estrategia farmacológica orientada tanto a corregir un defecto en la homeostasis del Ca^{2+} como aquellas orientadas a inhibir a mTOR podrían ser un nuevo enfoque terapéutico para en la DM1.

Calcio y mTOR: los compuestos ahulkenoides

Los compuestos ahulkenoides **(Figure I8)** son una nueva serie de nuevas entidades químicas diseñadas y caracterizadas como estabilizadores de la unión entre RyR y calstabina-2 y por lo tanto con actividad normalizadora de los niveles de calcio.

La reducción en la afinidad de la unión de RyR por calstabina hace que el canal no se cierre por completo y se incremente la concentración intracelular de calcio (Bellinger *et al.*, 2008a; Guerrero-Hernández *et al.*, 2014). Se ha determinado que este fenómeno ocurre en varias condiciones como durante el ejercicio crónico, el envejecimiento y las distrofias musculares (Andersson *et al.*, 2011; Bellinger *et al.*, 2009; Bellinger *et al.*, 2008b; Liu *et al.*, 2012; Reiken *et al.*, 2003). En este contexto, el AHK6 ha sido testado en un modelo murino de Duchenne (*mdx*) demostrando una mejora de la fuerza de agarre, la histopatología del músculo EDL y el diafragma (Aldanondo, 2017).

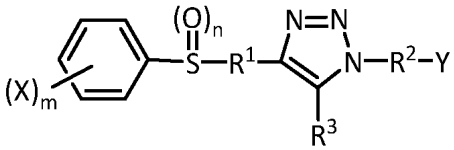

Figura I8. Estructura química general de los ahulkenoides. Los diferentes miembros de la familia de los ahulkenoides han sido generados mediante sustituciones en los radicales R^1, R^2, R^3, X e Y.

En base a todos los resultados experimentales obtenidos con los ahulkenoides tanto a nivel muscular como de SNC (datos no publicados), estos compuestos podrían tener un efecto anti-envejecimiento a través de dos mecanismos no excluyentes y complementarios. Por un lado, por su efecto canónico sobre la nitrosilación de los receptores RyR y la estabilización del complejo RyR-calstabina, evitando así la salida del calcio al citoplasma y, por otro lado, por la modulación de la unión de la calstabina a mTOR sobre el que ejercería una modulación en función del nivel de actividad previo y tendría un efecto inhibidor de esta vía.

Hipótesis

Los pacientes con DM1 presentan un envejecimiento acelerado a nivel biológico y celular lo que se traslada a la presencia de síntomas, síndromes y patologías asociadas al envejecimiento a nivel clínico.

Las terapias con compuestos con potencial efecto antienvejecimiento supondrán un beneficio terapéutico sistémico para los pacientes con DM1.

Objetivos

1. Identificar los procesos moleculares implicados en el envejecimiento acelerado en la cohorte de Guipúzcoa de pacientes con DM1 de Guipúzcoa.
   - **1.1.** Determinar el riesgo a desarrollar cáncer y su patrón molecular.
   - **1.2.** Estudiar la longitud telómerica.
   - **1.3.** Caracterizar las rutas de proliferación, división celular y senescencia.
   - **1.4.** Caracterizar el metabolismo celular.

2. Estudiar el impacto de nuevas estrategias terapéuticas frente a la DM1.
   - **2.1.** Analizar el efecto de los compuestos senolíticos en la DM1.
   - **2.2.** Analizar el efecto de la metformina en la DM1.
   - **2.3.** Analizar el efecto de los compuestos ahulkenoides en la DM1.

Materiales y métodos

1. Muestras y líneas celulares humanas

1.1. Consideraciones éticas

El presente estudio ha sido aprobado por el Comité de Ética en Investigación Clínica del Área Sanitaria de Guipúzcoa **(Anexo 1)** y se adecúa a la Ley 14/2007 de Investigación Biomédica y a los principios éticos de la declaración de Helsinki. Todos los participantes han firmado un consentimiento informado **(Anexo 2)** y han recibido una hoja de información al paciente **(Anexo 3).**

En el presente estudio se han utilizado muestras humanas derivadas de células mononucleares de sangre periférica, fibroblastos primarios y mioblastos primarios **(Tablas M1 y Anexo 5).** Todas las muestras usadas han sido utilizadas tras su depósito en el Biobanco Vasco de Investigación.

1.2. Muestras de sangre y aislamiento de PBMC

El aislamiento de células mononucleares de sangre periférica (PBMC) de pacientes con DM1 y de sujetos sanos se realizó mediante un procedimiento estándar usando un gradiente de *Ficoll*. La extracción de sangre (4 mL totales de sangre) se realizó por venopunción en el Servicio de Neurología del Hospital Universitario Donostia. La sangre se recogió en tubos *Vacutainer EDTA K3* y se mantuvo a temperatura ambiente hasta su procesamiento (menos de 30 min tras la extracción). Posteriormente, se diluyó en proporción 1:1 en tampón fosfato salino (PBS). Después, se añadió en un tubo cónico con 15 mL de Ficoll, sobre el que se incorporó muy suavemente la mezcla de sangre y PBS y, a continuación, se centrifugó a 400 g durante 30 min a 20ºC sin el mecanismo de frenado. Una vez centrifugadas las muestras, se aisló cuidadosamente la interfase que contenía la fracción de células mononucleares y se lavó dos veces con PBS centrifugándolas a 100 g durante 10 min a 20ºC hasta obtener un *pellet* celular. Las muestras fueron mantenidas a –80ºC hasta su uso y procesadas acorde a su uso final según la realización de la extracción de proteínas o de ARNm.

En la **Tabla M1** se muestra un resumen de las muestras derivadas de PBMC utilizadas y en el Anexo 4 se encuentra el listado completo.

Tabla M1. Resumen de muestras humanas usadas en el presente estudio.

Muestras de ARNm				Muestras de proteína			
Hombres	**Número**	**Edad**	**CTGn**	**Hombres**	**Número**	**Edad**	**CTGn**
Controles	12	43	–	Controles	2	35	–
DM1	27	43	756	DM1	3	46	667
Mujeres	**Número**	**Edad**	**CTGn**	**Mujeres**	**Número**	**Edad**	**CTGn**
Controles	10	47	–	Controles	5	44	–
DM1	28	43	968	DM1	2	41	500

1.3. Aislamiento y cultivo de fibroblastos primarios

Para el aislamiento de fibroblastos humanos **(Tabla M2)** se utilizó la técnica de explante. Se obtuvieron biopsias de piel por punción en el Servicio de Neurología del Hospital Donostia. Posteriormente, se trocearon en fragmentos de 1 mm. Los explantes se lavaron repetidamente en PBS y se depositaron en un frasco de cultivo de 25 cm^2, previamente humedecido con medio de cultivo DMEM (11594486, Gibco, del inglés *"Dulbecco's Modified Eagle Medium"*) suplementado con 10% de suero fetal bovino (FBS, 10270106, Gibco), 2 mM de L-glutamina (25030024, Gibco), 100 U/mL de penicilina y 100 µg/mL de estreptomicina (15140122, Gibco), lo que denominamos medio completo. A continuación, se procedió a incubar el frasco durante 4 h en posición vertical a 37ºC y un 5% de CO_2 en atmósfera húmeda en un incubador del tipo Steri-Cycle (Thermo Scientific). Una vez transcurrido dicho tiempo se procedió al cultivo en posición horizontal. En una semana, aproximadamente, se visualizó la migración de células adherentes sobre la superficie de cultivo. Cuando las células tapizaban la totalidad de la superficie, se consideró que ya estaba formada la monocapa de células y el cultivo se encontraba confluente, procediéndose al subcultivo de estas células en frascos de cultivo de 75 cm^2 con 10 mL de medio completo y empleando tripsina-EDTA al 0.05% (25300054, Gibco) durante 5 min a 37ºC para despegar las células 1 vez cada 7-10 días.

Tabla M2. Listado de líneas de fibroblastos usados en el presente estudio.

Línea Celular	Estatus	Género	Edad (biopsia piel)	MIRSa	CTG (n)[b]	Edad (al diagnóstico)
C1	Control	Hombre	49			
C2	Control	Mujer	48			
C3	Control	Hombre	27			
C4	Control	Hombre	29			
C5	Control	Hombre	46			
DM1-F1	DM1	Hombre	71	3	167	53
DM1-F2[c]	DM1	Hombre	50	5	233	20
DM1-F3	DM1	Hombre	56	3	333	33
DM1-F4	DM1	Mujer	45	2	333	41
DM1-F5	DM1	Mujer	44	2	833	21
DM1-F6	DM1	Hombre	56	3	1333	20
DM1-F7	DM1	Mujer	34	4	1650	12

[a] La debilidad muscular se ha evaluado mediante el uso de la escala MIRS. (0-sin afectación muscular, 5-debilidad proximal grave).

[b] La determinación del tamaño de la expansión CTG se ha realizado en sangre al diagnóstico de DM1.

[c] Las líneas celulares DM1-F2 (fibroblastos) y DM1-M2 (mioblastos, provienen del mismo paciente).

1.4. Cultivo de mioblastos primarios

Se obtuvieron biopsias musculares en el Servicio de Neurología del Hospital Donostia y se procedió al aislamiento de mioblastos primarios que fueron purificados usando el marcador de superficie CD56 **(Tabla M3)**.

Los mioblastos fueron cultivados en frascos de cultivo de 75 cm^2 previamente tratados con 0.5% de gelatina y con 7 mL de su medio de cultivo compuesto por un 65% de DMEM y un 21% de M-199 (BE12-117F, Lonza), suplementado con un 10% de FBS, un 1% de insulina, un 1% de glutamina, 100 U/mL de penicilina, 100 µg/mL de estreptomicina, 10 µg/mL de EGF y 25 µg/mL de FGF. El subcultivo de los mioblastos se realizó cada 7-10 días empleando tripsina al 0.25% (Gibco) durante 5 min a 37ºC para desprender los mioblastos de la superficie de cultivo.

Tabla M3. Listado de líneas de mioblastos usados en el presente estudio.

Línea Celular	Estatus	Género	Edad (biopsia piel)	MIRSa	CTG (n)[b]	Edad (al diagnóstico)
C-M1	Control	Hombre	26			
C-M2	Control	Mujer	36			
DM1-M1	DM1	Mujer	35	3	1400	15
DM1-M2[c]	DM1	Hombre	46	5	233	20

[a] La debilidad muscular se ha evaluado mediante el uso de la escala MIRS (0-sin afectación muscular, 5-debilidad proximal grave).
[b] La determinación del tamaño de la expansión CTG se ha realizado en sangre al diagnóstico de DM1.
[c] Las líneas celulares DM1-F2 (fibroblastos) y DM1-M2 (mioblastos, provienen del mismo paciente).

Compuestos utilizados

Los tratamientos fueron dispuestos 14-16 h tras la siembra de cada experimento y a la concentración y la duración mostrada en la **Tabla M4**.

Tabla M4. Listado de compuestos usados en el presente estudio.

Compuesto	Disolvente	Dosis	Función	Referencia
Metformina	Agua	1 µM 72 h	Fármaco anti-diabético a través de AMPK	PHR1084 Sigma-Aldrich
Ahulken-6 (AHK6)	Agua	0.1 µM 72 h	Estabilizador del canal de calcio rianodina-calstabina	Síntesis propia
Ahulken-7 (AHK7)	Agua	0.1 µM 72 h	Estabilizador del canal de calcio rianodina-calstabina	Síntesis propia
Doxorrubicina (DOX)	Agua	1 µM 16-18 h	Agente que induce daño en el ADN	44583 Sigma-Aldrich
Quercetina	DMSO	5, 10 y 15 µM 72 h	Inhibidor de estrés oxidativo e inflamación	Q4951 Sigma-Aldrich
Navitoclax (ABT-263)	DMSO	0.1, 1 y 10 µM 72 h	Inhibidor de BCL-XL, BCL2 y BCL-W	S1001 Selleckchem
Dasatinib	DMSO	0.1 y 0.5 nM 72 h	Inhibidor de Src/Bcr-Abl	SML2589 Sigma-Aldrich

2. Modelos animales

2.1. Modelo en mosca

En este estudio, mediante una colaboración con el Prof. Rubén Artero (Universidad de Valencia), hemos podido acceder a un modelo de DM1 en *Drosophila melanogaster*. En concreto, hemos usado la cepa MHC-Gal4 UAS-CTG480 1.1 (García-López *et al.*, 2008), que expresa 480 repeticiones CTG interrumpidas cada 20 repeticiones CTG por la secuencia *"CTCGA"*. Este modelo se encuentra bajo el promotor de la cadena pesada de la miosina (MHC), dirigiendo la expresión de las 480 repeticiones a la musculatura.

Las moscas se mantuvieron a 23ºC con un 70% de humedad y con ciclos de 12 h de luz y oscuridad. Todos los cruces fueron llevados a cabo en estas condiciones y las moscas fueron alimentadas con comida estándar para *Drosophila* suplementada con 5 mM de quercetina o con 50 nM de dasatinib.

Para el *ensayo de locomoción* se emplearon moscas de 5, 10, 15, 20, 25 y 30 días de vida. En concreto, se emplearon 5 moscas por cada grupo experimental y entre 7 y 9 grupos experimentales para cada edad, repitiéndose la prueba entre 2 y 3 veces. Para ello, se definió una distancia de 8 cm en vertical y se contabilizó el número de moscas capaces de recorrer dicha distancia durante 10 s de observación.

Para el *análisis de longevidad*, se partió de 50 moscas hembra cuya supervivencia fue testada cada dos días. Los resultados fueron representados usando el estimador de Kaplan-Meier y se analizó su significación estadística empleando el test de Log-rank con la corrección de Bonferroni para múltiples comparaciones.

Para el *ensayo de expresión de ARNm* se diseccionó el tórax de 12 moscas representativas de cada condición y se procedió a la extracción del ARNm mediante el reactivo *TRI Reagent Solution* (AM-9738, Life Technologies), previa disgregación del tejido mediante el procesador de tejidos TissueLyser (85210, Qiagen).

2.2. Modelo en ratón

Por otro lado, mediante una colaboración con la Dra. Geneviève Gourdon (INSERM, París) hemos accedido al modelo de ratón DMSXL de DM1. Este modelo está compuesto por una secuencia genómica de más de 45 kB compuesta por los genes DMPK, DMWD y SIX5, además de poseer más de 1.000 repeticiones CTG de manera multisistémica (Huguet *et al.*, 2012). Dicho modelo DMSXL deriva de un modelo previo (DM300) (Seznec *et al.,* 2000) en el que, tras sucesivas generaciones, de manera natural se produjo una fuerte expansión en el tamaño del triplete dando como resultado al modelo DMSXL.

La secuencia genética del modelo DM300 deriva de una familia que presenta grandes cambios intergeneracionales en la herencia del tamaño de la expansión. En dicha familia, el padre se encontraba ligeramente afecto y poseía entre 50-70 repeticiones, la hija heredó 500 repeticiones y dos nietas, hijas de la madre afecta, desarrollaron una forma congénita de la enfermedad con más de 2.000 repeticiones (Seznec *et al.,* 2000).

Para el *ensayo de expresión de ARNm* se obtuvieron muestras del músculo tibial anterior de ratones de dos meses de edad y se procedió a la extracción del ARNm mediante el reactivo *TRI Reagent Solution*, previa disgregación del tejido mediante el procesador de tejidos TissueLyser.

3. Ensayos funcionales en cultivos celulares

3.1. Ensayo de proliferación

Se sembraron por duplicado $3x10^4$ fibroblastos en placas de 9.5 cm^2 (placas de 6 pocillos) en un volumen final de 2 mL. Las células fueron despegadas con tripsina y contadas mediante una cámara de Neubauer (717805, Brand) a día 1, 3 y 5 tras la siembra del experimento.

3.2. Ensayo 3T3 de inducción de senescencia

Los fibroblastos fueron cultivados siguiendo el protocolo 3T3 para evaluar su entrada en senescencia por agotamiento replicativo. Los fibroblastos fueron sub-cultivados de manera sucesiva cada 3 días a una densidad celular de $3x10^4$ células en placas de 9.5 cm^2 (placas de 6 pocillos) en un volumen final de 2 mL. Este procedimiento se mantuvo hasta el agotamiento del cultivo o hasta superar un 60% de células positivas para la actividad β-galactosidasa a pH subóptimo. Para la cuantificación del resultado, se procedió al cálculo del número total de veces que los fibroblastos habían doblado su número o *PDL* (del inglés, "*Population Doublings*") mediante la siguiente fórmula: PDL= Log10 (nº de fibroblastos a día 3 / nº de fibroblastos a día 0) / Log10 (2).

3.3. Ensayo de actividad β-galactosidasa

Para la medida de la senescencia celular se utilizó un kit comercial de actividad β-galactosidasa (9860S, Cell Signaling). Este kit detecta la actividad β-galactosidasa de las células a pH 6, es decir, a pH subóptimo, una característica distintiva de las células senescentes. En el ensayo, el X-Gal es metabolizado en las células senescentes produciéndose un producto de color azul e insoluble (5,5'-dibromo-4,4'-dicloro-*índigo*) que es visible en las células mediante microscopía óptica. Se sembraron $0.5x10^4$ fibroblastos en pocillos de cámaras de inmunofluorescencia de 0.7 cm^2 (Nunc Lab-Tek II Chamber Slide, Thermo-Scientific) en 0.5 mL de su medio de cultivo y tras 48 h se retiró el medio de cultivo, se hicieron dos lavados con una solución de PBS y se procedió a la fijación y tinción de acuerdo a las instrucciones del fabricante. Se incubaron con la solución de tinción durante 15-18 h a 37ºC en un incubador sin CO_2. Posteriormente, las células fueron observadas en un microscopio óptico invertido, capturándose fotografías de manera aleatoria para obtener el número de células positivas o senescentes por cada condición experimental. En el caso de ensayos con compuestos, el compuesto se aplicó durante 72 h a las dosis indicadas, 24 h tras la siembra.

3.4. Ensayo de viabilidad celular MTT

Con el objetivo de evaluar el efecto de la metformina y de los compuestos ahulken sobre los fibroblastos humanos, se realizó el ensayo colorimétrico de viabilidad celular basado en el metabolismo del bromuro 3-(4,5 dimetil 2-tiazolil)-2,5-difeniltetrazolio (MTT). Este ensayo se basa en la medición del color púrpura del formazán producido por la reducción del reactivo MTT por parte del enzima mitocondrial succinato deshidrogenasa de las células viables.

Para ello, se sembraron $0.1x10^4$ fibroblastos por pocillo en placas de 0.32 cm^2 (96 pocillos) por sextuplicado y tras 24 h fueron tratados con los diferentes compuestos a las concentraciones indicadas en cada experimento. Como control se usó el disolvente de cada compuesto. Una vez transcurridas 72 h, se añadió el reactivo MTT (M2128, Sigma-Aldrich) a una concentración final de 0.25 mg/mL en cada pocillo y se incubó durante 2.5 h a 37ºC y con un 5% de CO_2. A continuación, se aspiró el sobrenadante de cada pocillo y se añadieron 150 µL de solución de DMSO (D1650, Sigma-Aldrich) para disolver los cristales de formazán. Posteriormente se midió la absorbancia a 570 nm en un lector de placas Halo LED 96 (Dynamica). Se asignó el 100% de viabilidad al valor medio de la absorbancia de los pocillos control (células tratadas con vehículo) y, respecto a ese valor, se calcularon los porcentajes de viabilidad de las células sometidas a las diferentes concentraciones de cada compuesto.

3.5. Medida de la producción de peróxido de hidrógeno

Para la medida de la producción de peróxido de hidrógeno (H_2O_2) se utilizó el kit comercial basado en bioluminiscencia *ROS-Glo™ H_2O_2 Assay* (G8820, Promega). Para ello se sembraron $0.1x10^4$ células en 100 µL en pocillos de 0.32 cm^2 (placas de 96 pocillos) por duplicado en su medio habitual. A las 24 h tras la siembra, 50 µL del sobrenadante de cada muestra en estudio fue incubado con un sustrato que se caracteriza por reaccionar directamente con el H_2O_2, generando un precursor de la luciferina. Después, se añadió una solución de detección que convierte dicho precursor en luciferina y contiene luciferasa, por lo que se produce una señal luminiscente, que es proporcional al nivel de H_2O_2 presente en la muestra. Dicha señal fue medida en un luminómetro *PHERAstar* (BMG LABTECH) usando placas de 96 pocillos opacas y blancas. La actividad luciferasa fue normalizada frente a la del control sano y expresada como actividad relativa. En el caso de ensayos con tratamientos, el compuesto se administró durante 72 h a la dosis indicada **(Tabla M4)**, 24 h tras la siembra.

3.6. Determinación del potencial de membrana mitocondrial, cantidad de mitocondrias y cantidad de ros producido por mitocondrias

Para las tres determinaciones se sembraron $20x10^4$ fibroblastos en placas de 60 cm^2 (placas tipo p100) y al día siguiente fueron procesadas para el análisis de los tres parámetros mencionados de manera independiente mediante citometría de flujo. El procesamiento consistió en tripsinizarlas y centrifugarlas a 2.000 g durante 5 min a 20ºC. Las células se incubaron utilizando las sondas especificadas en la **Tabla M5**, y tras el tiempo indicado de incubación, se lavaron con una solución de PBS, con la salvedad de que para la tinción con *MitoSOX* se usó durante todo el procesamiento una solución de HBSS en lugar de PBS. La suspensión celular fue analizada usando un citómetro de flujo *Guava Easy Cyte 8HT* (Millipore). Se determinó la intensidad media de fluorescencia de los tres parámetros analizados independientemente: *MitoSOX*, *MitoTracker Red* y *Rhodamine 123*. Posteriormente, los valores de *MitoSOX* fueron normalizados con los valores de *MitoTracker* para determinar la producción de especies reactivas de oxígeno por cada mitocondria individual.

El reactivo *Rhodamine 123* es una sonda que atraviesa la membrana mitocondrial y se acumula dentro de ella, pero cuando la mitocondria experimenta una pérdida en su potencial de membrana la sonda deja de acumularse y se pierde la tinción. El reactivo *MitoTracker Red FM* es una sonda que atraviesa pasivamente la membrana mitocondrial y se acumula dentro de las mitocondrias sanas, mientras que el reactivo *MitoSOX Red* es una sonda que también penetra la membrana mitocondrial pero que se oxida rápidamente por los superóxidos.

Tabla M5. Listado de sondas usadas para citometría de flujo.

Sonda	Función	Uso	Excitación	Emisión	Referencia
Rhodamine 123	Determinación del potencial de membrana mitocondrial	1 µM 15 min	511 nm	534 nm	R8004 Sigma-Aldrich
MitoTracker Red FM	Determinación de la cantidad de mitocondrias	0.2 µM 30 min	581 nm	644 nm	M22425, Invitrogen
MitoSOX Red	Determinación de especies reactivas de oxígeno, superóxidos específicos de mitocondria	5 µM 30 min	510 nm	580 nm	M36008, Invitrogen

3.7. Estudio del metabolismo mitocondrial

Para el estudio del metabolismo mitocondrial **(Figura M1)** se usó el analizador *XF96 Extracellular Flux Analyzer* (Seahorse Bioscience). Dicho instrumento se caracteriza por la medición simultánea de la tasa de consumo de oxígeno y de la acidificación del medio extracelular (OCR y ECAR, respectivamente). Para ello se usó el kit *XF Cell Mito Stress* (103015, Seahorse Bioscience), que nos proporciona la información de los siguientes parámetros mitocondriales:

- **Respiración Basal:** Es una aproximación del consumo de oxígeno utilizado para satisfacer la demanda celular de ATP resultante de la fuga de protones mitocondriales. Muestra la demanda energética de la célula en condiciones basales.
- **Producción de ATP:** Se mide como la disminución en el consumo de oxígeno tras la inyección del inhibidor de la ATP sintasa, la oligomicina. Representa la proporción de la respiración basal que se estaba utilizando para impulsar la producción de ATP. En otras palabras, muestra la cantidad de ATP producida por las mitocondrias que contribuye a satisfacer las necesidades energéticas de la célula.
- **Fuga de protones:** Se mide como la respiración basal no acoplada a la producción de ATP.
- **Respiración Máxima:** Se mide como el consumo de oxígeno tras la inyección del desacoplante FCCP. Simula una demanda energética exacerbada para que la mitocondria produzca la máxima cantidad de energía posible. En otras palabras, es la tasa máxima de respiración que puede producir una célula.
- **Capacidad Respiratoria Disponible:** Se define como la capacidad de una célula para responder a una demanda energética.
- **Respiración No Mitocondrial:** Se mide como el consumo de oxígeno producido tras la adición de los 4 inhibidores mitocondriales, es decir, el consumo de oxígeno que se produce por vías no mitocondriales.

Para ello se sembraron $1x10^4$ fibroblastos en placas de *SeaHorse XF96* pocillos ($11.40\ mm^2$), es su medio de cultivo, previamente tratadas con colágeno (354236, BD). Al día siguiente se reemplazó el medio de cultivo por medio de ensayo *Mito Stress* ajustado a pH 7.4 **(Tabla M6)**. Se hicieron tres mediciones basales tras las cuales el sistema inyectó tres inhibidores mitocondriales de manera secuencial: primero oligomicina (un inhibidor de la ATP sintasa), en segundo lugar FCCP (un desacoplante que colapsa el gradiente de protones) y, finalmente, un mix de rotenona y antimicina (inhibidores de los complejos I y III, respectivamente) **(Tabla M7 y Figura M1)**. Se hicieron tres mediciones después de la inyección de cada compuesto, determinándose en cada inyección el OCR y el ECAR.

Tabla M6. Composición del medio de cultivo para el ensayo *Mito Stress.*

Compuesto	Concentración final	Referencia
DMEM (en polvo)	0.83 g / 100 mL	D5030, Sigma-Aldrich
Glucosa	10 mM	G8769, Sigma-Aldrich
Piruvato	1 mM	11360070, Gibco
L- Glutamina	2 mM	25030024, Gibco

En el caso de experimentos donde las células fueron pre-tratadas con diferentes compuestos, se sembraron 0.5x104 fibroblastos por pocillo, tras 14-18 h se cambió el medio por medio con tratamiento y se procedió a la incubación durante 72 h adicionales, tras las cuales se analizó el metabolismo mitocondrial.

Tras las mediciones, se procedió a la normalización de los resultados en función del número de células en cada pocillo. Para ello, éstas fueron teñidas con una solución compuesta por 0.5 % de cristal violeta (HT90132, Sigma) y 4% de formaldehído (818708, Merck Millipore) durante 20 min. Posteriormente se retiró la tinción y se lavaron los pocillos. Una vez seca la placa, se procedió a disolver el cristal de violeta con una solución del 5% de ácido acético glacial (100063, Merck Millipore) y se midió la absorbancia a 570 nm en un lector de placas Halo LED 96 (Dynamica). Los valores de OCR y ECAR fueron normalizados con los valores de absorbancia obtenidos, expresándose en unidades arbitrarias (a.u.).

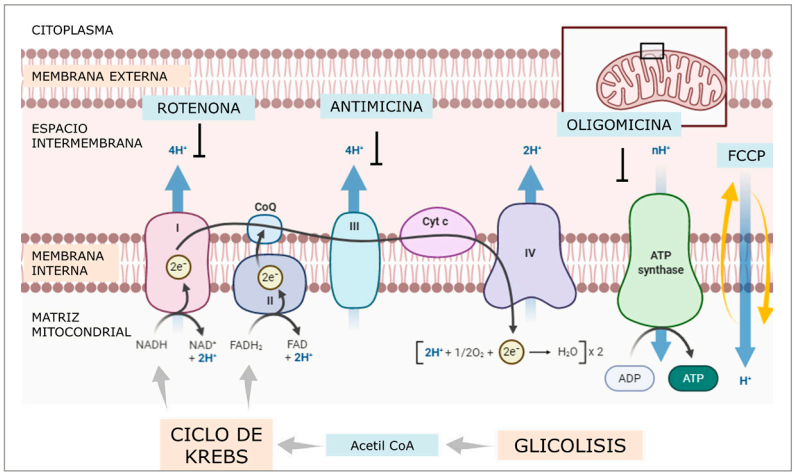

Figura M1. En la imagen se representa de manera esquemática la cadena de transporte de electrones mitocondrial. Para su correcto funcionamiento requiere de NADH y $FADH_2$ producidos en el ciclo de Krebs. Se representan los distintos inhibidores usados para evaluar la respiración mitocondrial: la rotenona es un inhibidor del complejo I; la antimicina es un inhibidor del complejo III; la oligomicina es un inhibidor de la ATP sintasa y el FCCP, desacomplante que colapsa el gradiente de protones.

Tabla M7. Listado de inhibidores usados para el ensayo *Mito Stress*.

Inhibidor	Función	Uso	Orden	Ref.
Oligomicina	Inhibe la ATP sintasa o complejo V mitocondrial. Provoca una reducción en el OCR que es proporcional a la producción de ATP.	1 µM	1	75351, Sigma-Aldrich
Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP)	Agente desacoplante que colapsa el gradiente de protones y destruye el potencial de membrana mitocondrial. Como resultado, el flujo de electrones no se inhibe y el consumo de oxígeno por el complejo IV alcanza el máximo.	1 µM	2	C2920, Sigma-Aldrich
Rotenona	Inhibidor del complejo I. Junto con la antimicina inhiben por completo la respiración mitocondrial y sirve para la medición de procesos no mitocondriales.	0.5 µM	3	R8875, Sigma-Aldrich
Antimicina	Inhibidor del complejo III. Junto con la rotenona inhiben por completo la respiración mitocondrial y sirve para la medición de procesos no mitocondriales.	0.5 µM	3	A8674, Sigma-Aldrich

3.8. Estudio de la glicolisis

Para el estudio de la glicolisis **(Figura M2)** se usó también el analizador *XF96 Extracellular Flux Analyzer* (Seahorse Bioscience), pero, en este caso, se empleó el kit *XF Glycolysis Stress* (103020, Seahorse Bioscience), siguiendo las instrucciones del fabricante. Para ello se sembraron $1x10^4$ fibroblastos en placas de 96 pocillos (11.40 mm^2) previamente tratadas con colágeno (354236, BD) en su medio de cultivo normal. Al día siguiente se reemplazó el medio de cultivo por medio de ensayo *Glyco Stress* ajustado a pH 7.4 **(Tabla M8)**. Se hicieron tres mediciones basales tras las cuales se procedió a la inyección de tres compuestos de manera secuencial: en primer lugar, glucosa, seguido por oligomicina y, finalmente, 2-deoxi-D-glucosa **(Tabla M9)**. Se hicieron tres mediciones después de la inyección de cada compuesto, determinándose en cada inyección el OCR y el ECAR. Tras las mediciones, se procedió a la normalización de los resultados en función del número de células en cada pocillo, estimado mediante la tinción con cristal violeta.

Tabla M8. Composición del medio de cultivo para el ensayo *Glyco Stress.*

Compuesto	Concentración final	Referencia
DMEM	0.83 g / 100 mL	D5030, Sigma-Aldrich
L- Glutamina	2 mM	25030024, Gibco

El diseño de este ensayo es el siguiente. En un primer momento, los fibroblastos están incubados con el medio de cultivo *Glyco Stress* sin glucosa ni piruvato. La primera inyección, de glucosa a concentraciones saturantes, se realiza para activar la **glicolisis basal** y con ello la producción de piruvato, ATP, NADH, H_2O y protones. Estos últimos serán los responsables del aumento del ECAR. La segunda inyección es de oligomicina, un inhibidor de la ATP sintasa, con lo que se va a inhibir la producción de ATP por vía mitocondrial y se desplazará toda esta producción a la vía de la glicolisis, lo que informará sobre la **capacidad máxima de la glicolisis.** Finalmente, se inyecta 2-deoxi-D-glucosa, que es un análogo de la glucosa que va a saturar al enzima glucosa hexoquinasa y, por lo tanto, va a inhibir la glicolisis, asegurando que la medición del ECAR en el experimento es debido exclusivamente a la ruta de la glicolisis. Por último, la diferencia entre la glicolisis máxima y la basal es la **reserva glicolítica** de la célula para enfrentarse a un episodio de estrés.

Tabla M9. Listado de inhibidores usados para el ensayo *Glyco Stress*.

Inhibidor	Función	Uso	Orden	Referencia
Glucosa	Sustrato principal de la glicolisis.	10 mM	1	G8769, Sigma-Aldrich
Oligomicina	Inhibe la ATP sintasa o complejo V mitocondrial. Provoca una reducción en el OCR que es proporcional a la producción de ATP.	1 µM	2	75351, Sigma-Aldrich
2-Deoxi-D-Glucosa	Análogo de la glucosa.	50 mM	3	D6134, Sigma-Aldrich

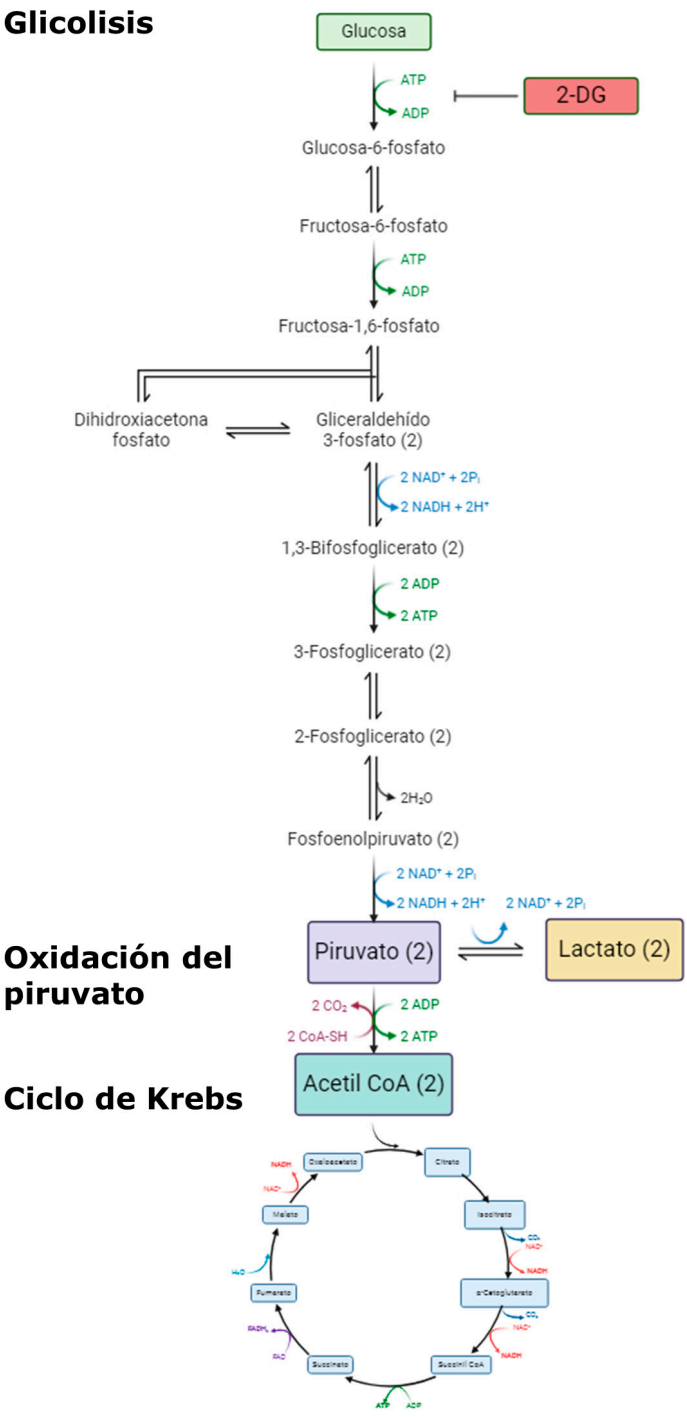

Figura M2. Representación esquemática de la glicolisis, la oxidación del piruvato y del ciclo de Krebs. Se representa el inhibidor 2-DG sobre el paso donde actúa el enzima hexoquinasa I.

3.9. Modulación de la expresión génica mediante infección lentiviral

Para la ***sobrexpresión de BMI1*** se usaron lentivirus portadores de un plásmido con la secuencia codificante de BMI1 (pCCL-BMI1, amablemente cedido por la Dra. Jacqueline Lees, MIT) y lentivirus portadores del mismo plásmido con la secuencia codificante de la proteína verde fluorescente (GFP) (pCCL-GFP) a modo de control.

Para el ***silenciamiento de BRCA1*** se usó la estrategia de "*short-hairpin RNA*" mediante la infección con lentivirus portadores de un plásmido con la secuencia de silenciamiento de BRCA1 (pLKO-shBRCA1, 44594, Addgene) y lentivirus portadores del plásmido control (pLKO.1 puro, 8453, Addgene).

Previamente a la generación de lentivirus ambos plásmidos fueron verificados mediante digestión por corte con enzimas de restricción. Para el diseño de la digestión se usó la plataforma *Restriction Mapper v3*.

Se sembraron $20x10^4$ fibroblastos en frascos de cultivo tipo 75 cm^2. Transcurridas 24 h se realizaron las infecciones con una MOI de 10 (lo que se traduce a 10 unidades infectivas por célula) en medio DMEM en ausencia de suero y empleando polibreno (H9268, Sigma-Aldrich) a una concentración final de 4 µg/mL. A las 6 h, se retiró el medio que contenía las partículas víricas y se añadió medio DMEM suplementado con FBS.

Transcurridas 48 h desde la infección, se procedió a la verificación de la misma. En el caso de la sobrexpresión de BMI1, mediante la observación de fibroblastos positivos para GFP (epifluorescencia). Además, dicha infección se verificó adicionalmente mediante la medición de la expresión proteica de GFP tanto por "*western-blot*" como por inmunofluorescencia. En el caso del silenciamiento de BRCA1, se procedió a la selección de las células resistencias a puromicina (2 µg/mL, durante 48 h).

4. Análisis de la expresión proteica

4.1. Extracción y cuantificación de proteínas

Los fibroblastos fueron recogidos y centrifugados a 8.000 g durante 5 min y lavados con PBS para la obtención de un pellet celular. Los precipitados celulares se lisaron en 80 µL de tampón de lisis (1% NP-40, 150 mM NaCl, 5 mM EDTA, 50 nM NaF, 30 mM Na4P2O7, 1 mM Na3VO4, 50 mM Tris-HCl pH 7.4), suplementado con una mezcla de inhibidores de proteasas (P8340, Sigma-Aldrich), de fosfatasas (P5726, Sigma-Aldrich), y el inhibidor de serina proteasas PMSF (P7626, Sigma-Aldrich) y se mantuvieron en hielo durante 30 min. Posteriormente fueron centrifugados a 13.000 g durante 10 min a 4ºC para precipitar el material no solubilizado. Los sobrenadantes fueron trasvasados a nuevos tubos para ser cuantificados.

La cuantificación de la cantidad de proteína de los extractos celulares se determinó mediante el método del ácido bicinconínico empleando el kit *Pierce BCA Protein Assay* (23227, Thermo Scientific). Este compuesto es capaz de formar un complejo púrpura con iones Cu+ en un medio alcalino en el que los enlaces peptídicos de las proteínas reducen Cu^{2+}. La absorbancia del compuesto será proporcional a la concentración de proteína presente. Según esto, se incubó 1 µL de extracto celular con BCA durante 20 min a 37ºC y se cuantificó la intensidad colorimétrica de las muestras en un lector de placas *MultiSkan Ascent* (Thermo Scientific) a 570 nm. Se emplearon muestras de albúmina de suero bovino a diferentes concentraciones conocidas para realizar una recta patrón en la que se interpoló la absorbancia de las muestras para obtener sus concentraciones.

4.2. Western-blot

Para la preparación de las muestras se tomó el volumen correspondiente a los microgramos de proteína deseados (generalmente 20 µg) y se le añadió una quinta parte de tampón de carga 5X (312.5 mM Tris pH 6.8, 10% SDS, 50 % glicerol, 0.5 % azul bromofenol y 5 % β-mercaptoetanol). Las muestras fueron incubadas a 95ºC durante 1 min para su completa desnaturalización y posteriormente fueron separadas por su peso molecular en geles de poliacrilamida mediante electroforesis del tipo SDS-PAGE. Se realizaron geles de poliacrilamida de 1.5 mm de grosor compuestos por una porción de gel concentradora del 4.5 % y otra porción inmediatamente inferior del 10 o 15 %, según el tamaño molecular de la proteína de interés. La electroforesis fue realizada en tampón de electroforesis (20 mM Tris, 0.2 M glicina, 0.1 % SDS y pH 8.3) usando una fuente de alimentación *BioRad HC Power Supply* durante 15 min a 70 V y, posteriormente, 2 h a 120 V. Las proteínas fueron transferidas a una

membrana de nitrocelulosa de 0.2 µm de tamaño de poro (10600001, Amersham), la cual tiene carga positiva de forma que aplicando una diferencia de potencial las proteínas migran del gel a la membrana y quedan inmovilizadas en ella. Se realizó mediante transferencia húmeda en tampón de transferencia (25 mM Tris, 192 mM glicina, 20% metanol y pH 8.6, 0.05% SDS) durante 90 min a 220 mA y a 4ºC. A continuación, la membrana se incubó con solución de bloqueo (tampón TBS-T: 0.1M Tris-HCl, 0.01 % Tween 20, suplementado con 5 % leche en polvo) durante 1 h a temperatura ambiente para evitar uniones inespecíficas de los anticuerpos primarios.

Tabla M10. Listado de anticuerpos primarios y secundarios usados para Western-blot.

Anticuerpos primarios				
Nombre	**Dilución**	**Origen**	**Fabricante**	**Referencia**
β-actina	1:20.000	Ratón	Sigma-Aldrich	AC-15
GFP	1:1.000	Cabra	Abcam	ab6673
Anticuerpos frente a la DM1				
DMPK	1:500	Ratón	Santa Cruz	sc-134319
MBNL1	1:1.000	Conejo	Abcam	ab45899
Anticuerpos para evaluar la proliferación y senescencia celular				
$p16^{INK4A}$	1:500 1:50	Conejo Ratón	Abcam	ab108349 ab16880
$p21^{CIP1}$	1:500	Cabra	Santa Cruz	sc-397
$p27^{KIP1}$	1:500	Ratón	BD Laboratories	BD610241
BMI1	1:500	Ratón	Millipore	05-637
Fosfo AKT Ser473	1:250	Conejo	Cell Signalling	9271
AKT 1/2/3	1:500	Conejo	Santa Cruz	sc-8312
Anticuerpos para evaluar el estrés celular				
Fosfo p38 MAPK Thr180/ Tyr182	1:500	Conejo	Cell Signaling	9211
p38 α/β	1:250	Ratón	Santa Cruz	sc-7972
Anticuerpos secundarios				
Cabra anti-Conejo HRP	1:1.000	Cabra	Cell Signalling	7074S
Cabra anti-Ratón HRP	1:1.000	Cabra	Cell Signalling	7076S
Asno anti-Cabra HRP	1:1.000	Asno	Santa Cruz	sc-2020

Tras el bloqueo se procedió a la incubación de las membranas de nitrocelulosa con la dilución correspondiente de cada anticuerpo primario en solución de bloqueo en agitación a 4ºC durante 15-18 h **(Tabla M10)**. Posteriormente las membranas se lavaron 3 veces durante 5 min con tampón TBS-T y se incubaron con el anticuerpo secundario correspondiente conjugado a peroxidasa en solución de bloqueo en agitación a temperatura ambiente durante 1 h. Tras la incubación las membranas fueron lavadas 3 veces con solución de TBS-T.

La detección de las proteínas se realizó mediante los sustratos *Luminata Crescendo Western HRP substrate* (WBLUR0100, Merck Millipore) o *SuperSignal West Femto Maximum Sensitivity Substrate* (34095, Thermo Scientific), basados en la reacción quimioluminiscente de la oxidación del luminol por parte de la peroxidasa conjugada a los anticuerpos secundarios. Se empleó el sistema de adquisición de imágenes de quimioluminiscencia iBright FL-1000 (Invitrogen).

4.3. Inmunofluorescencia

Para determinar la expresión y/o localización subcelular de las proteínas de interés se sembraron $1x10^4$ fibroblastos (o $0.5x10^3$ fibroblastos en el caso de tratamientos) en su medio de cultivo en pocillos de cámaras de inmunofluorescencia de 0.7 cm^2 (Nunc Lab-Tek II Chamber Slide, Thermo-Scientific) y tras 24 h fueron fijados con formaldehído al 4% durante 10 min. Tras la fijación se lavaron 3 veces con una solución de PBS, se bloquearon y permeabilizaron con una solución de PBS al 0.3 % de Tritón X-100 (T8787, Sigma-Aldrich) suplementado con 5 % de FBS durante 1 h a temperatura ambiente. Tras el bloqueo las muestras fueron incubadas con el anticuerpo primario a la dilución correspondiente con una solución de PBS al 0.3 % de Tritón X-100 durante 15-18 h a 4ºC **(Tabla M11)**. Posteriormente fueron lavadas 3 veces con solución de PBS para ser incubadas con el correspondiente anticuerpo secundario conjugado a un fluoróforo en solución de PBS al 0.3 % de Tritón X-100 a una dilución 1:500 durante 1 h a temperatura ambiente y en oscuridad. Se volvieron a lavar 3 veces con solución de PBS y se procedió a teñir la cromatina con *Hoechst* (33342, Sigma-Aldrich) durante 10 min. Posteriormente se retiraron los compartimentos y se realizó el montaje de las preparaciones con medio de montaje *Fluoro-Gel* (17985-10, Aname). Las preparaciones fueron fotografiadas en un microscopio Nikon Eclipse 80i o en un microscopio confocal Zeiss LSM 900 y procesadas con los softwares *NIS Elements Advances Research* y *Zen Blue,* respectivamente.

Tabla M11. Listado de anticuerpos primarios y secundarios usados para inmunofluorescencia.

Anticuerpos primarios				
Nombre	**Dilución**	**Origen**	**Fabricante**	**Referencia**
Anticuerpos frente a la DM1				
DMPK	1:200	Ratón	Santa Cruz	sc-134319
CUGBP1	1:500	Conejo	Abcam	ab9549
Anticuerpos para evaluar la proliferación y senescencia celular				
Fosfo Histona H3 Ser10	1:2.000	Ratón	Abcam	ab14955
Ki-67	1:500	Conejo	Abcam	ab15580
Anticuerpos para evaluar el daño en el ADN y la apoptosis				
γ-H2AX Ser139	1:250	Ratón	Millipore	05-636
Fosfo ATM Ser1981	1:250	Conejo	Cell Signalling	13050
53BP1	1:250	Conejo	Novus-Biologicals	NB100-304
PARP	1:700	Conejo	Abcam	ab32064
Capasa 3 activa	1:500	Ratón	R&D	AF835
Anticuerpos para evaluar la mitocondria				
TOM20	1:350	Conejo	Proteintech	11802-1AP
PGC1-α	1:250	Conejo	Novus Biologicals	NBP1-04676
Anticuerpos secundarios				
Alexa Fluor 555 Cabra anti-Ratón IgG (H+L)	1:500	Cabra	Invitrogen	A21422
Alexa Fluor 488 Cabra anti-Conejo IgG (H+L)	1:500	Cabra	Invitrogen	A32731

4.3.1. Medición de la intensidad de fluorescencia

La medición de la intensidad de fluorescencia se realizó mediante el programa Image J. En el caso de las proteínas γ-H2AX, 53BP1 y CUGBP1 se procedió a la medición de la intensidad nuclear de al menos 100 células por cada condición experimental y en el caso de las proteínas DMPK y MBNL1 se procedió a la medición de la fluorescencia total. Para establecer el umbral de la señal de fluorescencia se procedió a la medición de una zona del mismo tamaño en el exterior celular. Posteriormente, mediante el programa Image J se procedió al cálculo de la "*densidad de fluorescencia integrada*" restando a cada medición la fluorescencia umbral previamente medida.

4.3.2. Tinción con EdU

Para evaluar la habilidad de la célula de proliferar se utilizó la técnica basada en la detección de la incorporación de EdU. El EdU (5-etinilo-2´-deoxiuridina) es un análogo del nucleótido timina y, por lo tanto, se va a incorporar a la molécula de ADN cuando ésta se replique. Posteriormente, el EdU incorporado es detectado usando fluorescencia. Se usó el kit comercial *Click-IT EdU Imaging* (C10338, Invitrogen), de acuerdo a las instrucciones del fabricante.

Para ello se sembraron $0.5x10^3$ fibroblastos (o $0.5x10^3$ fibroblastos en el caso de tratamientos) en su medio de cultivo en pocillos de cámaras de inmunofluorescencia de $0.7cm^2$ y tras 12-18 h se procedió a cambiar el medio de cultivo por medio con tratamiento con metformina, para su posterior incubación durante 72 h adicionales, siendo las 24 h finales la incubación correspondiente con 10 µM de EdU.

Tras las incubaciones correspondientes, las células fueron fijadas con formaldehído al 4% durante 15 min. Tras la fijación se lavaron 3 veces con PBS al 3% BSA y se permeabilizaron con PBS al 0.3% Tritón X-100 durante 20 min a temperatura ambiente. Tras la permeabilización se lavaron 2 veces con PBS al 3 % BSA y se incubaron con el cóctel de reacción **(Tabla M12)** durante 30 min. Posteriormente fueron lavadas 1 vez con PBS-3% BSA y se procedió a teñir la cromatina con *Hoechst* durante 10 min. Por último, se retiraron los compartimentos del porta y se realizó el montaje con medio de montaje Fluoro-Gel. Las preparaciones fueron fotografiadas en un microscopio Nikon Eclipse 80i y procesadas con el software *NIS Elements Advances Research*.

Tabla M12. Composición del cóctel de reacción.

Componente	Cantidad/pocillo
Click-IT Buffer	172 uL
$CuSO_4$	8 uL
Alexa Fluor Azida	0.48 uL
Buffer aditivo	20 uL

4.4. Hibridación fluorescente *in situ* (FISH)

Para determinar la presencia de agregados CTG en forma de *foci* nucleares se sembraron $1x10^4$ fibroblastos en su medio de cultivo en pocillos de cámaras de inmunofluorescencia de 0.7 cm^2. Tras 12-14 h, los fibroblastos fueron fijados con formaldehído al 4% durante 15 min para ser posteriormente lavados con solución de PBS.

Tras la fijación se lavaron 3 veces con una solución de PBS y se procedió a su permeabilización con etanol al 70 % (ET00111000, Scharlau) durante 15-18 h a 4ºC. Posteriormente, las preparaciones fueron rehidratadas durante 5 min a temperatura ambiente con tampón salino de citrato de sodio 2X (S6639, Sigma-Aldrich) al 50% de formamida (47671, Sigma-Aldrich). Antes de la hibridación, tanto las preparaciones como la sonda, de manera independiente, fueron incubadas durante 5 min a 80ºC. Después, se procedió a la preparación de la solución de hibridación compuesta por: tampón salino de citrato de sodio 2X suplementado al 10% de sulfato de dextrano (D8906, Sigma-Aldrich), 2 mM de ribonucleótidos de vanadilo (94742, Sigma-Aldrich), 0.02 % de albúmina de suero bovino (A9647, Sigma-Aldrich), 50 % de formamida junto con 0.1 µM de la sonda específica (CAG)x10 conjugada al fluoróforo Alexa-488 (Thermo-Fisher). Las preparaciones fueron hibridadas durante 15-18 h a 37ºC en una atmósfera saturada de humedad. Posteriormente, las muestras fueron lavadas dos veces con tampón salino de citrato de sodio 2X suplementado al 50% de formamida durante 30 min a 37ºC y en agitación. Se procedió a teñir la cromatina con *Hoechst* durante 10 min a temperatura ambiente, se retiraron los compartimentos y se realizó el montaje de las preparaciones con medio de montaje *Fluoro-Gel*. Las preparaciones fueron fotografiadas en un microscopio confocal Zeiss LSM 900 (aumento 63X) y procesadas con el software *Zen Blue*.

5. Array de citoquinas

Los niveles de citoquinas inflamatorias secretadas por los fibroblastos fueron determinados usando el *array* de citoquinas *Human Inflammation Array C3* (AAH-INF-3, RayBiotech), el cual está basado en inmunodetección. Para ello se sembraron $20x10^4$ fibroblastos en placas 60 cm^2 (placas tipo p100) y tras 14-18 h se lavaron e incubaron en su medio de cultivo sin FBS durante 48 h para generar el medio condicionado (CM), que fue recogido y congelado a –80ºC hasta su uso. Además, se contó el número de células para normalizaciones posteriores. El CM fue descongelado y centrifugado a 9.000 g durante 5 min. Las membranas del *array* fueron bloqueadas con el reactivo de bloqueo durante 1 h, incubadas con el CM durante 15-18 h a 4ºC, lavadas 5 veces con el reactivo de lavado e incubadas con el cóctel de anticuerpos conjugados a biotina durante 15-18 h a 4ºC. Después se lavaron 5 veces con el reactivo de lavado y se incubaron con estreptavidina conjugada a peroxidasa durante 2 h a temperatura ambiente. Finalmente, los *arrays* fueron documentados con el sistema de quimioluminiscencia *iBright FL1000 system*. La señal correspondiente a cada citoquina se normalizó en función del número de células y fue analizada usando el programa *ImageJ* y la herramienta desarrollada por Carpentier, 2010.

6. Análisis de la expresión génica

6.1. Extracción de ARN total

Para el aislamiento del ARN total celular las células fueron lisadas mediante *TRI Reagent Solution* (AM-9738, Life Technologies). En el caso de las muestras de tejido muscular provenientes tanto del tórax de *D. melanogaster*, como del músculo tibial anterior del ratón DMSXL se procedió a su lisis usando el aparato disociador de tejido TissueLyser (85210, Qiagen), mediante bolas de acero inoxidable.

Tras la lisis completa, al lisado se le añadieron 0.2 mL de cloroformo (C2432, Sigma-Aldrich), se homogeneizó y se incubó durante 10 min a temperatura ambiente para la formación de fases. Posteriormente se centrifugó a 13.000 g durante 10 min a 4ºC. Una vez centrifugado se procedió a la separación de la fase acuosa en tubos tratados con DEPC (159220, Sigma-Aldrich), se añadieron 0.5 mL de 2-propanol (I9516, Sigma-Aldrich) y 1 µL de glicógeno a 5 µg/µL (AM9510, Ambion). Esta mezcla se incubó durante 10 min a temperatura ambiente para precipitar el ARN y se centrifugó a 13.000 g durante 10 min a 4ºC. Tras la centrifugación, se descartó el sobrenadante y se añadió 1 mL de etanol al 75% y se centrifugó a 7.000 g durante 5 min a 4ºC para lavar el ARN precipitado. Por último, se descartó de nuevo el sobrenadante y el ARN precipitado se resuspendió en agua libre de ARNasas (10977035, Invitrogen). La concentración y pureza del ARN aislado se determinó mediante la medición de la absorbancia a 260 y 280 nm en un espectrofotómetro Nanodrop-1000 (Thermo-Fisher). Las muestras de ARN fueron almacenadas a -80ºC hasta su uso y aquellas con un ratio 260/280 inferior a 1.80 fueron descartadas.

6.2. Transcripción reversa

Para la obtención del ADN complementario (ADNc) de cadena simple se realizó la retro-transcripción (RT) mediante el método de cebadores aleatorios y el kit *Maxima First Strand Complementary DNA* (K1671, Thermo Scientific), que incorpora un tratamiento con *ADNasa* para eliminar las posibles contaminaciones con ADN genómico. Las muestras de ARN fueron procesadas siguiendo las instrucciones del fabricante en un termociclador BioRad C1000 con el siguiente protocolo: una incubación de 10 min a 25ºC seguida de 30 min a 50ºC y 5 min a 85ºC. El ADNc se diluyó en agua libre de ARNasas (10977049, Invitrogen) a una concentración de 2 ng/µL y fue almacenado a –20ºC hasta su uso.

En el caso de análisis de microARNs se realizó una transcripción reversa específica para cada microARN usando 50 ng de ARN, una sonda específica *Taqman* de RT (Thermo-Fisher) y el kit *High-Capacity cDNA Reverse Transcription* (4368814, Applied Biosystems), siguiendo el siguiente protocolo: 30 min a 16ºC, 30 min a 42ºC y 5 min a 85ºC.

6.3. PCR cuantitativa a tiempo real

Para determinar los niveles de expresión de los genes de interés se utilizó la reacción en cadena de la polimerasa a tiempo real o cuantitativa (qRT-PCR) empleando el agente fluorescente *SybrGreen* (4368706, Applied Biosystem) para la detección del producto amplificado. Se emplearon 10 ng de ADNc como molde de la reacción, dos cebadores específicos para cada gen y una mezcla de reacción. Las reacciones se realizaron en un equipo *CFX384 Touch Real-Time PCR Detection System* (Bio-Rad) con el siguiente protocolo: 1 ciclo de 120 s a 50ºC, 1 ciclo de 600 s a 95ºC; 41 ciclos de 15 s a 95ºC y 60 s a 60ºC y finalmente 1 ciclo de 10 s a 95ºC seguido por 60 s a 60ºC y 1 s a 97ºC.

Como control interno para corregir las variaciones en los niveles de ADNc se usó la amplificación del gen de expresión endógena GAPDH (gliceraldehído 3 fosfato deshidrogenasa) para las muestras humanas. En el caso de las muestras derivadas del ratón DMSXL se procedió a la evaluación de 4 genes tomados de la bibliografía como controles de expresión endógena tanto en modelos de ratón como en músculo: Hprt, 18S, β-microglobulina y actina. En el caso de las muestras derivas de *D. melanogaster* se procedió a la evaluación de los endógenos rp49 y tubulina. La elección del mejor candidato se llevó a cabo mediante el algoritmo "*NormFinder*" (Andersen *et al.*, 2004), que arrojó como mejor opción actina en ratón y rp49 en mosca.

Las secuencias de los cebadores específicos de cada gen fueron buscadas en bibliografía, en la base de datos *Primer-Bank* (https://pga.mgh.harvard.edu/ primerbank), en la base de datos *Fly Primer Bank (https://www.flyrnai.org/ FlyPrimerBank)*, o diseñadas usando la herramienta *Primer 3 v4.1.0* (http://primer3.ut.ee). En todos los casos se verificó la especificidad de cada pareja de cebadores usando *Primer Blast* (https://www.ncbi.nlm. nih.gov/tools/primer-blast) y a través del análisis de la curva de disociación generada tras la reacción de la qRT-PCR. En los casos donde fue posible se diseñaron los cebadores frente a secuencias entre exones con el objetivo de evitar la amplificación de ADN genómico.

Para el análisis de los niveles de expresión génica de microARNs se utilizó la tecnología *TaqMan* (4440040, Applied Biosystem), que se basa en el uso de dos cebadores y una sonda MGB específica. Se usaron 5 ng de ADNc como molde de la reacción y cada muestra se analizó en triplicado en un equipo *7900HT Fast Real-Time PCR System* (Applied Biosystems) con el siguiente protocolo de reacción: 1 ciclo de 120 s a 50ºC, 1 ciclo de 600 s a 95ºC y 40 ciclos de 15 s a 95ºC y 60 s a 60ºC. Como control interno para corregir las variaciones en los niveles de ADNc se usó la expresión de *RNU48* (Schwarzenbach *et al.*, 2015).

Para la cuantificación relativa de los niveles de expresión génica se utilizó la fórmula $2^{-\Delta\Delta Ct}$, consistente en calcular la expresión relativa del gen de interés respecto a su expresión en una de nuestras muestras que consideramos de referencia (Livak and Schmittgen, 2001; Schmittgen and Livak, 2008).

6.4. Análisis de *splicing* alternativo

Para el análisis de variantes de *splicing* en *MBNL2* se usó la técnica de PCR semicuantitativa mediante la polimerasa *BIOTAQ DNA* (BIO-21040, Bioline) y el ADNc de interés como molde de la reacción. El protocolo y programa de PCR fueron descritos por Lin *et al*., 2006. Los cebadores **(Tabla M11)** fueron diseñados de manera que la diferencia entre la inclusión y exclusión del exón de interés tuviera una diferencia en pares de bases de entre un 10 y 25% respectivamente. La reacción se llevó a cabo durante 24 ciclos siguiendo el siguiente protocolo: 1 ciclo de 300 s a 95ºC; 24 ciclos de 30 s a 95ºC, 30 s a 60ºC y 60 s a 72ºC; y finalmente 1 ciclo de 300 s a 72ºC. Los productos de PCR se migraron en un gel de agarosa teñido con *GelRed Nucleic Acid Gel Stain* (41001, Biotium) y se visualizó en el sistema *iBright FL1000 system*.

Tabla M11. Listado de cebadores para variantes de *splicing* usados en el presente estudio.

Gen	Exón	Secuencia Forward (5' → 3')	Secuencia Reverse (5' → 3')
MBNL2	7	ACAAGTGACAACACCGTAACCG	TTTGGTAAAGGATGAAGAGCACC

6.5. Microarrays de expresión génica

Se han realizado dos microarrays de expresión génica, el primero de ellos en muestras de PBMC y el segundo en muestras de fibroblastos. Para el primero se usaron 0.3 µg de ARN obtenido de PBMC de 10 pacientes con DM1 y 10 controles sanos **(Anexo 5)**, empleando el chip *Human Gene 1.0* (Affymetrix). El posterior filtrado y análisis de expresión diferencial se llevó a cabo en función del sexo de los pacientes. En el segundo microarray se analizaron 0.3 µg de ARN extraído de fibroblastos en cultivo de 6 pacientes con DM1 y 4 controles sanos **(Anexo 5)** usando el chip *Human Clariom D* (Affymetrix). En ambos casos se procedió al análisis de la integridad del ARN mediante el kit Agilent RNA 6000 Nano, siendo usadas aquellas muestras con un valor de integridad superior a 7.

6.6. RNA-Seq

Se realizaron tratamientos convencionales con AHK7 (0.1 µM, 72 h) en tres líneas de fibroblastos DM1 y se analizó el transcriptoma mediante RNA-Seq. Se procedió al aislamiento del ARN mediante *TRI Reagent Solution* y al correspondiente análisis de la integridad del ARN mediante el kit Agilent RNA 6000 Nano. Se seleccionaron las muestras con un valor de integridad superior a 9.

La preparación de las librerías junto con la secuenciación se realizó con la empresa CD Genomics (Nueva York, EE.UU.). Para la preparación de las librerías se usaron

200 ng de ARN, tras el tratamiento correspondiente con ADNasa y el aislamiento del ARNm se realizó mediante bolas magnéticas con oligo (dT). Posteriormente, el ARNm es fragmentado, se sintetiza el ADNc mediante hexámeros aleatorios y se purifica. Para la cuantificación y el control de calidad de las correspondientes librerías se utilizaron los equipos *Agilent 2100 Bioanaylzer* y *ABI StepOnePlus Real-Time PCR System*. La secuenciación se realizó en un secuenciador Illumina Nova Seq 6000 en configuración *"paired end"* 150 pares de bases. Para la obtención de las firmas moleculares, se seleccionaron los 500 genes más diferencialmente expresados para cada muestra y se usó la colección C5 (GO Biological Process) de la base de datos MSigDB.

7. Análisis estadístico

Los datos mostrados en el presente trabajo representan la media ± error estándar de la media (SEM), con el número de experimentos (n) indicado en cada experimento en el pie de figura. Si no se indica lo contrario, las medias fueron comparadas mediante el test estadístico t de Student y los datos se representaron usando GraphPad 8. Los asteriscos indican los diferentes grados de significación estadística: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Los análisis de correlación en muestras de PBMC se basaron en el coeficiente de Pearson cuando las muestras estaban normalmente distribuidas o el coeficiente de Spearman cuando no estaban normalmente distribuidas El análisis de la normalidad se realizó mediante el test de Kolmogorov-Smirnov, usando el software GraphPad 8.

Resultados

Capítulo 1. Cancer risk in DM1 is sex-related and linked to miRNA-200/141 downregulation

Artículo publicado en Neurology
Factor de Impacto 8.68 - Decil 1

Fernández-Torrón R*, **García-Puga M*,** Emparanza JI, Maneiro M, Cobo AM, Poza JJ, Espinal JB, Zulaica M, Ruiz I, Martorell L, Otaegui D, Matheu A, López de Munain A.
Cancer risk in DM1 is sex-related and linked to miRNA-200/141 downregulation.
Neurology. 2016 Sep 20;87(12):1250-7.

doi: 10.1212/WNL.0000000000003124. Epub 2016 Aug 24.
Submitted: 17 February 2016. Accepted: 8 June 2016. Published: 24 August 2016

Cancer risk in DM1 is sex-related and linked to miRNA-200/141 downregulation

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Search Terms: Myotonic dystrophy, Steinert's disease, Cancer risk, miRNA200, miRNA141

Resumen

En este trabajo describimos el mayor riesgo a desarrollar cáncer en la cohorte de pacientes con DM1 de Guipúzcoa, que está compuesta por 424 pacientes seguidos entre los años 1985-2013. En dicha cohorte encontramos 70 tumores en 62 pacientes, lo que arrojó un incremento de 1.8 en el riesgo a desarrollar cáncer (95% CI= 1.37-2.36), siendo este riesgo mayor en mujeres que en hombres. El desarrollo de neoplasias fue la tercera causa de muerte, por detrás de las complicaciones respiratorias y las cardiacas. Las mujeres con DM1 tenían un riesgo mayor a desarrollar cáncer de ovario (SIR= 8.33, 95% CI= 1.72- 24.31) y endometrio (SIR= 6.86, 95% CI= 2.23-16.02). En cambio, en ambos sexos, el mayor riesgo estuvo en cáncer de tiroides (SIR= 23.33, 95% CI= 9.38-48.08) y de cerebro (SIR= 9.80, 95% CI= 3.18-22.88).

Por otra parte, realizamos un estudio transcriptómico en muestras de PBMC de pacientes con DM1, con el objetivo de establecer las rutas moleculares que estaban implicadas en el mayor riesgo a desarrollar cáncer en dichos pacientes. Encontramos que la ruta oncogénica compuesta por el miRNA 200c-141–BMI1–p16^{INK4a} presentaba una fuerte alteración en pacientes con DM1, y que esta alteración era más marcada en mujeres que en hombres.

En este trabajo describimos el mayor riesgo oncológico en pacientes con DM1, especialmente en mujeres. Además, identificamos que la familia de supresores tumorales del miRNA200-miRNA141 tiene una expresión reducida en mujeres.

Contribución

Mi contribución en este artículo ha sido la realización del array de expresión génica y su posterior validación en el resto de la cohorte, los estudios de expresión tanto génica como de proteína de la ruta miRNA 200c-141–BMI1–p16^{INK4a}, el análisis de los datos y la escritura del manuscrito.

Abstract

Objective. Describe the incidence of cancer in a large cohort of myotonic dystrophy type 1 (DM1) patients and to unravel the underlying molecular mechanisms.

Patients and Methods. Standardized incidence ratios (SIRs) were calculated in the Gipuzkoa DM1 cohort (1985-2013), dividing observed numbers by expected numbers for all cancers combined and stratified by sex. An estimation of the expected incidence was achieved by multiplying the specific age and sex incidence rates from the basque population cancer registry to the person-years observed in the study cohort. Large scale gene expression of PBMC samples derived from 10 DM1 individuals (5 men, 5 women), and 10 healthy matched controls were analyzed by the Human Gene 1.0 ST Affymetrix microarray.

Results. During 18796 person-years of follow-up, corresponding to 424 DM1 patients, we observed 70 cancer in 62 patients giving a 1.81-fold risk (95% CI= 1.37-2.36) which was stronger in women than in men. Ovary (SIR= 8.33, 95% CI= 1.72-24.31) and endometrium (SIR= 6.86, 95% CI= 2.23-16.02) in women and thyroid (SIR= 23.33, 95% CI= 9.38-48.08) and brain (SIR= 9.80, 95% CI= 3.18-22.88) in both sexes were tumor sites with significant higher risks in DM1. There were differences in gene expression between healthy and DM1 patients and between DM1 men and women; all DM1 patients combined, and female DM1 patients displayed significant downregulation of the miRNA200c-141 tumor suppressor family.

Conclusion. Oncologic risk is increased in DM1, especially in women and for gynaecologic, brain and thyroid cancer. The expression of miRNA200- miRNA141 tumor suppressor family is decreased in DM1 women.

Introduction

Myotonic Muscular Dystrophies are a group of autosomal dominant, multi-system diseases encompassed by two subtypes. Myotonic Dystrophy type 1 (DM1), also known as Curschmann-Steinert's disease (OMIM: 160900), is caused by the expansion of unstable trinucleotide (CTG) repeat expansion in the 3' untranslated region of a *DMPK* kinase gene located in chromosome 19. The type 2 (DM2, OMIM 602668) is originated by a tetra-nucleotide (CCTG) repeat expansion in intron 1 of the *CNBP* gene. DM1 displays a more severe phenotype than DM2 and represents the most common adult muscular dystrophy, with an estimated prevalence ranging from 0.5 to 18/100.000 people (1), although the disease prevalence is higher in some regions such the province of Gipuzkoa in the Spanish Basque Country (2).

The DM1 phenotype is grossly related to the size of CTG expansion and shows an extremely wide variability ranging from very mild forms with CTG repeats less than 50 and usually associated with cataracts developed in pre-senile age, to severe neonatal forms with more than CTG 1000 repeats, associated with severe developmental delays. Life expectancy is reduced due to complications derived from muscle weakness, respiratory and cardiac involvement, neoplasms and metabolic disturbances like hypercholesterolemia or diabetes (3,4).

In 1965, Cantwell & Reed first reported an association between DM1 and pilomatricoma, a rare and benign cutaneous tumor (5). Since then, several case reports describing benign and malignant neoplasms in virtually any location have been published (6). Furthermore, in the last years, large epidemiological studies performed in population or clinical-based cohorts provided evidence of increased risk of malignant tumours in DM patients (7-10). Excess risks for endometrium, ovarian, thyroid, skin, eye and colon cancer were observed in two of the three studies; while brain cancer excess risk was observed in one study. A follow-up study including 911 DM patients showed that females with DM1 were more likely to develop cancers (11). Several studies have shown no association between the size of leukocyte repeat expansion and cancer risk in those patients (6) and the underlying causes and the biological mechanisms of the susceptibility for developing tumors are still unknown.

In this study, we quantified cancer risk in the clinically and genetically well-characterized Gipuzkoa Myotonic Dystrophy Cohort, and used gene expression analysis to identify possible molecular mechanism of cancer susceptibility in those patients. The cohort includes all patients diagnosed with DM in the past 30 years in an area with one of the largest DM1 prevalence worldwide.

Results

Cancer incidence rate in DM1 patients

The study included 424 DM1 patients; 214 (50.5%) of them were women. Mean CTG repeat expansion size at the time of DM1 diagnosis was 684 ± 535 CTG triplet expansions (range: 43-2000 CTG repeats). 137 patients were deceased at the time of analysis. Additional demographic and clinical features of the patients are shown in **Table 1.** The most common causes of death were diseases of the respiratory system (38%), the circulatory system (24.1%), and neoplasms (15.3%); causes of death were unknown in 15.3% of the patients **(Table e-1).**

During a 18,796 person-years of follow-up, we observed 70 cancers in 62 DM1 patients (32 women, 30 men, **Table e-2**) We observed multiple primary cancers in 7 patients (6 patients had 2 cancers and one patient had 3 different cancers) **(Table e-3).** Mean age at cancer diagnosis for all 62 patients was 46.6 ± 14.7 years. Mean CTG repeat expansion size in DM1 patients with cancer was 629.

Table 1. Clinical and molecular characteristics of DM1 cohort.

Number of patients	424
Sex	214 women (50.5%) 210 men (49.5%)
Mean CTG repeat expansion size	684 ± 535.6 CTG repeats (43-2.000 CTG repeats)
Transmission	Paternal 65%
MIRS (n=217)	2.54 ± 1.21
Age at last visit	50.7 ± 15.61 years
Respiratory symptoms	86 patients (20.3 %)
Cardiac disease	184 patients (43.4%)
Patients with cancer	62 (14.6%)
Death	137 patients
Mean Age of death	60.25 ± 13.11 years
Follow up days	18313. 6 ± 5739.2 days

Table 2. SIRs by anatomical site. (O: Observed cancer in DM1 cohort; E: Expected cancer in the basque general population adjusted per sex and age; Asterisks (*), indicate statistical significance $p < 0.05$).

Cancer	Age at diagnosis of cancer	O	E	SIR	95%CI
Women					
Ovary	48 (16-63)	3	0.36	8.33	1.72-24.31*
Endometrium	53 (38-59)	5	0.73	6.86	2.23-16.02*
Breast	57 (56-69)	3	2.88	1.04	0.21-3.04
Men					
Prostate	75 (73-77)	2	4.33	0.46	0.06-1.67
Testes	15	1	0.07	14.25	0.35-79.6
Both sexes					
Thyroid	51 (40-67)	7	0.30	23.33	9.38-48.08*
Brain	53 (30-56)	5	0.51	9.80	3.18-22.88*
Kidney	53.5 (40-67)	2	0.79	2.53	0.31-9.15
Non-H Lymphoma	65.5 (61-70)	2	0.70	2.86	0.35-10.32
Liver	69 (66-72)	2	0.79	2.54	0.31-9.15
Colorectum	59 (41-86)	9	4.36	2.06	0.94-3.92
Stomach	56.5 (55-58)	2	1.27	1.57	0.19-5.69
Lung	59.5 (47-73)	4	3.40	1.18	0.32-3.01
Leukemia	57	1	0.26	3.80	0.09-21.43
Head & Neck	43	1	1.07	0.94	0.02-5.21
Melanoma	34	1	0.58	1.72	0.04-9.61
Urinary bladder	64	1	1.56	0.64	0.02-3.57

± 79 CTG repeats and there were no statistical differences between the length of the CTG expansion in DM1 patients with or without cancer (p>0.05). Although we only had MIRS data available at the time of the study in 217 patients, there were no statistical differences either regarding cancer status (p>0.05). Digestive organs (24.8 %), genitourinary system (21.4 %), skin (12.8 %) and thyroid gland (11.4%) were the most frequent malignant tumors (Table e-2).

When compared to the general population from Gipuzkoa, we detected an approximately two-fold increase in risk for cancer (SIR=1.81, 95% CI= 1.37-2.36). This risk increase was stronger in women (SIR= 2.71, 95% CI= 1.77-3.97) than in men (SIR= 1.40, 95% CI= 0.94- 2.01). When we compared the anatomical site of the cancer in the DM1 population with the general population stratified by sex and age, we found a high risk for developing ovary (SIR= 8.33, 95% CI= 1.72-24.31) and endometrium (SIR= 6.86, 95% CI= 2.23-16.02) cancer in women and thyroid (SIR= 23.33, 95% CI= 9.38-48.08) and brain (SIR= 9.80, 95% CI= 3.18-22.88) cancer in both sexes (**Table 2**). Besides these malignant tumors, only colorectal cancer nearly reached statistical significance (SIR= 2.06, 95% CI= 0.94-3.92).

Transcriptomic analysis of DM1 patients

As previously described (16), we observed differences in gene expression between healthy men and women **(Fig. 1A,B)**. Similar sex-differences were detected in the transcriptome of DM1 patients **(Fig. 1A-C)**. Compared to healthy controls, DM1 patients had an upregulation of RNA5SP211, an unknown pseudogen, and downregulation of TAS2R13, a subtype of taste receptors.

When we analysed the results per gender, we found that EMR4P (a hormone receptor), CD24 (a glycoprotein expressed on mature granulocytes and B cells), PLA2G7 (a platelet activating factor) and caspase-5 (a gene implicated in apoptosis) were significantly upregulated in men **(Fig. 1B,C)**. Moreover, we identified 11 genes differentially expressed in DM1 women including: upregulation of a subtype of histone (HIST1H2AK) and pyruvate dehydrogenase kinase 4 (PDK4), and down-regulation of three miRNAs precursors (pre-miR-3978, pre-miR-141 and pre-miR-200c), a transcription factor (ZEB2), an olfactory receptor (OR52K2), an inhibitory receptor of myeloid cells (CLEC12B), Myoferlin (a protein that play a role in calcium-mediated membrane fusion events, membrane regeneration and repair and subsequently in muscle weakness), a death-associated protein kinase (DAPK1), and MS4A4E **(Fig 1B,C)**. The same results were observed in an independent validation set of patients. **(Fig. 1D,E)**. Specifically, the differentially expressed levels of 13 out of 17 genes detected in the array, were confirmed in the validation cohort.

Identification *of miR-200c/miR-141* cluster associated to the cancer susceptibility phenotype in DM1

The miR-200c/miR-144 and miR-3978 precursors were downregulated in the DM1 women group in the array and validated in the extended cohort **(Fig. 2A,B).** Strikingly, the mature forms miR-200c-5p, miR-141-3p, miR-141-5p and miR-3978 were downregulated on the cohort of DM1 women **(Fig. 2C).** On the contrary, the expression was slightly elevated in men revealing a sex differential expression of those miRs in DM1 **(Fig 2C,D).**

As *BMI1* and *ZEB* family member oncogenes are reported as miR-200 target genes (17-22), we measured their expression finding that ZEB1and ZEB2 and were decreased whilst BMI1 levels were upregulated in the female group of DM1 patients **(Fig. 2E).** Moreover, the expression of $p16^{Ink4a}$ tumor suppressor, a known target of BMI1 epigenetic silencing, was lower at both mRNA and protein level in the same group of DM1 females **(Fig. 2F,G).**

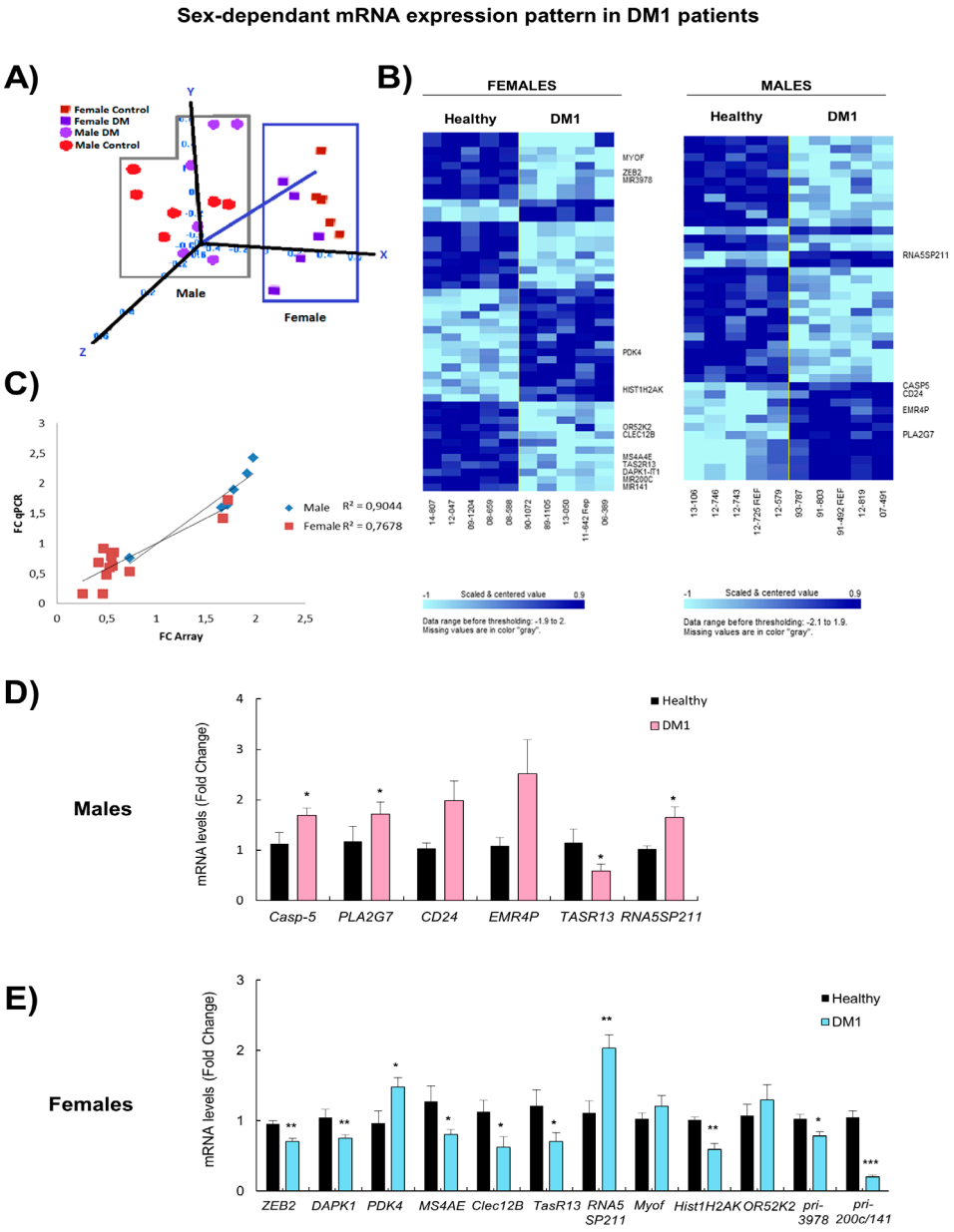

Figure 1. Sex-dependent mRNA expression pattern in DM1 patients.

A) Dendrogram explaining the gender differences between the expression patterns. B) Heat-map with the genes differentially expressed between DM1 patients (n=10) and healthy controls (n=10) in females (left, n=5) and males (right, n=5). C) Chart of the validation data (fold change from arrays versus fold change from RT-qPCR) in females (red) and males (blue). D) mRNA levels of genes altered in male cohort (n=16) in comparison with healthy controls (n=5). E) mRNA levels of genes altered in female cohort (n=25) in comparison with healthy controls (n=5).

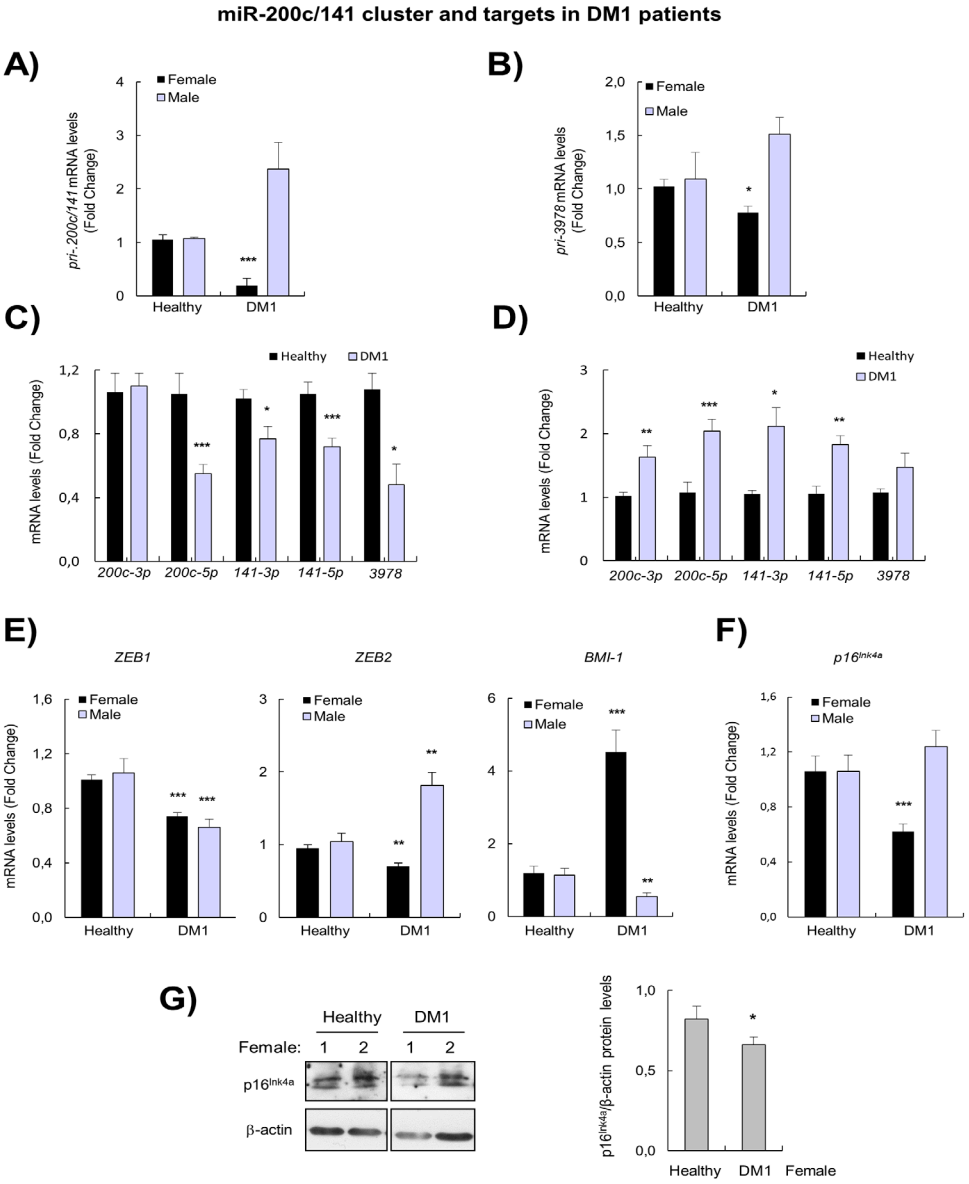

Figure 2. miR-200c/141 cluster and its targets in DM1 patients.

A, B) mRNA expression of two miRNA precursor, *miR-200c/141* and *miR-3978,* which are downregulated in DM1 females (n=25) and upregulated in DM1 males (n=16). C, D) Mature forms of these miRNAs are downregulated in DM1 females (left, n=25) and upregulated in DM1 males (right, n=16). E) In these group of patients *ZEB1* is downregulated in both sexes, while *ZEB2* only in DM1 females. On the contrary, *BMI1* is upregulated in DM1 females and downregulated in DM1 males. F) *p16^{INK4A}* expression is decreased in DM1 females (n=25). G) Representative Immunoblots and quantification of p16^{INK4A} derived from two different blood samples of healthy controls and DM1 females (n=4).

Discussion

There is recent accumulating evidence that patients with DM1 are at high risk of developing cancers. Confirming these findings in an independent study and in different DM population is important for patient clinical management. Using data from a large molecularly confirmed cohort of DM1 patients, we showed a statistically significant excess risk of endometrium, ovary, thyroid, and brain cancer. In the largest epidemiological cohort published so far accounting more than 1600 myotonic dystrophy 1 and 2 patients, Gadalla *et al.,* found a clear high risk for cancers of the endometrium, brain, ovary and colon, and possibly thyroid and choroidal melanoma and non-melanoma skin (7). In smaller studies, Win et al. reported a higher risk (8) for developing thyroid cancer and choroidal melamona whereas Mohamed et al. (9) concluded that there was a high risk for thymoma, gynecologic and lung cancer. Recently in a small published cohort, Bianchi et al (10) found that skin, thyroid, ovary and breast cancers were most frequent in DM patients.

Our results are coincidental with that reported by Gadalla et al. in a population-based study of more than 1600 DM patients (7) with the exception of colorectal cancer. However, the CI (0.94-3.92) of this type of cancer was near to significance values and the smaller sample size in our cohort could explain this result. Interestingly, colorectal cancer was also the most frequent among those patients that had two or more cancers. The excess risks of thyroid (8,10), endometrium and ovary (9,10) were also found in smaller studies. Of importance, as have been also pointed out by Gadalla et al (4,7), we did not find an overrepresentation of screening-related cancers (i.e. breast and prostate), strongly suggesting that the results are genuine and not biased by the close medical surveillance in DM patients. The fact that there are no incidence and mortality cancer differences between the Basques and other regions in Spain (23), lead us to discard that the excess of cancer in this population could be explained due to other genetic factors rather than the DM1 condition itself.

Our data shows that malignancies developed at a mean age of 46.6 ± 14.7 years. In agreement with previous reports (3,24) cancers represented the third leading cause of death after respiratory and circulatory diseases in our DM1 population. A progressive increase in cumulative incidence of cancer mortality has been previously reported (2% by age 50 to 6% by age 70) (4).

Other striking feature is the apparent absence of a correlation between cancer risk and nucleotide repeat length. This result could be potentially biased by methodological reasons because CTG repeat expansion size was measured years before cancer emergence and also because of the somatic instability that characterizes DM1. Based on the differences found between tumoral and healthy tissues in DM1 patients, some authors have suggested that there might be an underlying independent

mechanism of somatic instability in the tumoral tissue (25-27). However, the later contrast with the recent finding that MBLN1, a splicing factor sequestered in the nuclear foci of DM1 cells which correlates with CTG expansion size, could be implicated in the physiopathology of the progression of certain cancers (28).

Our results showing a differential transcriptional profiling of several genes previously linked to cancer such as PDK4 (29), DAPK1 (30), Caspase 5 (31) or PLA2G7 (32) genes in DM1 patients compared to healthy controls, open the door to a mechanistic explanation of this increased oncogenic risk in DM1. In addition, we observed a downregulation of miR-200c, miR-141 and miR-3978 mature and precursor forms in DM1 female patients. Of note, two of them, miR-200c and miR-141, belong to the same miR-200 tumor suppressor cluster Lower levels of the miR-200c family members were detected in tumor tissues, and blood-derived samples in a wide range of cancer types (33). This decline was associated with tumor progression and poor prognosis including metastasis (34,35).

miR-200 family has several well described oncogenes as downstream targets likely ZEB family and the polycomb group gene BMI1 becoming crucial targets (18-21,36). Our results show that these genes are altered in both sexes of DM1 patients supporting the relevance of this molecular signaling on the disease. In particular, DM1 women patients express elevated BMI1 levels, whilst ZEB1 and 2 are decreased. The existence of such alterations in patients with severe disease and no cancer prompted us to hypothesize that the downregulation of miR-200 cluster members play a role in cancer susceptibility and consequent upregulation of BM1 instead of through ZEB may be required for its development. In support of this idea (I) ZEB factors are EMT inducers and their role has been associated to metastasis rather than tumor formation; (II) The expression of $p16^{INK4a}$ tumor suppressor, a critical target of BMI1, inversely correlates with BMI1 levels in DM1 patients; (iii) The role of miR-200 and BMI1 in the maintenance of adult stem cells and tumor initiating cells in many organs. Of note that many of the cancers detected in DM1 patients emerge in tissues with a high rate of cellular replications.

Taken together, our findings support a model through a coordinated action between the miR-200 expression and its downstream targets ZEB1/2 and BMI1. In support of this notion there is a close functional link between miR-200 family and ZEB factors and BMI1 in a double-negative feedback loop respectively. Thus, the activation of one of them affects the expression and activity of the others (37,38).

Our study strength includes the population-based design (all DM1 patients in the Gipuzkoa population were included), and therefore no selection bias exists. We are limited by the lack of information of known cancer risk factors such as smoking, diet, lifestyle, environmental influences or alcohol intake among others. DM1 patients

have more obesity and the prevalence of tobacco smoking is higher than in general population (39) so these risk factors might have played a confounder role in our results and should be taken into account when planning longitudinal studies. However, a recent study suggested that lifestyle factors in DM1 patients do not explain the observed excess risk of cancers (10).

We believe that our study provides independent cancer site-specific confirmation of recently-reported excess cancer risks as part of the DM1 phenotype, especially for women, and suggests that this association could be more related to a transcriptomic regulation of oncogenic pathways than a direct consequence of the DM genomic signature.

Material and Methods

Study population

Data from patients were retrospectively obtained from the medical records of the Gipuzkoa historical myotonic dystrophy cohort, established in 1985. We identified 503 patients with molecularly confirmed DM diagnosis between 1985 and 2013. We excluded 4 patients with DM2 diagnosis and 75 DM1 patients due to the lack of data or lost to follow-up.

From patients medical records we extracted the following information: sex, age at the time of the study enrollment and age of death, nucleotide expansion size (CTG triplets), calendar year of diagnosis, disease severity assessed using the Muscular Impairment Rating Scale (MIRS) scale (12) at the time of the last visit, age and calendar year of cancer diagnosis and cancer anatomic site. Diagnoses of all types of malignant neoplasia were coded using the International Classification of Diseases (ICD) Revision 9. Death causes were coded regarding ICD-10 (2014 version) (13).

Genetic analysis

CTG repeat was measured at the time of DM1 diagnosis by conventional PCR and Southern Blot. Conventional PCR was performed with 100 ng of genomic DNA using gene specific primers flanking the *DMPK* CTG repeat. All normal homozygotes and expanded alleles were confirmed with Southern Blot. We included patients with 40 CTG repeats or more due to the demonstrated instability of the fragment size from this threshold, independently of the presence or absence of clinical manifestations assuming that if we eliminate the cases considered as premutations, we neglect a possible impact of genomic condition over cancer prone.

Statistical Analysis

Follow-up started at date of DM1 diagnosis and ended at date of first cancer diagnosis, death, or last visit. We calculated standardized incidence ratios (SIRs) by dividing the observed numbers of cancer by the expected for all cancers combined and cancer-specific anatomic sites, overall and stratified by sex. Expected numbers were calculated by multiplying the age- and sex- specific incidence rate from the Basque population cancer registry (14) to the person-years of the study cohort. SIRs were obtained for all types of cancers except for basal cell carcinoma of the skin because this type of cancer is not collected in the registry. Confidence intervals were calculated using the Poisson distribution.

Mean repeat length was compared for patients with and without cancer using Student t-test. All tests were considered statistically significant if $p<0.05$. Statistical analysis was performed using STATA-SE 12 (StataCorp LP, College Station, TX).

Data are presented as mean values ± S.E.M., with the number of experiments (n) in parentheses. Unless otherwise indicated, statistical significance (p-values) was calculated using the Student´s t -test. Asterisks (*, **, and ***) indicate statistical significance ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

Transcriptomic analysis

This analysis included 10 DM1 patients (5 women, 5 men; mean age: 43.4 ±5.16 years; expansion size: 750 ± 306 CTG triplets) who had moderate and severe clinical manifestations (MIRS median= 3, range 3-5) and no malignancy prior or at time of blood collection and 10 age and sex-matched healthy controls. We extracted RNA from Peripheral blood mononuclear cells using the LeucoLOCKTM Total RNA Isolation System (Life Technologies, Carlsbad, California, USA). For the RNA extraction, we use a two-step protocol, first with the miRNesay Mini Kit followed by automated RNA extraction in the QIAcube. Large scale gene expression was measured by the Human Gene 1.0 ST Affymetrix microarray (Affymetrix, Santa Clara, California, USA). RNA integrity was checked with Agilent RNA 6000 Nano kit. Those samples with an RNA Integrity Value (RIN) above 7 were accepted to be processed. 300 ng of total RNA were used for microarray analysis following the manufacturer's instructions.

We analyzed gene expression differences in DM1 patients and healthy controls and in a second step; we studied the differences between DM1 patients by sex. Results of the microarray data were extended by RT-PCR in additional DM1 male (n=16) and female (n=25) subset of patients.

Western Blot analysis

We performed a Western Blot of the protein products of the targets altered in the transcriptomic analysis. Immunoblots were performed as previously reported (15). We used ab16123 (Abcam) for $p16^{INK4a}$ detection and A-5441 (Sigma) for β-actin and HRP-linked anti-rabbit or anti-mouse (Santa Cruz Biotechnology) secondary antibody at a 1:2000 dilution. Detection was accomplished by chemiluminescence using NOVEX ECL Chemi Substrate (ThermoFisher).

Standard protocol approvals, registrations and patient consents

This study was approved by the Donostia Universitary Hospital Ethical Board and conducted in accordance with the Declaration of Helsinki ethical standards.

Acknowledgements and Funding

We thank S Gadalla (NIH) for reviewing this manuscript and for her helpful suggestions, L. Manterola for miRNA analysis and all of the myotonic dystrophy patients. RF-T was recipient of a Rio Hortega from the Instituto de Salud Carlos III. AM was granted with an Ikerbasque Research Fellow position. MG-P was recipient of a predoctoral fellowship from UPV/EHU (PIF 15/245). This work was supported by CIBERNED, and grants from Isabel Gemio Foundation, Diputacion Gipuzkoa (OF30/2014), Ministry of Economy and Competition and FEDER Funds to AL-M (FIS PS 09–00660 and PI147436) and AM (PI13/02277), and European Union (Marie Curie CIG 2012/712404).

Author contributions

RF-T; MD. had the original idea, collected clinical data from the patients, evaluated the patients, did the clinical and statistical analysis and wrote the manuscript. MG-; BSc did the transcriptomic analysis and wrote the manuscript. JI-E; MD., PhD, did the statistical analysis, reviewed the Ethics and wrote the manuscript. AMC; BSc, PhD, did the molecular analysis and improved the manuscript. MZ; BSc, collected clinical and molecular data, did the transcriptomic analysis and improved the manuscript. JJP, JBE and MM; MD. collected clinical data, evaluated the patients and improved the manuscript. IR; MD., did the anatomopathologic analysis and improved the manuscript. LM; BSc, PhD, did the molecular analysis and improved the manuscript. DO; BSc, PhD, did the transcriptomic analysis and improved the manuscript. AM; BSc, PhD, had the original idea, did the transcriptomic analysis and wrote the manuscript. AL-M; MD., PhD, had the original idea, collected clinical data from the patients, followed and evaluated them, did the clinical analysis and wrote the manuscript.

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Supplementary materials

Table e-1. Causes of death in DM1 patients regarding ICD-10.

Causes of death	n (%)
Certain infectious and parasitic diseases (A00 B99)	**4 (2.9%)**
A 41 Sepsis	3
B 20 HIV	1
Neoplasms (C00-D48)	**21(15.3%)**
Diseases of the nervous system (G00-G99)	**1(0.7%)**
G 61 Guillain-Barré síndrome	1
Mental and Behavioral disorders (F00-F99)	**1(0.7%)**
F 00 Dementia in Alzheimer's disease	1
Diseases of the circulatory system (I00-I99)	**33 (24.1%)**
I 21 Acute myocardial infarction	1
I 26 Pulmonary embolism	3
I 46 Cardiac arrest	6
I 47 Paroxysmal tachycardia	3
I 50 Heart failure	15
I 61 Intracerebral haemorrhage	1
I 62 Subdural hematoma	1
I 63 Cerebral infarction	3
Diseases of the respiratory system (J00-J99)	**52 (37.9%)**
J 15 Bacterial pneumonia, not elsewhere classified	11
J 69 Pneumonitis due to food and vomit	14
J 96 Respiratory failure	27
Diseases of the digestive system (K00-K93)	**2 (1.4%)**
K 23 Duodenal ulcer with acute hemorrhagic complication	1
K 56 Paralytic ileus and intestinal obstruction without hernia	1
Injury, poisoning and certain other consequences of external causes (S00-T98)	**1(0.7%)**
T 17.9 Foreign body in respiratory track	1
External causes of morbidity and mortality (V01-Y98)	**1(0.7%)**
V01-V99 Transport accidents	1
Unknown	**21 (15.3%)**
Total	**137**

Table e-2. Location and cancer histological subtypes.

Cancer location site	Sex		Total
	Female	Male	
Digestive Organs and Peritoneum			**17**
Colon adenocarcinoma	4	5	9
Rectum adenocarcinoma	2	1	3
Stomach adenocarcinoma	1	2	3
Liver hepatocarcinoma	0	2	2
Genitourinary Organs			**15**
Uterus adenocarcinoma	5	0	5
Kidney white cells	2	1	3
Ovary	3	0	3
Prostate adenocarcinoma	0	2	2
Seminoma	0	1	1
Bladder	0	1	1
Bone, Connective tissue, skin			**10**
Basocelular skin cancer	6	2	8
Melanoma	1	0	1
Maxilar carcinoma	0	1	1
Thyroid			**8**
Papillar	3	2	5
Anaplasic	0	2	2
Folicular	1	0	1
Lung			**4**
Non small cell	0	3	3
Small cell	0	1	1
Breast	4	0	**4**
Brain and nervous system			**5**
Glioblastoma multiforme	2	0	2
Anaplastic astrocytoma	1	1	2
Schwanoma	1	0	1
Other endocrine glands and related structures			**4**
Multiple endocrine neoplasia	1	0	1
Carcinoid tumor	0	1	1
Hypophisis macroadenoma	0	1	1
Pheochromocytoma	1	0	1
Lymphatic and Hematopoietic tissue			**3**
Non Hodking lymphoma	0	2	2
Chronic lymphoid leukemia	0	1	1
Total			**70**

Table e-3. List of cancer location and subtypes of those patients with more than one cancer.

Sex	CTG expansion size	1st cancer type and location	Age when first cancer	2nd cancer type and location	Age when second cancer	3rd cancer type and location	Age when third cancer
Female	1333	Papilar thyroid cancer	43	Renal white cells	50		
Female	1500	Papilar thyroid cancer	40	Colon adenocarcinoma	51		
Male	97	Melanoma	52	Basoceular skin cancer	61	Thyroid anaplasic	62
Male	80	Renal white cells	67	Colon adenocarcinoma	69		
Male	80	Papilar thyoid	50	Colon adenocarcinoma	51		
Female	80	Colon adenocarcinoma	51	Breast carcinoma	57		
Female	1833	Uterus adenocarcinoma	53	Stomach adenocarcinoma	59		

CAPÍTULO 2. Leukocyte telomere length in patients with myotonic dystrophy type I: a pilot study

Artículo publicado en
Annals of Clinical and Translational Neurology
Factor de Impacto 4.65 - Cuartil 1

Wang Y, Best A, Fernández-Torrón R, Alsaggaf R, **García-Puga M,** Dagnall CL, Hicks B, Thompson M, Matheu A, Zulaica M, Greene MH, López de Munain A, Gadalla SM. **Leukocyte telomere length in patients with myotonic dystrophy type I: a pilot study.** Ann Clin Transl Neurol. 2020 Jan;7(1):126-131. doi: 10.1002/acn3.50954. Epub 2019 Dec 5.

Submitted: 16 October 2019. Accepted: 31 October 2019. Published: 7 December 2019.

Leukocyte telomere length in patients with myotonic dystrophy type I: a pilot study

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Resumen

En este trabajo analizamos la longitud telomérica relativa (RTL) en 361 pacientes de la cohorte de pacientes con DM1 de Guipúzcoa y en 223 controles sanos que poseían un grado de parentesco con los pacientes DM1. Aunque no encontramos diferencias en la RTL entre controles y pacientes DM1, sí encontramos un acortamiento acelerado con el tiempo en la RTL en DM1, tanto en la cohorte de descubrimiento (cambio de −0.013 en pacientes con DM1 vs. −0.005 en controles sanos, P = 0.04), como en la cohorte de validación (cambio de −0.005 en pacientes con DM1 vs. −0.002, en controles sanos P = 0.33).

Nuestros resultados sugieren que el acortamiento telomérico no juega un papel directo en la etiología de la DM1, pero puede ser una consecuencia de alteraciones moleculares que se dan con el tiempo en pacientes con DM1.

Contribución

Mi contribución en este trabajo ha sido la preparación del ADN de la cohorte de pacientes con DM1 y de los controles sanos, además de la correlación entre los datos clínicos (expansión CTG, escala MIRS, edad y sexo) con la RTL.

Abstract

Myotonic dystrophy type I (DM1) is an autosomal dominant disease of which clinical manifestations resemble premature aging. We evaluated the contribution of telomere length in pathogenesis in 361 DM1 patients (12 with serial measurements) and 223 unaffected relative controls using qPCR assay. While no differences in baseline leukocyte relative telomere length (RTL) was noted, the data suggested an accelerated RTL attrition in DM1 (discovery cohort: T/S change/year = −0.013 in DM1 vs. −0.005 in controls, P = 0.04); similar trend was noted in validation cohort. Further investigations are needed to examine the role of TL in the pathophysiology of DM1.

Introduction

Myotonic dystrophy type 1 (DM1) is an autosomal dominant multisystem disorder resulting from an unstable trinucleotide (CTG)n repeat expansion in the 3' untranslated region of the dystrophia myotonica-protein kinase (DMPK) gene[1]. Clinical manifestations of DM1 include progressive muscle weakness and wasting, myotonia, premature cataracts, cardiac conduction defects, gastrointestinal/ endocrine abnormalities, and increased risk of certain cancers[2, 3]. It has been suggested that the DM1 phenotypes may resemble a premature aging syndrome[4].

Telomeres are tandem hexanucleotide (TTAGGG)n repeats and protein complexes at the end of chromosomes, maintaining genomic stability[5]. They shorten with each cell division[6] and are marker of cellular aging. Few studies suggested a link for telomere length in the pathogenesis of Duchenne (DMD) or limb girdle muscular dystrophies[7, 8]. Telomere length studies in DM1 were limited; an in vitro investigation using DM1 muscle precursor cells from three fetuses with congenital DM1 showed abnormal accumulation of $p16^{INK4a}$ and higher telomere attrition per cell division compared with controls[9].

Here we compared leukocyte relative telomere length (RTL) in a large cohort of genetically-confirmed DM1 patients and DM1 mutation-free clinically healthy relative controls and evaluated telomere length attrition overtime in a subset of patients with serial blood samples.

Results

Characteristics of study participants

DM1 patients and controls showed similar ages (median age = 42.0 and 43.0 years in DM1 vs. controls, respectively) and sex distributions (proportion of males = 45.7% and 43.9% in DM1 vs. controls, respectively). No age or sex differences were noted between DM1 patients in the two study subsets, or between patients and relatives in each set ($P > 0.05$). Patients with DM1 in the discovery and validation cohorts also had similar CTG repeat size (median repeat size = 433 and 500 for discovery vs. validation cohorts, respectively, $P = 0.37$) **(Table S1)**.

In DM1 patients, the size of CTG repeat was inversely correlated with age at genetic testing in both discovery ($r = -0.26$, $P = 0.0003$) and validation cohorts ($r = -0.21$, $P = 0.02$) **(Fig. S1A-B)**.

The patients with a second blood sample showed similar age, and sex distribution. However, despite of lack of statistical significance, the data suggested that patients with subsequent sample may have a less severe disease as evident by shorter CTG repeat sizes (median = 167 vs. 500, in those with and without subsequent sample, respectively) and less Muscular Impairment Rating Scale (MIRS) (0% vs. 24% with MRIS > 3, respectively) **(Table S2)**.

Telomere length in DM1 patients and controls

RTL was inversely correlated with age among both DM1 patients and controls (discovery cohort: $r = -0.39$, $P < 0.0001$ for DM1 and $r = -0.51$, $P < 0.0001$ for controls; validation cohort: $r = -0.27$, $P = 0.002$ for DM1 and $r = -0.23$, $P = 0.07$ for controls; **Fig. S2A-B**).

No differences were observed in RTL between DM1 patients and controls at baseline: discovery cohort: (mean T/S ± SD) = 0.62 ± 0.20 and 0.60 ± 0.16 for DM1 and controls, respectively, $P = 0.13$; or in the validation cohort: (mean T/S ± SD)=0.42 ± 0.12 and 0.43 ± 0.12, respectively, $P = 0.78$, **Fig. 1A and B**). Age- and sex-adjustment did not alter the results. In DM1 patients, RTL was not associated with CTG repeat expansion size, nor with MIRS in models adjusted for age and sex **(Fig. 2A and B)**.

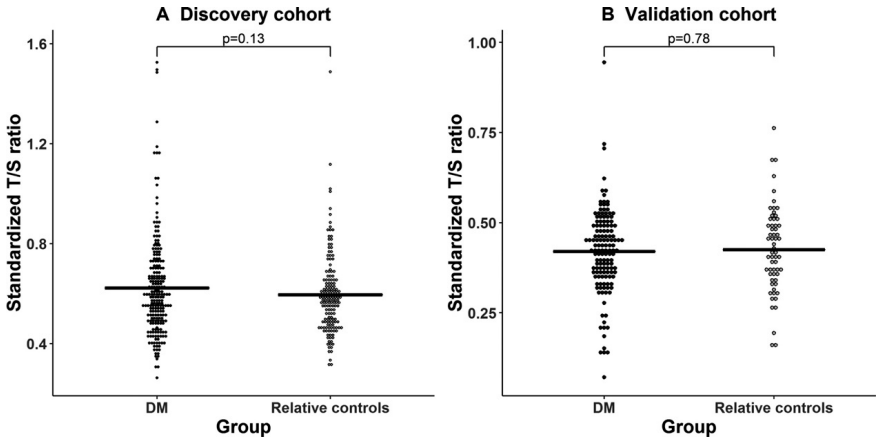

Figure 1. Comparison of relative telomere length between DM1 patients and their healthy relative controls. (A) Discovery cohort and (B) Validation cohort.

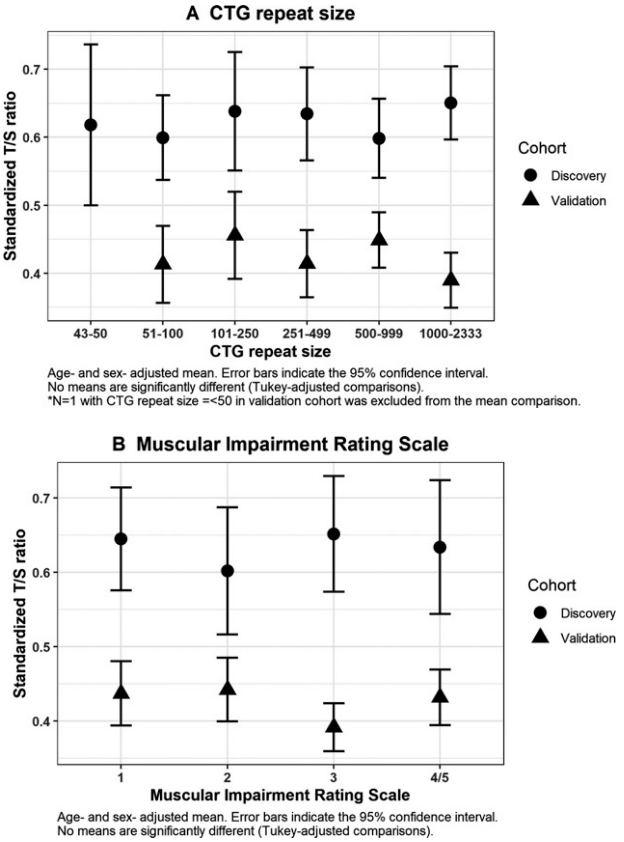

Figure 2. Relative telomere length in DM1 patients by CTG repeat size and by Muscular Impairment Rating Scale (MIRS) in age- and sex-adjusted models. (A) CTG repeat size, (B) Muscular Impairment Rating Scale.

Telomere length attrition overtime in patients with DM1

Baseline RTL was similar in patients with and without repeated RTL measurements (median T/S = 0.58 vs. 0.52, P = 0.27).

In the repeated RTL measurement analyses, and when compared with expected based on RTL attrition from relatives' cross-sectional data, a more rapid RTL decline was noted in DM1 in the discovery cohort (T/S decline/year = −0.013 vs. −0.005 for DM1 and controls, respectively, P = 0.04). A similar (non-significant) trend was noted in the validation cohort (T/S decline/year = −0.005 vs. −0.002, P = 0.33) **(Fig. 3A and B, Table S3)**.

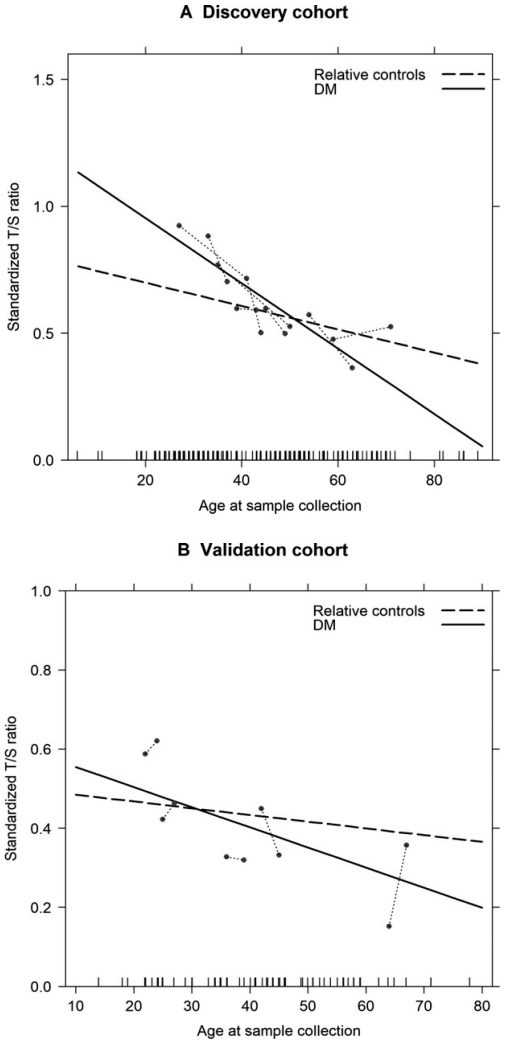

Figure 3. Comparison of annual relative telomere length attrition between DM1 patients and their healthy relative cont rols. (A) Discovery cohort, (B) Validation cohort.

Discussion

In this analysis of a large cohort of patients with genetically confirmed DM1 and clinically healthy relative controls, we observed no difference in leukocyte RTL measured at genetic testing between patients and controls, yet, a higher rate of annual telomere length attrition was noted in DM1 patients.

Prior DM1 telomere length studies have been limited to few small in-vitro investigations focusing on its possible role in muscle precursor cell dysfunction. In agreement with our results of similar leukocyte RTL in DM1 and controls, a study comparing telomere length in distal (with clinical and pathological alterations) and proximal muscles (relatively spared) from four DM1 patients showed no telomere length difference[14]. Yet, we showed a higher rate of RTL attrition overtime in DM1 patients than expected based on RTL attrition rate from controls. Our findings were also consistent with a study of muscle satellite cells in fetuses/infants with congenital DM1 which showed a faster telomeric DNA loss per cell division (171.9 ± 17 base pairs) when compared with control cells (108.1 ± 10 base pairs); the authors concluded that large CTG expansion repeats may interfere with telomere homeostasis in those patients[9]. Of note, we found no association between patients' leukocyte RTL and CTG repeat size; suggesting no direct role for repeat expansion size in the observed telomeric attrition. However, our data were limited by the unavailability of progenitor repeat size. Also, testing this hypothesis in blood cell (not known to be affected in DM1), may not be directly applicable to somatic changes in affected organs such as the muscles. Somatic mosaicism and instability in repeat expansion size are well-described in DM1[15]. It is possible that the accelerated telomere attrition observed in our DM1 patients is a consequence of the cellular exposure to oxidative stress. The triple-guanine sequence in telomeric DNA (TTAGGG)n is very sensitive to oxidative damage[16], resulting in accelerated telomere shortening[17]. Previous studies have shown a significant increase in blood level of free radicals and oxidative stress biomarkers in DM1 patients[18, 19], suggesting a role for oxidative stress in disease progression.

The observed differences in the rate of TL attrition between the discovery and validation cohort could be affected by the difference in the time interval between samples (median interval = 9 years in the discovery, and 3 years in the validation). It has been shown that TL elongation in longitudinal studies is mostly artifact related to measurement errors in studies with short follow-up (≤5 years) where TL changes are usually small and hard to capture[20].

Our study strengths include a relatively large sample size, the genetic diagnosis confirmation, the objectively healthy status of controls, and the availability of repeat expansion size. Limitations include the small sample size of patients with serial samples, who were not systematically sampled. Due to short time period between

blood sampling, observed TL elongation in some patients could be artifactual20. Our control data were based on baseline measurement of RTL in relatives because of the unavailability of serial samples; a prior study has shown small differences in TL attrition rate calculated from cross-sectional or longitudinal data in the general population (24.6 vs. 31 bp per year)[20]. The qPCR assay is sensitive to pre-analytic processing, notably DNA extraction method. Consequently, we presented our results stratified by DNA extraction method. Although similar trends were observed in the two datasets, we cannot rule out the possibility that the difference in DNA extraction methods and/or time between serial samples contributed to the observed difference in the rate of TL attrition between the two sets.

In conclusion, our results suggest that telomere shortening does not play a direct role in DM1 etiology but may be a consequence of molecular alterations over time, possibly secondary to oxidative stress. It is possible that accelerated telomere shortening may mediate the age-related phenotypic presentation of DM1.

Materials And Methods

Study participants

We used data and samples from 379 DM1 patients and 235 DM1-free relative controls from the Spanish Guipúzcoa historical myotonic dystrophy cohort10. Participants' demographics and clinical information were obtained through medical records review. We excluded 30 individuals (28 missing age, one twin, and one with failed quality control metrics for RTL assay). The final analysis included 361 DM1 patients and 223 controls. Twelve participants provided a second sample for RTL measurement, as convenience permitted.

The study was approved by the Donostia University Hospital Ethical Board and by the Office of Human Subjects Research Protections at the National Institutes of Health.

DNA extraction and telomere length measurement

Blood DNA was extracted using the salting out procedure or Qiagen Flexigen DNA kit (QIAGEN, Germantown, MD). We measured RTL after adapting the originally published quantitative polymerase chain reaction (qPCR) method11. Method details are available elsewhere[12]. Briefly, qPCR assay measures a ratio between telomere repeats amplification (T) to that of an autosomal single-copy gene (S; *36B4*). The T/S ratio then get normalized using internal QC calibrator samples to yield a standardized T/S ratio. All samples were assayed in triplicate. The coefficient of variation for internal control samples was 4.5%.

Statistical analysis

All analyses were stratified by DNA extraction method to account for known variable qPCR sensitivity to extraction method[13]. Samples extracted by salting out (*N* = 223 patients and 162 controls) served as a discovery cohort, and those extracted by Qiagen Flexigen DNA kit (*N* = 138 patients and 61 controls) served as a validation cohort. RTL data from serial samples extracted by the same method were available for 12 DM patients (*N* = 7 for discovery, *N* = 5 for validation cohort).

We used generalized linear models to compare the mean RTL between DM1 and controls, and in DM1 patients by CTG repeat size categories (40–50, 51–249, 250–499, ≥500). A random intercept linear mixed model was used for repeated measurement analysis; RTL annual attrition rate in DM1 patients with repeated samples (median time between samples = 9 years, range = 4-17 years in the discovery, and median time = 3 years, range = 2–3 years in the validation) was compared with that expected from regression models of RTL and age using control cross-sectional data using R package 'lme4'. Differences in RTL attrition rates between DM1 patients and controls were assessed by an interaction term between DM1-control status and age. Statistical analyses were conducted using SAS version 9.4 and R version 3.4.4.

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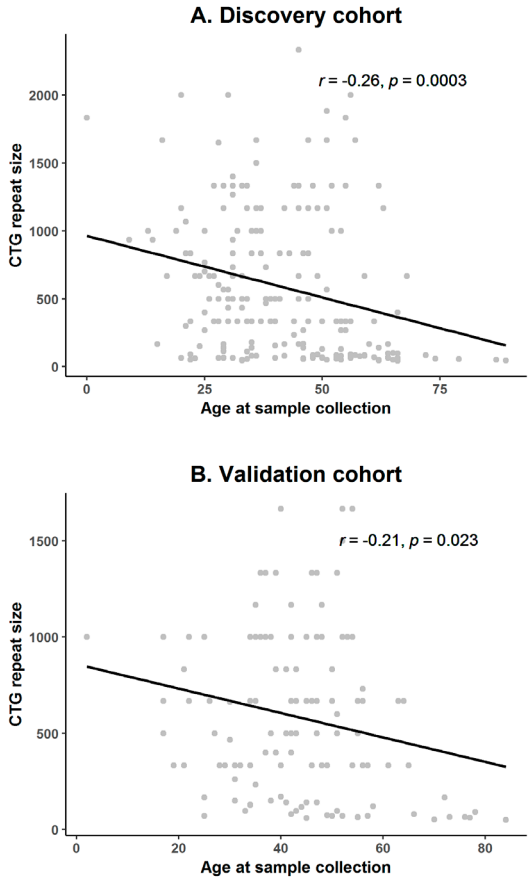
Acknowledgments

We thank the patients and their family members for their participation. The authors declare no conflict of interest.

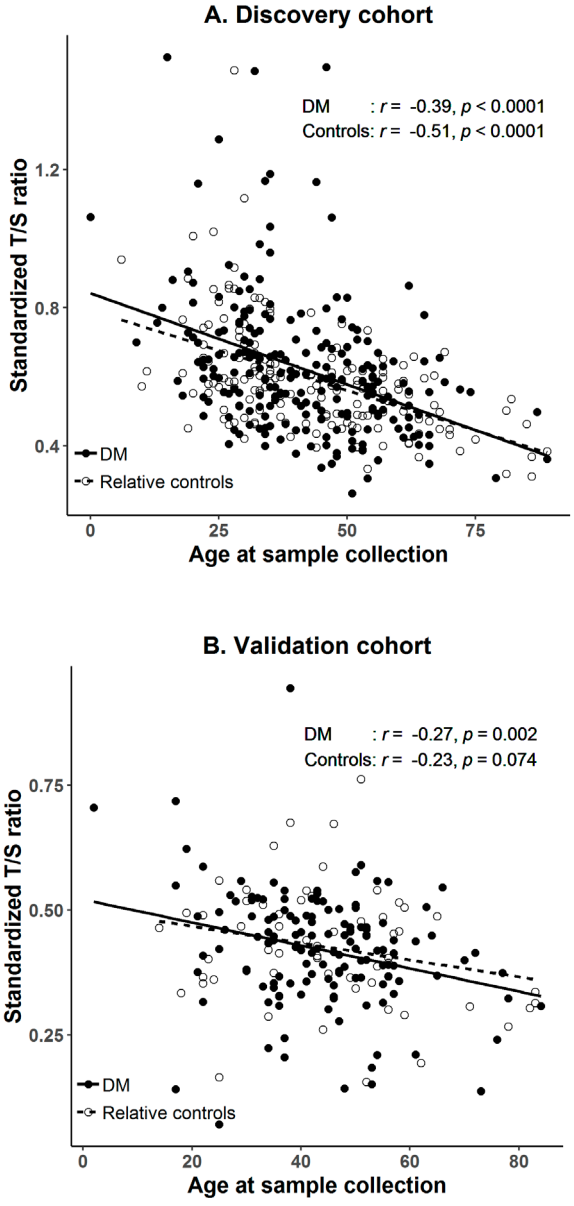
Funding information

The study was supported by the Intramural Research Program of the Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health.

Supplementary materials



Supplemental Figure 1. Correlation between CTG repeat size and age in DM1 patients. A. Discovery cohort, B. Validation cohort.



Supplemental Figure 2. Correlation between relative telomere length and age. A. Discovery cohort, B. Validation cohort.

Supplemental Table 1. Baseline characteristics of DM1 patients and their unaffected relative controls, stratified by DNA extractioan method.

Characteristics	Discovery cohort		Validation cohort	
	DM (n=223)	Relative controls (n=162)	DM (n=138)	Relative controls (n=61)
Age, median (range)	40 (0-89)	38.5 (6-89)	43.5 (2-84)	44 (14-83)
CTG repeat size, median (range)[1]	433 (43-2333)		500 (50-1667)	
Muscular Impairment Rating Scale[2]				
1	38 (33%)		25 (19%)	
2	25 (22%)		26 (20%)	
3	30 (26%)		46 (35%)	
4	22 (19%)		32 (24%)	
5	1 (1%)		2 (2%)	
Male, n (%)	98 (44%)	67 (41%)	67 (49%)	31 (51%)
Calendar year at sample collection, n (%)				
1989-2001	60 (27%)	54 (33%)	4 (3%)	0 (0%)
2002-2005	82 (37%)	48 (30%)	0 (0%)	0 (0%)
2006-2011	65 (29%)	53 (33%)	15 (11%)	13 (21%)
2012-2016	16 (7%)	7 (4%)	119 (86%)	48 (79%)

[1] CTG repeat size are unknown in 43 DM1 patients (discovery cohort n=26, validation cohort n=17).

[2] Muscular Impairment Rating Scale (MIRS) was unknown or not clearly defined in 114 DM1 patients (discovery cohort n=107, validation cohort n=7).

Supplemental Table 2. Comparison of baseline characteristics of DM1 patients with and without repeated relative telomere length measurement.

Characteristics	DM1 patients				p
	No subsequent sample (N=349)		With subsequent sample (N=12)		
Age, median (min, max)	42.0	(0, 89.0)	37.5	(22.0, 59.0)	0.50[1]
CTG repeat size[2], median (range)	500.0	(43.0, 2333.0)	167.0	(45.0, 1333.0)	0.17[1]
Muscular Impairment Rating Scale[3]					0.28[4]
1	58	(25%)	5	(42%)	
2	48	(20%)	3	(25%)	
3	72	(31%)	4	(33%)	
4	54	(23%)	0	(0%)	
5	3	(1%)	0	(0%)	
Male, n (%)	158	(45%)	7	(58%)	0.37[5]
Calendar year at sample collection, n (%)					0.01[4]
1989-2001	60	(17%)	4	(33%)	
2002-2005	78	(22%)	4	(33%)	
2006-2011	76	(22%)	4	(33%)	
2012-2016	135	(39%)	0	(0%)	

[1] Wilcoxon rank-sum test.
[2] CTG repeat size are unknown in 43 DM patients (discovery cohort N=26, validation cohort N=17).
[3] Muscular Impairment Rating Scale (MIRS) was unknown or not clearly defined in 114 DM1 patients (discovery cohort n=107, validation cohort n=7).
[4] Fisher's exact test.
[5] Chi-square test.

Supplemental Table 3. Telomere length attrition in DM1 patients and controls.

	Discovery cohort (DM1 N=7, controls N=162)			Validation cohort (DM1 N=5, controls N=61)		
Effect	ß	SE	p	ß	SE	p
Intercept	0.798	0.029	<.0001	0.526	0.046	<.0001
DM1 *vs.* controls	0.420	0.155	0.031	0.104	0.130	0.468
Age	-0.005	0.001	0.0001	-0.002	0.001	0.134
DM*Age	-0.008	0.003	0.036	-0.003	0.003	0.333
Sex	-0.017	0.021	0.437	-0.047	0.029	0.178

* Models included age, DM, age-DM interaction term and sex.

CAPÍTULO 3. Whole transcriptome analysis and functional studies reveal that senescence plays a pivotal role in Myotonic Dystrophy type 1

Artículo en revisión en Nature Communications
Factor de Impacto 11.88 - Decil 1

García-Puga M, Saenz-Antoñanzas A, Gerenu G, Arrieta-Legorburu A, Fernández-Torrón R, Zulaica M, Saenz A, Araúzo-Bravo M, López de Munain A, Matheu A. **Whole transcriptome analysis and functional studies reveal that senescence plays a pivotal role in Myotonic Distrophy type 1.**

Submitted: 18 November 2019. Accepted with major revision: 26 February 2020.

Whole transcriptome analysis and functional studies reveal that senescence plays a pivotal role in Myotonic Dystrophy type 1

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Resumen

En este trabajo, mediante la realización de un estudio transcriptómico en fibroblastos derivados de pacientes con DM1 hemos descrito la alteración de las vías moleculares implicadas en la regulación del ciclo celular, la división celular y la respuesta al daño en el ADN. Estas alteraciones moleculares estarían favoreciendo el desarrollo de una senescencia prematura en los fibroblastos DM1. Estudios de pases seriados *in vitro* confirmaron el desarrollo prematuro de senescencia y la adquisición de un fenotipo secretor asociado a dicha senescencia en los fibroblastos DM1.

Mediante estudios funcionales describimos la implicación de la ruta BMI1/p16^{INK4a} en los fenotipos celulares asociados a la DM1. Los resultados de los análisis del transcriptoma en fibroblastos se corroboraron en mioblastos humanos y en muestras de sangre, así como en modelos de la enfermedad en ratón y en mosca. Por otra parte, el tratamiento con compuestos senolíticos como quercetina, dasatinib y navitoclax revierte el fenotipo de envejecimiento acelerado *in vitro*, pero también en un modelo de DM1 de *Drosophila melanogaster.*

Hemos identificado la acumulación de procesos relacionados con la senescencia celular como mecanismos alterados en la DM1, los cuales son revertidos mediante terapia con compuestos senolíticos.

Contribución

Mi contribución en este trabajo ha sido la realización de todos los experimentos a excepción del análisis original del microarray de expresión y los ensayos en *Drosophila melanogaster*. Además, he contribuido al análisis de los resultados generados y en la escritura del manuscrito.

Abstract

Myotonic dystrophy type 1 (DM1; MIM #160900) is an autosomal dominant disorder, clinically characterized by progressive muscular weakness and multisystem degeneration. The broad phenotypes observed in DM1 patients resemble the appearance of an accelerated aging process. However, the molecular mechanisms underlying these phenotypes remain largely unknown.

Transcriptomic analysis of fibroblasts derived from DM1 patients and healthy individuals revealed a decrease in cell cycle activity, cell division, and DNA damage response in DM1, all of which facilitated the accumulation of cellular senescence. Serial passage studies in vitro confirmed the accelerated increase in senescence and the acquisition of a senescence-associated secretory phenotype in DM1 fibroblasts.

Moreover, functional studies highlighted the impact of BMI1/p16^{INK4a} pathway deregulation in DM1-associated cellular phenotypes. The data from transcriptome analyses were corroborated in human myoblasts and blood samples as well as in mouse and Drosophila models of the disease. Importantly, treatment with the senolytic compounds, Quercetin, Dasatinib, or Navitoclax, reversed the accelerated aging phenotypes in both DM1 fibroblasts in vitro and in Drosophila in vivo.

Our results identified the accumulation of senescence-related processes as a major driver of DM1 pathophysiology and therefore, demonstrated the efficacy of senolytic compounds in the pre-clinical setting.

Introduction

Myotonic dystrophy (DM) is an autosomal dominant multisystem form of muscular dystrophy, with an estimated incidence of 1 in 8,000 people worldwide[1]. Based on genetic alterations, DM is clinically classified in two distinct forms. DM type 1 (DM1; MIM #160900), also known as Steinert's disease, is caused by the accumulation of CTG (cytosine-thymine-guanine) trinucleotide repeat expansions in the 3′ untranslated region of the dystrophia myotonic-protein kinase (*DMPK*) gene on chromosome 19q13.3[2]. DM type 2 (DM2; MIM #602668) results from a tetra-nucleotide CCTG expansion in the zinc finger protein 9 (*ZNF9*) gene on chromosome 3q21.3[3]. At the molecular level, when these genes are transcribed, pathogenic transcripts are generated and these are retained in the nuclei of cells as ribonuclear aggregates. These aggregates interfere with and disrupt RNA-binding proteins that regulate alternative splicing[4]. The accumulation of RNA toxicity plays a major role in the pathology of the disease, due to microsatellite expansion in noncoding regions that can be transcribed into pathogenic transcripts. This alters alternative splicing processes, modifies the bioavailability of splicing factors, and increases the number of fetal mRNA isoforms. Consequently, there are deregulated patterns of protein expression, with negative effects on multiple tissues, thus contributing to the multisystem pathogenesis of DM[5].

DM1 is more common than DM2 and represents a more severe phenotype[6]. In DM1, mutant *DMPK* transcripts accumulate in ribonuclear foci that interfere with two families of proteins that regulate alternative splicing, muscleblind-like (MBNL) and CUGBP Elav-like family (CELF). The MBNL family comprises MBNL1 and MBNL2 (jointly denoted MBNL1/2), which show overlapping patterns of expression in skeletal muscle, heart, and the central nervous system, and have partially redundant functions. In these tissues, the function of MBNL1/2 is reduced, while CELF1 is activated, by aberrant binding to CTG expansions. These changes maintain the fetal alternative splicing patterns of transcripts in adults, resulting in specific symptoms[7]. Importantly, the length of the CTG expansion increases with each generation and this is associated with an earlier age of onset of the disease and a more severe phenotype[8]. Thus, unaffected individuals carry less than 50 triplet repeats, whereas expansions ranging between 50 and 4.000 CTG repeats are present in affected individuals[8].

DM1 patients present a multisystem degenerative process that includes progressive muscular weakness and atrophy, myotonia, cardiomyopathy, insulin-resistance, cataracts, increased cancer incidence, dementia-related Tau protein deposits in the brain, metabolic syndrome, and premature death. Respiratory distress and heart failure are the most frequent causes of death[9,10]. Different pathogenic mechanisms, including alteration of autophagy[11,15], mitochondrial dysfunction[12,14,16,17], senescence

accumulation and telomere shortening[18-23], and genomic instability[24] have been associated with DM1 phenotypes. However, the detailed experimental validation of these mechanisms remains incomplete and the extent to which each mechanism contributes to the development of the disease has not yet been clearly defined.

In order to understand the underlying pathogenic mechanism of DM1, we performed a transcriptomic analysis of primary fibroblasts derived from healthy donors and DM1 patients. We found that DM1 cells showed impaired cell cycle progression, cell division, and DNA damage response (DDR), as well as increased accumulation of senescent cells. These results were validated in patient-derived myoblasts and peripheral blood mononuclear cells (PBMCs) as well as in *DMSXL* mouse[25] and REC2 *Drosophila*[26] models of the disease. Remarkably, treatment of human fibroblasts and myoblasts as well as the *Drosophila* model with senolytic compounds (Quercetin, Dasatinib, and Navitoclax) resulted in the recovery of multiple DM1-related phenotypes and may represent a potential therapy for these patients.

Results

DM1-derived fibroblasts resemble the molecular characteristics of DM1 patient´s pathophysiology

Fibroblasts constitute a well-established model for the study of cell aging processes *in vitro*[27]. Since DM1 is a multisystem disease that resembles accelerated aging[28], fibroblasts might be a good cellular model to understand the pathophysiology of the disease. With this purpose, we established fibroblast cultures from 7 different DM1 patients and 5 healthy donors **(Table 1).**

First, we determined whether fibroblasts recapitulated the molecular alterations observed in DM1 patients. Western blot, immunofluorescence and qPCR revealed that the expression of *DMPK*, *MBNL1*, and *SIX5* (a homeodomain-encoding gene adjacent to *DMPK*) was reduced at the mRNA and protein level, whereas CUGBP1 was elevated in DM1-derived fibroblasts **(Figure 1A-C)**. In addition, we detected the accumulation of nuclear foci **(Figure 1D)** and the expression of the neonatal isoform of *MBNL2*, with the inclusion of exon 7, in DM1 fibroblasts **(Figure 1E)**. Finally, there was a correlation between the severity of the molecular alterations and both the number of CTG expansions and the Muscular Impairment Rating Scale (MIRS) score **(Figure 1F-I)**. These results indicate that patient-derived fibroblasts maintain the altered molecular and genetic patterns of DM1 patients.

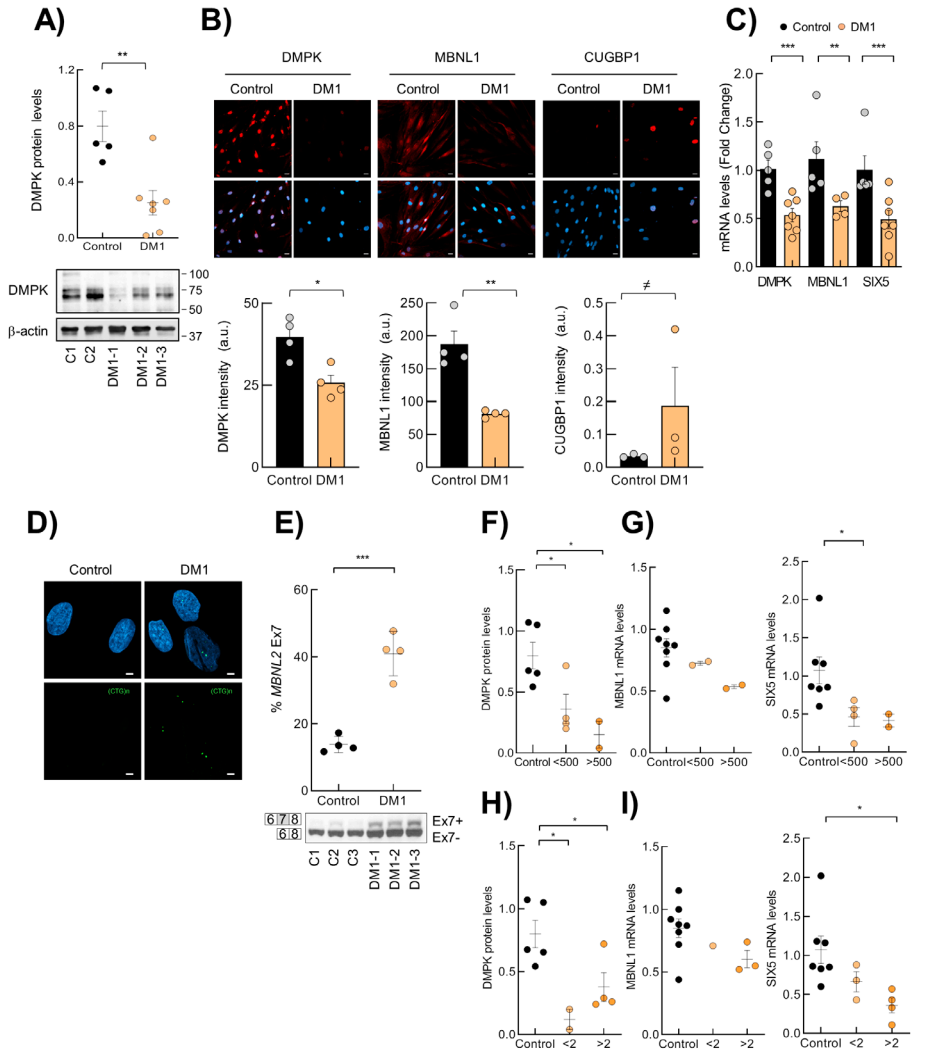


Figure 1. The molecular characteristics of DM1-derived fibroblasts resemble those of DM1 patient pathophysiology.

A) Representative image and quantification of DMPK protein levels in fibroblasts derived from DM1 patients and controls. B) Representative image and quantification of DMPK, MBNL1, and CUGBP1 immunofluorescence in fibroblasts derived from DM1 patients (n=3) and controls (n=3). C) *DMPK, MBNL1,* and *SIX5* mRNA levels in DM1 (n=7) and control fibroblasts (n=5). D) Representative fluorescence of FISH images showing (CAG)n *foci* in DM1 (n=4) and control fibroblasts (n=4) scale bar: 5 µm E) RT-PCR and representative image of alternative splicing changes of the *MBNL2* transcript in DM1 (n=4) and control (n=4) fibroblasts. Upper band represents the exon inclusion isoform (Ex7+) and lower band represents the exon exclusion isoform (Ex7-). F) DMPK protein levels in DM1-derived fibroblasts stratified by CTG expansion. G) *MBNL1* and *SIX5* mRNA levels in DM1-derived fibroblasts stratified by CTG expansion. H) DMPK protein levels in DM1-derived fibroblasts stratified by Muscular Impairment Rating Scale (MIRS). I) *MBNL1* and *SIX5* mRNA levels in DM1-derived fibroblasts stratified by MIRS. *In this figure each dot represents one patient and is the average of at least n=2 experiments.*

Transcriptomic study reveals multiple processes differentially altered in DM1 fibroblasts

Next, we performed transcriptomic studies in DM1 and control fibroblasts. We identified 378 genes that were significantly altered in DM1 fibroblasts, including 292 downregulated and 86 upregulated transcripts **(Table 2 and 3)**. Gene Ontology (GO) enrichment analysis showed that cell cycle, cell division and replication, and DDR were the processes most significantly altered in DM1-derived fibroblasts, all of which were downregulated **(Figure 2A,B)**.

Within the cell cycle-associated genes, there were genes involved in phase transition, such as *CDK2*, *CDK6*[29] and *FOXM1*[30]; regulators of cyclin-dependent kinase (CDK) inhibitors, such as *HMGA2*[31]; and proliferation markers, such as *Ki67*. We validated by qPCR the lower levels of *CDK2* and *HMGA2* in DM1 fibroblasts (Figure 2C). Among cell division, decreased expression of genes involved in DNA replication such as *CDC45*, *MCM3*, *MCM4*, *MCM2-7* and *GINS1*[32,33]; genes required to preserve the centromere position, such as *CENP-A*[34]; members of the constitutive centromere-associated network, such as *CENP-E*, *CENP-I*, *CENP-M* and *CENP-W*[35]; regulators of DNA replication and repair, like *TRAIP*[36]; and genes involved in chromosome condensation and mitosis, such as *AURKB*[37], were detected. qPCR confirmed the lower levels of *CENP-A*, *CENP-E*, *CENP-I*, *CDC45*, *MCM2*, *GINS1* and *AURKB* in DM1 fibroblasts **(Figure 2D)**. The expression of several DDR genes was also decreased including *BRCA1*, *BRCA2*, *RAD51*, *RAD51AP*, *FANCA* and *POLQ1,* which were validated by qPCR **(Figure 2E).**

On the contrary, processes involving extracellular matrix and potassium-related pathways were elevated in DM1 fibroblasts. Among them, the increased levels of *ABCC9, KCND2* and *SFRP2* expression were confirmed by qRT-PCR in DM1 cells **(Figure 2F)**. Taken together, our results reveal that critical processes for cell homeostasis, such as the cell cycle, cell division, DNA replication, and DDR are deregulated in DM1 cells.

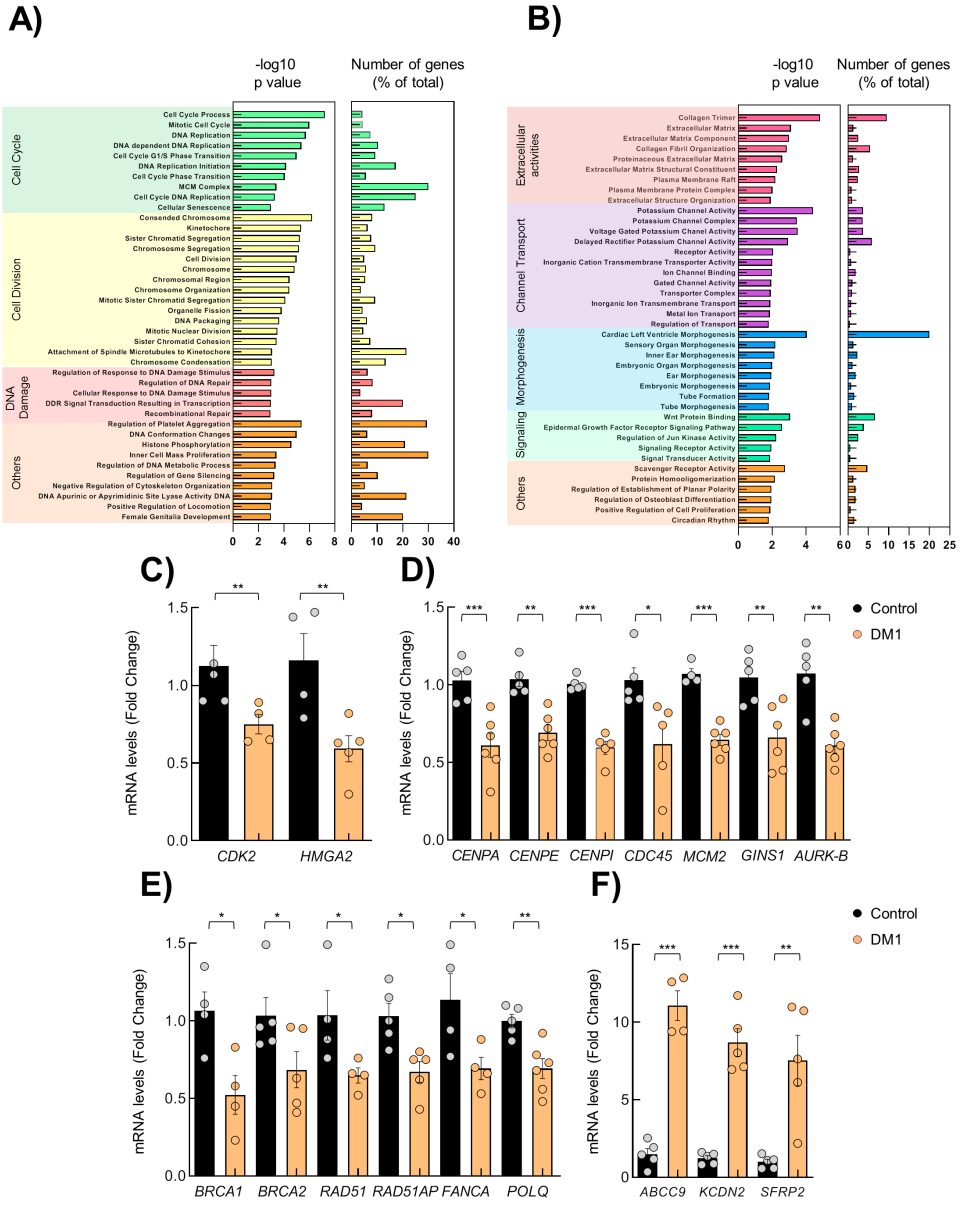


Figure 2. Transcriptomic studies reveal transcripts associated with multiple processes differentially expressed in DM1 fibroblasts.

A-B) Bar plot of the -log10(p-value) and percentage of genes altered in each term of the significantly **A)** downregulated and **B)** upregulated terms in DM1 fibroblasts (n=6) compared to controls (n=3). Longer bars correspond to greater statistical significance of the enrichment. qPCR analysis of the indicated genes related to **C)** Cell cycle; **D)** Cell division; **E)** DNA damage repair; and **F)** Extracellular matrix and potassium pathways in DM1 and control fibroblasts. *In this figure each dot represents one patient and is the average of at least n=2 experiments.*

In vivo validation in DM1-derived blood samples and muscle physiology

Next, we investigated whether these results could be translated to additional disease models and the clinical setting. Therefore, we first measured the expression levels of several of the aforementioned genes in peripheral blood mononuclear cells (PBMCs) from a cohort of DM1 patients established in Guipúzcoa (Basque Country, Spain)[38,39,23] (detailed information in **Table 4**). In agreement with previous studies[40], we found decreased levels of *DMPK1* and *MBNL1* when compared to a group of healthy individuals of similar age **(Figure 3A)**. Interestingly, DM1 samples displayed lower expression levels of *CDK2* and *HMG2A*, involved in the cell cycle, *AURKB, CDC45*, and *CENPA,* in cell division and *BRCA1*, *RAD50* and *RAD51*, in DDR **(Figure 3B)**. On the contrary, the levels of *KCND2* were elevated in blood samples from DM1 patients **(Figure 3C)**. We also compared the expression of those genes taking into account the gender and the absence or presence of cancer, which have been shown to be important features in DM1 patients[39], obtaining similar pattern of expression in the majority of cases except *HMG2A*, *AURKB* and *RAD50* **(Figure Suppl 1)**. Correlation analysis confirmed significant positive associations between *MBNL1* and *DMPK1* and between them and *CDK2*, *HMG2A* and *RAD51* in some cases **(Figure 3D)**.

Next, we studied whether those pathways could be linked to muscle biology. For this, we measured the expression of the previously identified genes in myoblasts isolated from the same patients and also in the tibialis anterior muscle from *DMSXL* mouse model carrying >1.000 CTG repeats[25]. Of note, we detected the same expression pattern in both human myoblasts **(Figure 3E)** and tibialis anterior muscle from *DMSXL* mouse model **(Figure 3F,G)**, in which genes associated with cell cycle, cell division, cell replication and DDR significantly were significantly decreased in DM1 samples. These data translate the results of the transcriptome analysis to patient blood samples and to muscle pathophysiology.

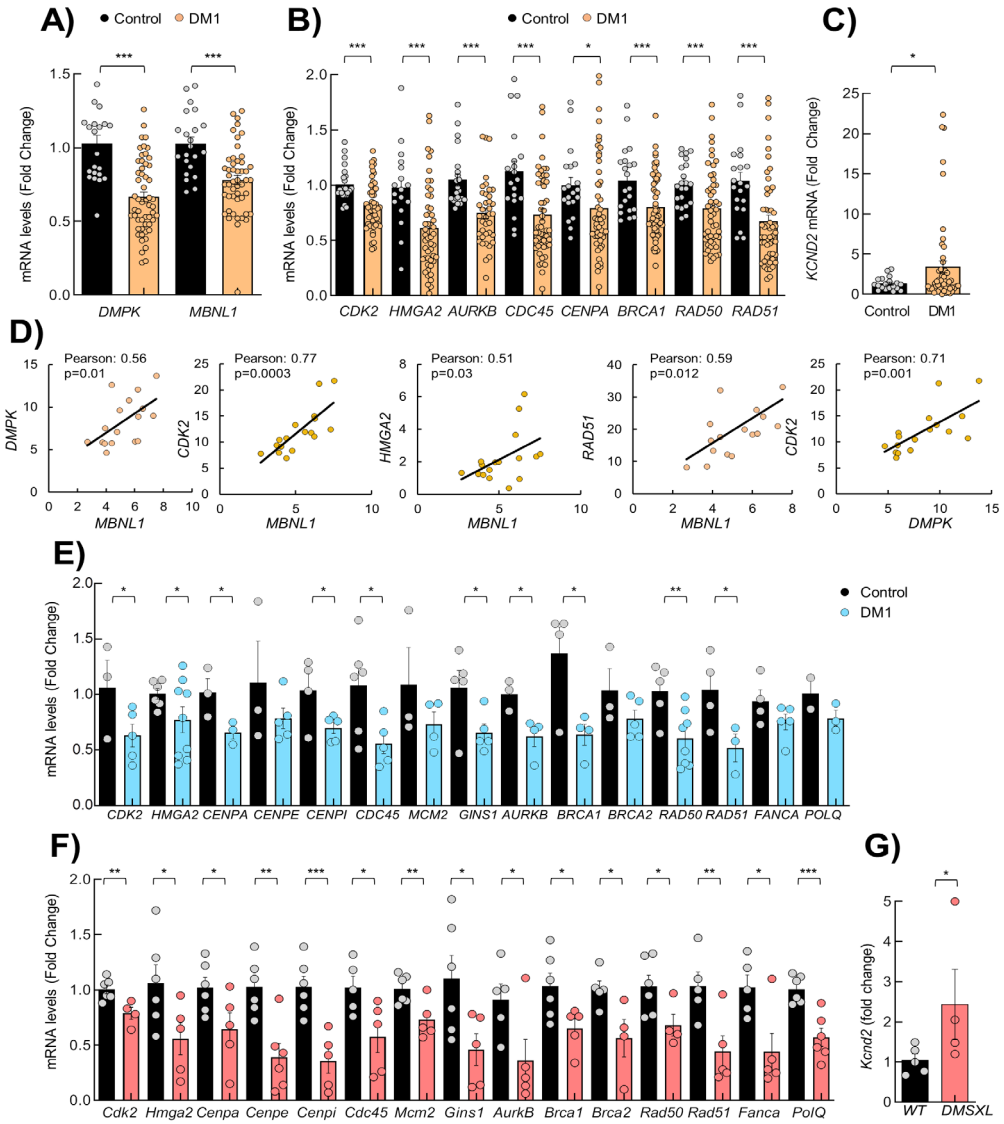


Figure 3. Transcriptomic findings are extended to additional DM1 models *in vitro* and *in vivo.* **A)** Measurement of *DMsPK* and *MBNL1* mRNA levels by qPCR in PBMCs derived from DM1 patients (n≥56) and controls (n≥22). **B)** qPCR analysis of the indicated genes in PBMCs from DM1 and controls. **C)** qPCR analysis of *KCND2* in PBMCs. **D)** Correlation analysis between indicated genes in DM1 samples (n=17). Numbers indicate cycles of difference against internal control. *A-D each dot represents one patient.* **E)** Measurement of mRNA levels of indicated genes by qPCR in early passage myoblasts (passage≤7) derived from DM1 patients (n=2) and controls (n=2) (each point represents one independent experiment). **F-G)** Measurement of mRNA levels of indicated genes by qPCR in tibialis anterior muscle of 2 months-old *DMSXL* and control mice (n≤6, each dot represents one mouse).

Proliferation and DNA damage response are impaired in DM1-derived fibroblasts

The results presented above underscore the importance of cell proliferation and cell repair processes in DM1 pathology. Since previous studies have shown contradictory results in regards to cell growth and cell proliferation in DM cells[22,41,42], we performed cell proliferation and DDR assays, to functionally validate and extend our investigations. First, we found a significant decrease (50%) in the growth of DM1-derived fibroblasts, compared to control fibroblasts **(Figure 4A)**. This was accompanied by a remarkable reduction in the number of cells positive for phospho-histone H3 (pH3), which is a well-established marker of mitosis and cell division[43], and ethynyl-labeled deoxyuridine (EdU) **(Figure 4B-C)**. In line with the lower proliferative capacity of DM1 fibroblasts, myoblasts also showed lower number of Ki67 positive cells **(Figure 4D)**. Finally, we detected an inverse correlation between the proliferative capacity of DM1 fibroblasts and both the number of CTG expansions and the MIRS score, but no clear association with donor age **(Figure Suppl 2A-C)**. These data show that DM1 cells display decreased proliferation.

To analyze cell repair and DDR processes, we determined the number of cells positive for γ-H2AX, a well-established marker of the response to DNA damage[44]. DM1 fibroblasts tended to have a higher number of γ-H2AX-positive cells at early passage **(Figure 4E,F)**. Importantly, treatment with doxorubicin, an inducer of DNA damage, increased foci of γ-H2AX in both control and DM1 cells, but to significantly higher levels in the latter **(Figure 4E,F)**. In line with this, phospho-ATM levels were also elevated after doxorubicin treatment of DM1 fibroblasts **(Figure 4G)**. These results indicate that DM1 cells accumulate higher amounts of DNA damage after the induction of stress.

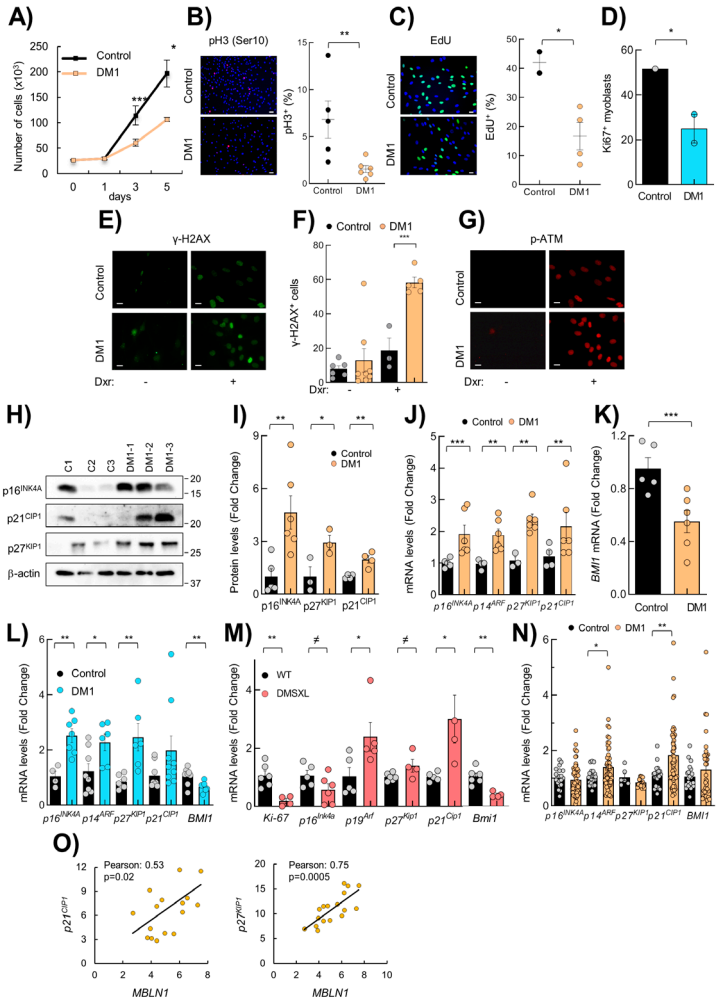


Figure 4. Proliferation and DNA damage response are impaired in DM1-derived cells.
A) Growth of DM1-derived fibroblasts (n=6) compared to control fibroblasts (n=4). **B)** Representative image and quantification of phospho-Histone H3 Ser10 positive cells in controls (n=5) and DM1 fibroblasts (n=6). Scale bar: 100 µm. **C)** Representative image and quantification of EdU positive cells in controls (n=2) and DM1 fibroblasts (n=4). Scale bar: 50 µm. **D)** Quantification of Ki-67 positive cells in controls (n=1) and DM1 myoblasts (n=2). **E-F)** Representative image and quantification of γ-H2AX-positive cells in controls and DM1 fibroblasts in the absence or presence of doxorubicin (n≥3). Scale bar: 50 µm. **G)** Representative image of phospho-ATM in controls and DM1 fibroblasts in the absence or presence of doxorubicin (n=3). Scale bar: 50 µm. **H-I)** Representative immunoblot and quantification of p16^{INK4a}, p21^{CIP1}, and p27^{KIP1} protein levels in DM1 compared to control fibroblasts. **J)** mRNA levels of *p16^{INK4a}*, *p14^{ARF}*, p27^{KIP1} and *p21^{CIP1}* and **K)** *BMI1* in fibroblasts (controls n=4 and DM1 n=6). **L)** mRNA levels of indicated genes in myoblasts from DM1 patients (n=2) and controls (n=2) (each dot represents one experiment). **M)** Measurement of mRNA levels of indicated genes in tibialis anterior muscle of 2 months-old *DMSXL* and control mice (n≤6, each dot represents one mouse). **N)** Measurement of mRNA levels of indicated genes in PBMCs from DM1 patients (n≥56) and controls (n≥22). **O)** Correlation between *MBNL1* and *p21^{CIP1}* and *p27^{KIP1}* in DM1 samples (n=17). *If nothing is indicated, each dot represents one patient and is the average of n≥2.*

Cell cycle and cell proliferation are regulated by CDK inhibitors. Thus, we studied $p16^{INK4a}$, $p14^{ARF}$, $p21^{CIP1}$ and $p27^{KIP1}$ and found that all four factors had elevated expression, at both the protein and mRNA level in DM1 fibroblasts (Figure 4H-J). Additionally, *BMI1*[45], a member of the Polycomb family that plays a central role in cell proliferation through the repression of $p16^{INK4a}$, $p14^{ARF}$, and $p21^{CIP1}$ showed significantly reduced expression in DM1 cells **(Figure 4K)**. A similar pattern of expression of *CDK* inhibitors and *BMI1* were obtained in myoblasts **(Figure 4L)**. We also detected elevated levels of $p19^{Arf}$, $p21^{Cip}$ and $p27^{Kip}$ and reduced levels of *Bmi1* and *Ki-67* in muscle from *DMSXL* mouse model (**Figure 4M**), whilst $p14^{ARF}$ and $p21^{CIP1}$ presented elevated levels in DM1 PBMCs (**Figure 4N**). In contrast, $p16^{INK4a}$ and *BMI1* expression was altered according the gender and the presence of cancer in DM1 samples **(Figure 4N and Figure Suppl 3)**, being decreased and elevated, respectively, in female patients with increased cancer risk, but the opposite in males, in agreement with a previous study[39]. Finally, we detected a positive correlation between the expression of *MBNL1* and both $p21^{CIP1}$ and $p27^{KIP1}$ **(Figure 4O)**.

DM1 fibroblasts undergo premature senescence

Cellular senescence is a state of irreversible arrest of the cell cycle, in which cells remain metabolically active, but they can no longer replicate after a limited number of cell divisions[46]. Next, we investigated whether the accumulated deregulation of cell cycle, replication, division and DDR could trigger the acquisition of a permanent senescent state in DM1. Thus, we performed a serial passage protocol, which is a well-established methodology to study senescence *in vitro*[47] and counted the number of population doublings after approximately 40 passages. DM1 fibroblasts displayed a decreased division rate compared to control cells and, interestingly, they also required a lower number of divisions and passages to undergo senescence and stop dividing **(Figure 5A)**. The premature cessation of dividing was accompanied by the presence of a higher number of senescence-associated β-galactosidase (SA-β-Gal)-positive DM1 fibroblasts **(Figure 5B,C)**. Remarkably, DM1 fibroblasts showed increased levels of this senescence biomarker in early passages and the proportion of SA-β-Gal-positive cells increased to over 60% of cells in late passages **(Figure 5B,C and Figure Suppl 4)**.

Senescent cells display important morphological and molecular features[27] and therefore, we evaluated these features in control and DM1 fibroblasts. First, we found that DM1 cells were more enlarged adopted a flattened morphology and had a larger volume than control fibroblasts at advanced passage **(Figure 5B)**. At the molecular level, the accumulation of components of the $p16^{INK4a}$/*Rb* and $p14^{ARF}$/*p53*/$p21^{CIP1}$ pathways is required to reduce cell cycle progression and cell division and to maintain a senescent state[48]. Thus, we studied $p16^{INK4a}$, $p14^{ARF}$, $p21^{CIP1}$ and $p27^{KIP1}$ expression

and found that their levels increased at late passages, with higher levels in DM1 fibroblasts than in control cells **(Figure 5D-G)**. On the contrary, the levels of *BMI1* were lower in DM1 cells **(Figure 5H)**.

Persistent DNA damage signalling triggers the secretion of several senescence-associated pro-inflammatory cytokines[49], a phenotype termed Senescence-Associated Secretory Phenotype (SASP). Interleukin 6 (IL6) plays a particularly relevant role in this phenotype[50]. To evaluate and compare the inflammatory state of control and DM1 fibroblasts, we performed a cytokine array analysis of the supernatants of both genotypes. Interestingly, we found a completely different pattern of the secretory phenotype between control and DM1 fibroblasts, with elevated expression of multiple pro-inflammatory cytokines (IL6, IL1α, TNFα, IL1β, IFNγ), chemokines (CCL5) and other factors in DM1 cells at early passage **(Figure 5I)**. We used qPCR to confirm the elevated expression of *IL6, TNFα*, and *CCL5* at the mRNA level in DM1 fibroblasts **(Figure 5J)**. Further analysis of the cytokine and chemokine expression profile showed the elevation in *IL6, TNFα*, and *CCL5* mRNA levels in late passages, which was particularly exacerbated in DM1 fibroblasts **(Figure 5J)**, thus providing additional evidence of the accumulation of senescent cells and the acquisition of the SASP in DM1 fibroblasts.

In order to determine the translation of these results from *in vitro* to *in vivo* samples, we measured the expression of *IL6* in blood samples and found higher levels of *IL6* in DM1 patients-derived PBMCs than from healthy donors **(Figure Suppl 5A,B)**, which correlated with *MBNL1* levels **(Figure Suppl 5C)**. Taken together, our results illustrate the existence of the SASP in DM1 cells.

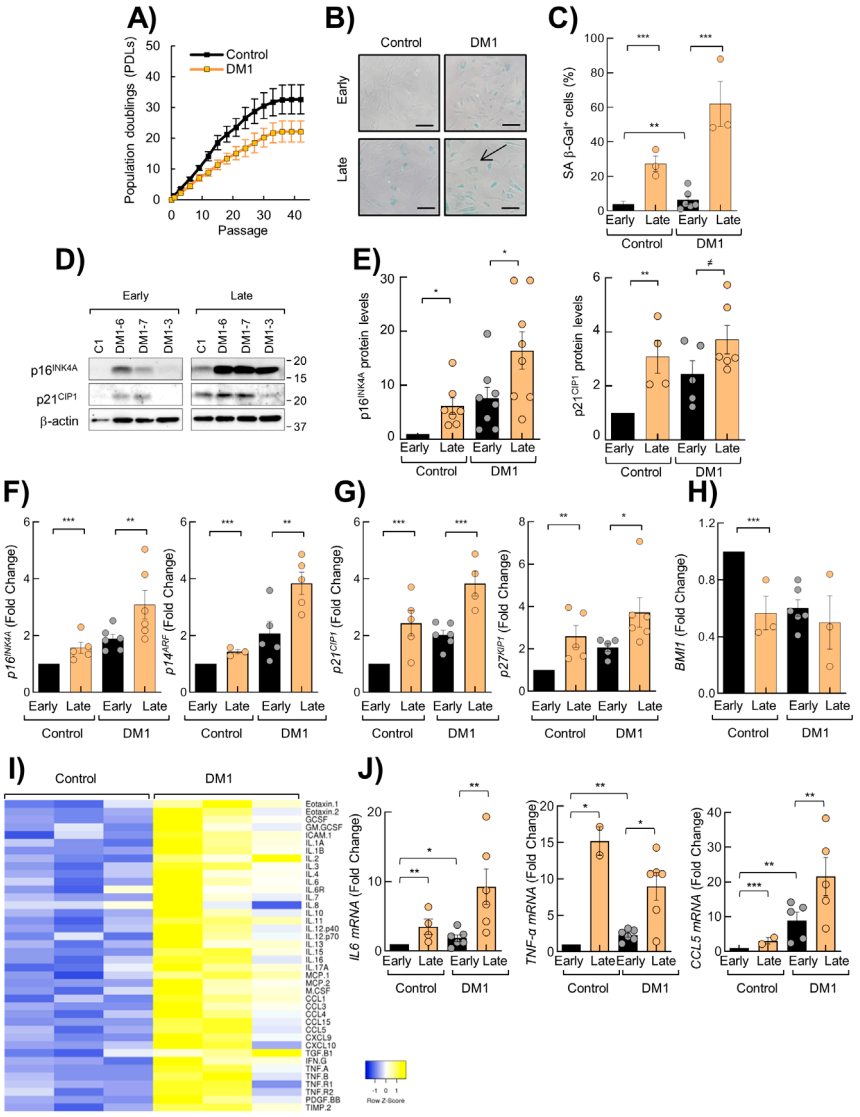


Figure 5. DM1 fibroblasts undergo premature senescence.

A) Results of 3T3 protocol where replicative senescence was induced in fibroblasts by seeding the same density of cells every 3 days. Population doubling (PDL) of DM1 cells (n=4) and controls (n=4) was calculated at each passage. **B-C)** Representative images and quantification of SA β-Galactosidase positive cells at early (between 3-10) and late passage (between 35-40) in control and DM1 fibroblasts (n≥5). Scale bar: 100 µm **D-E)** Representative immunoblot and quantification of $p16^{INK4a}$ and $p21^{CIP1}$ proteins at early and late passages in DM1 relative to control fibroblasts (n≥4). **F-H)** mRNA levels of *$p16^{INK4a}$*, *$p14^{ARF}$*, *$p21^{CIP1}$*, *$p27^{KIP1}$* and *BMI1* in DM1 and controls (n≥4), at early and late passages. **I)** Antibody array of soluble factors secreted by control and DM1 fibroblasts. For each cell culture, control signals were averaged and used as the baseline. The heat map indicates fold changes from baseline between DM1 (n=3) and control fibroblasts (n=3). **J)** *IL-6, TNF-α*, and *CCL5* mRNA levels at early and late passages (n≥4). *In this figure each dot represents one patient and is the average of at least n=2 experiments.*

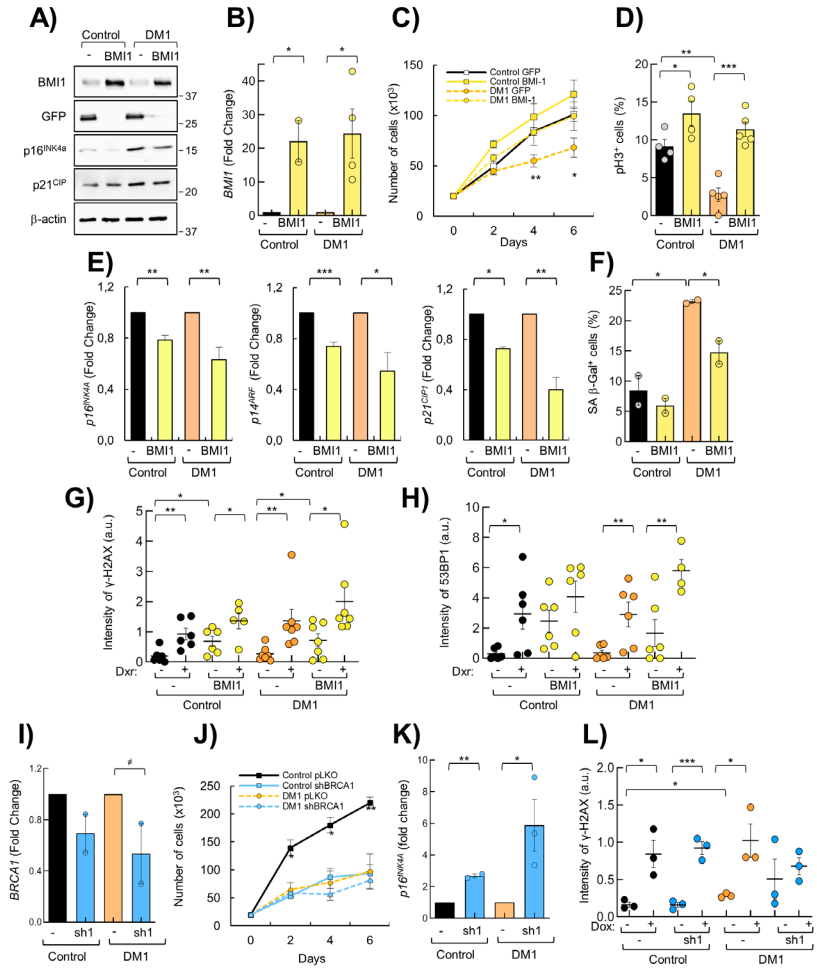


Figure 6. BMI1 pathway and DNA Damage Response regulate several phenotypes of DM1 fibroblasts.

A) Western blot of indicated proteins and B) mRNA levels of *BMI1* in controls and DM1 fibroblasts infected with an empty vector (*GFP;* -) or with a construct encoding *BMI1.* (n=3 experiments with 2 independent individuals in controls and 4 independent patients in DM1). C) Cell growth of different conditions at the indicated time points (controls n=4; DM1 n= 5). D) Quantification of pH3 positive cells in the indicated conditions (controls and DM1 n=4). E) mRNA levels of indicated genes in DM1 (n=3) and control fibroblasts (n=2). F) Quantification of SA β-Galactosidase positive cells in indicated conditions. Results are average of 1 experiment with 2 independent samples each. Quantification of G) γ-H2AX and H) 53BP1 integrated fluorescence density at indicated conditions in the absence or presence of doxorubicin (controls n=6; DM1 n=7, each dot represents one independent sample). I) *BRCA1* mRNA levels in DM1 and control fibroblasts infected with an empty vector (*pLKO;* -) or with a short hairpin vector against BRCA1 (*shBRCA1*) (controls n=2; DM1 n=2). J) Cell growth of different conditions at the indicated time points (controls n=2; DM1 n=3). K) mRNA levels of *p16^{INK4a}* in DM1 and control fibroblasts of the indicated conditions (controls n=2; DM1 n=3). L) Quantification of γ-H2AX integrated fluorescence density in controls and DM1 fibroblasts in the absence or presence of doxorubicin (controls n=2; DM1 n=3). *In this figure each dot represents one experiment.*

BMI1 over-expression rescues impaired phenotypes of DM1 fibroblasts

Our previous results show that *BMI1* expression is decreased in DM1 cells *in vitro*. To determine whether BMI1 was involved in the reduced proliferative capacity of DM1 cells, we over-expressed it in control and DM1 fibroblasts, via lentiviral infection of a construct encoding the full-length of the *BMI1* sequence. After confirming the increase in *BMI1* expression at the protein (**Figure 6A**) and mRNA level (**Figure 6B**), we performed functional studies and found that BMI1 restoration rescued the impaired cell growth and proliferation of DM1 fibroblasts, to a similar extent to control cells (**Figure 6C,D and Figure Suppl 6A**). In parallel, DM1 fibroblasts over-expressing BMI1 had significantly reduced levels of $p16^{INK4a}$, $p14^{ARF}$, and $p21^{CIP1}$ (**Figure 6A,E**). Moreover, we also found that *BMI1* overexpression decreased the number of SA-β-Gal-positive cells (**Figure 6F**) and increased the number of γ-H2AX and 53BP1 positive cells after treatment with doxorubicin (**Figure 6G,H and Figure Suppl 6B,C**). These results reveal that ectopic BMI1 restored DM1-associated cellular and molecular alterations.

To determine whether impaired DNA repair machinery could be responsible of the phenotypes observed in DM1 cells, we knocked down BRCA1 expression with a short hairpin (*shBRCA1*) via lentiviral infections in control and DM1 fibroblasts. Silencing of *BRCA1* (**Figure 6I**) promoted a significant decrease in cell growth (**Figure 6J**), and an increase in $p16^{INK4a}$ and γ-H2AX levels in both control and DM1 fibroblasts (**Figure 6K,L Fig. Suppl 6D**). Moreover, several genes involved in cell cycle, cell division and DDR were significantly altered in DM1 cells with *BRCA1* knockdown (**Fig. Suppl 7A-C**). These results show that impaired DNA repair machinery is responsible, at least in part, of the phenotypes in DM1 cells.

Senotherapy rescues DM1 associated impairments *in vitro* and *in vivo*

Senotherapy is a novel therapeutic strategy that aims to permanently remove senescent cells, clear immune-mediated senescent cells, and neutralize the SASP[51]. Given the premature and enhanced senescence of DM1 cells, we investigated the efficacy of this therapy against DM1. We treated late-passage DM1 and control fibroblasts with three independent senolytic compounds, Quercetin, Dasatinib and Navitoclax for 72 hours. First, we quantified SA-β-Gal staining and observed a dose-dependent decrease in the number of positive cells in control and DM1 fibroblasts after treatment with each of the three senolytic compounds independently with 10 µM Quercetin or Navitoclax or 0.5 nM Dasatinib resulting in over 50% reduction in SA-β-Gal-positive cells in DM1 and around 40% in controls **(Figure 7A-C, Fig. Suppl 8A)**. We also found that the number of pH3 positive cells decreased **(Fig. Suppl 8B)**, and cleaved Caspase-3 and PARP-positive cells increased significantly in response to senolytic treatment, thus confirming the death of senescent DM1 fibroblasts **(Figure 7D, Fig. Suppl 8C)**. These functional data were further validated at the molecular level, as *IL-6* expression decreased significantly to approximately 50%, whereas Caspase 5 expression increased up to 4-fold after the treatment of DM1 and control fibroblasts with senolytics **(Figure 7E,F)**. These results confirm that senolytics selectively killed senescent cells in control and, particularly, DM1 fibroblasts.

We performed similar experiments in early passage fibroblasts.Senolytic treatment also decreased the number of SA-β-Gal positive cells **(Figure 7G Fig. Suppl 9A)**, and increased the number of Caspase 3 and PARP positive cells to a similar extent in control and DM1 cells **(Figure 7H, Fig. Suppl 9B)**. In line with this, Quercetin also increased the number of apoptotic cells in myoblast cultures **(Figure Suppl 9C-E)**.

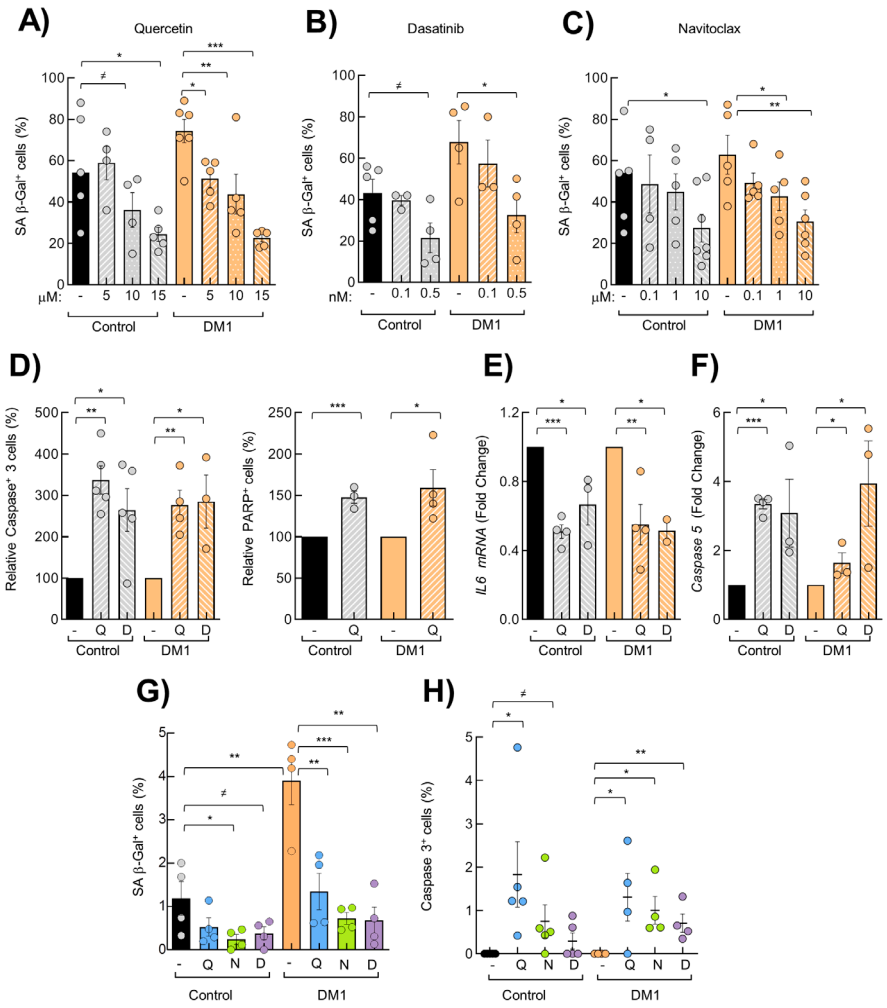


Figure 7. Senotherapy restores multiple deficiencies in DM1 fibroblasts in vitro.
A-C) Quantification of SA β-Galactosidase-positive cells, in absolute numbers, in late-passage (n≥40) DM1 (n=5) and control fibroblasts (n=5) treated for 72 hours with **A)** 5, 10, or 15 μM Quercetin (Q); 15 μM of Q treatment decreased the number of positive cells from 58% to 36% in controls and from 74 to 22% in DM1. **B)** 0.1 or 0.5 nM Dasatinib (D); 0.5 nM of D decreased them from 43% to 26% in controls and from 67 to 32% in DM1. **C)** 0.1, 1, or 10 μ M Navitoclax (N). 10 μ M of N decreased them from 50% to 27% in controls and from 62 to 26% in DM1. **D)** Quantification of apoptosis measured as active caspase-3 (left) and cleaved PARP (right) positive cells at late passage (n≥40) after treatment with Q or D for 72 hours (n=3). **E-F)** mRNA levels of *IL6* and *caspase-5* in late-passage fibroblasts treated with Q or D for 72 hours (n=3). **G)** Quantification of SA β-Galactosidase-positive cells, in absolute numbers, in early passage (passage-5-10) in DM1 and control fibroblasts treated for 72 hours (n=4) with 15 μM of Q, 10 μM of N or 0.5 nM of D. **H)** Quantification of caspase 3 positive cells in early passage (passage 5-10) DM1 and control fibroblasts treated for 72 hours (n≥4) with 15 μM of Q, 10 μM of N or 0.5 nM of D. *In this figure each point represents one experiment.*

Next, we aimed to validate the *in vitro* results in a *Drosophila melanogaster* model of DM1 that expresses 480 interrupted CTG repeats[26]. First, we found that DM1 flies present impaired locomotor activity **(Figure 8A)** and reduced lifespan **(Figure 8B)** compared to control flies lacking CTG repeats, indicative of an accelerated aging phenotype. In line with this idea, the expression of the markers of senescence *Dacapo* (homolog of human $p21^{CIP}$/$p27^{KIP}$) and *Psc* (homolog of BMI1), was elevated and reduced, respectively, in DM1 flies (Figure 8C). In this context, DM1 flies showed reduced levels of *Mbnl*, *String* (CDC45), *Cid* (CENPA) and *Cana* (CENPE) **(Figure 8D)**, further validating the relevance of the results obtained in the transcriptome study. Interestingly, 5 mM Quercetin rescued the expression of *Mbln*, *String*, *Cid and Cana* **(Figure 8E,F)**. Similarly, the levels of *Dacapo* and *Psc* were restored after treatment with Quercetin **(Figure 8G)**. These results confirm that senolytic treatment recovers molecular alterations of DM1 also *in vivo*.

Finally, we measured locomotor activity and longevity in the absence or presence of 1 and 5 mM Quercetin and 50 nM Dasatinib. Interestingly, Quercetin and Dasatinib significantly increased the motility of DM1 flies at all studied times **(Figure 8H)**. In particular, at 25 days, the percentage of flies moving was 9% in non-treated DM1 *vs* 71% with 5 mM Quercetin and 42% with 50 nM Dasatinib **(Figure 8I)**. Of note, 1 mM of Quercetin promoted similar positive effect on locomotor activity of DM1 flies, whereas none of those concentrations altered the motility of *wt* flies **(Figure 8I)**. Moreover, the senolytics extended the longevity of DM1 flies by double increasing the median survival from 30 to 59 days with 5mM Quercetin (Log-rank test; $p<0.0001$) and to 47 with 50 nM Dasatinib ($p<0.0001$) **(Figure 8J,K)**. As with locomotor activity, senolytics did not significantly affect the survival of *wt* flies **(Figure 8J, Fig. Suppl 10)**. These results confirm that senotherapy efficiently and selectively rescues the phenotypic spectrum of DM1.

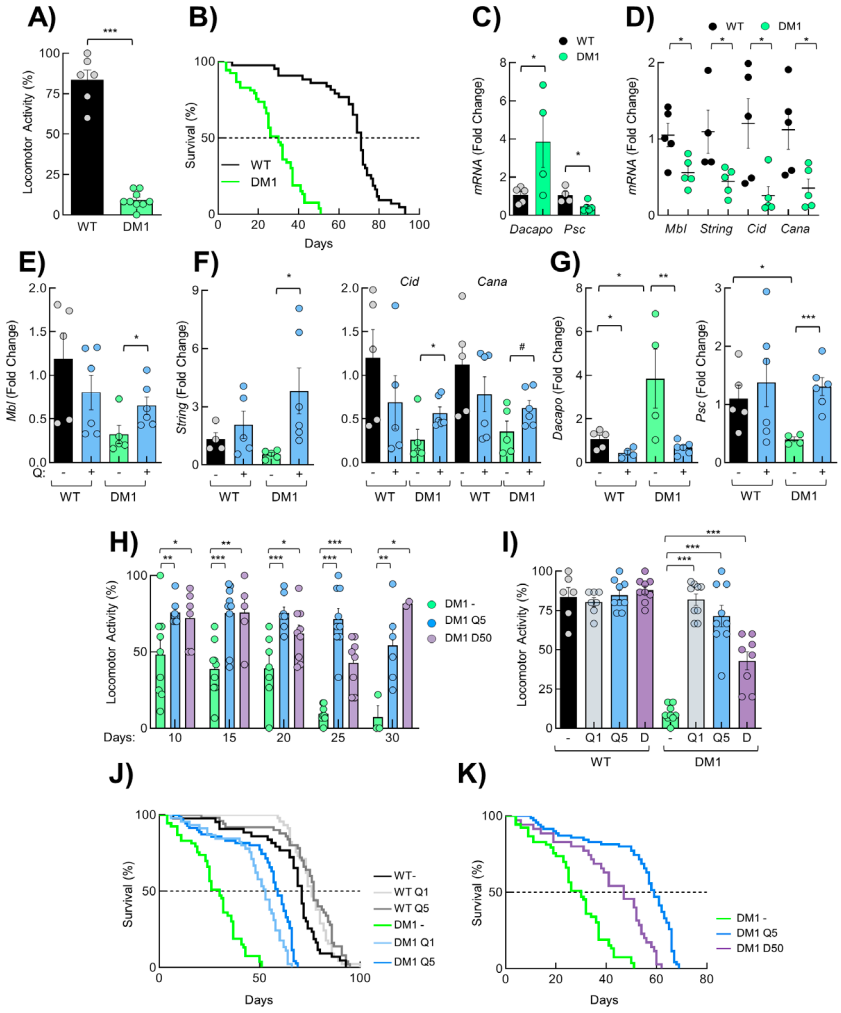


Figure 8. Senotherapy restores multiple deficiencies in DM1 fruit flies in vivo.
A) Locomotor activity obtained in *Drosophila* model of DM 1 (n=53) and controls (n=46) at 25 days. B) Survival curve of DM1 flies and controls. Average is 30 *vs.* 71 days and p-value<0.0001. C) mRNA levels of *Dacapo* and *Psc* in thorax derived from DM1 and control fruit flies (n≥4, each dot represents a pool of 12 flies). D) mRNA levels of indicated genes in thorax derived from DM1 and control fruit flies (n≥4, each dot represents a pool of 12 flies). E-G) mRNA levels of indicated genes in thorax derived from DM1 and control fruit flies (n≥4, each dot represents a pool of 12 flies) in the absence or presence of 5 mM of Quercetin. H) Locomotor activity obtained in *Drosophila* DM1 model non-treated (n=55) or in presence of 5 mM Quercetin (n=46) and 50 nM Dasatinib (n=9) at the indicated time points. I) Locomotor activity of non-treated DM1 (n=9) and control (n=10) flies or in the presence of 5 mM Quercetin (control n=9 and DM1=9) and 50 nM Dasatinib (control n=9 and DM1=8). J) Survival curve of non-treated control (n=45) and DM1 (n=53) flies or in presence of 1 mM (DM1, n=45 and control, n=50) and 5 mM Quercetin (DM1, n=70 and control, n=45) and p-value<0.0001 in the two doses of Quercetin in DM1 flies. K) Survival curve of DM1 flies non-treated (n=53) or in presence of 5 mM Quercetin (n=70) and 50 nM Dasatinib (n=36).

Discussion

Myotonic dystrophy is a rare, clinically variable disease with no currently available treatment to slow or stop the progression of the disease. Supportive treatments, preventative measures and clinical surveillance are the only options available for DM1 patients[52]. The results of this study demonstrate the impact of senescence in DM1 pathogenesis and the efficacy of senotherapy in the pre-clinical setting.

The variety of symptoms that develop DM1 patients strongly resemble signs of accelerated aging[22,28,39,53]. Similarly, deregulation of several hallmarks of aging has been associated with the pathophysiology of DM1 in different human cell types[28]. In our study we established cultures of fibroblasts derived from DM1 patients that recapitulated the major molecular and genetic characteristics of DM1 pathophysiology. This indicates that skin fibroblasts are a useful source to study the pathogenesis of DM1. This is advantageous, since a skin biopsy represents a much less invasive procedure than the isolation of myoblasts or satellite cells from muscle biopsies. Importantly, transcriptomic analysis and functional studies revealed that DM1 fibroblasts show impaired proliferation, increased senescence and enhanced accumulation of DNA damage. Moreover, our data revealed a more complex *status quo* and identified dysregulation in pathways from various steps of cell cycle, cell division, cell replication, and cell repair. Importantly, the decreased expression of critical genes of those processes, such as *CDK2*, *HMGA2*, *CDC45*, *CENPA*, *AURKB*, *BRCA1*, *RAD51* was also detected in myoblasts and PBMCs from human patients as well as in muscle from *DMSXL* mice and thorax of *REC2 Drosophila* model, rendering the results from *in silico* transcriptome analysis and cultured cells to muscular pathology and clinical samples. The detailed impact of such deregulation on DM1 pathophysiology requires further investigation. Preliminary evidence indicates that some of them, such as DNA repair, play a role in DM1 pathogenesis since it affects repeat instability[54] and mediates several cellular phenotypes as shown with *BRCA1* knockdown.

Previous studies have detected normal cell growth[41,42] or reduced proliferative capacity and premature senescence in DM cells *in vitro*[19,20,22]. We found that DM1 fibroblasts and myoblasts displayed reduced proliferation, and this correlated with CTG repeat expansion. The isolation of cells from DM1 or DM2 patients and the expansion of the triplet might be the main features that explain the differences between studies[22]. Moreover, the underlying molecular mechanism also seems to be related to the same features, since *p16^{INK4a}* accumulation is not seen in DM2 cells[20] or its elevation is significant in cells with longer repeats[22]. We found that DM1 fibroblasts and myoblasts had elevated levels of p16^{INK4a} from early passages, but its accumulation was more exacerbated in DM1 cells at late passages, when cells had been exposed to continuous and intense doses of culture stress[47]. Moreover, our transcriptomic and

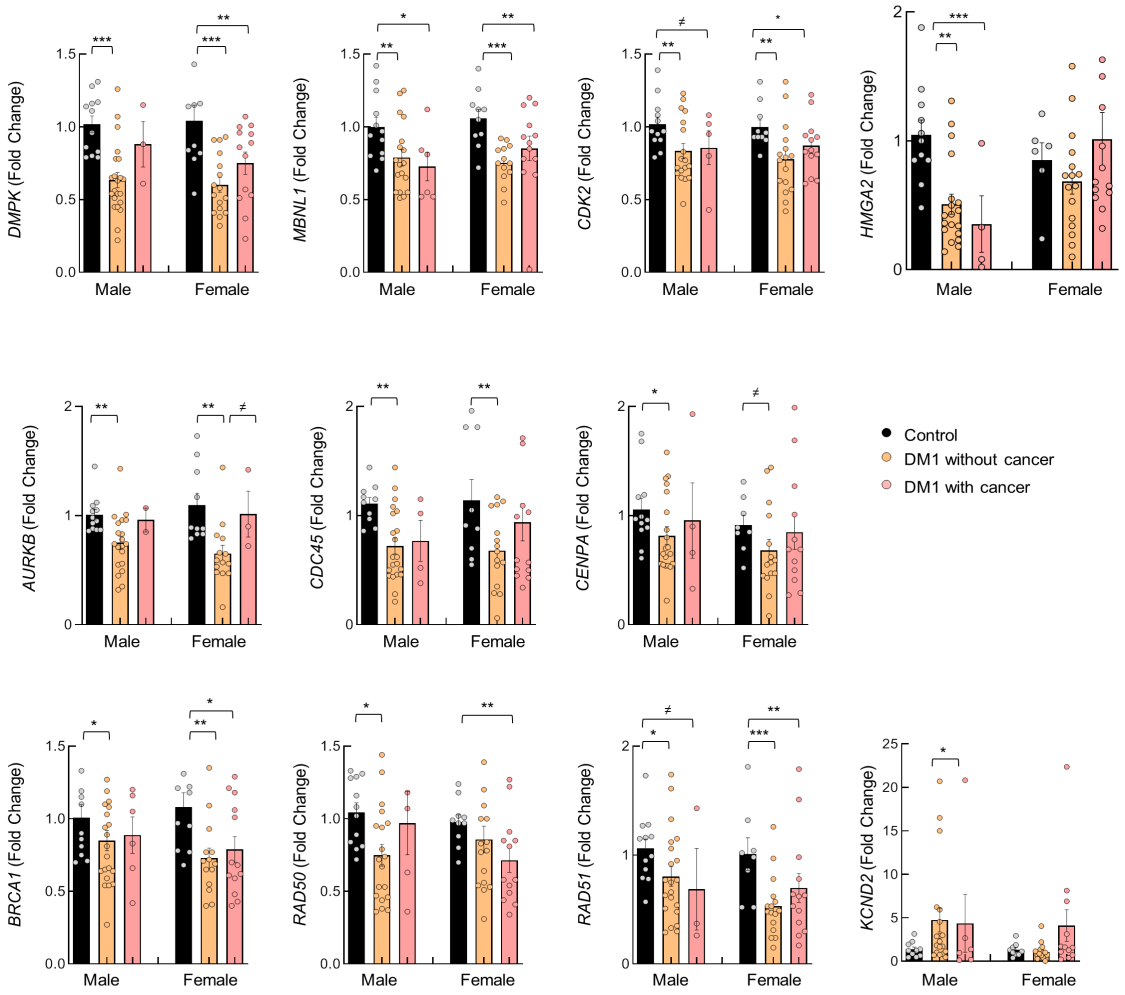
functional studies revealed a complex situation with increased number of cell cycle control pathways dysregulated in DM1 such as (I) reduction of upstream regulators of $p16^{INK4a}$ expression as BMI1 and HMG2A[31,45]; (II) decline of direct targets involved in the G0/S phase transition as CDK6 and CDK2; and (III) elevation of additional CDK inhibitors, such as $p14^{ARF}$, $p21^{CIP1}$, and $p27^{KIP1}$, and differences between *in vitro* and *in vivo* samples or between genders in the case of BMI1-$p16^{INK4a}$. Indeed, their levels were elevated and decreased respectively in DM1 female patients with cancer but the opposite in males[39]. Therefore, BMI1-$p16^{INK4a}$ activity might be context and time dependent in DM1 and it is likely to reflect their roles in senescence, aging and cancer processes.

Our results identified the impairment of cell cycle control, cell division, and DNA replication and repair, as required steps for the accumulation of senescence, which we experimentally demonstrate as a critical step in the pathophysiology of DM1. Cellular senescence is a trigger of tissue remodeling that acts during normal embryonic development and upon tissue damage[55]. However, senescence may not be efficiently completed in aged tissues, and consequently, senescent cells accumulate with age and this is accompanied by an enhanced pro-inflammatory secretory phenotype, which leads to tissue dysfunction[55]. Consequently, senescence has been shown to participate in and trigger physiological aging and a wide spectrum of age-related diseases[55,56,57]. Our results demonstrated an increase in the accumulation of senescent cells in DM1 fibroblast cultures, in which BMI1-$p16^{INK4a}$ pathway plays a role. Moreover, there was an increase in the accumulation of additional biomarkers of senescence such as SA-β-Gal, CDK inhibitors as $p21^{CIP1}$, and several cytokines, including IL6, which indicated the presence of SASP, which might chronically signal the immune system. In support of this idea, activation of the IL6 signaling pathway also contributes to impaired myocyte maturation and muscle atrophy in congenital DM1[58]. Nevertheless, our results in fibroblasts and myoblasts cultures and tissues from mouse and *Drosophila* animal DM1 models, as well as human PBMCs, provide evidence that DM1 cells undergo premature senescence and accelerated aging.

A number of potential therapeutic strategies have been identified in DM1, such as small molecule therapeutics, antisense oligonucleotide-based therapies and genome editing. However, these strategies have achieved limited success in preclinical and/or clinical trials[7,59.] Senolytics are agents that induce apoptosis of senescent cells. Among them, Quercetin is a flavonoid found in many fruits and vegetables, whereas Dasatinib and Navitoclax were originally developed as anti-cancer drugs. Importantly, the US Food and Drug Administration has already approved the treatment of Quercetin and Dasatinib and preliminary results have shown that both are well-tolerated and are effective in elderly individuals. Thus, the combination of these senolytics alleviate physical dysfunction in patients with idiopathic pulmonary fibrosis[61] and decrease the senescent cell burden in individuals with chronic kidney

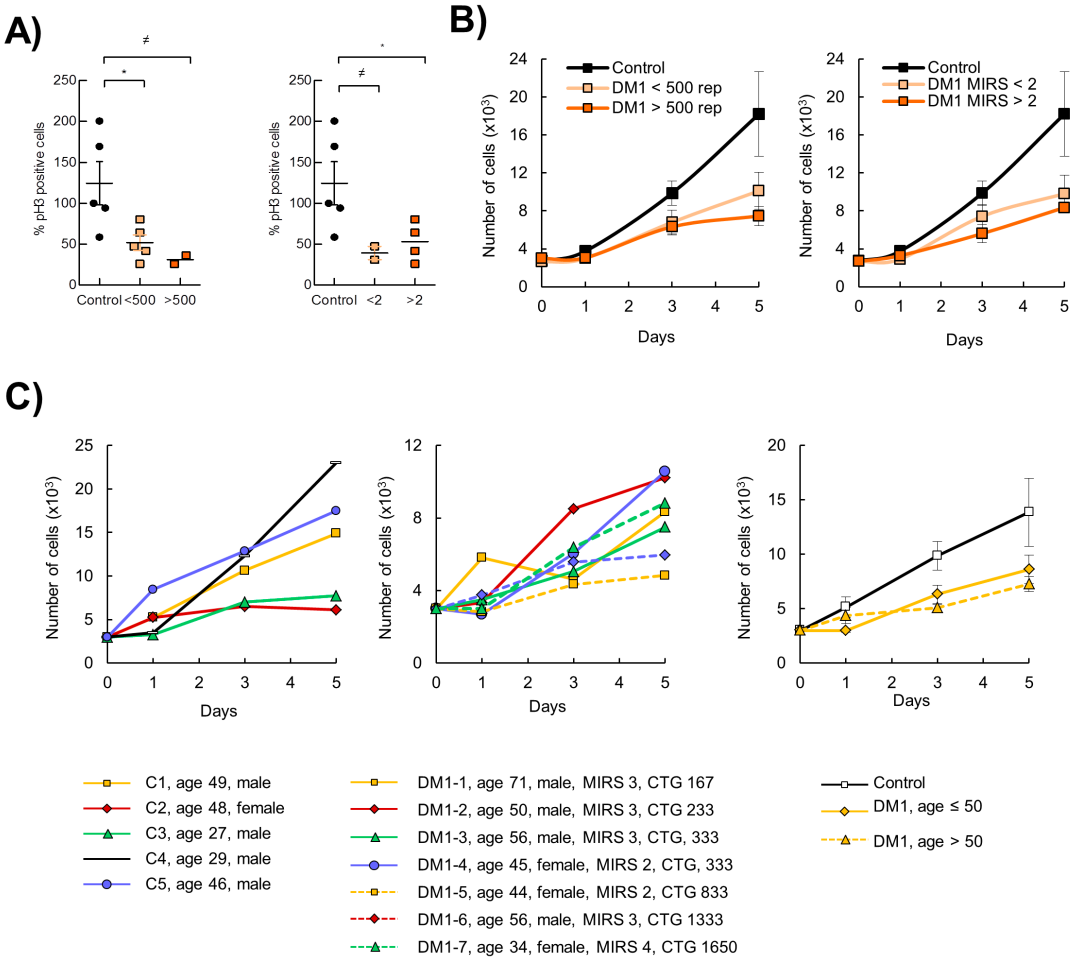
disease[62]. Moreover, combined treatment with Quercetin and Dasatinib improves the health and lifespan of aged mice[60]. Our results showed that both Quercetin and Dasatinib individually, as well as Navitoclax, (I) selectively eliminated senescent cells; (II) decreased the expression of SASP-related factors; and (III) rescued multiple phenotypes, including very significant improvements in locomotor activity and longevity in the *REC2 Drosophila* model of DM1. Thus, our study provides evidence that targeting senescent cells improve multiple cellular, molecular, and functional phenotypes, including both health and lifespan, in DM1 disease.

In summary, whole transcriptome analysis of fibroblasts derived from DM1 patients revealed impairments of cell cycle, cell division, cell replication and DNA damage response, which facilitates the accumulation of cellular senescence. These alterations were further confirmed in myoblasts and blood human samples as well as in mouse and *Drosophila* models of the disease. Importantly, treatment with senolytic compounds reversed multiples phenotypes in both DM1 fibroblasts and myoblasts *in vitro* and in an *in vivo Drosophila* model, thus providing the proof of concept of the efficacy of senolytic compounds for the treatment of DM1 in the pre-clinical setting. Senotherapy warrants further assessment as a novel candidate strategy for DM1 treatment.

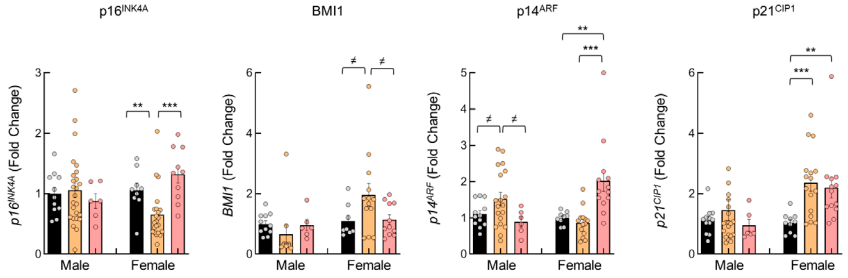


Supplementary Figure 1. Genes involved in cell cycle, cell division, cell replication and DDR are diminished in PBMCs of DM1 individuals.

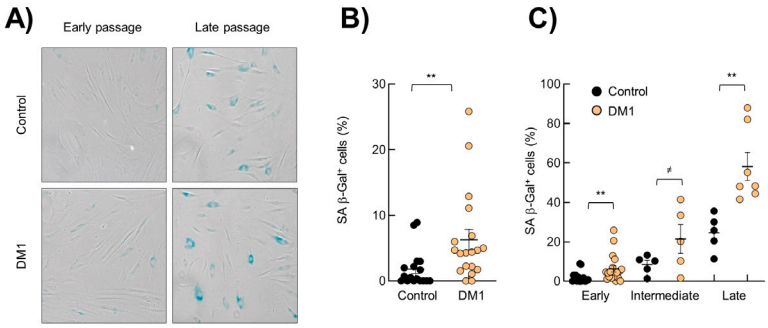
Measurement of mRNA levels of indicated genes by qPCR in PBMCs derived from DM1 patients (n≥56) and controls (n≥22). Both groups were divided by gender: DM1 male (n=27) and female (n=29) and controls male (n=12) and female (n=10). DM1 patients were divided by the development of cancer: in red with cancer (male n=6 and female n=13) or in orange without cancer (male n=21 and female n=16). Each dot represents one patient.



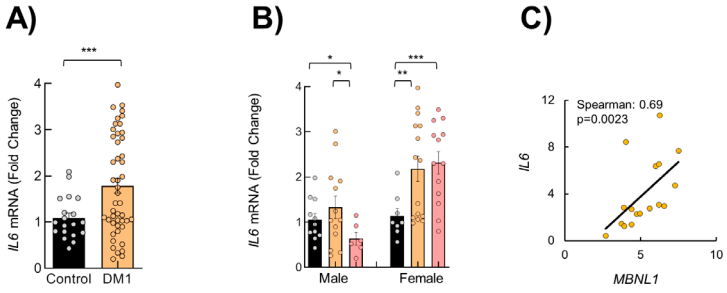
Supplementary Figure 2. Proliferation is impaired in DM1 derived fibroblasts.
A) Quantification of phospho-Histone H3 (Ser10) in DM1 derived fibroblasts stratified by CTG expansion and MIRS scale. B) Cellular growth in DM1 derived fibroblasts compared to controls analyzed as a number of total cells from day 0 to day 5 stratified by CTG expansion and MIRS scale. C) Cellular growth in control and DM1 fibroblasts does not seem to be associated to the age of individuals.



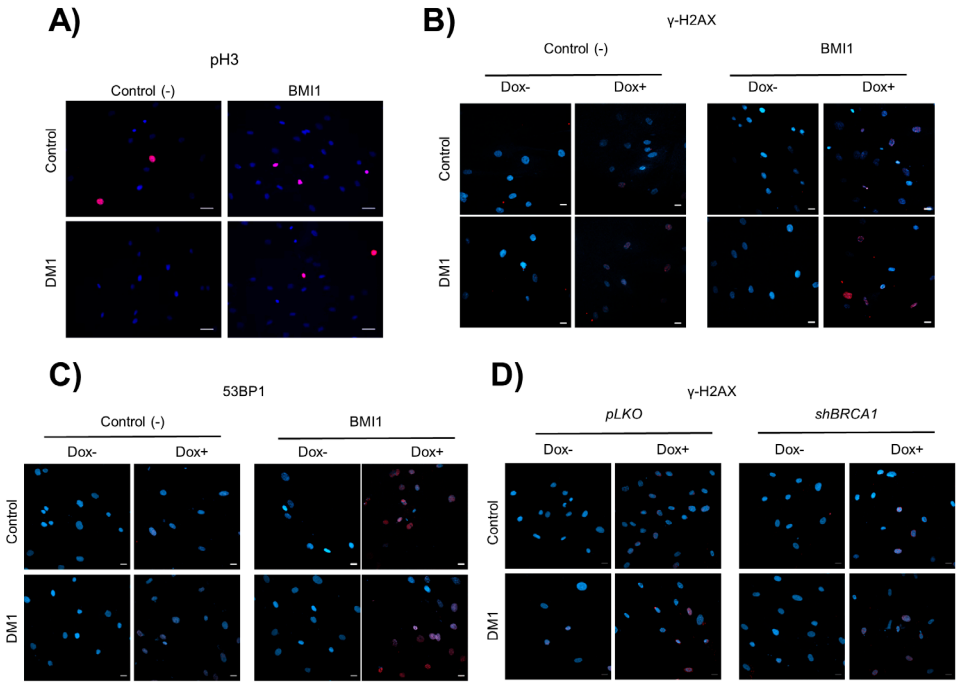
Supplementary Figure 3. Expression of CDKIs is altered in PBMCs of DM1 individuals.
Measurement of mRNA levels of indicated genes by qPCR in PBMCs derived from DM1 patients (n≥56) and controls (n≥22). Both groups were divided by gender: DM1 male (n=27) and female (n=29) and controls male (n=12) and female (n=10). DM1 patients were divided by the development of cancer: in red with cancer (male n=6 and female n=13) or in orange without cancer (male n=21 and female n=16). Each dot represents one patient.



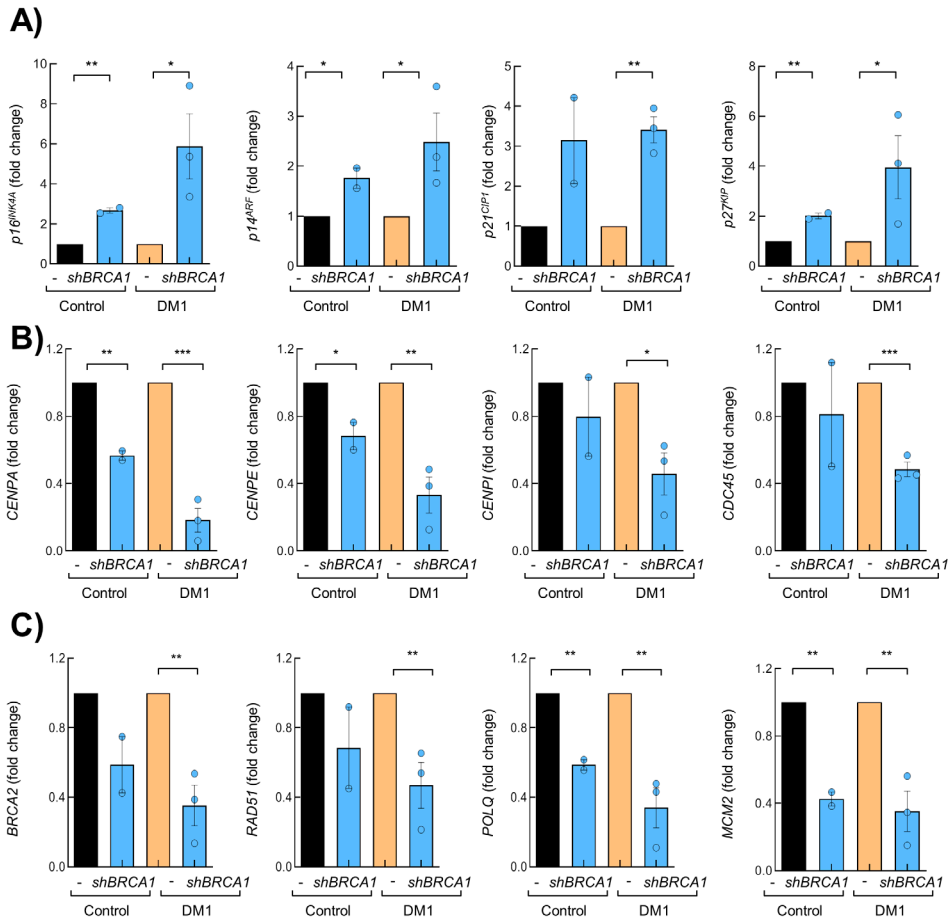
Supplementary Figure 4. SA β-galactosidase positive cells are elevated in DM1.
A) Representative images and **B)** Quantification of SA β-galactosidase positive cells at early (between 0-10) passage in control and DM1 fibroblasts (n≥10). **C)** Comparison of SA β-galactosidase positive cells at early (between 0-10), intermediate (10-20) and late (between 35-40) passagE (n≥5).



Supplementary Figure 5. Expression of IL6 is elevated in PBMCs of DM1 individuals.
A) Measurement of *IL6* mRNA levels by qPCR in PBMCs derived from DM1 patients (n≥56) and controls (n≥22). **B)** Both groups were divided by gender and by the development of cancer as in Suppl Fig 1. **C)** Correlation analysis between *MBNL1* and *IL6* in DM1 samples (n=17). Numbers indicate cycles of difference against internal control.

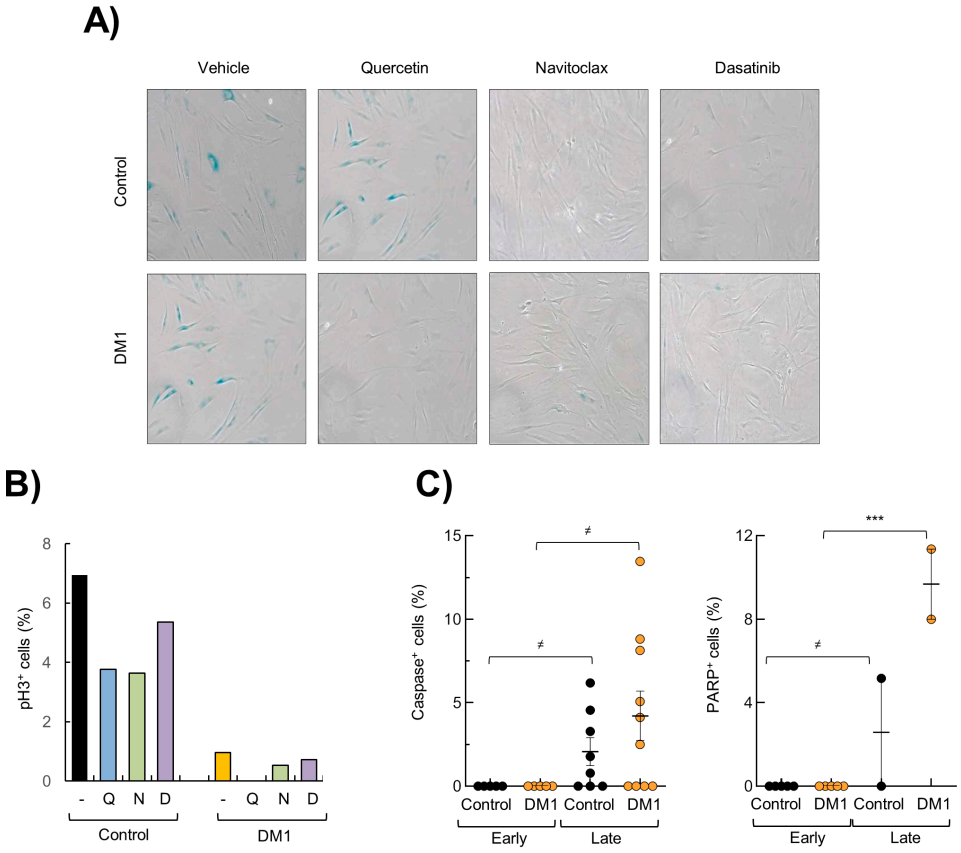


Supplementary Figure 6. BMI1 and DDR play a role in DM1 cellular phenotypes.
A) Representative image of phospho-Histone H3 (pH3) positive cells in controls and DM1 fibroblasts infected with empty vector (GFP; -) or with a plasmid encoding *BMI1* (BMI1). B) Representative image of γ-H2AX in controls and DM1 fibroblasts infected with empty vector (GFP; -) or *BMI1* in the absence (Dox-) or presence of doxorubicin (Dox+). C) Representative image of 53BP1 detection in controls and DM1 fibroblasts infected with empty vector (GFP; -) or a plasmid encoding BMI1 in the absence (Dox-) or presence of doxorubicin (Dox+). D) Representative image of γ-H2AX-positive cells in controls and DM1 fibroblasts infected with empty vector (*pLKO*) or with a short hairpin against BRCA1 (*shBRCA1*) in the in the absence (Dox-) or presence of doxorubicin (Dox+).

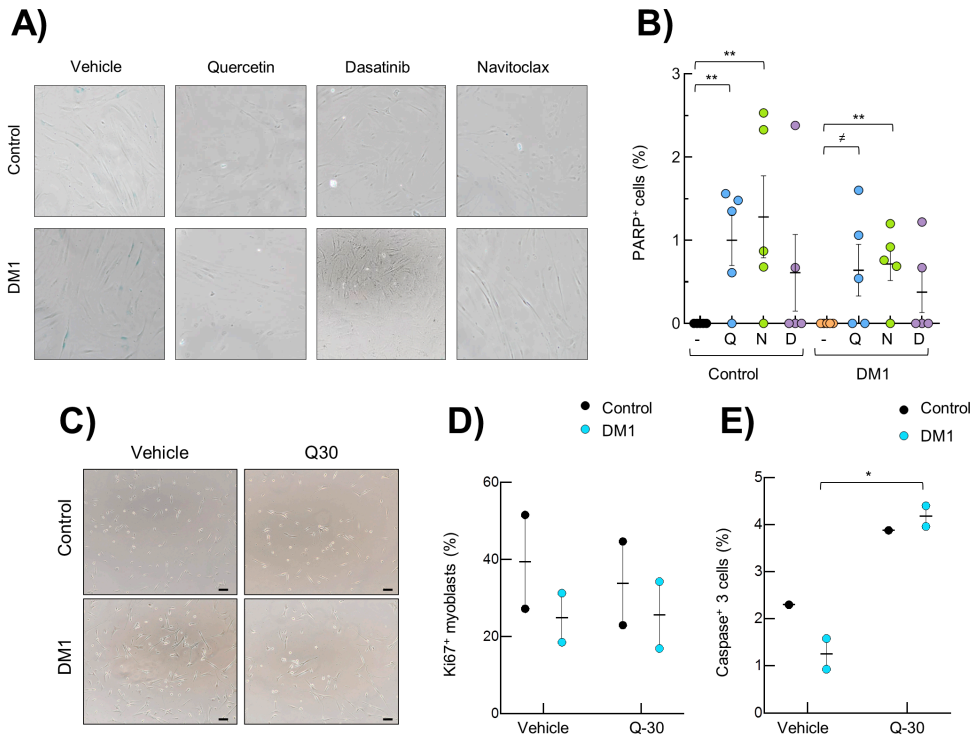


Supplementary Figure 7. BRCA1 regulates the expression of relevant genes involved in cell cycle, cell division and DDR.

A-C) mRNA levels of genes involved in cell cycle, cell division and replication and DDR in DM1 and control fibroblasts of the indicated conditions (controls n=2 and DM1 patients n=3).

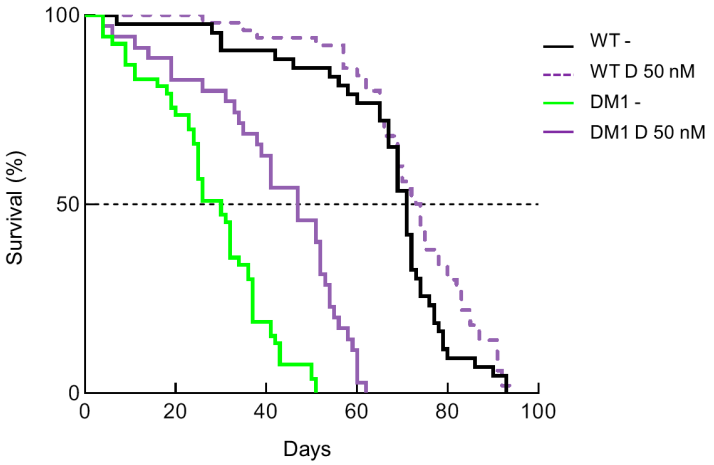


Supplementary Figure 8. Senotherapy kills senescent cells in DM1 fibroblasts at late passage. **A)** Representative images of SA β-Galactosidase in control and DM1 fibroblasts at late passage in the absence or treated with 15 µM of Quercetin, 0.5 nM of Dasatinib or 10 µM of Navitoclax for 3 days. **B)** Quantification of pH3 positive cells in fibroblasts in the absence or treated with 15 µM of Quercetin, 0.5 nM of Dasatinib or 10 µM of Navitoclax (controls and DM1 n=1). **C)** Quantification of active Caspase 3 and cleaved-PARP positive cells in fibroblasts at early (between 0-10) and late passage (over 40) (controls and DM1 n≥2).



Supplementary Figure 9. Senotherapy kills DM1 cells at early passage.

A) Representative images of SA β-Galactosidase in control and DM1 fibroblasts at early passage (1-5) in the absence or treated with 15 µM of Quercetin, 0.5 nM of Dasatinib or 10 µM of Navitoclax for 3 days. **B)** Quantification of PARP positive cells in fibroblasts at early passage in the absence or treated with 15 µM of Quercetin, 0.5 nM of Dasatinib or 10 µM of Navitoclax. **C)** Representative images of SA β-Galactosidase in myoblasts at early passage (3-5) in the absence or treated with 30 µM Quercetin. DM1 myoblasts present few SA β-Galactosidase, which are not detected with Quercetin treatment (controls n=2 and DM1 n=2). Scale bar: 100 µM **D)** Quantification of Ki67 positive cells in myoblasts at early passage (3-5) in the absence or treated with 30 µM Quercetin for 3 days (controls n=2 and DM1 n=2). **E)** Quantification of active Caspase 3 positive cells in myoblasts at early passage in the absence or treated with 30 µM Quercetin for 3 days (controls n=1 and DM1 n=2).



Supplementary Figure 10. Dasatinib significantly extends longevity in REC2 Drosophila DM1 model in vivo.

Survival curve of non-treated wt (n=45) and DM1 flies (n=53) or in presence of 50nM Dasatinib (Control, n=48 and DM1, n=36). Average survival in days is 73 *vs.* non-defined in wt (non-treated ***vs***. Dasatinib, p>0.05) and 30 ***vs.*** 47 in DM1 flies (non-treated *vs.* Dasatinib, p<0.0001).

Methods

Primary human fibroblast isolation and culture

For the isolation of human primary fibroblasts from healthy donors (age range 27 to 49) and DM1 patients (age range, 34 to 71), punch skin biopsies were cut into 2–3 mm^3 fragments and placed on a surface moistened with modified Eagle's medium, containing 13% newborn calf serum, 0.4% penicillin/streptomycin (Gibco) and 2 mM L-glutamine (Gibco). Flasks were incubated vertically for 3–6 h at 37ºC under 5% CO_2 and then returned to the horizontal position. Human fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA), 1% L-glutamine (Gibco), and 1% penicillin–streptomycin (Gibco). Fibroblasts were tested regularly for mycoplasma contamination and were negative. 7 cultures from different DM1 patients and 5 from healthy controls were established and used for qPCR validation and functional studies (see **Table 1** for patient characteristics).

Primary human skeletal muscle culture

Human proximal muscle biopsies were processed and cultured in monolayer. To obtain highly purified myoblasts, primary cultures were sorted by immunomagnetic selection based on the presence of the early cell surface marker CD56 (separator and reagents from Miltenyi Biotec). CD56-positive cells were seeded at 2.500–3.000 cells/cm^2 in culture medium for the myoblast stage, in cell culture plates previously treated with 0.5 % of gelatin. Myoblast culture medium was composed of 65% of DMEM and 21% of M-199 (Sigma-Aldrich) and supplemented with 10% of FBS, 1% of insulin, 1% of glutamine, 1% of penicillin–streptomycin, 10 µg/µL of EGF and 25 µg/µL of FGF. Control donors were healthy individuals that underwent surgery for bone fractures and the muscle biopsies were obtained during this surgery. Samples were obtained from proximal limb muscles (biceps, deltoids and triceps). For mRNA analysis we performed 3 independent experiments at passage 4, 5 and 7.

Blood samples and patient information

Blood samples from healthy controls and DM1 patients were obtained between 2012 and 2015. They were stored in the Basque Biobank (https://www.biobancovasco.org/en/) and RNA extraction from peripheral blood mononuclear cells (PBMCs) was performed between 2016 and 2017, using the miRNesay Mini Kit followed by automated RNA extraction in the QIAcube (Qiagen). Data from DM1 patients were retrospectively obtained from the medical records of the Guipúzcoa historical myotonic dystrophy cohort, established in 1985. From patients medical recorded, we selected sex, age at the time of the study enrollment, nucleotide expansion size (CTG triplets), disease severity assessed using the Muscular Impairment Rating Scale (MIRS) scale 63 at the time of the last visit, and cancer diagnosis. See Table 4 for summary of patient characteristics.

Animal models

Transgenic *Drosophila melanogaster* Mhc-Gal4 UAS-i(CTG)480 flies[26] were kindly gifted by Dr. Rubén Artero (Valencia University). Flies without any marker or balancer were selected for experiments to ensure the expression of the nucleotide repetition. Control flies were obtained crossing UAS-+-+ flies (kindly gifted by Angel Cedazo-Mínguez, Karolinska Institute, Sweden) and Mhc-Gal4 flies (purchased from Bloomington Stock Center) to obtain UAS-+-MhcGal4 flies. Flies were housed at 23°C, 70% humidity and 12h/12h light/darkness cycle. All crosses were carried out at these standard conditions with standard fly food or plus 1 or 5 mM Quercetin and 50 nM Dasatinib for treated groups. For mRNA experiments we isolated RNA from thorax of 12 flies per condition.

For longevity assay, around 50 flies (5 females/tube) were selected from each group of study. Dead flies were counted every 2 days. Kaplan-Meier method was used to plot the results. A log-rank test was used to analyze results and Bonferroni correction was used for multiple comparisons. For locomotor activity, 5, 10, 15, 20, 25 and 30 days-old flies were analyzed in groups of five. They were placed in a tube with an 8 cm mark from the bottom. Assays were recorded and archived. The number of flies that passed the 8 cm mark in 10 seconds was counted (3 times/tube).

DMSXL mice were previously described[25]. Tibialis anterior muscle from 6 *DMSXL* and 6 control littermates of 2 month-old were a kind gift of *Dr.* Geneviéve Gourdon (INSERM, Paris).

Transcriptomic study and data analysis

Microarrays were performed with RNA extracted from the fibroblasts from 6 DM1 patients (3 male and 3 female; age from 34 to 59) and 3 controls (2 male and 1 female; age from 46 to 52) at early passage using TRIzol (Life Technologies, Carlsbad, CA, USA). Large-scale gene expression analysis was performed using the Human Clariom D microarray (Affymetrix, Santa Clara, CA, USA). RNA integrity value above 9 was confirmed using an RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA, USA). A total of 300 ng of RNA from each sample was used for microarray analysis, following the manufacturer's instructions.

Microarray data analysis: Arrays normalization was performed using the Affymetrix Robust Multi-array Analysis (RMA) background correction model. To find the statistically significant differentially expressed genes (DEGs) between two groups of samples we calculated the mean values of each probe across all the samples of each of the two groups. Next, we filtered out all the probes whose absolute value of difference of mean values between the two groups was less than a selection threshold $\theta DEG = 2$ (that corresponds to a fold change of 1 in log2 scale), and we applied the Student's t-test. The multitest effect influence was tackled through control of the False Discovery Rate (FDR) using the Benjamini-Hochberg method for correcting the initial p-values with significance threshold $\alpha DEG = 0.01$. The GO terms were taken from the curated collection of molecular signatures (gene set collection C5) of version 3.0 of the Molecular Signatures Database (MSigDB). The significance of DEGs was analyzed using an enrichment approach based on the hypergeometric distribution to estimate the significance (*p*-value) of the gene set enrichment. Data post-processing and graphics were performed with in-house developed functions in Matlab (MathWorks™).

Data availability statement

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO series accession number GSE142542. Additional data that support the findings of this study are available from the corresponding author upon reasonable request.

Proliferation and senescence studies

For *cell growth* assays, 3×10^4 early-passage fibroblasts were seeded in 6-well plates in complete medium and counted at day 1, 3, and 5 using a light microscope.

To assess *senescence*, a serial passage protocol was performed. Human fibroblasts were cultured and passaged every 3 days. Population doublings (PDLs) were calculated using the formula PDL = log10 (nº of cells at day 3/nº of cells at day 0)/$\log_{10}$. Additionally, senescence was studied at early (5 to 10), intermediate (10-25) and late passages (35 to 40) by measuring SA-β-Gal activity using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology), according to the manufacturer's protocol. At least 4 controls and 4 DM1 samples were cultured simultaneously to complete these experiments.

Treatment with senolytics

Quercetin (Sigma-Aldrich), Navitoclax (Selleckchem), and Dasatinib (Sigma-Aldrich) were used to treat fibroblasts. For SA-β-Gal measurement, 0.5×10^4 cells were seeded in immunofluorescence chambers and then treated with 5, 10 and 15 µM Quercetin; 0.1, 1 and 10 µM Navitoclax or 0.1 and 0.5 nM Dasatinib for 72 hours. For mRNA expression analysis, 10×10^4 fibroblasts were seeded in p100 plates and then incubated with 15 µM Quercetin, 10 µM Navitoclax or 0.5 nM Dasatinib for 48 hours. In these experiments early passage was considered between 1 and 5 whereas late passage was over 40. Experiments at early passage were performed in 1 control and 1 DM1 patients and experiments al late passage were performed in more than 3 controls and 3 DM1 patients.

mRNA expression analysis

Total RNA was extracted using TRIzol (Life Technologies) and tissues from *D. melanogaster* (12-thorax per condition) and DMSXL mice (tibialis anterior) were previously homogenized in TRIzol using a tissue lyser (85210, Qiagen). Reverse transcription was performed using random priming and the Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase (Thermo Fisher Scientific), according to the manufacturer's guidelines. Quantitative PCR (qPCR) was performed using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 10 mM each primer and 20 ng of cDNA, in a CFX384 thermocycler (Bio-Rad). Variations in RNA input were corrected by analyzing the expression of *GAPDH* (human), *Actin* (DMSXL mice) or *rp49* (*D. melanogaster)* as a housekeeping gene. The $\Delta\Delta C_T$ method was used for relative quantification. Primers sequences will be given upon request to the corresponding authors.

Splicing assays were performed by reverse-transcription PCR (RT-PCR), using BIOTAQ DNA Polymerase (Bioline). The primers used have been described previously and were selected to give a length difference of 10%-25% between exon-inclusion

and exon-exclusion products[64]. PCR amplification was performed for 20–24 cycles and PCR products were resolved on agarose gels, stained with GelRed Nucleic Acid Gel Stain (Biotium), visualized using an iBright FL1000 system (Invitrogen), and quantified using ImageJ.

Western blotting and immunofluorescence analysis

For ***immunofluorescence assays,*** $12x10^3$ fibroblasts were seeded in immunofluorescence chambers and after 14-18h were processed following standard procedures previously described[56]. Nuclear DNA was stained with Hoechst 33342 (Sigma-Aldrich) and slides were examined using Zeiss LSM 900 confocal microscope or Nikon Eclipse 80i microscope.

For ***western-blot assays,*** $20x10^4$ fibroblasts were seeded in T75 flasks and after 14-18h were processed following standard procedures previously described[56]. HRP-linked secondary antibodies were used (Cell Signaling Technology), and detection was performed by chemiluminescence using the Novex ECL Chemiluminescent Substrate Reagent Kit (Thermo Fisher Scientific) in an iBright FL1000 system (Invitrogen).

To evaluate ***DM1 alterations*** we use the primary antibodies against MBNL1 (1:500, ab45899; Abcam, Cambridge, UK), anti-DMPK (1:200 and 1:500, sc-134319; Santa Cruz Biotechnology) and anti-CUGBP1 (1:500, ab9549; Abcam).

To evaluate ***cell proliferation,*** we use primary antibodies against phospho Histone H3 Ser10 (1:2000, ab14955, Abcam), anti-Ki67 (1:500, ab15580; Abcam), anti-$p27^{KIP1}$ (1:500, BD610241; BD Biosciences, Franklin Lakes, NJ, USA), anti-$p16^{INK4a}$ (1:200, ab108349, Abcam) and $p21^{CIP1}$ (1:500, sc-397, Santa Cruz Biotechnology) and anti-BMI1 (1:500, 05-637; Millipore).

To evaluate ***DNA damage and apoptosis,*** we use the primary antibodies against γ-H2AX Ser139 (1:250, 05-636; Millipore, Burlington, MA, USA), anti-53BP1 (1:250, NB100-304; Novus Biologicals), anti-phospho-ATM Ser1981 (1:250, 13050, Cell Signaling Technology), anti-cleaved PARP1 (1:700, ab32064; Abcam) and anti-active-Caspase 3 (1:500, AF835; R&D Systems).

Other antibodies used were anti-GFP (1:1.000, ab-6673; Abcam) and anti-β-actin (1:10.000, AC-15; Sigma-Aldrich).

For the fluorescence intensity measurement of γ-H2AX, 53BP1, DMPK, MBNL1 and CUGBP1, at least 100 cells were measured and an outline of the same size around the nucleus was used as the background fluorescence. Using Image J, we calculated the integrated intensity subtracting the background fluorescence for each fibroblast. For γ-H2AX, 53BP1 and CUGBP1 we measured nuclear fluorescence and for DMPK and MBNL1 we measured nuclear and cytoplasmic fluorescence.

Fluorescent in situ hybridization (FISH)

$5x10^3$ fibroblasts were plated in 8-chamber slides. After an over-night (o/n) incubation, fibroblasts were fixed in 4% of formaldehyde (Sigma-Aldrich) for 15 min, washed and permeabilized by treatment with 70% ethanol o/n at 4ºC. Cells were rehydrated for 5 min at room temperature, in 2x SSC (Sigma-Aldrich), 50% formamide (Sigma-Aldrich) and then incubated for 5 min at 80ºC prior to hybridization. Then, cells were hybridized in an atmosphere saturated with 100% humidity o/n at 37ºC. Hybridization buffer was composed of 10% dextran sulfate (Sigma-Aldrich), 2 mM vanadyl-ribonucleoside complex (Sigma-Aldrich), 0.02% RNAse-free BSA (Sigma-Aldrich), 2x SSC, 50% formamide and 0.1 µM of Alexa-488 (CAG)x10 probe (Thermo-Fisher). Then, fibroblasts were washed twice for 30 min with 2x SSC, 50% formamide at 37ºC, stained with DAPI (1:600), mounted using Fluoro-Gel (17985-10 Aname) and examined using Zeiss LSM 900 confocal microscope. Images were processed using Zen Blue and Image J software's.

Lentiviral transductions

For stable over-expression of BMI1, lentiviral transduction of primary fibroblasts was performed using a pCCL-BMI1 construct (a gift from Jacqueline Lees's group, Institute of Integrative Cancer Research, MIT, US) and pCCL-GFP as a control. After incubation with polybrene (Sigma-Aldrich), cells were infected at a multiplicity of infection of 10 for 6 hours and then medium without serum was replaced with normal medium66. For BRCA1 silencing, lentiviral transduction was performed using pLKO-shBRCA1#1 (this construct was a gift from Eros Lazzerini Denchi, Addgene plasmid #44594) and the corresponding pLKO.1 puro (gift from Bob Weinberg, Addgene, plasmid#8453).

Cytokine antibody arrays

Fibroblast cultures were washed and incubated in serum-free DMEM for 48 hours to generate conditioned medium (CM), which was then collected and cells were counted. CM was frozen at −80 °C and analyzed using the Human Inflammation Array C3 (RayBiotech), following the manufacturer's instructions. Briefly, CM was thawed and centrifuged for 5 min at 15.000 rpm. Array membranes were preincubated with 2 mL of blocking solution, incubated with CM overnight at 4 °C, washed 5 times, and finally incubated with a biotin-conjugated antibody cocktail overnight at 4 °C. After 5 washes, membranes were incubated with HRP-streptavidin for 2 hours at room temperature and then visualized using chemiluminescence detection in an iBright FL1000 system (Invitrogen). Signals were analyzed and normalized using ImageJ.

Statistics

Data are presented as mean values ± S.E.M., with the number of experiments (n) in parentheses. Unless otherwise indicated, statistical significance (p-values) was calculated using the Student´s t -test. Asterisks (*, **, and ***) indicate statistical significance ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively). Correlation analyses were done using Pearson coefficient when samples were normally distributed or Spearman coefficient when samples were not normally distributed using GraphPad. Log-Rank test was used for longevity studies.

Study approval

This study was approved by the Donostia University Hospital Ethical Board (approval number 15-57) and was conducted in accordance with the Declaration of Helsinki´s ethical standards. All subjects gave written informed consent before sample donation.

Acknowledgements and Funding

MG-P and AS-A are recipient of predoctoral fellowships from the University of the Basque Country (PIF 15/245) and Carlos III Institute (FI17/00250) respectively. This work is supported by grants from the Instituto Salud Carlos III and FEDER funds (CP16/00039, PI16/01580, PI17/01841, DTS18/00181) and Health department from Basque Country (2017222021).

Author contributions

MG-P, AS-A, and AA performed all the experiments in fibroblasts. GG performed experiments in fruit flies. AS and MG-P performed experiments in myoblasts. MG-P analysed the results and completed the figures. RF-T and MZ collected clinical data and samples from the patients, evaluated the patients and did the clinical experiments. MA-B performed the transcriptomic analysis. AM and ALM directed the project, contributed to data analysis and wrote the manuscript.

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CAPÍTULO 4. Myotonic Dystrophy type 1 cells display impaired metabolism and mitochondrial dysfunction that are reversed by metformin

Artículo publicado en Aging
Factor de Impacto 5.52 - Cuartil 1

García-Puga M*, Saenz-Antonñanzas A, Fernández-Torrón R, López de Munain A, Matheu A. **Myotonic Dystrophy type 1 cells display impaired metabolism and mitochondrial dysfunction that are reversed by metformin.** Aging (Albany NY). 2020 Apr 8;12(7):6260-6275. doi: 10.18632/aging.103022. Epub 2020 Apr 8.

Submitted: 1 December 2019. Accepted: 2 March 2020. Published: 8 April

Myotonic Dystrophy type 1 cells display impaired metabolism and mitochondrial dysfunction that are reversed by metformin

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Key words:

Myotonic Dystrophy type 1, cellular metabolism, OXPHOS activity, mitochondria, metformin, fibroblasts

Resumen

En este trabajo caracterizamos el impacto del metabolismo celular y de la función mitocondrial en pacientes con DM1, tanto en muestras de fibroblastos como de PBMC derivadas de pacientes con DM1.

Nuestros resultados describen un descenso en la fosforilación oxidativa con un menor nivel de respiración basal, así como de producción de ATP por la vía mitocondrial. Por otro lado, también observamos una alteración en los procesos de fusión y fisión mitocondrial, así como en la producción de ROS total y específica de mitocondria.

Además, el tratamiento con metformina revirtió el descenso en el metabolismo mitocondrial, además de otros procesos como la proliferación celular, la producción de ROS y los procesos de fusión y fisión mitocondrial. Todos ellos procesos que se encuentran alterados durante el envejecimiento fisiológico.

En este trabajo identificamos al metabolismo mitocondrial como uno de los mecanismos implicados en la DM1 y cómo el tratamiento con metformina lo revierte.

Contribución

Mi contribución en este trabajo ha sido la realización de toda la experimentación, además del análisis de los resultados generados y la contribución en la escritura del manuscrito.

Abstract

Myotonic dystrophy type 1 (DM1; OMIM #160900) is an autosomal dominant disorder, clinically characterized by progressive muscular weakness and multisystem degeneration. The broad phenotypes observed in patients with DM1 resemble the appearance of a multisystem accelerated aging process. However, the molecular mechanisms underlying these phenotypes remain largely unknown.

In this study, we characterized the impact of metabolism and mitochondria on fibroblasts and peripheral blood mononuclear cells (PBMCs) derived from patients with DM1 and healthy individuals. Our results revealed a decrease in oxidative phosphorylation system (OXPHOS) activity, oxygen consumption rate (OCR), ATP production, energy metabolism, mitochondrial dynamics in DM1 fibroblasts, as well as increased accumulation of reactive oxygen species (ROS). PBMCs of DM1 patients also displayed reduced mitochondrial dynamics and energy metabolism.

Moreover, treatment with metformin reversed the metabolic and mitochondrial defects as well as additional accelerated aging phenotypes, such as impaired proliferation, in DM1 derived fibroblasts. Our results identified impaired cell metabolism and mitochondrial dysfunction as important drivers of DM1 pathophysiology and therefore, revealed the efficacy of metformin treatment in a pre-clinical setting.

Introduction

Myotonic dystrophy is the most common type of muscular dystrophy in adults and is inherited in an autosomal dominant manner [1]. There are two clinically similar but genetically distinct types: DM type 1 (DM1, also known as Steinert's disease; OMIM #160900), caused by an unstable expansion of a CTG trinucleotide repeat in the non-coding region of the dystrophia myotonic-protein kinase gene (*DMPK*) [2], and DM type 2 (DM2; OMIM #602668), caused by a tetra-nucleotide repeat CCTG expansion in the zinc finger 9 (*CNBP*) gene [3]. CTG and CCTG mutations lead to formation of transcript aggregates in the nucleus, which interfere with proteins that play an important role in RNA metabolism, including members of the muscleblind (MBNL) and CUGBP RNA-Binding Protein Elav-Like Family Member 1 (CELF1) family of RNA-binding proteins [4]. Both diseases are characterized by missplicing of several downstream effector genes with negative effects on multiple tissues, thus contributing to the multisystem pathogenesis of DM [5]. DM1 is more common than DM2 and represents a more severe phenotype. In DM1, unaffected individuals carry less than 50 triplet repeats, whereas expansions ranging between 50 and 4000 CTG repeats have been found in affected individuals [6].

Patients with DM1 present a multisystem degenerative process that includes progressive muscular weakness and atrophy, myotonia, cardiomyopathy, insulin-resistance, cataracts, increased cancer incidence, neurodegeneration, metabolic syndrome, or premature death. This multisystem degenerative process strongly resembles an accelerated aging process [7,8]. From a cellular point of view, different pathogenic mechanisms, such as alteration of autophagy, increased senescence, telomere shortening, or genomic instability, all of them hallmarks of aging [9], have been proposed to explain how the expansion in the genome of affected patients leads to the DM multisystem phenotypes [7]. However, detailed experimental validation of these mechanisms remains incomplete and has not yet been clarified.

It is well known the existence of several metabolic alterations, which accumulate over time, and affect longevity, aging and neurodegeneration [10,11]. As a consequence, deregulated nutrient sensing and mitochondrial dysfunction have been proposed as hallmarks of aging [9] and metabolism is a pillar of aging [12]. In DM1, patients present several metabolic defects such as hyperinsulinemia, glucose resistance, and in some cases, diabetes mellitus [7]. Moreover, muscle samples *in vitro* and blood samples *in vivo* show reduced Coenzyme Q10 (CoQ10) levels, a component of the electron transport chain that participates in aerobic cellular respiration [13,14], indicative of mitochondrial dysfunction. However, the role of metabolism and mitochondria in the pathogenesis of DM1 has not been addressed in detail.

In this work, we studied their contribution using human primary fibroblasts and PBMCs derived from healthy donors and patients with DM1 as models. Our results indicated that DM1 fibroblasts showed impaired metabolism and mitochondrial dysfunction resulting in lower levels of ATP production and increased reactive oxygen species (ROS) production. PBMCs from DM1 patients also showed impaired mitochondrial dynamics and energy homeostasis. Interestingly, treatment with metformin resulted in the restoration of these phenotypes.

Results

DM1-derived fibroblasts present impaired metabolism

To investigate the role of cellular metabolism in the pathogenesis of DM1, we first measured the oxygen consumption rate (OCR) in the fibroblasts of patients with DM1 and healthy donors. DM1 fibroblasts showed a 40% and 50% reduction in basal respiration and maximal respiration, respectively, compared to controls, which leads to a 50% reduction in ATP production via the Mitochondrial Oxidative Phosphorylation System (OXPHOS) activity **(Fig 1 A,B)**. Next, we hypothesized that the reduction in OXPHOS activity could be responsible for a reduction in the glycolysis pathway. To examine this hypothesis, we measured extracellular acidification (ECAR) as a measure of glycolysis [15]. We did not find any alteration in the glycolysis pathway **(Fig Suppl 1)**, suggesting that all glucose taken by DM1 fibroblasts was coupled to pyruvate production. In the canonical pathway, pyruvate is converted to acetyl-CoA and transported to the mitochondria, but in some pathological conditions it could be transformed to lactate. We measured the levels of lactate in DM1 cells and we found an elevation of 1.8-fold of this metabolite **(Fig 1F)**.

The addition of carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) simulates an exacerbated physiological energy demand by stimulating the respiratory chain to operate at maximum capacity. DM1 cells were not able to respond to this stress as efficiently as controls, indicating impaired maximal respiration **(Fig 1 A,B)**. However, we did not find any difference in the proton-leak nor the coupling efficiency **(Fig 1A-C)**. Therefore, it seems that all the protons generated are coupled to ATP production. Moreover, DM1 fibroblasts have a more quiescent metabolism compared to healthy controls and simulate a stress condition. DM1 fibroblasts could not switch to a more energetic metabolism **(Fig 1D,E)**, resulting in a lower metabolic potential. Consistent with these results, DM1 fibroblasts presented lower AKT activation (measured as phosphorylated AKT) **(Fig 2A,B)**, which is the central mediator of the PI3K pathway that serves a key role in multiple cellular processes, including glucose metabolism [16]. In summary, DM1-derived fibroblasts present decreased metabolism.

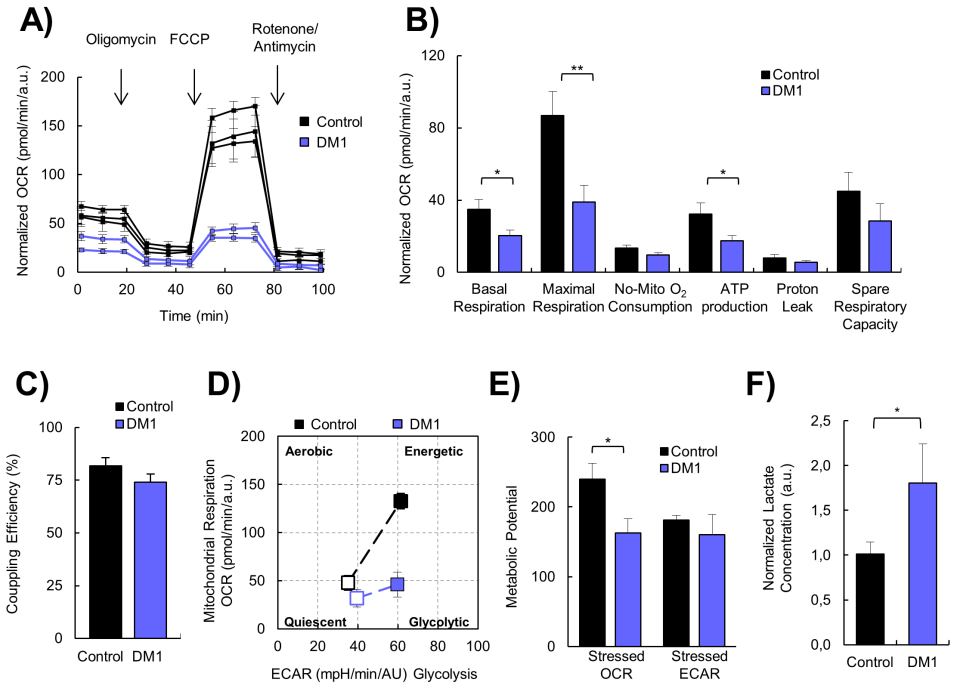

Figure 1. DM1-derived fibroblasts present impaired metabolism.

A) Kinetic normalized OCR response in DM1 and control fibroblasts in basal conditions and after consecutive addition of Oligomycin 1.5 µM, FCCP 1.5 µM and Antimycin-A/Rotenone 1.5 µM. A representative experiment out of 3 is shown with 3 independent control and 2 DM1. **B,C)** Quantification of mitochondrial respiratory functions and coupling efficiency in DM1 (n=7) and control fibroblasts (n=3). **D)** Representative energy map and **E)** Quantification of metabolic potential of DM1 and control fibroblasts. *Stressed* indicates the values of OCR and ECAR after the injection of Oligomycin and FCCP simultaneously in DM1 (n=5) and control fibroblasts (n=3). **F)** Lactate production in DM1 (n=4) and control fibroblasts (n=2).

Correlation between impaired metabolism and markers of disease pathophysiology

Next, we attempted to associate the impaired metabolism of DM1-derived fibroblasts with several pathophysiological characteristics of the disease. First, we found that the decrease in AKT phosphorylation in DM1-derived fibroblasts correlated with lower expression of DMPK and MBNL1, both at the protein **(Fig 2A,B)**, and mRNA levels **(Fig 2C)**. Moreover, we examined whether there was a correlation between the severity of the metabolic alterations and both the number of CTG expansions and the Muscular Impairment Rating Scale (MIRS) score. We did not detect significant differences in basal and maximal respiration or in ATP production when fibroblasts were divided into those with less or more than 500 CTG repeats and 3 MIRS score **(Fig 2D,E and Fig Suppl 2)**. Moreover, there were no marked differences between cells obtained from DM1 patients of different ages, although the cells from a 71-year-old patient showed slightly higher impairment than others **(Table 1, Fig Suppl 3)**. Overall, metabolic dysfunction in fibroblasts derived from patients with DM1 seems not to be significantly altered by the repeat expansion of patients with DM1.

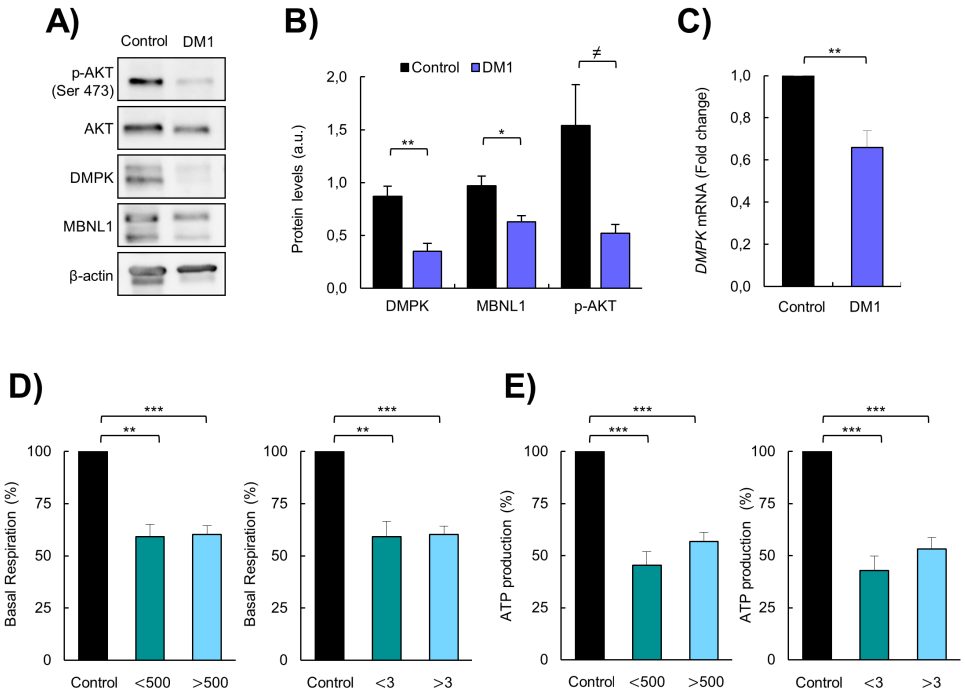

Figure 2. Correlation between impaired metabolism and markers of disease pathophysiology. **A)** Representative immunoblots and **B)** Quantification of phospho-AKT, AKT, DMPK and MBNL1 protein levels (n=4). **C)** mRNA levels of *DMPK* in DM1 fibroblasts (n=7) and controls (n=3). **D)** Basal respiration levels in controls (n=3) and DM1 fibroblasts stratified by CTG expansion in <500 (n=4) and >500 (n=3) (left) and MIRS scale in <3 (n=2) and >3 (n=5) (right). **E)** ATP production levels using the same stratification by CTG expansion and MIRs scale.

DM1-derived fibroblasts display mitochondrial dysfunction but no changes in mitochondrial biogenesis

The results presented above indicate that the mitochondria of patients with DM1 could function normally, but with a reduced OXPHOS activity. We further investigated this by examining the biogenesis of mitochondria [17]. First, we evaluated the levels of two markers of mitochondrial content and biogenesis such as TOMM20 and PGC1-α. Immunofluorescence showed that expression of these two markers was not markedly altered in cells from DM1 compared to healthy controls **(Fig 3A, B)**. In addition, flow cytometry was used to analyze another marker of mitochondrial content, MitoTracker, obtaining similar results. Indeed, no differences were detected in the mitochondrial content in DM1 and control cells **(Fig 3C)**. Moreover, the mitochondrial membrane potential remained elevated in DM1 cells **(Fig 3D)**. However, the expression of the mitochondrial transcription factor A (*TFAM*), gene that participates in the regulation of the mitochondrial genome [18], was reduced by 50% in DM1 fibroblasts **(Fig 3E)**. These results suggest that the impaired cellular bioenergetics were not related to substantial alterations in mitochondrial biogenesis and content.

Mitochondria are organelles with high dynamic plasticity to rapidly adapt in response to stress situations. Mitochondrial dynamic is regulated by a machinery of pro-fusion and pro-fission proteins, which constitutes an important part of the mitochondria quality control as it facilitates the elimination of damaged mitochondria by mitochondrial selective autophagy (mitophagy) [19]. We studied the expression of *OPA1*, *MFN1* and *MFN2* fusion related genes, *DRP1* fission related gene, and *PARKIN*, which is involved in mitophagy [20]. Interestingly, the levels of *OPA1*, *MFN2*, *DRP1,* and *PARKIN* were decreased in DM1-derived fibroblasts **(Fig 3F)**. Overall, DM1-derived fibroblasts show mitochondrial dysfunction.

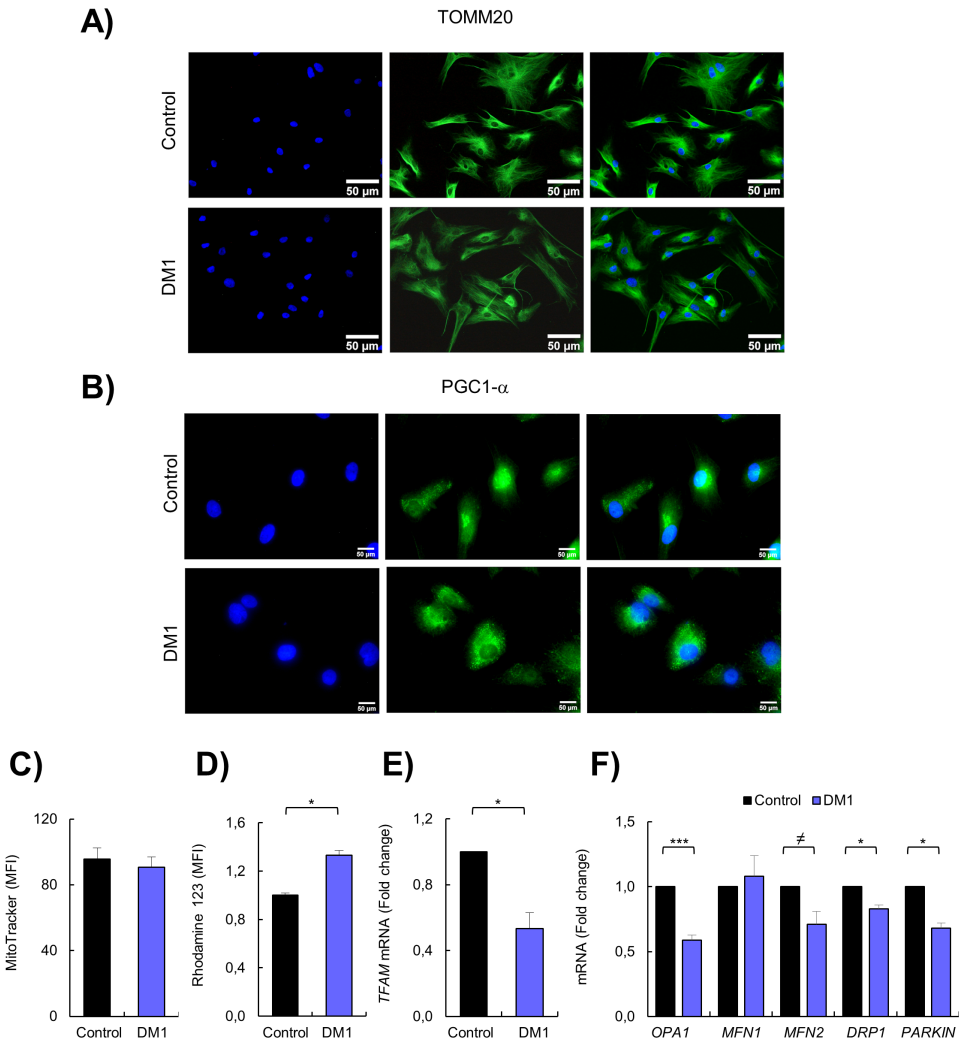

Figure 3. DM1-derived fibroblasts have no changes in mitochondria biogenesis.
A,B) Representative images of immunofluorescence of TOMM20 and PGC1-α in DM1 (n=3) and control fibroblasts (n=3). Medium fluorescence intensity of **C)** MitoTracker Red FM in control (n=3) and DM1 cells (n=5); and **D)** Rhodamine 123 in DM1 (n=3) and control fibroblasts (n=3). **E)** mRNA levels of *TFAM* transcription factor in control (n=3) and DM1 cells (n=3). **F)** mRNA levels of *OPA1, MFN1, MFN2, DRP1* and *PARKIN* in DM1 (n≥2) and control fibroblasts (n=3).

DM1-derived fibroblasts present accumulation of ROS and p38MAPK activation

Production of ROS is enhanced in several pathological conditions in which the respiratory chain is impaired [23]. Therefore, we measured ROS production and found a 50% increase in total ROS in DM1 cells compared to controls **(Fig 4A)**. Similar results were obtained when specific ROS produced by the mitochondria were measured in DM1 fibroblasts and compared to controls **(Fig 4B)**. Further, the expression of glutathione peroxidase 1 (*GPX1*) antioxidant gene was decreased by 50% in DM1 cells **(Fig 4C).** In agreement with metabolic studies, we did not observe differences in ROS accumulation when fibroblasts were divided based on the number of CTG repeats and MIRS score **(Fig 4D,E)**.

p38MAPK is a stress-activated protein kinase, which accumulates with aging and it is activated by the presence of ROS [24, 25]. Consequently, we measured the different p38 isoforms and we found that all isoforms, p38-α, $-\beta$, $-\Delta$, $-\gamma$, were elevate in DM1 cells **(Fig 4F)**. In addition, the total levels of p38MAPK and its phosphorylated form (P-p38MAPK) were an over 2-fold increase in the levels of P-p38MAPK in DM1-derived fibroblasts compared to control cells **(Fig 4G,H)**. In summary, DM1 fibroblasts display increased ROS production, which is associated with an enhanced activation of p38MAPK.

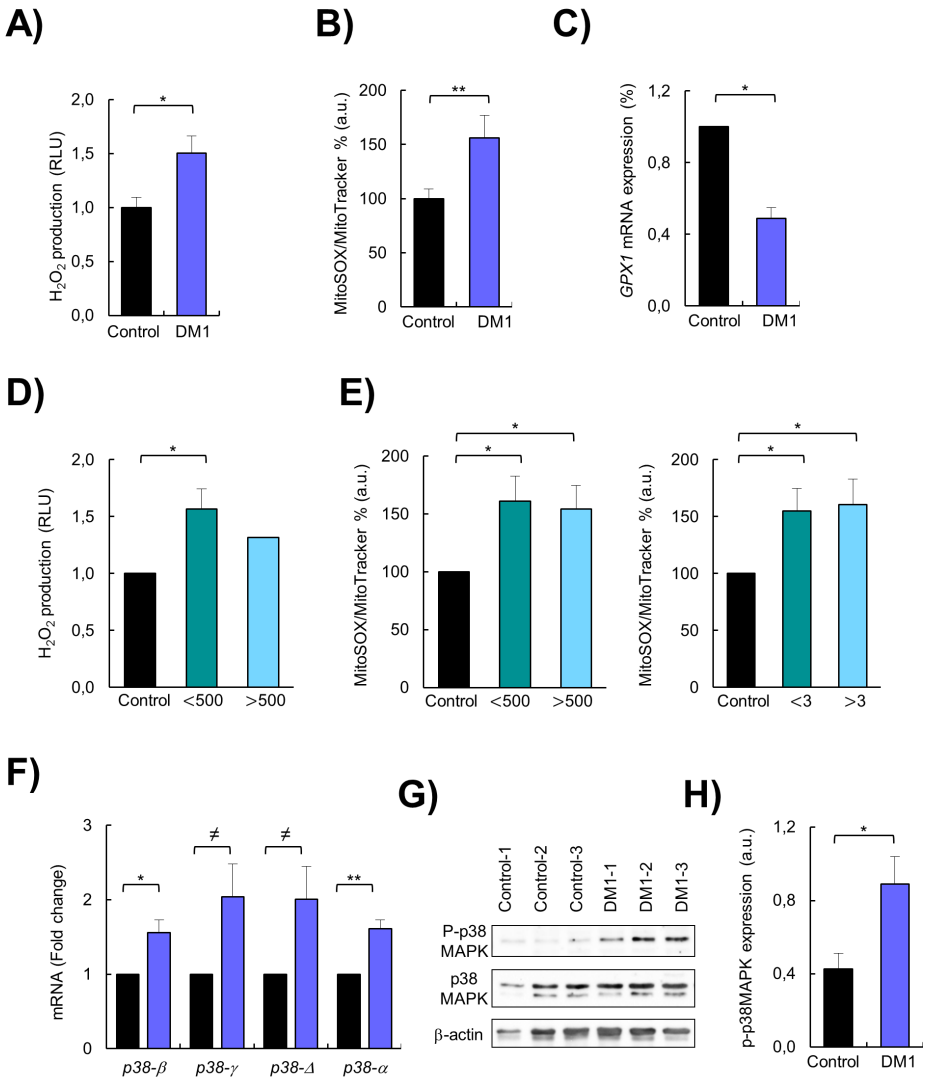

Figure 4. DM1 fibroblasts present accumulation of ROS and p38MAPK activation.
A) Luminescence signal proportional to H_2O_2 production in DM1 (n=4) and control fibroblasts (n=3). B) Medium fluorescence intensity of MitoSOX+ values normalized to mean fluorescence of MitoTracker values in controls (n=3) and DM1(n=5). C) *GPX1* mRNA levels in DM1 (n=2) and control fibroblasts (n=2). D) Luminescence signal proportional to H_2O_2 production in controls (n=3) and DM1 fibroblasts stratified by CTG expansion in <500 CTG (n=3) and >500 CTG (n=1). E) Medium fluorescence intensity of MitoTracker Red FM in controls (n=3) and DM1 stratified by CTG expansion in <500 (n=3) and >500 (n=2) (left) and MIRS scale in <3 (n=2) and >3 (n=3) (right). F) mRNA levels of the different p38 isoforms in DM1 (n=3) and control fibroblasts (n=3). G) Representative immunoblot and H) Quantification of P-p38MAPK and p38MAPK protein levels in DM1 and control fibroblasts (n=3).

DM1-derived blood samples show mitochondria dysfunction

Next, we investigated whether these results could be translated to the clinical setting. Therefore, we measured the expression levels of several of the aforementioned genes in peripheral blood mononuclear cells (PBMCs) from a cohort of patients with DM1 established in Guipúzcoa (Basque Country, Spain) [36]. Interestingly, we found lower expression levels of *SIRT1,* a key metabolic sensor that modulates a large variety of cellular processes such as energy metabolism stress response and aging [22], *OPA1* and *TFAM* (Figure 5), further supporting the relevance of the results obtained in cell culture and highlighting the importance of metabolism and mitochondria for the disease.

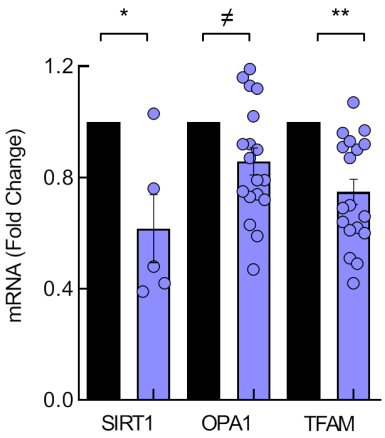

Figure 5. DM1-derived blood samples also show mitochondria dysfunction.
mRNA levels of *SIRT1, OPA1* and *TFAM* in PBMCs derived from DM1 (n≥12) and controls (n=4).

Metformin restores metabolism and mitochondria activity

Metformin is a first-line anti-diabetic agent that functions mainly through the suppression of glucose production and alleviation of insulin resistance and has recently been shown to improve mitochondrial respiratory activity [26, 27]. We examined whether metformin could improve the impaired OXPHOS activity in patients with DM1. To test this idea, we treated DM1 and control fibroblasts with 1 mM of metformin for 72 hours and evaluated cellular metabolism and mitochondrial activity. Interestingly, metformin improved the basal oxygen consumption rate and maximal respiration of DM1 fibroblasts by more than twice (**Fig 6A-C**), which resulted in an increased ATP production via OXPHOS (**Fig 6A,D**). Moreover, it increased the levels of *OPA1*, *MFN1*, *MFN2*, *DRP1* and *TFAM* in DM1 cells by at least 1.5-fold (**Fig 6E**).

Next, we measured ROS production and found that treatment with metformin significantly decreased ROS production in control as well as DM1 fibroblasts (**Fig 6F**)**.** In accordance, the levels of *GPX1* and *PARKIN* were elevated in the presence of metformin in DM1 cells (**Fig 6G**)**.** The levels of *PGC1*α and *SIRT1*, critical downstream targets, were also induced in DM1 cells cultured in the presence of metformin, validating the effect of metformin in metabolic pathways (**Fig 6G**).

In summary, metformin restores the impaired metabolism and mitochondrial activity in DM1 fibroblasts.

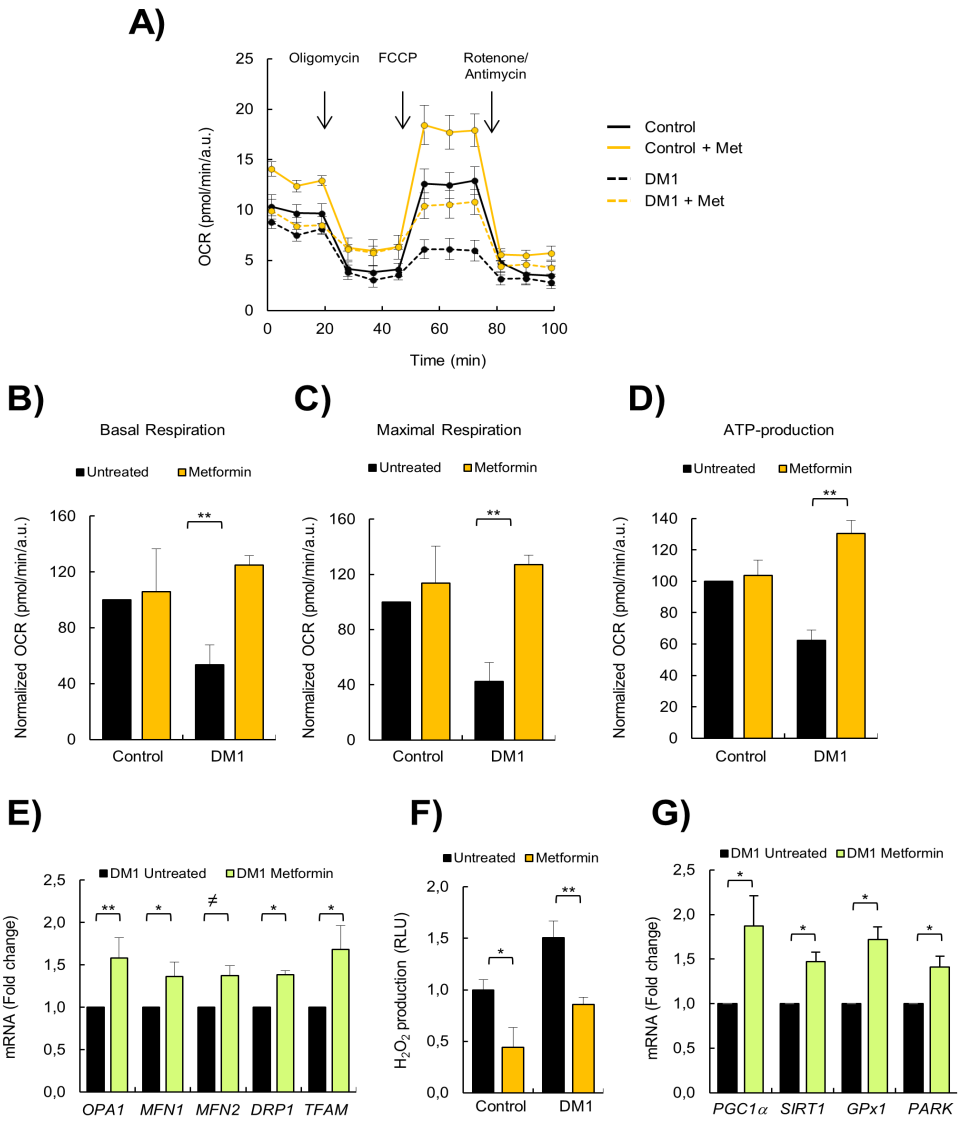

Figure 6. Metformin restores OXPHOS activity and ROS production in DM1 fibroblasts.
A) Representative kinetic normalized OCR response in DM1 (n=6) and control fibroblasts (n=3) after treatment with 1mM of metformin for 72 h. DM1 and control fibroblast were plated at 5.000 cells/well 24 hours prior to the assay. A representative experiment out of 3 is shown. B-D) Quantification of mitochondrial basal respiration, maximal respiration, and ATP production, respectively, after treatment with 1 µM of metformin for 72h in controls (n=3) and DM1 fibroblasts (n=6). E) mRNA levels of *OPA1, MFN1, MFN2, DRP1* and *TFMAN* after treatment with 1 µM of metformin 72 h (n≥2). F) H_2O_2 production after treatment with 1mM of metformin for 72 h (n=3). G) mRNA levels of *PGC1α, SIRT1, GPX1* and *PARKIN* in DM1 and control fibroblasts (n=3) after treatment with 1 µM of metformin 72 h (n≥2).

Metformin restores additional DM1 associated phenotypes

Metformin exerts a potent anti-aging activity, in part by increasing proliferation and inhibiting senescence [28,29]. It has been previously reported that DM1 fibroblasts display decreased cell proliferation and enhanced senescence accumulation [8,30]. Next, we investigated the impact of metformin in the proliferative potential of DM1 fibroblasts. For this, we treated DM1 and control fibroblasts with 1 and 10 mM of metformin and measured cell viability. As expected, DM1 cells had lower viability than controls but, importantly, the treatment increased significantly the viability of DM1 fibroblasts, reaching almost the levels of control cells **(Fig 7A and Fig Suppl 4)**. Moreover, we measured the number of cells positive for phospho-Histone H3, Ki-67 and EdU, which are well-established markers of mitosis and cell division, and found reduced numbers in those markers in DM1 cells **(Fig 7B-G)**. Importantly, metformin increased the number of pH3, Ki-67 and EdU positive cells by almost 2-fold in DM1 cells (**Fig 7C,E,G**).

These functional results were further validated at the molecular level. Metformin modulated the expression of critical genes involved in cell proliferation and cell cycle activity such as BMI1, $p16^{INK4a}$ and $p21^{CIP}$. In particular, treatment for 72 h increased the levels of *BMI1,* and partially decreased the levels of $p16^{INK4a}$ and $p21^{CIP}$ cell cycle inhibitors **(Fig 7F)**. Finally, we also detected that metformin restored by 1.5-fold the levels of *DMPK* and *MBNL1* **(Fig 7G)**. Overall, metformin rescues multiple phenotypes associated to DM1 cells.

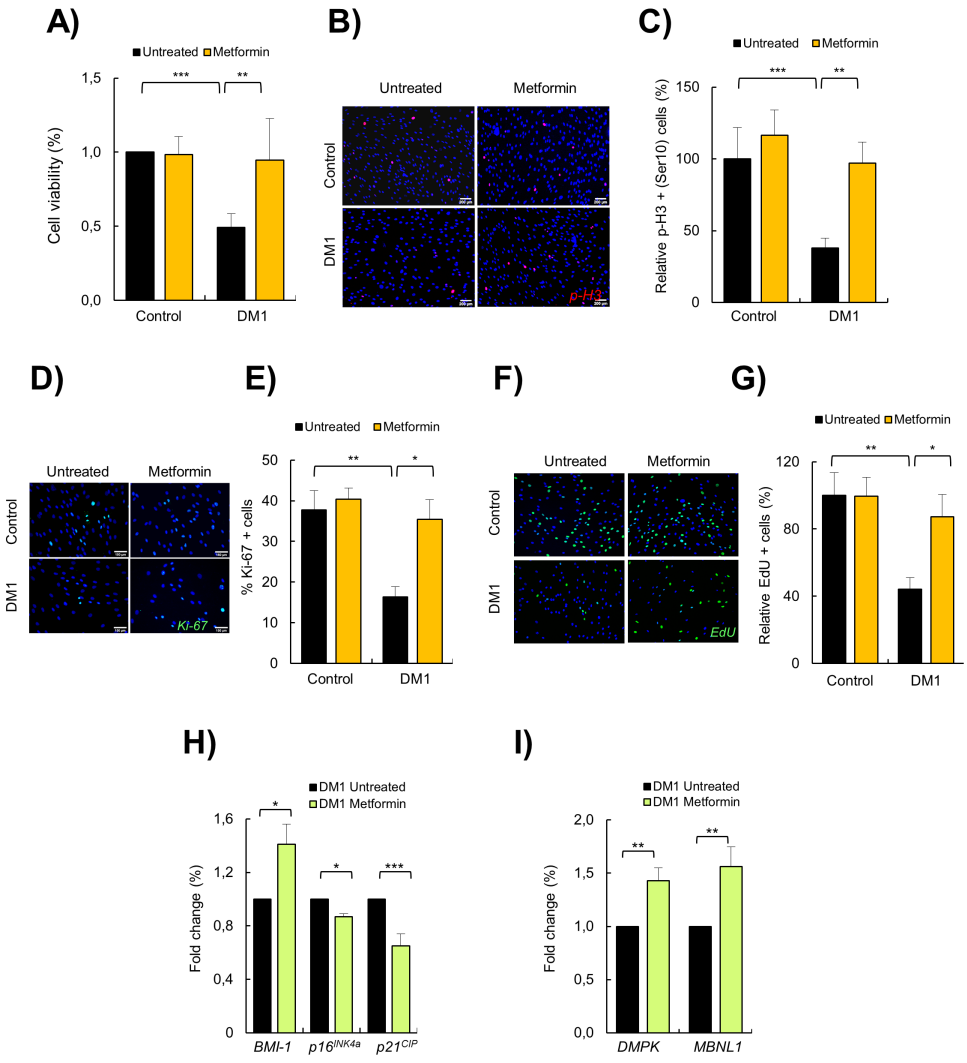

Figure 7. Metformin restores cell viability and proliferation in DM1 fibroblasts.
A) Cell viability of DM1 (n=5) and control (n=3) fibroblasts measured in MTT studies after treatment with 1 µM of metformin for 72 h. **B,C)** Representative image and quantification of phospho-Histone H3 (Ser10) staining in controls (n=3) and DM1 fibroblasts (n=7). **D,E)** Representative image of Ki-67 staining and quantification in controls (n=2) and DM1 cells (n=3). **F,G)** Representative image of EdU staining and quantification in controls (n=2) and DM1 cells (n=3). **H)** mRNA levels of *BMI1*, *p16*^{INK4a} and *p21*^{CIP} in cells treated or not with 1 µM of metformin 72h (n≥2). **G)** mRNA levels of *DMPK* and *MBNL1* in same conditions (n=3).

Discussion

We established primary cultures of fibroblasts derived from patients with DM1 and found that they display impaired metabolism and mitochondrial dysfunction. In particular, DM1 fibroblasts present lower production of ATP by OXPHOS, less efficient mitochondrial electron transport chain, impaired mitochondrial dynamics, and higher production of ROS compared with healthy control-derived fibroblasts. Interestingly, some of these defects, such as energy homeostasis and mitochondrial dynamics, were also detected in PBMCs from patients with DM1, together revealing the impact of metabolism and mitochondrial function on the pathophysiology of DM1.

These results show that fibroblasts, which are a well-established model for cell aging studies *in vitro* [31], might be a good cellular model to characterize the pathophysiology of the disease, as they resemble multiple molecular and cellular phenotypes of the disease. However, we did not detect a correlation between the severity of the phenotypes and the number of CTG repeats. This result might be potentially biased by methodological reasons because CTG was measured several years before isolation of skin fibroblasts, from blood samples, and could be also related because of experiments were performed at early passage.

Our results reveal novel processes involved in the pathophysiology of the disease. Indeed, the role of mitochondria in DM1 remained practically unknown. A previous study observed an inverse correlation between the expression of Coenzyme Q10, an electron carrier in the mitochondrial respiratory chain, and lactate production with CTG expansion in PBMC samples [14], whereas mitochondrial dysfunction was suggested to occur in muscles of patients with DM1 as well [32]. The results of these studies are in line with our data and are indicative of mitochondrial dysfunction in DM1. The lower mitochondrial efficiency detected in our study could be due to the conversion of pyruvate, generated during glycolysis, to lactate instead of acetyl CoA, which is transported to mitochondria and enters into the Krebs cycle. Moreover, our results show that mitochondrial biogenesis seems to be normal in DM1 cells, but they are not able to maintain the metabolic state as a consequence of unbalance remodeling of mitochondrial network morphology, which is not correctly controlled by the machinery of pro-fusion and fission proteins, and impaired elimination through mitophagy.

DM1 patients develop a large variety of symptoms in multiple systems that strongly resemble the clinical signs of accelerated aging, including some related to metabolism and mitochondria dysfunction such as insulin resistance, glucose intolerance, hyperinsulinemia, and increased risk of type 2 diabetes [33, 34]. Our results shed light in the underlying molecular mechanisms of these symptoms. Given that mitochondria is the main energy hub of the cell and the main intracellular source of

ROS, our results might be extended to additional DM1 symptoms, particularly in the muscle, since a shift in energy production anticipates muscle atrophy with aging. Finally, our results further support the link between DM1 and accelerated aging [8], since cellular metabolism and mitochondrial dysfunction are critical mechanisms in aging.

DM1 is a rare, clinically variable disease with no currently available treatment to slow or stop disease progression. Supportive treatments, preventative measures and clinical surveillance are the only options available for patients with DM1 [35]. Metformin is a synthetic biguanide that is currently one of the most recommended medications for type 2 diabetes treatment around the world. Interestingly, studies in both vertebrates and invertebrates have shown that metformin delays aging and increases longevity [29]. Moreover, a meta-analysis has suggested that metformin reduces all-cause mortality and aging-related diseases in humans independent of its effect on diabetes [36]. We show here that metformin improves ATP production by OXPHOS and decreases the production of ROS in DM1 cells even at a much lower concentration compared to its current therapeutic dose (1 mM *vs* 75 mM). In line with our results, it has been recently shown that mitochondria might be a target of metformin [27]. We also found that metformin treatment reverses additional DM1-related phenotypes such as impaired proliferation, suggesting that its mechanism of action in DM1 is wider. In support, low doses of metformin may also correct several alternative splicing defects in DM1 myoblasts *in vitro* [37], the use of metformin reduced the risk of cancer in patients with DM1 having diabetes [38], and also improved mobility of DM1 patients in a small randomized clinical trial [39]. If the hypothesis of an accelerated aging in patients with DM1 is validated, our results add to the potential benefits of expanding metformin use in DM1, outside of the management of T2D, to include cancer prevention [38] and also phenotypes associated with aging. In summary, our results showed the efficacy of metformin in a pre-clinical setting and suggest that it warrants further assessment as a candidate drug for DM1 treatment.

Table 1. Characteristics of human primary fibroblasts used in the present study.

Fibroblasts	Status	Gender	Age biopsy (years)	MIRS	CTG (n) in blood	Age diagnosis (years)
C1	Control	M	49			
C2	Control	F	48			
C3	Control	Unknown	53			
DM1-1	DM1	M	71	3	167	53
DM1-2	DM1	F	45	2	333	41
DM1-3	DM1	F	59	3	333	26
DM1-4	DM1	F	44	2	833	27
DM1-5	DM1	M	56	3	1333	20
DM1-6	DM1	F	34	4	1650	12
DM1-7	DM1	M	50	5	233	20

MIRS: Muscle impairment rating scale.

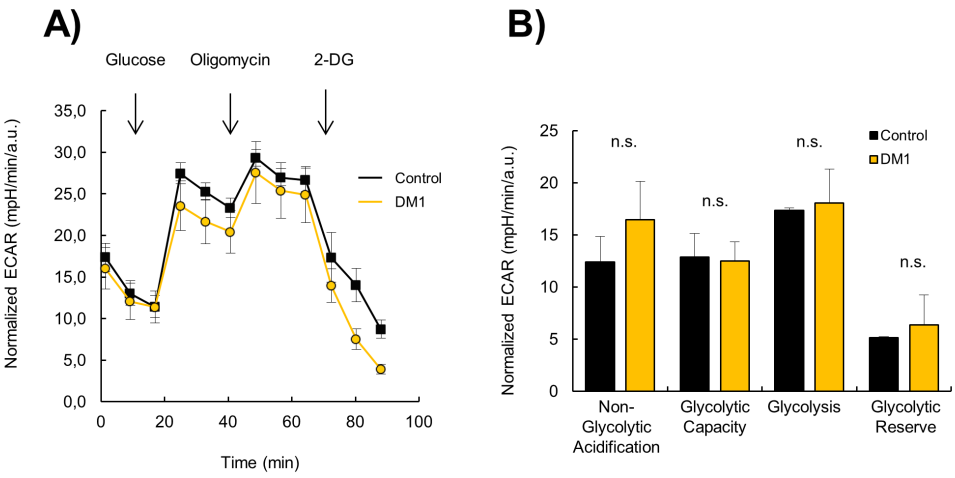

Figure Supplementary 1. DM1-derived fibroblasts have no changes in glycolysis.
A) Kinetic normalized ECAR response in DM1 and control fibroblasts obtained in basal condition and after consecutive addition of Glucose 10 mM, Oligomycin 1 µM and 2-D-Deoxy-Glucose 50 mM. DM1 and control fibroblast were plated at 5.000 cells/well, in XF96 cell culture plates, 24–28 hours prior to the assays. The assay medium was the substrate-free base medium supplemented with 2 mM glutamine. Upon completion of an assay, cells were normalized using violet crystal. A representative experiment is shown here (n=3 controls and n=4 DM1 patients). B) Quantification of glycolytic functions in DM1 and control fibroblasts (n=3 controls and n=4 DM1 patients).

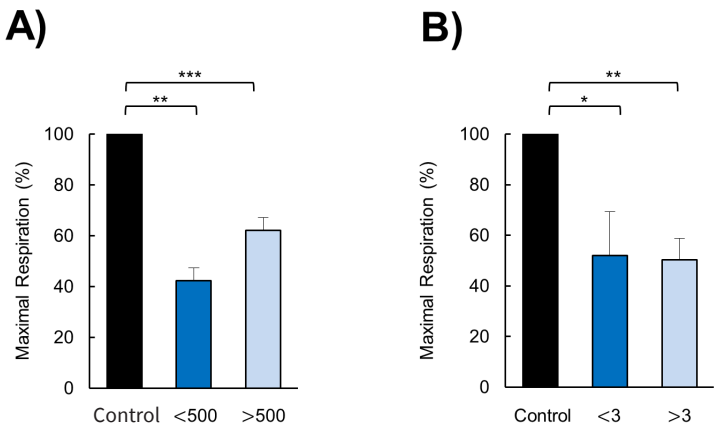

Figure Supplementary 2. DM1-derived fibroblasts have no changes in maximal respiration after stratification by CTG amplification and MIRS scale.
Maximal respiration in DM1 derived fibroblasts stratified by **A)** CTG expansion (n=3 controls; n=4 <500 CTG and n=3 >500 CTG DM1 patients) and **B)** MIRS scale (n=3 controls; n=2 MIRS <3 and n=5 MIRS >3 DM1 patients).

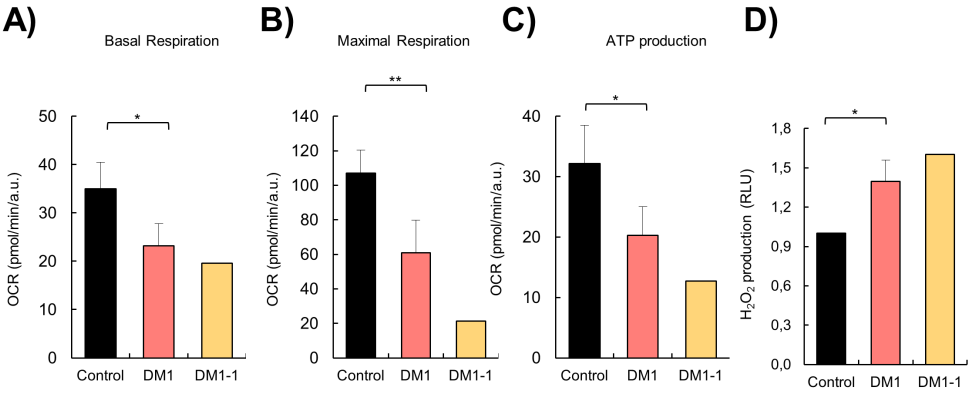

Figure Supplementary 3. **A)** Basal, **B)** Maximal respiration, **C)** ATP production and **D)** H_2O_2 production in control fibroblasts (first bar), in DM1 derived fibroblasts excluding the case with 71 years old (second bar) and in the specific case of the patient with 71 years old (third bar).

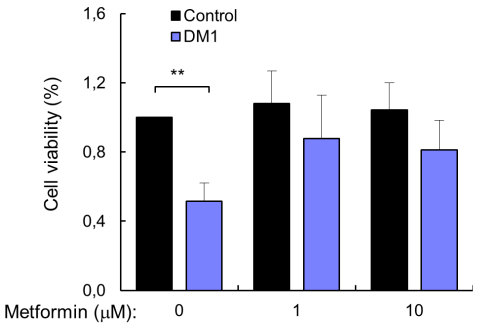

Figure Supplementary 4. Metformin restores cell viability in DM1-derived fibroblasts.
Cell viability of DM1 and control fibroblasts after treatment with 1 and 10 μM of metformin 72 h (n=3 controls and n=5 DM1 patients).

Materials and Methods

Study approval

This study was approved by the Donostia University Hospital Ethical Board (approval number 15-57) and was conducted in accordance with the Declaration of Helsinki's ethical standards. All subjects gave written informed consent before sample donation.

Reagents and Cell culture

For the isolation of primary fibroblasts, punch skin biopsies were chopped into 2–3 mm^3 fragments and placed on a surface moistened with modified Eagle's medium containing 13% newborn calf serum, 0.4% penicillin/streptomycin (Gibco, Waltham, MA, USA) and 2 mM L-glutamine (Gibco). Flasks were incubated vertically for 3–6 hours at 37 ºC in a 5% CO_2 atmosphere and then returned to the horizontal position. Human fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA), 1% L-glutamine (Gibco) and 1% penicillin/streptomycin (Gibco). 7 independent cultures from different patients with DM1 and 3 from healthy controls were established (see Table 1 for patient characteristics). When indicated, fibroblasts were treated with metformin (Sigma-Aldrich) for 72 hours. Experiments were performed in early passage cultures (range 5 to 10 passages).

PBMCs sample extraction and RNA isolation

Peripheral blood samples (PBMCs) were stored in the Basque Biobank. Briefly, blood sample was extracted by venipuncture and PBMCs were immediately isolated using Ficoll. Total RNA was isolated using RNeasy Mini Kit (Qiagen) following the manufacturer´s instructions. RNA samples were then resuspended in DNase/RNase-free water and maintained at -80°C until use. RNA concentration and purity were measured using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific).

Metabolic measurements

Measurement of OCR and ECAR were performed in XF96 plates with *XF Extracellular Flux Analyzer* (Seahorse Bioscience). Fibroblasts were seeded in collagen (BD Biosciences) coated XF 96-well plates (Seahorse/Agilent) in octuplicates at 1.2×10^4 cells/well in 100 µl growth medium. In the metformin treatment experiments, cells were plated at 5×10^3 cells/well 24–28 hours prior to the assay.

Mitochondrial activity was evaluated using the *Seahorse XF Cell Mito stress Test Kit,* according to manufacturer's instructions (Agilent). Oligomycin (75351, Sigma-Aldrich), FCCP (C2920, Sigma-Aldrich), and Rotenone/Antimycin A (R8875 and A8674, Sigma-Aldrich) were used at 1.5 µM concentration, after a titration experiment.

Glycolytic activity was evaluated using the *XF Glycolysis Stress Test* according to manufacturer's instructions (Agilent). Glucose (G8769, Sigma-Aldrich) was used at 10 mM, oligomycin at 1 µM and 2-D-Deoxy-Glucose at 50 mM (D6134, Sigma-Aldrich).

Cell content was normalized using crystal violet. The post-normalization values of OCR and ECAR reflect both the metabolic activities of the cells and the number of cells being measured. Data were further processed according to manufacturer's instructions.

L-Lactate was quantified using L-lactate assay kit (ab65331, Abcam) according to the manufacturer's protocol. Briefly, fibroblasts were seeded at 20×10^4 cells in T75 flasks. After 48h, fibroblasts were detached and counted for posterior normalization. Cell pellet was homogenized in 50 µL of lactate assay buffer and deproteinized using TCA (ab204708, Abcam).

Total ROS measurement

A total of 1×10^3 fibroblasts were plated in 96-well plates and grown for 3 days. Afterwards, *ROS-Glo H_2O_2 Assay* (G8820, Promega) was performed according to the manufacturer's instructions. Briefly, a H_2O_2 substrate was employed that reacts directly with H_2O_2 to generate a luciferin precursor and, upon addition of a detection reagent, the precursor is converted to luciferin, which generates a luminescent signal that is proportional to the H_2O_2 concentration. White flat bottom plates (Corning) were used for final readout in a PHERAstar (BMG Labtech) luminometer plate reader.

Mitochondrial ROS production and mitochondrial content measurement

Mitochondrial ROS analysis was performed using the dye *MitoSOX* (M36008, Invitrogen). Mitochondrial content was assayed using the dye *MitoTracker FM* (M22425, Invitrogen), which passively diffuses across the plasma membrane and accumulates in active mitochondria.

20×10^4 fibroblasts per condition were grown for two days, reaching 70% confluence in p100 plates. Cells were detached using trypsin for 5 min at 37 °C. For MitoSOX staining, cells were washed once using warm HBSS, incubated with 5 µM MitoSOX in HBSS for 30 min at 37 °C, washed 3x using warm HBSS and suspended in HBSS. For MitoTracker staining, cells were washed with PBS, incubated with 0.2 µM MitoTracker for 30 min at 37 °C, washed 3x using warm PBS and suspended in PBS. Cells were directly analyzed via flow cytometry. In FSC and SSC, we first gated the population; next, two gates were set on SSC-A vs. SSC-H and SSC-A vs. SSC-W to exclude doublets. Based on an unstained control, MitoSOX+ and MitoTracker+ gates were set. Mean fluorescence of MitoSOX+ was normalized as a mean fluorescence of MitoTracker values, which represents ROS production per mitochondria. Antimycin was used as a positive control and FCCP as a negative control.

Mitochondrial membrane potential measurement

20×10^4 fibroblasts per condition were grown for 2 days, reaching 70% confluence in p100 plates. Cells were detached using trypsin for 5 min at 37 °C. We used 1 µM of *Rhodamine 123* (Invitrogen) for 15 min at 37 °C to measure the mitochondrial membrane potential. This probe is readily sequestered by functioning mitochondria and is easily washed out of cells once the mitochondria experience a loss in membrane potential.

Cell Viability

Fibroblasts were seeded in 96-well plates followed by treatment with metformin for 72 h. Viable cells were quantified using the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay in six replicates per condition.

Fibroblasts mRNA expression analysis

Total RNA was extracted using TRIzol (Life Technologies). Reverse transcription was performed using random priming and the Maxima First Strand cDNA Synthesis Kit for RT-qPCR, with dsDNase (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's guidelines. Quantitative PCR (qPCR) was performed using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 10 mM of each primer and 20 ng of cDNA, in a CFX384 thermocycler (Bio-Rad, Hercules, CA, USA). Primer sequences will be given upon request. Variations in RNA input were corrected by analyzing the expression of GAPDH as a housekeeping gene. The ΔΔCT method was used for relative quantification.

Western Blot and Immunofluorescence analysis

For ***immunofluorescence assays,*** 12×10^3 fibroblasts (or 5×10^3 with metformin treatment) were seeded in immunofluorescence chambers and after 24-28h were processed following standard procedures previously described [40]. To evaluate ***cell proliferation,*** we use primary antibodies against phospho Histone H3 Ser10 (ab14955, Abcam) and Ki67 (ab15580, Abcam). To evaluate ***mitochondria biogenesis,*** we use primary antibodies against TOMM20 (11802-1-AP, Proteintech) and PGC1-α (NBP1-04676, Novus Biologicals). For ***5-Ethynyl-2'-deoxyuridine (EdU) assay,*** Click-iTTM EdU Cell Proliferation Kit for Imaging, Alexa FluorTM 488 dye was used (C10337, Invitrogen) following manufacturer's protocol. Finally, cells were stained with Hoechst 33342 (Sigma-Aldrich) to stain nuclei and slides were mounted with Fluoro-Gel Mounting medium (#17985, Electron Microscopy Sciences). Immunofluorescence images were obtained with a Nikon Eclipse 80i microscope using NIS Elements Advances Research software.

For ***western-blot assays,*** 20×10^4 fibroblasts were seeded in T75 flasks and after 24-28h were processed following standard procedures previously described [57]. We use the following primary antibodies: phospho p38MAPK Thr180/Tyr182 (9211, Cell Signaling), p38MAPK (sc-7972, Santa Cruz Biotechnology), AKT1/2/3 (sc-8312, Santa Cruz Biotechnology), phospho AKT Ser 473 (9271, Cell Signaling), DMPK (sc-134319, Santa Cruz Biotechnology), MBNL1 (ab45899, Abcam) and β-actin (AC-15,

Sigma-Aldrich). We used HRP-linked antibodies (Santa Cruz Biotechnology) and the detection was performed by chemiluminescence using *Novex ECL Chemi Substrate* (Thermo Fisher).

Statistics

Data are presented as mean values ± S.E.M., with the number of experiments (n) in parentheses. Unless otherwise indicated, statistical significance (p-values) was calculated using the Student´s t -test. Asterisks (*, **, and ***) indicate statistical significance ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively). Correlation analyses were done using Pearson coefficient when samples were normally distributed or Spearman coefficient when samples were not normally distributed using GraphPad.

Acknowledgements and funding

MG-P and AS-A are recipients of predoctoral fellowships from the University of the Basque Country (PIF 15/245) and Carlos III Institute (FI17/00250) respectively. The Authors would like to thank Miren Zulaica for technical help with fibroblasts isolation and culture and the Cell Culture Platform of the Biodonostia Health Research Institute, Monica Zufiria and Maddalen Otaegi-Ugartemendia for their help with SeaHorse experiments. This work was supported by grants from the Instituto Salud Carlos III and FEDER funds (CP16/00039, PI16/01580, PI17/01841) and Health department from Basque Country (2017 and 2018-2017222021).

Author contributions

MG-P and AS-A performed all the experiments on fibroblasts. MG-P analyzed the results and completed the figures. RF-T and ALM collected clinical data and samples from the patients. AM and ALM directed the project, contributed to data analysis, and wrote the manuscript.

The authors declare no competing financial interests.

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CAPÍTULO 5. Ahulkenoids restore multiple phenotypes *in vitro* and *in vivo* in Myotonic Dystrophy type 1

Artículo en preparación

Ahulkenoids restore multiple phenotypes *in vitro* and *in vivo* in Myotonic Dystrophy type 1

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Resumen

En este trabajo testamos la eficacia de los compuestos de una nueva familia química (Ahulkenoides, AHK) en fibroblastos humanos derivados de pacientes con DM1. Esta familia de compuestos está integrada por una serie de triazoles con acción sobre la homeostasis del calcio intracelular a través de su interacción con el complejo rianodina/calstabina y ejerce también un efecto inhibitorio sobre mTOR.

Observamos que dos compuestos de esta familia, los denominados AHK6 y el AHK7 incrementan la viabilidad celular de manera más eficiente que el resto de compuestos AHK de la misma familia. Describimos igualmente que el tratamiento con AHK6 y AH7, además de rescatar la viabilidad celular en fibroblastos derivados de pacientes con DM1, también rescata la proliferación celular e incrementa la producción de energía a través de la vía mitocondrial. Además, AHK6 y AHK7 reducen la formación de las especies reactivas de oxígeno en fibroblastos DM1. Estudios transcriptómicos revelaron que los alhulkeoides inciden sobre los procesos de ciclo celular, metabolismo y señalización celular. Por último los compuestos AHK prolongan de manera significativa la longevidad del modelo de mosca de la enfermedad.

Nuestros resultados revelan que los ahulkenoides ejercen un efecto positivo frente a diferentes alteraciones de la DM1 y sugieren que podrían tener potencial terapéutico para los pacientes con DM1.

Contribución

Mi contribución en este trabajo ha sido la realización de los experimentos realizados con los fibroblastos, el análisis de los resultados y la redacción del borrador del manuscrito.

Abstract

Myotonic dystrophy type 1 (DM1; MIM #160900) is an autosomal dominant disorder, clinically characterized by a progressive muscular weakness with myotonia and multisystem degeneration. DM1 is a rare and clinically variable disease with no currently available treatment to slow or stop disease progression.

Calcium plays a vital role in a wide range of cellular processes and alterations in calcium homeostasis are associated with multiple diseases. Specifically, alterations in the ryanodine receptor RyR1 leads to a chronic increase in cytosolic calcium levels in DM1.

In this study, we characterized the impact of a new family of agents aimed to normalize the intracellular calcium levels modulating the RyR-calstabine interaction (known as ahulkenoids). Treatment with ahulkenoids reversed several phenotypes observed in DM1 derived primary fibroblasts. Thus, 2 different compounds restored the impaired cell viability, proliferation, metabolic and mitochondrial defects of DM1 cells. Importantly, treatment with AHKs significantly extended the longevity of a *Drosophila* model of the DM1 disease.

Our results revealed the benefits of a new family of ahulkenoids in the pre-clinical setting of DM1.

Introduction

Myotonic dystrophy (DM1) is one of the most common muscular dystrophies in adults with a worldwide prevalence of 1 in 8.000 people (Harper, 2002). DM1 is an RNA-dominant disorder caused by the expression of expanded microsatellite repeats located in the 3′ untranslated region (UTR) of the DM1 protein kinase (DMPK) gene (Fu *et al.*, 1992; Mahadevan *et al.*, 1992). Mutant transcripts containing an expanded CUG tract are retained within the nucleus as discrete *foci* (Davis *et al.*, 1997; Taneja *et al.*, 1995). Due to their high affinity for CUG expansions, RNA binding factors from the MBNL family are sequestered within the CUG-RNA *foci*, leading to their functional loss (Miller *et al.*, 2000), which is a central pathophysiologic mechanism in DM1 (Lee *et al.*, 2013).

Patients with DM1 present a multisystem degenerative process characterized by myotonia, progressive muscular weakness and atrophy, cardiomyopathy, insulin-resistance, cataracts, increased cancer incidence, neurodegeneration, as well as endocrine deficiencies, cognitive impairments or premature death (Mateos-Aierdi *et al.*, 2015; Thornton, 2014). In recent years, several compounds have been developed as

anti-DM1 therapy, but today, there is no treatment to cure or delay the course of this disease. Agents that reduce the production, accelerate the degradation, or alleviate the toxicity of repetitive RNA have shown encouraging results in preclinical models. The most promising therapy was anti-sense oligonucleotides development for reducing mutant DMPK mRNA in DM1 cells. However ASO uptake in muscle was relatively low (Thornton *et al.*, 2017). Additionally preclinical therapies included, pentamidine and actinomycin D that reduce the expression of toxic CUG (Coonrod *et al.*, 2013; Siboni *et al.*, 2015); inhibitors of PKC and GSK3beta that leads to normalization of CELF protein levels, rescuing muscle wasting in mouse models (Jones *et al.*, 2012; Wang *et al.*, 2009); and small molecules inhibit the interaction between MBNL and CUG-repeats (Konieczny *et al.*, 2017). It remains to be seen however, if any of these substances are safe and effective in DM1 patients and it will be necessary to consider the possibility that targeting one mechanism may fail to address or even exacerbate others. Therefore, it is necessary to develop new compounds with new potential therapeutic use in this disease.

DM1 patients have a dysregulation of calcium metabolism with a deregulated alternative splicing of several genes implicated in calcium homeostasis and muscle weakness. In this regard, abnormal skipping in the calcium channel CACNA1S aggravates myopathy in the HSA^{LR} mouse model of DM1 (Tang *et al.*, 2012). In addition, expression of fetal variants of other calcium-related genes has been reported. The fetal variants of ryanodine receptor type 1 (RyR1) which lacks residue 3481–3485, and SERCA1b which differs at the C-terminal were significantly increased in skeletal muscles from DM1 patients and in HSA^{LR} mouse (Kimura *et al.*, 2005; Vihola *et al.*, 2013). RyRs receptors are intracellular calcium-release channels involving in the calcium release from intracellular organelles and SERCA transports calcium from the cytosol into the sarcoplasmic reticulum. Additionally, DM1 patients have a chronic increase in cytosolic calcium levels (Benders *et al.*, 1997; Hlaing *et al.*, 2019; Jacobs *et al.*, 1990).

We have discovered a novel family of FKBP12 “reshapers” (AHK compounds or Ahulkenoids), originally designed to normalize calcium homeostasis by stabilizing the interaction between ryanodine and calstabine. In this work, we studied the contribution of AHK compounds in human primary fibroblasts of DM1 patients *in vitro* and *Drosophila* model of DM1 disease *in vivo*. Our results reveal that Ahulkenoids rescued multiples phenotypes previously linked to DM1 fibroblasts such as impaired proliferation and metabolism or elevated reactive oxygen species (ROS) production, targeting cell cycle regulation, metabolism and cell signaling. Strikingly, they also rescue the reduced longevity of DM1 flies.

Results

AHK compounds restore cell viability and proliferation

First, we examined the effect of AHK compounds in DM1 fibroblasts cell viability. For this, we cultured DM1 and control fibroblasts with a single dose of 0.1 µM of 12 AHK compounds for 72 hours and evaluated cell viability. Interestingly, the different AHK agents did not show effect in controls but improved the impaired cell viability in DM1 fibroblasts by more than 2-fold **(Fig 1A)**. Among them, AHK6 and AHK7 were the compounds that restored cell viability to the levels observed in control **(Fig 1A)**. Therefore, we treated cells with 0.1, 1 and 10 µM of AHK6 and AHK7 for 72 hours. These compounds did not exhibit cell toxicity in control or DM1 fibroblasts **(Fig 1B)**, but increased cell viability with a minimal dose of 0.1 µM in DM1 cells **(Fig 1C)**.

DM1 fibroblasts display decreased cell proliferation (García-Puga *et al.*, 2020a). We investigated the impact of AHK6 and AHK7 in the proliferative potential of DM1 fibroblasts. As previously described, DM1 cells had lower number of cells positive for phospho-Histone H3 (Ser10). Importantly, treatment with 0.1 µM of AHK6 and AHK7 increased the number of pH3 positive cells by almost 2 and 3-fold in DM1 cells, respectively **(Fig 1D,E)**, concluding that AHK6 and AHK7 rescued cell viability and proliferation in DM1 fibroblasts.

AHK6 and AHK7 restored cell metabolism and decreased ROS production in DM1 fibroblasts

We previously reported that DM1 fibroblasts display decreased oxidative phosphorylation (OXPHOS), impaired mitochondrial activity and enhanced ROS production (García-Puga *et al.*, 2020a). We wonder to know whether Ahulkenoids could improve these phenotypes. To test this idea, we treated DM1 and control fibroblasts with 0.1 µM of AHK6 and AHK7 for 72 hours and evaluated cellular metabolism and mitochondrial activity by SeaHorse. Interestingly, AHK6 and AHK7 improved thebasal oxygen consumption rate and maximal respiration of DM1 fibroblasts by more than twice **(Fig 2A-D)**, and also increased ATP production via oxidative phosphorylation **(Fig 2A,E)**, with a discrete effect in control fibroblasts. Thus, ahulkenoids restore the impaired metabolism and mitochondrial activity in DM1 fibroblasts. Next, we measured ROS production and found that treatment with 0.1 µM of AHK6 and AHK7 for 72 hours significantly decreased ROS production in control as well as in DM1 fibroblasts **(Fig 2F)**. In summary, AHKs rescued OXPHOS activity and reduced ROS production in fibroblasts. Next, we characterized the calcium levels in DM1 fibroblasts and compared to healthy controls. Surprisingly, we did not see changes in the calcium levels among DM1 patients and healthy controls **(Fig 2G)**. These results might suggest that some of the effects of Ahulkenoids are independent of calcium activity.

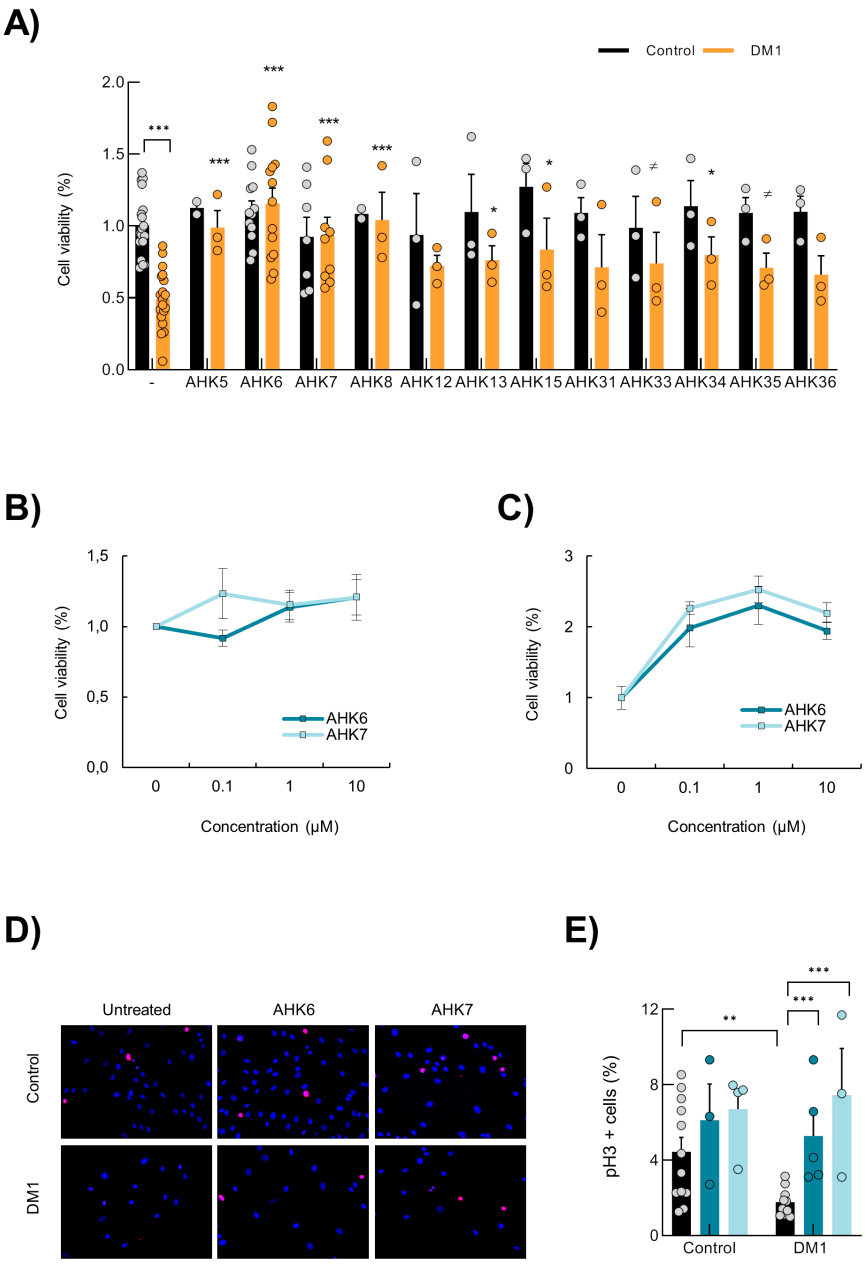

Figure 1. AHK compounds restore cell viability and proliferation.
A) Cell viability of DM1 (n=5) and control (n=3) fibroblasts after treatment with 0.1 µM of different AHK for 72 h. **B,C)** Cell viability of control (n=3) (left) and DM1 (n=5) (right) fibroblasts after treatment with 0.1, 1 and 10 µM of AHK6 and AHK7 for 72 h. **D,E)** Representative image and quantification of phospho-Histone H3 (Ser10) in controls (n=3) and DM1 fibroblasts (n=5) after treatment with 0.1 µM of AHK6 and AHK7 for 72 h.

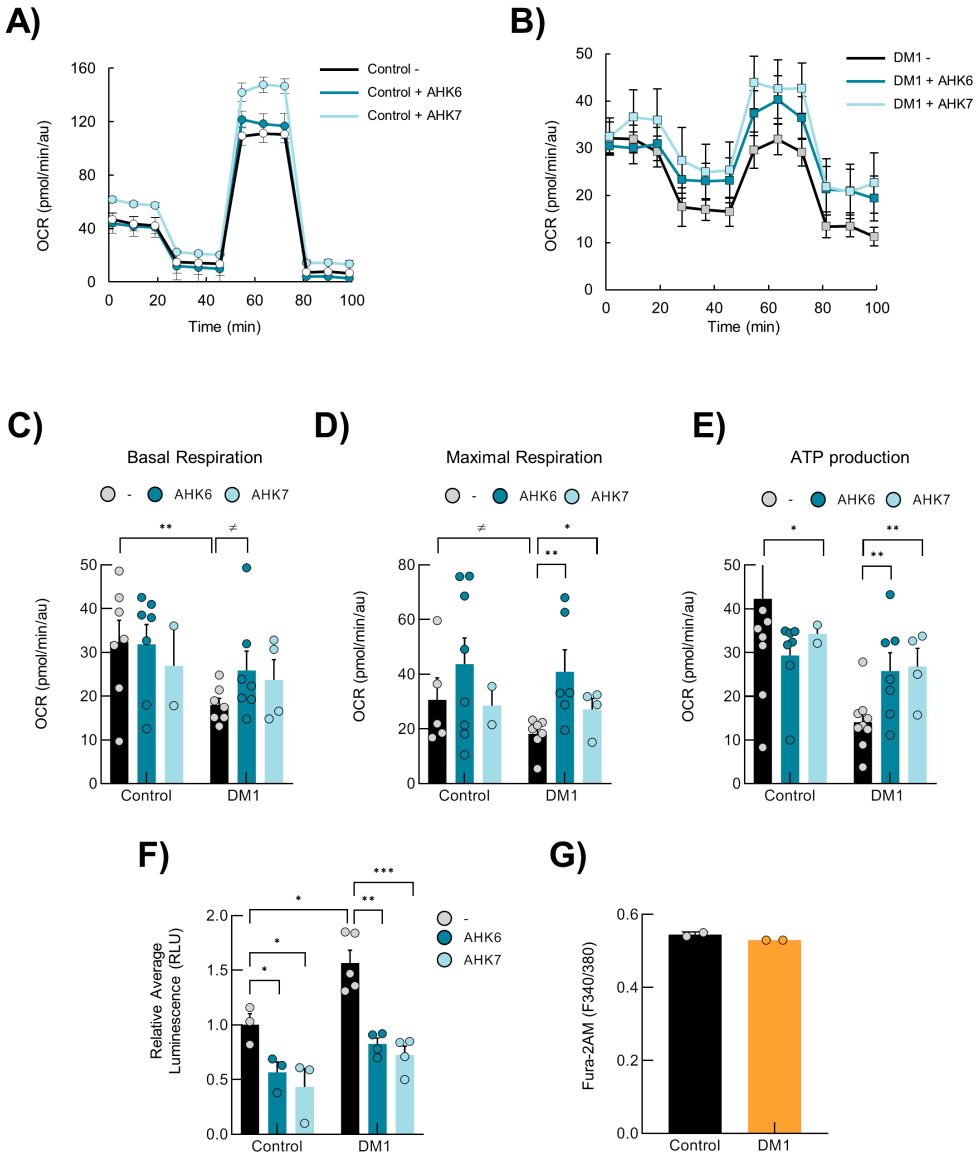

Figure 2. AHK6 and AHK7 restored cell metabolism and ROS production.
A,B) Representative kinetic normalized OCR response in control (n=3) and DM1 patients (n=5) after treatment with 0.1mM of AHK6 and AHK7 for 72 h. DM1 and control fibroblast were plated at 5.000 cells/well 24 hours prior to the assay. A representative experiment out of 3 is shown. **C-E)** Quantification of mitochondrial basal and maximal respiration, and ATP production, respectively, after treatment with 0.1mM of AHK6 and AHK7 for 72h in controls (n=3) and DM1 fibroblasts (n=5). **F)** H_2O_2 production after treatment with 0.1 µM of AHK6 and AHK7 for 72 h (n=3). **G)** Quantification of intracellular calcium levels measured with Fura-2AM fluorochrome showing the F340/380 fluorescence ratio (DM1 n=2 and controls n=2).

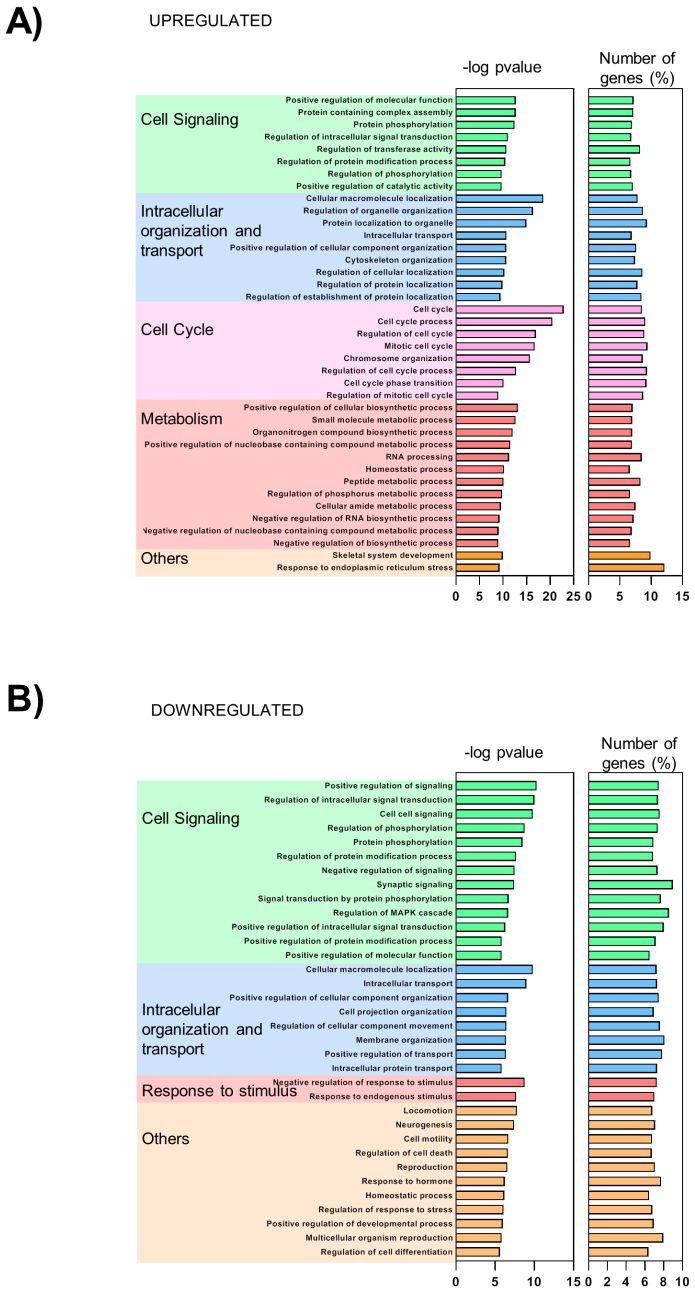

Figure 3. Transcriptomic study reveals that Ahulkenoids target multiple processes in DM1 fibroblasts.

Bar plot of the -log10(p-value) and percentage of genes altered in each term of the significantly **A)** upregulated and **B)** downregulated GO terms in DM1 fibroblasts treated with AHK7 (n=3). Longer bars correspond to greater statistical significance of the enrichment.

Transcriptomic study reveals that Ahulkenoids target multiple processes in DM1 fibroblasts

To understand the mode of action of Ahulkenoids an unbiased high-throughput RNA-sequencing (RNA-seq) was performed **(Figure 3A,B)**. This analysis was performed in three fibroblasts cell line treated with AHK7. We selected the top 500 most differentially expressed genes in each condition. Gene Ontology (GO) enrichment analysis showed that cell signaling, phosphorylation, intracellular organization and transport, cell cycle, and metabolism were the processes most significantly altered in DM1-treated fibroblasts, all of which were upregulated. On the other hand, we found that cell signaling, intracellular organization and transport and genes associated to response to stimulus were downregulated with the treatment of AHK7 **(Figure 3A,B)**.

Within the *cell cycle* there were genes implicated in phase transition, such as *CDK2* and *CDK10* and regulators of CDKs such as *cyclin D1*, *D2*, *I*, *G1* and *H*; and *BMI1* the negative regulator of $p16^{INK4a}$. We also showed that AHK7 regulated genes involved in cell division such as *GINS2*, *CDC20*, *CDC26* and *NEK6*. Surprisingly, some of these genes we previously reported to have a decreased expression in DM1 cells (García-Puga *et al.*, 2020a). Among genes implicated in *cell signaling* we found that AHK7 regulates *MAP4K5*, a mitogen-activated protein kinase (MAPK pathway); *PKIA,* a cAMP-dependent protein kinase inhibitor, some G protein-coupled receptor kinase such as *GRK2*, *4*, *5* and *6*; *RhoA*, a small GTPase protein involved in actin organization, cell development, transcriptional control and cell cycle maintenance; *PRKAG1,* a regulatory subunit of the AMP-activated protein kinase (AMPK); ERBB2 and several members of *FGF* and *TGF* pathways; and *CHUK,* an inhibitor of NF-kappa-B complex, among others. Interestingly, some of the genes involved in cell signaling also are regulating cell cycle transition and cell division such as *UHMK1*, that is a serine/threonine protein kinase that promotes cell cycle progression through G1 by phosphorylation of $p27^{KIP1}$; *TTK*, that is essential for chromosome alignment at the centromere during mitosis and required for centrosome duplication and *AKT3*, involved in cell proliferation. Additionally, we found several enzymes implicated in *cell metabolism* such as alanyl, phenylalanyl, cysteinyl, and threonyl tRNA synthetase, acetyl-CoA carboxylase (*ACACA*), arginase 2 (*ARG2*), glutamate dehydrogenase 1 (*GLUD1*), hexokinase 1 (*HK1*) and several members of ATP syntase (*ATP5F1C*, *ATP5MC1* and *ATP5PD*). Taken together, our results reveal that critical pathways are regulated by Alhukenoids in DM1, such as cell signaling, cycle and metabolism.

AHK significantly extends longevity *in vivo*

Next, we aimed to extend these results *in vivo* and characterized the effect of Ahulkenoids in a *Drosophila melanogaster* model of DM1, which express 480 interrupted CUG repeats (García-López *et al.*, 2008) and display significantly shorter lifespan than control flies {García-Puga, 2020 #666}. We selected AHK7 to studied longevity of DM1 flies because we previously shown that this compound had the strongest effect *in vitro* and crosses the blood brain barrier (data not shown).

Interestingly, AHK7 extended the longevity of DM1 flies treated with AHK7 in adult stage by more than double increasing the median survival from 60 to more than 70 days in females and from 50 to more than 70 days in male flies with 10 and 100 µM AHK7 (**Figure 4A**). Similar results were observed when DM1 flies were treated with AHK7 from the larval stage (**Figure 4B**). These results confirmed that ahulkenoids, rescued multiple phenotypes of DM1 disease in both patient-derived fibroblasts and in an *in vivo* model of the disease.

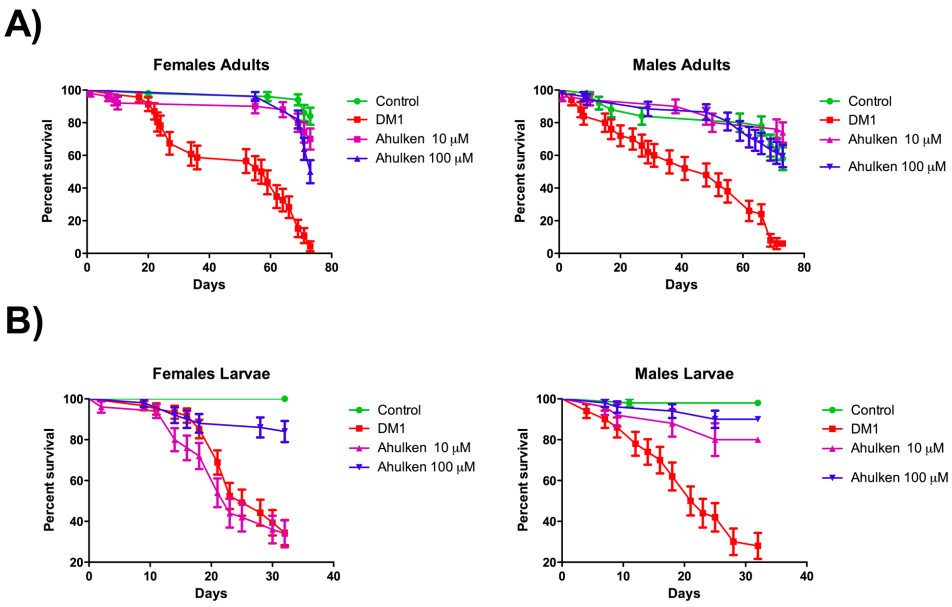

Figure 4. AHK7 increases longevity in DM1 *Drosophila melanogaster*.
Survival curves showing the fraction of 100 flies over time of control flies, DM1 flies non-treated and DM1 flies in presence of 10 or 100 mM of AHK7 treated from **A)** adult stage and from **B)** larvae stage; males are represented in the right and females in the left panels.

Discussion

Myotonic dystrophy is a rare, clinically variable disease with supportive treatments, preventative measures and clinical surveillance as the only options available for DM1 patients nowadays (Gutiérrez Gutiérrez *et al.*, 2019). In this work we reveal the beneficial impact of AHK compounds in DM1-derived fibroblasts and in a *Drosophila melanogaster* model of DM1.

Previous studies showed a reduction in the proliferative capacity of DM1 cells and we showed that AHK compounds rescued the impaired proliferative capacity and similarly the lower mitochondrial efficiency detected in DM1 patients (García-Puga *et al.*, 2020a). Ahulkenoids also reduced the production of ROS. Moreover, our results showed that Ahulkenoids rescued multiple phenotypes in vitro, but also in a *Drosophila* model of DM1 with very significant improvements in longevity.

It is necessary to know the molecular mechanism behind the reversal of these phenotypes by AHKs. The AHK compounds were originally designed to normalize calcium homeostasis by stabilizing the interaction between ryanodine and calstabine. However, we did not see differences in calcium levels, which suggest that AHK might have additional activities involved in rescuing DM1 phenotypes. In this line, we performed an RNA-seq analysis which revealed cell cycle, cell signaling and metabolism as the pathways involved in ahulkenoids activity, pathways we showed altered in DM1 (García-Puga *et al.*, 2020a; García-Puga *et al.*, 2020b). Moreover, ahulkenoids might inhibit the mTOR pathway (Gereñu *et al.*, manuscript in preparation). mTOR belongs to the phosphatidylinositol-3 kinases (PI3K)-related kinase (PIKK) family that is present as catalytic subunit in at least two protein complexes: mTORC1 and mTORC2. Inhibition of mTOR with rapamycin increase lifespan more than a double in *C. elegans* (Blackwell *et al.*, 2019), *D. melanogaster* (Bjedov *et al.*, 2010) and in mice (Anisimov *et al.*, 2010; Harrison *et al.*, 2009; Ramos *et al.*, 2012). Also, deleting the mTORC1 substrate S6K1 similarly increases lifespan in female mice (Selman *et al.*, 2009). Moreover, in DM1 decreased mTOR-dependent phosphorylation was observed *in vitro* (Denis *et al.*, 2013) and *in vivo,* transgenic DM1 flies with overexpression of mTOR rescued the impaired longevity of this model (Bargiela *et al.*, 2015). However, more experiments are necessary to know the molecular mechanism behind AHK activity.

In summary, our results showed the efficacy of AHK6 and AHK7 in a pre-clinical setting and suggest that it warrants further assessment as a candidate drug for DM1 treatment.

Methods

Study approval

This study was approved by the Donostia University Hospital Ethical Board (approval number 15-57) and was conducted in accordance with the Declaration of Helsinki's ethical standards. All subjects gave written informed consent before sample donation.

Cell isolation and culture

For the isolation of primary fibroblasts from healthy donors and DM1 patients (age range, 34 to 71), punch skin biopsies were cut into 2–3 mm3 fragments and placed on a surface moistened with modified Eagle's medium, containing 13% newborn calf serum, 0.4% penicillin/streptomycin (Gibco, Waltham, MA, USA) and 2 mM L-glutamine (Gibco). Flasks were incubated vertically for 3–6 h at 37°C under 5% CO_2 and then returned to the horizontal position. Human fibroblasts were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco) containing 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO, USA), 1% L-glutamine (Gibco), and 1% penicillin–streptomycin (Gibco). Fibroblasts were tested regularly for mycoplasma contamination. Seven cultures from different DM1 patients and five from healthy controls were established (see **Table 1** for patient characteristics).

Transgenic *Drosophila melanogaster*

We used the transgenic Mhc-Gal4 UAS-i(CTG)480 flies. Flies without any marker or balancer were selected for experiments to ensure de expression of the nucleotide repetition. Flies were housed at 23°C, 70% humidity and 12h/12h light/darkness cycle. All crosses were carried out at these standard conditions with standard fly food or plus AHK7 10 or 100 mM for treated group. Kaplan-Meier method was used to plot the results. A log-rank test was used to analyze results and Bonferroni correction for multiple comparisons.

Measurement of cell viability

Fibroblasts were seeded in 96-well plates followed by treatment with metformin for 72 h. Viable cells were quantified using the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (M5655, Sigma-Aldrich) assay in six replicates per condition.

Total ROS measurement

A total of $0.1x10^4$ fibroblasts were plated in 96-well plates and grown for 3 days. Afterwards, ROS-Glo H_2O_2 Assay (G8820, Promega) was performed according to the manufacturer's instructions. Briefly, a H_2O_2 substrate was employed that reacts directly with H_2O_2 to generate a luciferin precursor and, upon addition of a detection reagent, the precursor is converted to luciferin, which generates a luminescent signal that is proportional to the H_2O_2 concentration. White flat bottom plates (Corning) were used for final readout in a PHERAstar (BMG Labtech) luminometer plate reader.

Cell proliferation measurement

For immunofluorescence assays, 5x103 fibroblasts were seeded in immunofluorescence chambers and after 14-16 h were treated with 0.1 uM of metformin for 72 h. After that were processed following standard procedures previously described (Carrasco-García *et al.*, 2016). To evaluate cell proliferation, we use primary antibody against phospho Histone H3 Ser10 (ab14955, Abcam).

Metabolic measurements

Measurement of OCR, as well as ECAR, was performed in XF96 plates with *XF Extracellular Flux Analyzer* (Seahorse Bioscience). Fibroblasts were seeded in collagen (BD Biosciences) coated XF 96-well plates (Seahorse/Agilent) in octuplicates at 5x103 cells/well in 100 µl growth medium.

Mitochondrial activity was evaluated using the Seahorse XF Cell Mito stress Test Kit, according to manufacturer's instructions (Agilent). Oligomycin (75351, Sigma-Aldrich), FCCP (C2920, Sigma-Aldrich), and Rotenone/Antimycin A, (R8875 and A8674, Sigma-Aldrich) were used at 1.5 µM concentration, after a titration experiment. *Cell content* was normalized using crystal violet. The post-normalization values of OCR and ECAR reflect both the metabolic activities of the cells and the number of cells being measured. Data were further processed using the manufacturer's calculation matrix. Briefly, the determined parameters were calculated according to manufacturer's instructions.

Calcium measurement

Calcium measurements were performed as previously described (Toral-Ojeda *et al.*, 2016). Briefly, fibroblasts were loaded with 4 µM Fura 2-AM and 0.02% pluronic acid in culture medium for 30 min at 37°C. Cells were left in Ringer buffer (125 mM NaCl,

5 mM KCl, 1.2 mM MgSO4, 6 mM glucose, 2 mM CaCl2 and 25 mM HEPES, pH 7.4) for 20 min at room temperature to remove nonhydrolysed fluorophore and complete de-esterification of the dye. Image acquisition was performed using ECLIPSE Ti-S/L100 microscope (Nikon) with the Nis Elements-AR software. Intracellular calcium concentration was estimated by the ratio of Fura 2-AM fluorescence intensities at excitation wavelength 340 and 380 nm.

RNA-sequencing

Fibroblasts derived from 3 DM1 patients were treated with 0.1 µM of AHK7 for 72 h and RNA extracted using TRIzol. Library preparation, sequencing and bioinformatics analysis were performed following standard procedures at CD Genomics (NY, USA) using an Illumina NovaSeq PE150 sequencer. The GO terms were taken from the curated collection of molecular signatures (gene set collection C5) of version 3.0 of the Molecular Signatures Database (MSigDB).

Statistics

Data are presented as mean values ± S.E.M., with the number of experiments (n) in parentheses. Unless otherwise indicated, statistical significance (*p*-values) was calculated using the Student´s t -test. Asterisks (*, **, and ***) indicate statistical significance ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

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Discusión general

La DM1 es un tipo de distrofia muscular que se caracteriza por la afectación no sólo del sistema muscular sino también del resto de sistemas vitales, lo que la define como un desorden multisistémico complejo. En la DM1 los ARNs producto de las secuencias de ADN que contienen las expansiones CTG se retienen y se acumulan en el núcleo celular interfiriendo con los reguladores de *"splicing"* de las familias de MBNL y de CUGBP. La alteración en la función de estos dos genes, junto con la expresión reducida de *DMPK* y de los genes adyacentes *SIX5* y *DMWD*, va a desencadenar multitud de alteraciones moleculares en la célula.

La hipótesis de partida de esta tesis es que los pacientes con DM1 presentan a nivel celular una exacerbación o una aparición precoz de los procesos biológicos característicos del envejecimiento fisiológico (López-Otín *et al.*, 2013), que se basa en la observación clínica de múltiples signos de envejecimiento en pacientes DM1 de manera prematura.

En primer lugar, abordamos la caracterización de fibroblastos derivados de pacientes con DM1, que presentan los fenómenos de envejecimiento celular observados en dichos pacientes. Así, observamos, que los fibroblastos derivados de estos pacientes presentan alteraciones de procesos clave del envejecimiento como una disminución en la proliferación y en la división celular, una pérdida en la respuesta al daño en el ADN, un descenso en el metabolismo mitocondrial y una entrada prematura en senescencia. Por otra parte, también describimos un acortamiento telomérico exacerbado en muestras sanguíneas de pacientes con DM1. Además, a nivel clínico observamos que los pacientes con DM1 presentan un mayor riesgo a desarrollar cáncer, identificando, asimismo, las vías moleculares alteradas que se encuentran asociadas a este fenómeno.

El carácter multisistémico de la enfermedad se traduce en la aparición de un conjunto de rasgos como son la atrofia muscular, las cataratas, la alopecia, el síndrome metabólico, los trastornos cardíacos y las alteraciones en el SNC, entre otros. Todas estas alteraciones, tomadas globalmente, podrían ser caracterizadas como un proceso de envejecimiento prematuro. Los resultados de esta tesis confirman que los pacientes con DM1 presentan un envejecimiento celular prematuro y un mayor riesgo oncogénico. Por tanto, esta tesis describe los mecanismos biológicos que justifican, al menos en parte, la aparición de este proceso degenerativo.

En la actualidad no existe un tratamiento eficaz que revierta o detenga el desarrollo de la DM1. Las aproximaciones existentes están dirigidas a corregir alteraciones moleculares concretas de la enfermedad, sin una visión global de la entidad.

Empleando un enfoque novedoso, hemos empleado compuestos que han demostrado tener potencial antienvejecimiento, como son los compuestos senolíticos y la metformina, además de nuevas moléculas de desarrollo propio (los compuestos

ahulkenoides, AHK). En este sentido, observamos que estas tres nuevas aproximaciones terapéuticas son capaces de revertir, al menos parcialmente, y por mecanismos diferentes, los fenotipos asociados a la DM1, tanto en cultivos celulares como en un modelo animal (*Drosophila melanogaster*) de la enfermedad.

En resumen, en esta tesis constatamos la hipótesis de que los pacientes con DM1 presentan rasgos asociados a un envejecimiento prematuro y proponemos el tratamiento con compuestos antienvejecimiento como una alternativa terapéutica para esta entidad clínica.

1. Alteraciones de los mecanismos implicados en el envejecimiento en la DM1

1.1. Alteración en la respuesta al daño en el ADN, el ciclo celular y la división celular

Para la caracterización a nivel celular y molecular de la DM1, hemos establecido como modelo el cultivo primario de fibroblastos aislados de la piel, derivados de pacientes con DM1. Dicho modelo celular recapitula las alteraciones moleculares del proceso del envejecimiento (Tigges *et al.*, 2014).

Hemos observado que las diferentes alteraciones características de la DM1 son recapituladas por los fibroblastos derivados de pacientes con DM1, como son la reducción en la expresión tanto de DMPK, como de *SIX5*, la acumulación nuclear de CUGBP1, la presencia de la isoforma neonatal de *MBNL2*, la cual incluye el exón 7, y la presencia de *foci* nucleares. Estos datos indican que los fibroblastos constituyen un modelo apropiado para el estudio de la DM1.

Por otro lado, el uso de fibroblastos primarios tiene otras ventajas. Frente a las células inmortalizadas genéticamente, son más representativos de la fisiología celular, y frente a las células musculares como mioblastos o células satélite presentan la ventaja de que su obtención y aislamiento es más sencilla y menos invasiva.

Para caracterizar los procesos moleculares alterados en la DM1 realizamos un análisis del transcriptoma comparando fibroblastos primarios DM1 y fibroblastos control a pase temprano, así como diferentes ensayos funcionales. Describimos que los fibroblastos derivados de pacientes con DM1 presentaban una disminución de los procesos de proliferación y un incremento de la senescencia celular. Sin embargo, nuestro estudio pone de manifiesto la alteración de vías adicionales como la regulación del ciclo celular, la división y replicación celular, el metabolismo celular y la reparación del daño en el ADN. Estas vías moleculares se encuentran alteradas, además de en fibroblastos, también en mioblastos primarios DM1, en los tejidos musculares de dos modelos *in vivo* de la enfermedad, como el ratón DMSXL con más de 2.000 repeticiones CTG introducidas de manera multisistémica (Huguet *et al.*, 2012) y el modelo i(CUG)480 en *D. melanogaster* (García-López *et al.*, 2008), que posee 480 repeticiones en el tejido muscular, así como en las PBMC derivadas de los pacientes con DM1. El hecho de estas alteraciones se produzcan en varios tipos celulares, refuerzan la idea de que estos procesos son importantes en la enfermedad. Además, estas vías podrían proveer de nuevos biomarcadores útiles para monitorizar el éxito de las aproximaciones terapéuticas para la DM1 planteadas en esta tesis.

La señalización celular ante un daño en el ADN es uno de los procesos alterados a nivel molecular en los fibroblastos DM1. La ruta de BRCA1/2 - FANCA es la principal ruta que regula la respuesta celular ante el daño en el ADN, cooperando con proteínas señalizadoras de daño como ATM, y con otras proteínas de respuesta a este daño, como RAD51 y POLQ (D'Andrea and Grompe, 2003). En nuestro estudio describimos que los fibroblastos DM1 presentan una señalización anormal cuando se produce el daño en el ADN, y además, una menor reparación del mismo. Esta idea es corroborada funcionalmente en nuestro estudio, ya que, tras inducir un daño en el ADN mediante el tratamiento con el agente intercalante doxorrubicina, los fibroblastos DM1 acumulan mayores niveles de γ-H2AX, marcador de respuesta al daño en el ADN, y una mayor activación de fosfo-ATM **(Figura D1)**. Además, el silenciamiento de BRCA1 en fibroblastos DM1 disminuye dichos marcadores, tras la inducción de daño en el ADN y también reduce su capacidad proliferativa. Es importante destacar que existe una interconexión de esta ruta de señalización con los procesos de parada de la proliferación y división celular, ya que se ha observado que AURK activa a BRCA1/FA (Chun *et al.*, 2016) y que la inhibición de CDK2 resulta en un incremento en el daño al ADN (Liu *et al.*, 2020), entre otros ejemplos.

La contribución en la patogénesis de la DM1 de las proteínas implicadas tanto en la reparación como en la señalización tras un daño en el ADN no se había estudiado previamente en profundidad. Sin embargo, sí que había ciertos indicios de que, tanto en la DM1, como en otras enfermedades producidas por expansión de nucleótidos, los mecanismos de reparación del ADN juegan un papel importante en el desarrollo de la enfermedad (Jones *et al.*, 2017; Kosmider and Wells, 2006). Por un lado, DMPK se activa en respuesta a doxorrubicina a través de p53 (Itoh *et al.*, 2019). Además, otros autores han observado que la inestabilidad del triplete CTG está mediada por las proteínas Msh implicadas en la reparación del ADN (Seriola *et al.*, 2011; Tomé *et al.*, 2009; van den Broek *et al.*, 2002). En modelos de otras enfermedades causadas por expansión de trinucleótidos, como en la enfermedad de Huntington, se observa asimismo una acumulación de γ-H2AX (Enokido *et al.*, 2010; Illuzzi *et al.*, 2009).

Estudios previos habían investigado el potencial proliferativo de células derivadas de pacientes con DM, encontrando una proliferación normal (Hartwig *et al.*, 1982; Wertz *et al.*, 1981) o reducida, junto con una senescencia prematura (Bigot *et al.*, 2009; Denis *et al.*, 2013; Furling *et al.*, 2001; Renna *et al.*, 2014), aunque dichos estudios se habían realizado fundamentalmente en células con un origen muscular. Recientemente, un nuevo estudio ha descrito una proliferación reducida en células madre de origen muscular, rescatada mediante la sobreexpresión de MBNL1 y de mTOR (Song *et al.,* 2020). En nuestro estudio, describimos que tanto los fibroblastos como los mioblastos DM1 presentan una proliferación reducida y una entrada prematura en senescencia. Las diferencias entre nuestro estudio y los estudios previos pueden deberse al propio aislamiento y mantenimiento de los cultivos celulares,

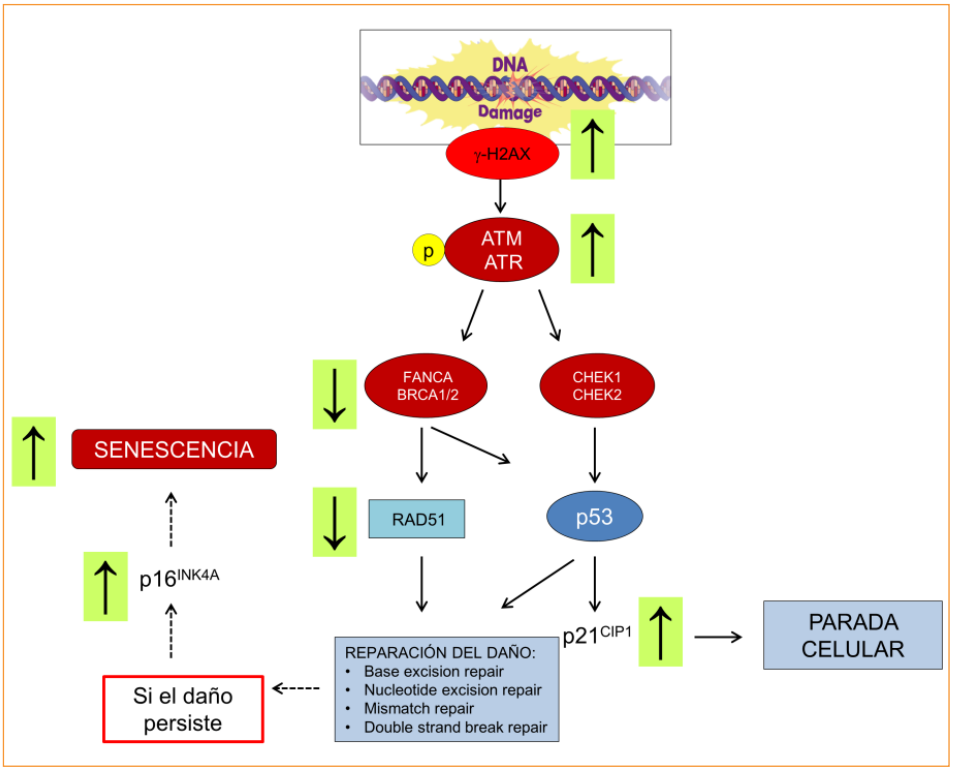

Figura D1. Los fibroblastos derivados de pacientes con DM1 poseen una disminución en la señalización celular tras un daño en el ADN y en la respuesta a la reparación de dicho daño. En la imagen se representa mediante flechas un resumen con las alteraciones principales identificadas en esta tesis; una mayor acumulación del marcador de daño en el ADN γ-H2AX junto con mayores niveles de fosfo-ATM. Además, se observa una disminución en la expresión de *BRCA1* y *2, FANCA* y *RAD51,* junto con *POLQ.* La presencia de daño en el ADN provocaría una activación de las rutas de reparación de dicho daño y una parada del ciclo celular.

así como al método empleado para evaluar la proliferación. En este sentido, en los estudios donde describen una proliferación normal (Hartwig *et al.*, 1982; Wertz *et al.*, 1981), la metodología difiere sustancialmente, ya que no mantienen una densidad celular constante a lo largo de los pases como es nuestro caso. Las diferencias también pueden deberse al tamaño de la expansión CTG, que no ha sido determinada en dichos estudios (Hartwig *et al.*, 1982; Wertz *et al.*, 1981). Otro punto diferencial de los estudios anteriores es que no determinan si los pacientes de los que habían sido aislados los fibroblastos corresponden a DM1 o DM2 (Hartwig *et al.*, 1982; Wertz *et al.*, 1981). En esta dirección, es importante destacar que el mecanismo molecular detrás de la reducción en la capacidad proliferativa parece ser diferente entre DM1 y DM2, ya que no se observa una alteración de $p16^{INK4a}$ en células DM2 (Meinke *et al.*,

2018; Renna *et al.*, 2014). En nuestro estudio describimos que los niveles elevados de $p16^{INK4a}$ se producen a pase temprano tanto en fibroblastos como en mioblastos, pero dichos niveles se exacerban en células DM1 a pase tardío, cuando las células han sido sometidas a intensas dosis de un estrés continuo por el mero hecho de mantenerlas en cultivo (Sherr and DePinho, 2000).

Por otro lado, el estudio transcriptómico y los ensayos funcionales describieron un mecanismo más complejo, con la alteración de varias rutas celulares que están implicadas en el control del ciclo celular. En este sentido, describimos niveles elevados de BMI1 y HMGA2, ambos reguladores de $p16^{INK4a}$ (Jacobs *et al.*, 1999; Nishino *et al.*, 2008); un descenso en los moduladores de la transición G0/S como CDK2 y CDK6; y una acumulación de los inhibidores de CDK como $p14^{ARF}$, $p21^{CIP1}$ y $p27^{KIP1}$. $p16^{INK4a}$ actúa a través de Rb, y $p14^{ARF}$ y $p21^{CIP1}$ son los reguladores y dianas principales de p53 (Carrasco-García *et al.*, 2017; Matheu *et al.*, 2008), lo que confirma que la reducción en la capacidad proliferativa de las células DM1 no está mediada únicamente por una de las vías.

Adicionalmente estudiamos la expresión de las vías alteradas en el transcriptoma en otros modelos de la DM1 como los mioblastos, el tejido muscular del ratón DMSXL y de *D. melanogaster*, así como las PBMC de pacientes con DM1 y describimos alteraciones similares a las observadas en los fibroblastos DM1 en la expresión de los genes implicados en el control del ciclo celular, la división celular y el daño en el ADN.

Nuestro estudio revela la implicación de la alteración de varias vías moleculares, posiblemente interconectadas, en la DM1, que convergen todas ellas en una pérdida en la capacidad proliferativa de las células DM1 **(Figura D2)**. En este sentido, las alteraciones propias de la DM1, como son una expresión reducida de DMPK, así como la acumulación de los transcritos mutados en la célula, entre otras muchas, podrían estar promoviendo un estrés adicional a la célula al que respondería con una parada en la proliferación celular.

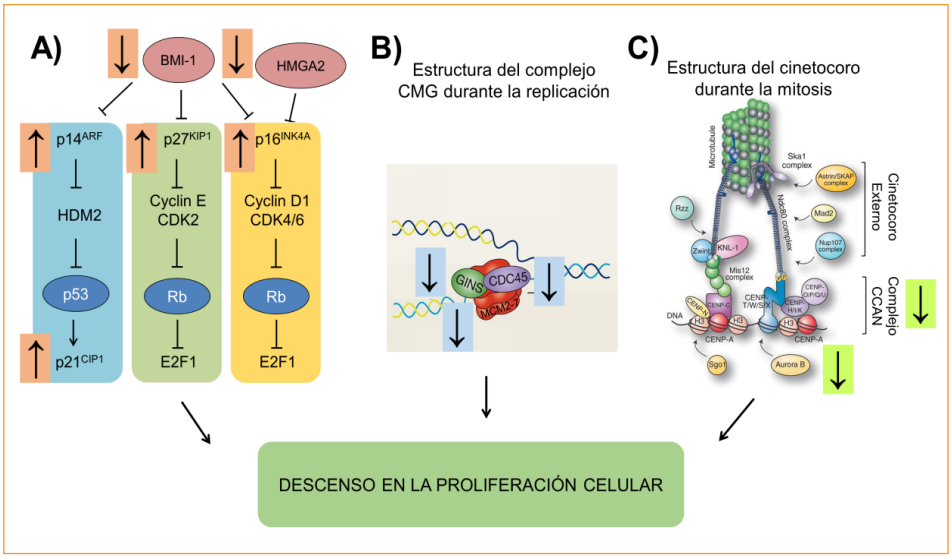

Figura D2. La alteración en la regulación del ciclo celular, junto con un descenso en la expresión de las proteínas que forman el complejo helicasa CMG y el nucleosoma provocan un descenso en la proliferación celular en fibroblastos DM1, que se manifiesta con un descenso en los marcadores de proliferación pH3, Ki-67 y EdU. **A)** Se representan los diferentes reguladores del ciclo celular alterados en fibroblastos derivados de pacientes con DM1, donde observamos un descenso en la expresión de BMI1 y *HMGA2* junto con una acumulación de $p16^{INK4a}$, $p14^{ARF}$, $p27^{KIP1}$ y $p21^{CIP1}$. **B)** El complejo CMG es una helicasa que está formada por las proteínas *CDC45, GINS* y el complejo de *MCM2-7,* cuya expresión se ve reducida en fibroblastos DM1. En la imagen se representa su estructura durante la replicación del ADN (modificado de Gaggioli and Zegerman, 2017). **C)** En la imagen se representa la estructura y organización del cinetocoro durante la mitosis. La fosforilación del complejo CDK/Ciclina B provoca la entrada en mitosis y la correcta organización del cinetocoro. En la imagen se representa una expresión reducida de la aurora kinasa B (*AURKB*), cuya función es evitar un ensamblaje incorrecto del cinetocoro a los microtúbulos, así como una reducción de *CENPA, CENPE* y *CENPI,* estos últimos pertenecientes al complejo asociado al centrómero (CCAN, del inglés *"Constitutive Centromere-Associated Network"*. Modificado de Cheeseman, 2014.

1.2. Entrada prematura en senescencia en fibroblastos DM1

La senescencia celular es un proceso biológico que provoca la incapacidad de las células para proliferar, quedándose retenidas en la fase G0 del ciclo celular, aunque éstas siguen siendo activas metabólicamente. Es un mecanismo fisiológico conservado que se encarga del remodelado tisular durante el desarrollo embrionario, pero que también se produce en la edad adulta cuando existe un daño (Muñoz-Espín and Serrano, 2014).

Nuestros resultados describen un descenso en los procesos del control del ciclo celular, de la división celular y de la replicación y reparación del ADN como eventos previos al desarrollo de una senescencia prematura en fibroblastos primarios DM1. El mantenimiento de las células primarias en cultivo en altas concentraciones de oxígeno conlleva un estrés que provoca que vayan perdiendo su capacidad proliferativa hasta entrar en senescencia (Hayflick and Moorhead, 1961), debido a la activación de las vías de p16/Rb, de p53 y al acortamiento telomérico que se produce en cada división celular (Gorgoulis *et al.*, 2019). En nuestro trabajo observamos que los fibroblastos DM1 experimentan el cese de la proliferación y la entrada en estado senescente, tras un número inferior de divisiones celulares respecto a los fibroblastos derivados de sujetos sanos. Además, este proceso se acompaña de alteraciones moleculares características, como una mayor expresión de $p16^{INK4a}$, $p14^{ARF}$, $p21^{CIP1}$ y $p27^{KIP1}$, y fisiológicas, como una actividad β-galactosidasa incrementada y un cambio en la morfología.

Las células senescentes se comunican con su entorno secretando un conjunto de factores solubles y citoquinas proinflamatorias. Se trata del fenotipo denominado *SASP* (del inglés *"Senescence Associated Secretory Phenotype"*) (Acosta *et al.*, 2013; Coppé *et al.*, 2008; Herranz and Gil, 2018; Kuilman *et al.*, 2010), mediante el cual las células senescentes interactúan con las células de su entorno a través de un mecanismo paracrino. En este sentido, observamos que los fibroblastos DM1 tienen un perfil de secreción inflamatorio diferente al de las células control, con una mayor secreción de distintas citoquinas proinflamatorias, entre ellas IL-1, IL-8, INF-γ, IL-6, TNF-α y CCL5. Validamos que los niveles de expresión de IL-6, TNF-α y CCL5 están aumentados en fibroblastos DM1 a pase temprano y especialmente a pase tardío. En este sentido, las células DM1 presentan un perfil de expresión de citoquinas diferencial que sugiere presencia de *SASP*. En esta dirección, se ha observado un aumento de la inflamación en pacientes con DM1, asociándose niveles elevados de TNF-α con la resistencia a la insulina, la dislipemia y la función cardiaca (Fernández-Real *et al.*, 1999; Johansson *et al.*, 2000; Mammarella *et al.*, 2002).

La expresión aumentada de $p16^{INK4a}$, $p14^{ARF}$, $p21^{CIP1}$ y $p27^{KIP1}$ se ha observado también en mioblastos y PBMC (que como se discutirá más adelante, presentan además un acortamiento telomérico prematuro), así como en los tejidos musculares de los modelos animales de DM1 en ratón y mosca, evidencias que sugieren la existencia de una

senescencia incrementada *in vivo* en células de DM1. IL-6 es la citoquina pro-inflamatoria clave en el desarrollo de la senescencia y característica del SASP (Mosteiro *et al.*, 2016). Los niveles aumentados en fibroblastos, así como en PBMC refuerzan la idea de una senescencia prematura en la DM1 y sugieren que podría estar jugando un papel importante en la patogénesis de esta enfermedad. En este sentido, se ha descrito una asociación entre los niveles elevados de IL-6 y el grado de inmadurez muscular en la forma congénita de la DM1. Además, se ha determinado que la introducción exógena de las repeticiones CTG y su consiguiente acumulación aumentan el estrés celular, y con ello la secreción de dicha citoquina, contribuyendo, a su vez, a una mayor atrofia y desgaste muscular (Muñoz-Cánoves *et al.*, 2013; Nakamori *et al.*, 2017).

Nuestros resultados demuestran que la alteración en el ciclo celular, la proliferación, la división celular, así como en la reparación del ADN, provocan que las células DM1 no puedan responder de manera adecuada al estrés fisiológico y, posteriormente, entren prematuramente en senescencia **(Figura D3)**.

Figura D3. En la parte superior se enumeran los procesos moleculares alterados en este estudio en fibroblastos, mioblastos, tejido muscular del ratón DMSXL y de la mosca DM1, así como en PBMC derivadas de pacientes con DM1. Estos procesos serían previos al desarrollo de la senescencia celular. En la parte inferior se enumeran las características que presenta un fibroblasto senescente derivado de pacientes con DM1: un incremento en su tamaño y cambios en su morfología, un arresto del ciclo celular mediante un aumento en la expresión de reguladores del ciclo celular como $p16^{INK4}$, $p14^{ARF}$, $p21^{CIP1}$ y $p27^{KIP1}$, y un descenso de BMI1, HMGA2 y CDK2, así como un aumento en la actividad SA β-galactosidasa y una secreción al entorno de factores y citoquinas pro-inflamatorias.

1.3. Disfunción mitocondrial en DM1

En nuestro estudio también observamos que los fibroblastos DM1 tienen una menor producción de ATP mediante fosforilación oxidativa, con una menor eficiencia en la cadena de transporte de electrones mitocondrial y una mayor producción de lactato. Además, observamos una mayor acumulación de ROS total, pero también un incremento de ROS producido específicamente por las mitocondrias, junto a una mayor acumulación de p38 MAPK, mediador principal de la respuesta al estrés celular (Cuadrado and Nebreda, 2010). Por otra parte, nuestro estudio revela que, aunque no haya cambios en la biogénesis de las mitocondrias en células DM1, sí existe una alteración en los procesos de fusión (*OPA1*, *MFN1* y *MFN2*) y fisión (*DRP1*) mitocondrial y de mitofagia (*PARK*), encontrándose todos ellos reprimidos en los fibroblastos DM1. **(Figura D4)**. Los procesos de fusión y de fisión, así como de autofagia mitocondrial son procesos esenciales para mantener una mitocondria sana y funcional (Sharma *et al.*, 2019). La fisión es esencial para la eliminación de las mitocondrias defectuosas mediante mitofagia y se ha observado que dicho proceso desciende con el envejecimiento (Sharma *et al.*, 2019). En cambio, los procesos de fusión mitocondrial ayudan a mitigar el estrés mitocondrial al fusionar el contenido de mitocondrias parcialmente dañadas con mitocondrias no dañadas (Sharma *et al.*, 2019). En este sentido, en nuestro estudio estaríamos en un escenario donde, aunque no observamos un cambio en el número total de mitocondrias, sí observamos un descenso en su funcionalidad.

Este estudio es el primero que involucra el estado funcional de la mitocondria con la fisiopatología de la DM1, ya que hasta la fecha el rol de la mitocondria en esta enfermedad era prácticamente desconocido. Existían algunos estudios correlativos donde se habían descrito mayores niveles de lactato en sangre (Siciliano *et al.*, 2001), así como en cerebro de pacientes con DM1 (Gramegna *et al.*, 2018) y una acumulación de mitocondrias en el músculo (Ueda *et al.*, 1999). Estos estudios, junto con el nuestro, ponen de manifiesto la implicación de la disfunción mitocondrial en la DM1, aspectos en el que futuros estudios deberán profundizar.

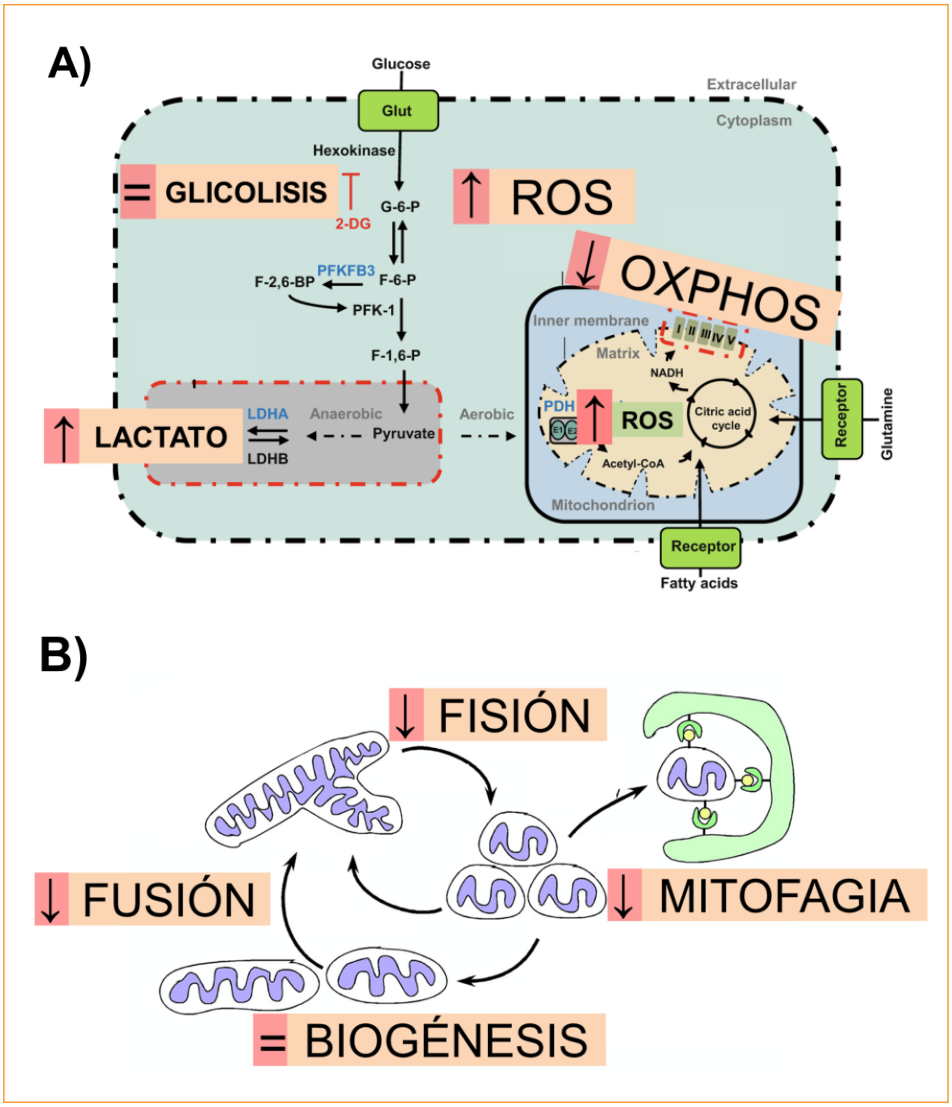

Figura D4. La respiración mitocondrial, la producción de ROS y los procesos dinámicos mitocondriales de fusión y fisión, además de la proliferación celular, se encuentran disminuidos en fibroblastos DM1. A) En la imagen se representa el metabolismo de la glucosa, donde en la glicolisis se produce piruvato que puede transformarse en lactato o entrar en la mitocondria hacia el ciclo del ácido cítrico para generar NADH, que se acopla a la producción de ATP mediante fosforilación oxidativa. Además, se muestra la producción de ROS total y mitocondrial. Se representa mediante flechas una mayor producción de lactato y de ROS, y una menor producción de ATP, todos ellos procesos alterados en fibroblastos DM1. Modificado de Yetkin-Arik *et al.,* 2019. B) En la imagen se representan los procesos de biogénesis, fusión, fisión y autofagia mitocondrial. Con flechas se indican aquellos procesos disminuidos en fibroblastos DM1. Modificado de Boland *et al.,* 2013.

1.4. Mayor acortamiento de los telómeros en DM1

El acortamiento telomérico es uno de los mecanismos moleculares más importantes del envejecimiento. Por ello estudiamos la longitud telomérica en PBMC de una cohorte compuesta por 379 pacientes con DM1 y 223 individuos sanos, observando que los pacientes con DM1 no presentaban cambios en la longitud telómerica. Además, no encontramos una asociación entre la longitud telomérica y el número de repeticiones CTG o la escala MIRS (Mathieu *et al.*, 2001). Estos resultados no fueron los esperados debido a la relevancia que tiene la longitud telomérica para el envejecimiento y la homeostasis. Sin embargo, están en línea con otros autores que tampoco encontraron diferencias o incluso observaron una mayor longitud telomérica *in vitro* en otras poblaciones celulares como en un número reducido de mioblastos y de células satélite derivados de pacientes con DM1 (Bigot *et al.*, 2009; Renna *et al.*, 2014; Thornell *et al.*, 2009).

Nuestro estudio completó un estudio longitudinal en un subgrupo de pacientes en los que se obtuvo una muestra sanguínea a dos tiempos sucesivos y pudimos analizar la longitud telomérica con el paso del tiempo. Mediante este abordaje, pudimos describir que los pacientes con DM1 poseían un acortamiento telomérico mayor que los controles sanos con el paso del tiempo. En línea con esto, sí que se había visto *in vitro* que las células satélite de formas congénitas de la DM1 presentaban un acortamiento telomérico mayor en cada división celular (Bigot *et al.*, 2009). De esta manera el acortamiento telomérico no sería un biomarcador diagnóstico de la enfermedad, pero si podría plantearse la tasa de acortamiento telomérico como un biomarcador de progresión para esta enfermedad.

En la actualidad se desconoce por completo el mecanismo por el cual se produce este defecto en pacientes con DM1, proponiéndose que las expansiones CTG podrían interferir en la homeostasis de los telómeros (Bigot *et al.*, 2009). Por otra parte, se ha propuesto que el estrés oxidativo podría estar jugando un papel importante en este proceso. Se sabe que los telómeros son muy sensibles a dicho estrés y que se produce un acortamiento telomérico mayor en presencia de daño oxidativo (Henle *et al.*, 1999; von Zglinicki, 2002). En este sentido, nuestros resultados en fibroblastos revelan elevados niveles de estrés oxidativo, y otros trabajos han identificado un aumento de radicales libres y de estrés oxidativo en muestras de sangre de pacientes con DM1, circunstancias que podrían estar erosionando al telómero (Ihara *et al.*, 1995; Toscano *et al.*, 2005).

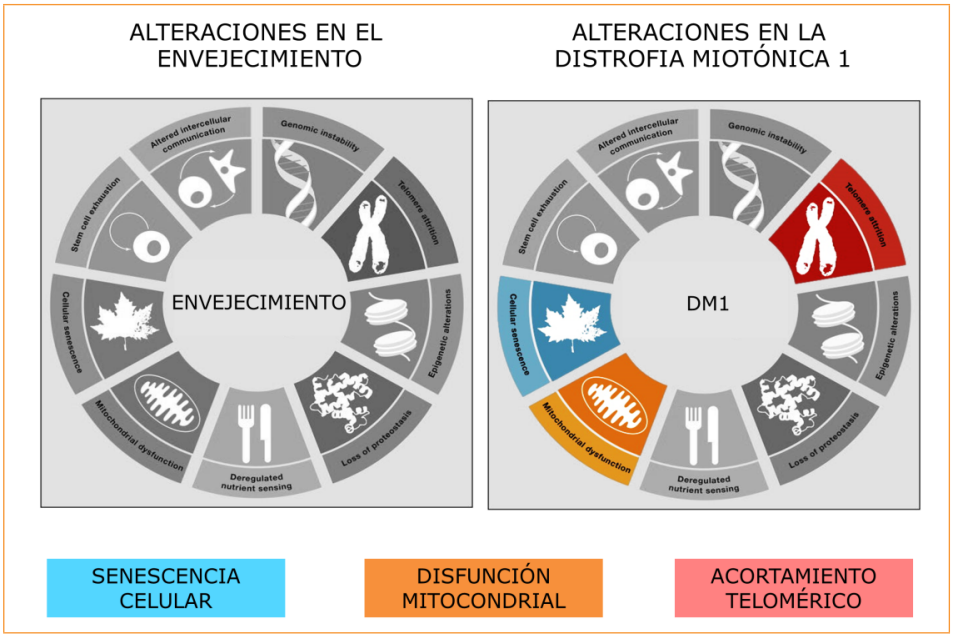

Figura D5. A la izquierda se representan las características moleculares implicadas en el proceso del envejecimiento y a la derecha, se resaltan en color aquellas que han sido estudiadas en el presente trabajo y que están alteradas en la DM1: la senescencia celular, el acortamiento telomérico y la disfunción mitocondrial. Modificado de López-Otín *et al.,* 2013.

El descenso en los procesos de división celular, la parada proliferativa, la acumulación de daño en el ADN, el aumento en la inflamación, el acortamiento telomérico, y la disfunción mitocondrial, son todos ellos procesos que ocurren durante el envejecimiento fisiológico (López-Otín *et al.*, 2013). En nuestro trabajo describimos como todos ellos se encuentran alterados de manera exacerbada en la DM1 **(Figura D5)**. Hemos identificado estas alteraciones tanto en fibroblastos como en mioblastos y en PBMC, pero también en el modelo murino DMSXL y en el modelo en mosca, presentando ambos modelos animales una supervivencia global comprometida (García-López *et al.*, 2008; Huguet *et al.*, 2012). Los resultados de esta tesis refuerzan la idea de considerar a la DM1 como una enfermedad multisistémica caracterizada por un envejecimiento prematuro (Mateos-Aierdi *et al.*, 2015; Meinke *et al.*, 2018).

1.5. Identificación del mayor riesgo oncogénico y sus vías moleculares implicadas en DM1

1.5.1. Identificación del mayor riesgo oncogénico en DM1

El envejecimiento es el factor de riesgo más importante para el desarrollo del cáncer (Niccoli and Partridge, 2012). En este trabajo estudiamos el riesgo a desarrollar cáncer en DM1, a través del análisis de nuestra cohorte compuesta por más de 400 pacientes con DM1 y observamos un mayor riesgo para el desarrollo de neoplasias malignas, siendo el riesgo superior en mujeres que en hombres.

Nuestros resultados replican los de otro estudio previo realizado en Suecia y Dinamarca, aunque dicho estudio incluía pacientes sin distinción entre DM1 y DM2 (Gadalla *et al.*, 2011). Además, otros estudios confirmaron posteriormente estos resultados en cohortes poblaciones adicionales de Francia y de distintas regiones de Estados Unidos (**Tabla D1**).

En el estudio original de Gadalla *et al.,* los pacientes con DM presentaban un mayor riesgo de desarrollar cáncer de endometrio, de cerebro, de ovario y de colon. De manera muy similar, en nuestra cohorte el riesgo fue mayor para los tumores de tiroides, de cerebro, de endometrio y de ovario. Además, no encontramos una correlación significativa entre el mayor riesgo a desarrollar cáncer con el número de repeticiones CTG y la escala MIRS. No obstante, cabe señalar que la determinación molecular de la expansión CTG se realizó en sangre y en el momento del diagnóstico de DM1, pero no en el momento del diagnóstico del cáncer ni en el propio tejido tumoral, pudiendo haber variaciones temporales y tisulares en ese intervalo de tiempo.

A día de hoy, además de nuestro trabajo, se han publicado 5 estudios adicionales de cohortes de pacientes con DM1 donde se analiza el riesgo de desarrollar cáncer (Abbott *et al.*, 2016; Alsaggaf *et al.*, 2018; Gadalla *et al.*, 2011; Mohamed *et al.*, 2013; Win *et al.*, 2012), y un metanálisis recopilatorio realizado por nuestro grupo (Emparanza *et al.*, 2018). En 4 cohortes se observa un mayor riesgo de desarrollar cáncer de tipo ginecológico, representado por cáncer de endometrio y/o de ovario, sugiriendo un riesgo mayor en mujeres que en hombres, coincidiendo con nuestros resultados. Por otro lado, las alteraciones en el tiroides y el desarrollo de melanoma se han observado en 2 estudios, seguido por el cáncer de cerebro. Los resultados del metanálisis (Emparanza *et al.*, 2018), confirmaron un mayor riesgo para las neoplasias anteriores, junto con el cáncer de testículos y colorrectal. Curiosamente, estos tipos tumorales no son los más frecuentes en la población general, a excepción del cáncer colorrectal.

Tabla D1. Se presentan los diferentes estudios que han analizado el riesgo de desarrollar cáncer en pacientes con DM1.

Tipo tumoral	Fernández-Torrón & García-Puga *et al.*, 2016	Emparanza *et al.*, 2019	Gadalla *et al.*, 2011	Win *et al.*, 2012	Mohamed *et al.*, 2013	Abbott *et al.*, 2016	Alsaggaf *et al.*, 2018
Tiroides	**23.33***	**8.52***	**7.10***	5.54		3.78	**15.93***
Cerebro	**9.80***	6.20	**5.30***	1.54			4.99
Ovario	**8.33***	**5.56***	**5.20***	1.66	**9.30***		**14.88***[a]
Endometrio	**6.86***	**7.48***	**7.60***	1.07	**21.70***	**6.98***	
Testículo	14.25	**5.95***	1.40	5.09		**10.74***	
Melanoma	1.72	**2.45***	2.30	**27.54***	7.10	0.89	**5.98***
Colorrectal	2.06	**2.20***		1.09			1.82
Ocular			**12.00***				
Otros del aparato reproductor femenino			**9.60***				
Páncreas			**3.20***				2.92
Colon			**2.90***		5.00	2.15	
Timoma					**100***		
Linfoma no-Hodgkin	2.86	2.75	1.80			**4.25***	
Riñón	2.53	2.12	1.70	2.39			2.26
Pulmón	1.18	1.72	1.70	0.86	6.00		1.21
Próstata	0.46	1.19		0.70		1.43	
Mama	1.04	1.11	1.20	0.95			0.52b
Nariz			9.00				
Hígado	2.54		4.20				
Esófago			3.40				
Recto			2.20			4.56	
Leucemia	3.80	2.38		2.19			
Leucemia aguda			2.00			8.75	
Leucemia crónica						12.70	
Vejiga	0.64			1.07			
Otros urinarios				0.40			
Mieloma múltiple				3.19			
Linfoma				2.41			
Faringe			1.80				
Cérvix			0.90				
Cabeza y cuello	0.94				5.90	1.50	
Estómago	1.57						

Se han propuesto varias hipótesis para explicar dicho riesgo a desarrollar cáncer que implican la presencia de mutaciones en la ruta de β-catenin, la inestabilidad genómica y la toxicidad celular provocada por las repeticiones CTG, y mutaciones en genes involucrados en la reparación del daño en el ADN (Mueller *et al.,* 2009; Rübben *et al.,* 2020). Sin embargo, la causa por la que estos pacientes tienen un mayor riesgo a desarrollar cáncer no se conoce con exactitud.

1.5.2. Identificación de las vías moleculares asociadas al mayor riesgo oncogénico en DM1.

La identificación de los mecanismos moleculares asociados al mayor riesgo de desarrollar cáncer en pacientes con DM1 era una cuestión por resolver al inicio de esta tesis. Para la identificación del mecanismo molecular responsable del mayor riesgo oncogénico realizamos un estudio de expresión génica en muestras de PBMC de pacientes con DM1 y de controles sanos. Encontramos una firma molecular asociada a pacientes con DM1 con un patrón de expresión característico de dos genes diferencialmente expresados: el pseudogén RNA5SP211 y el receptor del gusto TAS2R13. Sin embargo, cuando el análisis consideraba el sexo de los individuos, se detectaron alteraciones en la expresión en 4 genes adicionales en hombres y de otros 10 genes en mujeres.

De estos 14 genes diferencialmente expresados, 8 habían sido previamente asociados a la presencia de cáncer. Entre ellos, la glicoproteína CD24 (Kristiansen *et al.*, 2003; Li *et al.*, 2017; Ooki *et al.*, 2018; Wang *et al.*, 2017), la caspasa 5 (Flood *et al.*, 2015), la piruvato deshidrogenasa 4 o PDK4 (Leclerc *et al.*, 2017; Woolbright *et al.*, 2018), la proteína quinasa 1 asociada a muerte celular o DAPK1, la fosfolipasa PLA2G7 (Lehtinen *et al.*, 2017; Vainio *et al.*, 2011a; Vainio *et al.*, 2011b), el factor de transcripción ZEB2 (Caramel *et al.*, 2018; Goossens *et al.*, 2017) así como los miARNs miR-200c y miR-141 (Feng *et al.*, 2014; Huang *et al.*, 2019; Liu *et al.*, 2018).

En nuestro estudio encontramos una expresión aumentada de caspasa 5, PLA2G7 y CD24 en varones con DM1. En cambio, en mujeres observamos una expresión aumentada de PDK4 y reducida de ZEB2, DAPK1 y del precursor de los miR-200/141. En este sentido, niveles elevados de CD24, PDK4 y PLA2G7 se han asociado a una mayor agresividad tumoral, siendo factores oncogénicos. De entre ellos cabe destacar CD24, que es un marcador de célula madre tumoral en cáncer de ovario (Gao *et al.*, 2010) y PDK4, que posee un rol oncogénico tanto en cáncer de ovario como en glioblastoma (Liu *et al.*, 2014; Wang *et al.*, 2019a).

El miR-200c y el miR-141 se han asociado al desarrollo de cáncer, siendo biomarcadores tanto de diagnóstico como de pronóstico en varios tipos tumorales (Peter, 2009), y tienen como dianas a ZEB2, BMI1 y p16^{INK4a}. Junto con sus dianas, estos miR están involucrados en el control de la plasticidad celular y de la progresión del cáncer a través de la modulación del proceso de transición epitelio-mesénquima, de la regulación de la senescencia y de la actividad de las células madre tumorales, formando entre ellos un bucle autorregulatorio. En concreto, se han descrito unos niveles reducidos de los miARN, de ZEB y de p16^{INK4a}, y aumentados de BMI1, en muestras de tumores primarios, de metástasis y en la población de células madre tumorales.

Se ha descrito que ambos miR inhiben a sus dianas ZEB1 y ZEB2, pero de la misma manera se ha descrito que ZEB1 es capaz de regular a ambos miR. Así la activación de un grupo de factores afectará fuertemente a la expresión del otro grupo. Al ser los factores ZEB potentes inductores de la transición epitelio mesénquima, la sobreexpresión del miR-200 provocaría una represión de ZEB y con ello una mayor diferenciación epitelial, y viceversa (Brabletz *et al.*, 2011; Brabletz and Brabletz, 2010; Díaz-Riascos *et al.*, 2019; Feng *et al.*, 2014; Hill *et al.*, 2013).

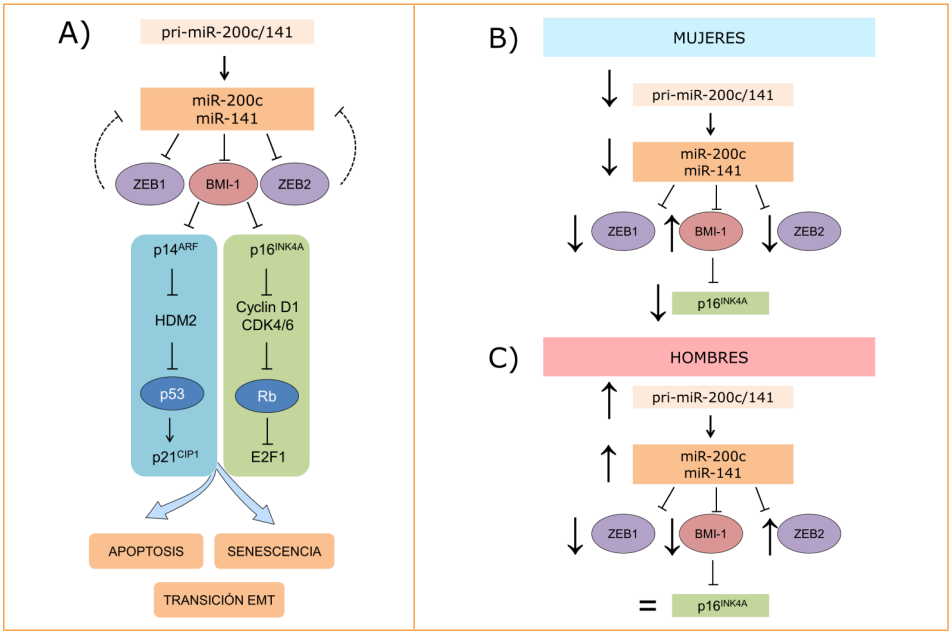

Figura D6. En la imagen se representan las vías moleculares alteradas en pacientes con DM1. **A)** En primer lugar, la inhibición de BMI1 y ZEB1/2 por parte de los miR-200c y miR-141. La inhibición de BMI1 provoca a su vez la inhibición tanto de p14^{ARF} como de p16^{INK4a}, y esto desencadenaría los procesos de senescencia y apoptosis. En las figuras **B)** y **C)** se representa patrón diferencial en la expresión encontrada entre mujeres y hombres con DM1.

En nuestra cohorte observamos unos niveles reducidos tanto del miR-200 como de los factores ZEB y $p16^{INK4a}$ en las mujeres con DM1, y una mayor expresión de BMI1, lo que apoyaría la observación de una mayor presencia de cáncer en mujeres con DM1. En cambio, en los varones con DM1 observamos unos niveles elevados de dicha familia de miARNs junto con niveles superiores de ZEB2 y $p16^{INK4a}$ y menores de BMI1 **(Figura D6)**.

Por otra parte, en estudios en células no tumorales se ha descrito que el miR-141 controla la proliferación y la entrada en senescencia de los fibroblastos a través de BMI1 y de $p16^{INK4a}$. En este sentido, se ha observado que la elevación del miR-141 inhibe la proliferación, aumenta el número de células senescentes y de las células con daño en el ADN. A su vez, la inhibición del miR-141 rescata todos estos procesos (Dimri *et al.*, 2013; Li *et al.*, 2019). Además, se ha propuesto a los miR-141 y miR-200c como marcadores de envejecimiento hepático, ya que se ha demostrado que su expresión aumenta con la edad (Capri *et al.*, 2017; Moimas *et al.*, 2019).

No obstante, se ha descrito que el cáncer de ovario está ligado a mutaciones en genes implicados en la reparación del daño en el ADN como BRCA1, BRCA2, ATM y RAD51; genes que hemos visto que se encuentran alterados en pacientes con DM1. Recientemente se han descrito mutaciones en β-catenin y ATM en tumores derivados de pacientes con DM1 (Rübben *et al.,* 2020). Por otra parte, entre los factores de riesgo más importantes para el desarrollo del cáncer de ovario se encuentran una baja maternidad, el desarrollo de abortos y el uso de una terapia hormonal tras la menopausia (Momenimovahed *et al.*, 2019), entre otros factores que están ligeramente alterados en pacientes con DM1 (Higgs *et al.*, 2019).

Estos datos sugieren que las vías que están implicadas en el envejecimiento celular también lo están en el desarrollo de tumores en pacientes DM1, debido a que hemos observado que la vía BMI1/$p16^{INK4a}$ se encuentra alterada durante el envejecimiento celular, pero también está implicada en el mayor riesgo de desarrollar cáncer de los pacientes con DM1. Su expresión se observa en diferente dirección según esté involucrada en el proceso de envejecimiento o de cáncer, coherente con la idea de que los procesos biológicos y los mecanismos moleculares implicados en el desarrollo tanto del cáncer como del envejecimiento tienen un origen común y están interconectados, aunque actúen de manera opuesta y con diferente intensidad según el contexto celular (Hanahan and Weinberg, 2000; López-Otín *et al.*, 2013).

2. Nuevas terapias en DM1

En los últimos años se han planteado una gran cantidad de estrategias terapéuticas frente a la DM1, pero a día de hoy, ninguna de ellas ha logrado pasar a la clínica e implantarse como terapia efectiva para estos pacientes. En este sentido, cabe destacar el gran esfuerzo que se ha realizado en la búsqueda de nuevas moléculas pequeñas (Konieczny *et al.*, 2017) y, por otro lado, en el desarrollo de oligonucleótidos antisentido (ASO) (Pandey *et al.*, 2015), aunque actualmente ninguna de estas aproximaciones ha superado los ensayos clínicos. Esto puede ser debido a que estas terapias han sido dirigidas a alteraciones puntuales de la enfermedad, como es el caso de los ASO, que interfieren en la expresión del ARN de DMPK, pero que no afectan, por ejemplo, a otros procesos implicados en la enfermedad como la expresión reducida de *SIX5* o *DMWD*, entre otros (Thornton *et al.*, 2017). Lo mismo sucede con otro tipo de estrategias, como las encaminadas a la inhibición del secuestro de MBNL1 por el transcrito mutado o las estrategias basadas en reducir los niveles de CUGBP1/CELF1 (Thornton *et al.*, 2017). Estas estrategias se centran en un mecanismo concreto de la enfermedad, lo que puede resultar insuficiente en una enfermedad tan compleja y de carácter multisistémico. Otro aspecto que puede haber contribuido a que dichas terapias no se hayan implantado puede ser debido a la deficiente distribución de estas moléculas en los tejidos diana, que en algunos casos se reveló muy escasa tras su administración sistémica (Sardone *et al.*, 2017).

Por todo ello, se requieren nuevas estrategias encaminadas a resolver los problemas celulares de una manera global. Dado que hemos observado que los pacientes con DM1 poseen alteraciones en varios de los mecanismos principales implicados en el envejecimiento fisiológico, valoramos el uso de compuestos que han demostrado un beneficio en el envejecimiento fisiológico, como es el caso de los compuestos senolíticos, la metformina o compuestos de nueva síntesis como los compuestos ahulkenoides.

2.1. Terapia senolítica en DM1

La senescencia celular es un proceso conservado implicado en la remodelación tisular durante el desarrollo embrionario pero que también se activa en la edad adulta cuando se produce un daño en el tejido con el objetivo de repararlo (Muñoz-Espín and Serrano, 2014). Sin embargo, es posible que su acción como remodelador tisular no se complete de manera eficiente en los tejidos envejecidos y, por lo tanto, provoca una acumulación de células senescentes en dichos tejidos (Childs *et al.*, 2015). Esto conduce a una disfunción tisular que desencadena el envejecimiento fisiológico, además de un amplio espectro de enfermedades relacionadas con la edad (Baker *et al.*, 2016; Baker *et al.*, 2011; Muñoz-Espín and Serrano, 2014).

Quercetina, dasatinib y navitoclax son los tres compuestos con acción senolítica más estudiados y que mejores resultados han proporcionado. Se ha demostrado en modelos animales que la quercetina y el dasatinib aumentan la supervivencia y reducen el riesgo de mortalidad, a la vez que mejoran la actividad funcional de ratones envejecidos naturalmente y la de ratones a los que se les han trasplantado células senescentes (Xu *et al.*, 2018). También revierten otros procesos implicados en el envejecimiento (Cavalcante *et al.*, 2020; Chandra *et al.*, 2020; Ogrodnik *et al.*, 2017), en todos los casos con una baja toxicidad. Además, se ha observado que el dasatinib reduce la fibrosis (Cavalcante *et al.*, 2020; Cruz *et al.*, 2016; Kanemaru *et al.*, 2018) y que el navitoclax rejuvenece el nicho de célula madre hematopoyética y muscular en modelos de ratón (Chang *et al.*, 2016). Asimismo, en humanos se ha observado que la quercetina y el dasatinib reducen el número de células $p16^{INK4a}$ positivas en epidermis y en tejido adiposo, mejorando la capacidad funcional pulmonar de pacientes con fibrosis pulmonar idiopática (Hickson *et al.*, 2019; Justice *et al.*, 2019).

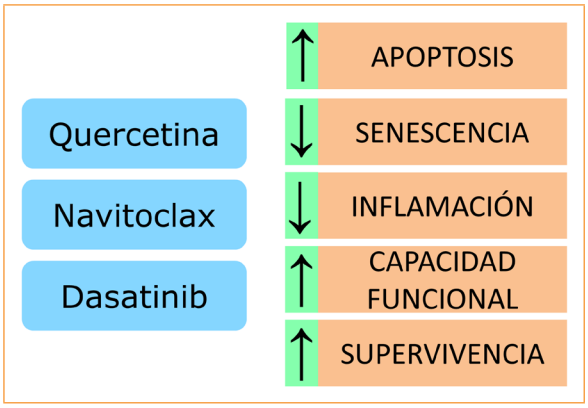

Figura D7. En la figura se enumeran los procesos sobre los que actúan los compuestos senolíticos en DM1, eliminando las células senescentes, con un aumento de la apoptosis y un descenso en la inflamación. Además en el modelo de mosca de DM1 mejoran tanto la capacidad funcional como su supervivencia.

Nuestros resultados demuestran que tres compuestos senolíticos, la quercetina, el dasatinib y el navitoclax promueven la eliminación de células senescentes mediante apoptosis y revierten parcialmente el *SASP*. Además, la quercetina rescata los niveles de *Mbnl*, *Dacapo* (homólogo de p27/p21) y *Psc* (homólogo de BMI1) en tejido muscular de mosca y tanto ésta como el dasatinib aumentan de manera considerable la supervivencia global de las moscas con DM1, así como su actividad locomotora **(Figura D7)**.

La acumulación de células senescentes se ha descrito en diferentes patologías asociadas a la edad como la diabetes mellitus tipo 2 (Chesnokova *et al.*, 2009; Sone and Kagawa, 2005), la sarcopenia (Cosgrove *et al.*, 2014; Du *et al.*, 2014; Sousa-Victor *et al.*, 2014) o el desarrollo de cataratas (Baker *et al.*, 2008), entre otras. Dichos proce-

sos se dan de una manera más frecuente en pacientes con DM1 y la terapia con compuestos senolíticos dirigida a la eliminación de células senescentes podría suponer un beneficio terapéutico para estos pacientes.

Por otra parte, es interesante mencionar que tanto el navitoclax como el dasatinib se desarrollaron como tratamientos antitumorales. En este sentido, el dasatinib ha mostrado efectividad en el tratamiento de la leucemia linfoblástica aguda (Rousselot *et al.*, 2016; Slayton *et al.*, 2018) y en el cáncer de mama (Tian *et al.*, 2018), mientras que el navitoclax es útil para el tratamiento del cáncer de pulmón (Gandhi *et al.*, 2011), lo que refuerza la idea de una complementariedad de los procesos moleculares implicados tanto en el cáncer como en el envejecimiento.

En definitiva, esta es la primera prueba de concepto en la que se describe que las terapias senolíticas tienen un efecto beneficioso para los pacientes con DM1, tanto en un modelo *in vitro* como en un modelo *in vivo* de la enfermedad, lo que abre las puertas a una nueva y prometedora aproximación terapéutica para el tratamiento de la DM1 que deberá ser validada en futuros ensayos clínicos.

2.2. Terapia con metformina en DM1

El tratamiento con metformina es otra alternativa como potencial tratamiento de la DM1 con resultados preliminares. En mioblastos DM1 se ha observado que la metformina es capaz de rescatar el *splicing* alternativo (Laustriat *et al.*, 2015). Por otra parte, se ha descrito que los pacientes con DM1 tienen una mayor incidencia de diabetes y que principalmente son tratados con metformina (Alsaggaf *et al.*, 2018), prescripción que se da también en poblaciones mayores sanas (Hostalek *et al.*, 2015). En este sentido, los pacientes con DM1 y diabéticos presentan un mayor riesgo a desarrollar cáncer, riesgo que se reduce significativamente por el tratamiento con metformina (Alsaggaf *et al.*, 2019). Además, en un ensayo clínico de 1 año con pacientes DM1 tratados con metformina se observó una mejora en su movilidad (Bassez *et al.*, 2018). Recientemente se ha descrito que la metformina inhibe la traducción no asociada a ATG (*RAN translation*), fenómeno presente en la DM1, en dos enfermedades producidas por expansiones de repeticiones como la demencia frontotemporal y la esclerosis lateral amiotrofia (Zu *et al.,* 2020).

En este estudio, describimos que la metformina incrementa la producción de ATP mediante la cadena de transporte de electrones mitocondrial y disminuye la producción de ROS en fibroblastos DM1. Por otra parte, observamos que este fármaco rescata otros fenotipos alterados en fibroblastos DM1, tales como la proliferación celular y la dinámica de fusión y fisión mitocondrial **(Figura D8)**. Resultados similares se han obtenido en fibroblastos derivados de pacientes con síndrome de Down, las cuales también presentan una disfunción mitocondrial (Izzo *et al.*, 2017) y en

hepatocitos sanos primarios (Wang *et al.*, 2019b), donde se observa que la metformina rescata tanto el metabolismo mitocondrial como los procesos de fisión y fusión mitocondrial. En esta línea, se ha observado que la metformina revierte los procesos de senescencia, aumenta la proliferación y reduce la apoptosis *in vitro* (Chen *et al.*, 2016; Mu *et al.*, 2018). Estas acciones podrían indicar que el efecto de la metformina en DM1 puede estar relacionado con su acción anti-envejecimiento.

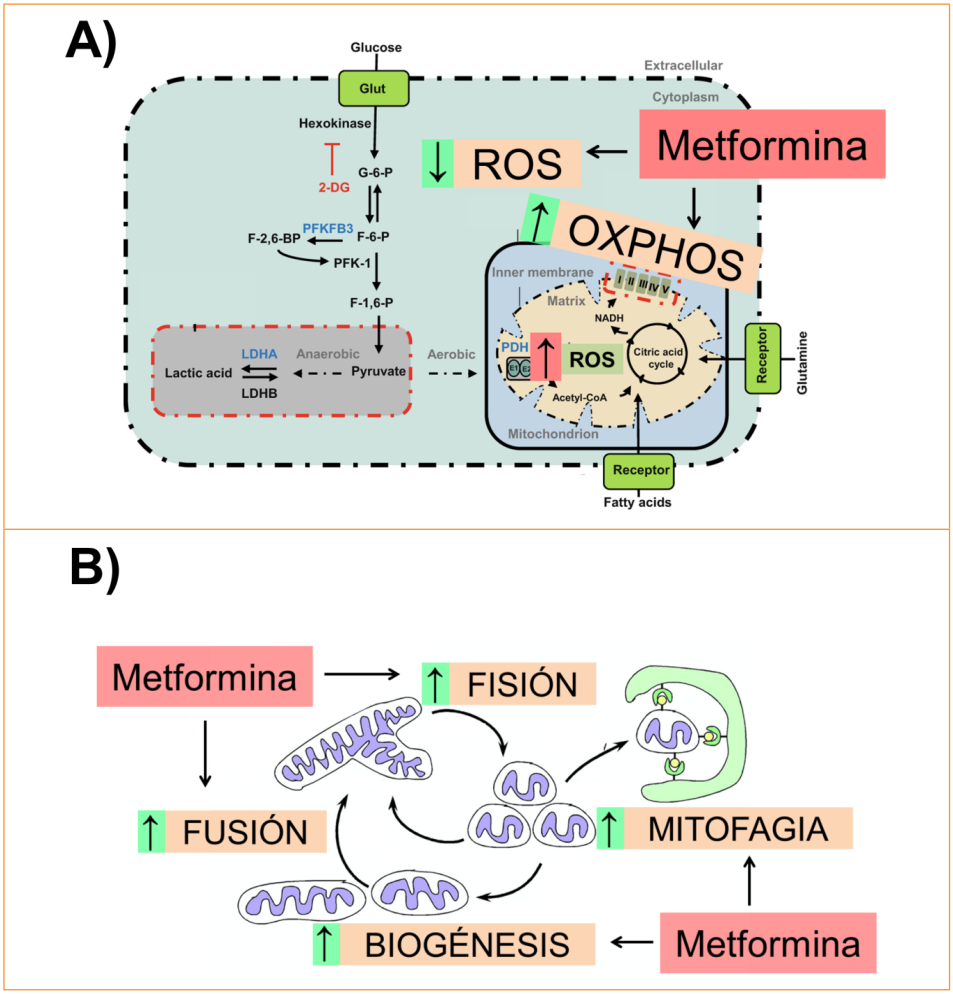

Figura D8. El tratamiento con metformina recupera la respiración mitocondrial, disminuye la producción de ROS, mejora los procesos dinámicos mitocondriales de fusión y fisión, además de la proliferación celular y de DMPK y MBNL1 en fibroblastos DM1. **A)** En la imagen se representan los procesos revertidos por el tratamiento con metformina como la producción de ROS y la producción de ATP por vía mitocondrial. Modificado de Yetkin-Arik *et al.,* 2019. **B)** El tratamiento con metformina también revierte los procesos de biogénesis, fusión, fisión y autofagia mitocondrial. Modificado de Boland *et al.,* 2013.

La acción antienvejecimiento de la metformina radica en múltiples procesos. Así, se ha observado que tiene efectos múltiples en la célula reduciendo los niveles de insulina a través de ERBB2 (Kim *et al.*, 2016), como inhibidor de mTOR (Amin *et al.*, 2019), reduciendo los niveles de inflamación asociados al proceso de senescencia a través del bloqueo de la ruta de NF-kB (Moiseeva *et al.*, 2013), reduciendo los niveles de ROS (Batandier *et al.*, 2006), activando AMPK (Howell *et al.*, 2017) y reduciendo el daño en el ADN (Algire *et al.*, 2012). En este sentido, hemos observado que la metformina provoca una reducción en los niveles de producción de ROS, activando la defensa antioxidante a través de GPX1, lo que refuerza la idea de que su acción es la suma de todos los mecanismos.

2.3. Terapia con compuestos AHK

Los compuestos AHK fueron diseñados en origen para normalizar la homeostasis del calcio celular mediante la estabilización de la interacción entre rianodina y calstabina en la distrofia muscular de Duchenne.

En este trabajo describimos el potencial de los compuestos AHK en la DM1. En el modelo de fibroblasto vemos que tanto el AHK6 como el AHK7 incrementan la proliferación celular y la producción de ATP por vía mitocondrial, y también reducen la producción de ROS, sin tener un efecto tóxico para la célula. A pesar de que en nuestro modelo no vemos una alteración en los niveles de calcio celular en fibroblastos derivados de pacientes con DM1, sí que observamos una clara reversión del fenotipo en estas células, sugiriendo una acción independiente de la homeostasis del calcio. En línea con esta idea, el AHK revierte de manera muy significativa la longevidad reducida del modelo de mosca.

El estudio de RNA-seq realizado está ayudando a identificar las vías de acción del AHK. En este sentido, dicho análisis sugirió la acción de los compuestos ahulkenoides a través de la señalización celular, la regulación del ciclo y la regulación del metabolismo celular. Tanto la regulación del ciclo celular como el metabolismo celular son dos de los procesos que hemos descrito como alterados en células DM1 y son rescatados parcialmente por los Ahulkenoides. Además, datos preliminares del grupo sugieren que estos compuestos actúan a través de la ruta mTOR (datos no publicados). Se ha descrito que la inhibición de mTOR con rapamicina incrementa la supervivencia en varios modelos animales como en *C. elegans* (Blackwell *et al.*, 2019), *D. melanogaster* (Bjedov *et al.*, 2010) y en ratón (Anisimov *et al.*, 2010; Harrison *et al.*, 2009; Ramos *et al.*, 2012). En DM1, se ha descrito *in vitro* un descenso de la fosforilación dependiente de mTOR y que su sobrexpresión en un modelo de mosca de DM1 rescata su supervivencia global (Bargiela *et al.*, 2015). Estos datos preliminares sugieren una acción sobre varios mecanismos en DM1.

Por todo ello, serían necesarios nuevos estudios que describan las vías moleculares moduladas por los compuestos AHK en DM1.

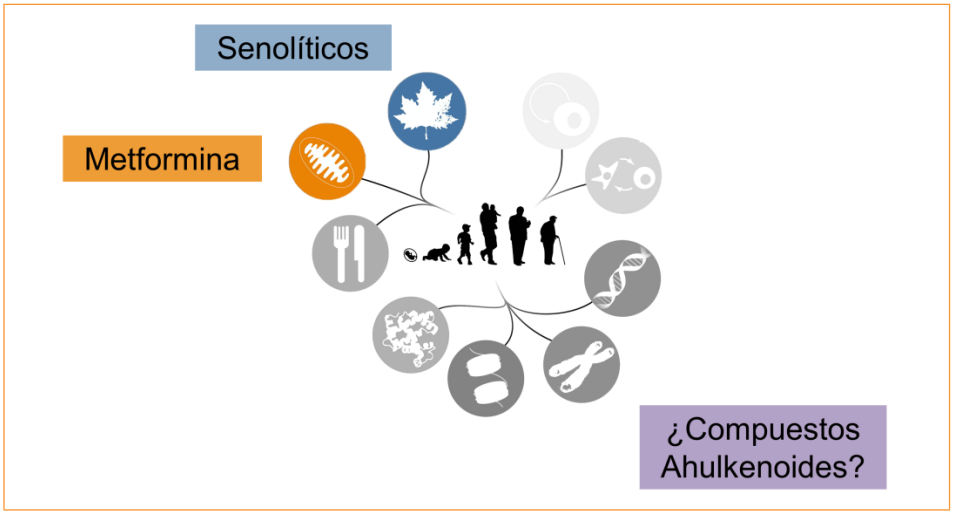

Figura D.9. En la figura se representan los procesos celulares implicados en el envejecimiento fisiológico y en color se representan aquellos que hemos demostrado que se encuentran alterados en DM1 y que tienen una intervención terapéutica. Los compuestos senolíticos eliminan a las células senescentes y la metformina incide sobre el metabolismo mitocondrial. Se representan también los compuestos ahulkenoides sin especificar un mecanismo concreto. Modificado de López-Otín *et al.,* 2013.

En definitiva, en esta tesis describimos la alteración de varios procesos implicados en el envejecimiento fisiológico que se producen de manera prematura en la DM1, como son la disminución en la regulación de los procesos de proliferación y división celular, una alteración en los mecanismos de respuesta al daño en el ADN, una entrada prematura en senescencia, así como una alteración en varios procesos mitocondriales y un acortamiento telomérico prematuro. Además, observamos un mayor riesgo de desarrollar cáncer en pacientes con DM1. Por otro lado, caracterizamos los efectos de tres estrategias terapéuticas dirigidas a revertir este envejecimiento prematuro que podrían ser beneficiosas para los pacientes con DM1 (**Figura D.9**).

Conclusiones

1. Fibroblastos y mioblastos derivados de pacientes con DM1 tienen disminuida la regulación del ciclo celular, la división celular y la respuesta al daño en el ADN, así como una entrada prematura en senescencia. Estas alteraciones son debidas, en parte, a la vía mediada por BMI1 y $p16^{INK4a}$.

2. Los fibroblastos derivados de pacientes con DM1 presentan una menor respiración y como consecuencia una menor producción de ATP por la vía mitocondrial, generando una mayor producción de ROS que se asocian a una activación de la vía de señalización de p38MAPK.

3. No existen diferencias en la longitud telomérica en muestras de PBMC entre pacientes con DM1 y controles sanos, pero el proceso de acortamiento telomérico a lo largo del tiempo está acelerado en los pacientes con respecto a los controles.

4. Los pacientes con DM1 tienen un riesgo dos veces superior que la población normal a desarrollar cáncer, especialmente de cáncer de tiroides, de cerebro, de ovario y de endometrio, siendo este riesgo mayor en mujeres que en hombres.

5. El mayor riesgo de desarrollo de cáncer se asocia con un patrón molecular diferencial, que en mujeres se caracteriza por una menor expresión de la familia del miR-200 y de $p16^{INK4a}$ y con una mayor expresión de BMI1.

6. Los compuestos senolíticos quercetina, dasatinib y navitoclax rescatan varios elementos constitutivos del fenotipo del envejecimiento prematuro característico de la DM1, como es la eliminación de células senescentes, además de un aumento significativo de la longevidad y la capacidad de locomoción en el modelo de DM1 en *Drosophila melanogaster.*

7. El tratamiento con metformina rescata los déficits mitocondriales presentes en los modelos celulares de DM1, además de otros elementos característicos del fenotipo de envejecimiento prematuro como es la capacidad de proliferación, la producción de ROS y la actividad de la vía BMI1/$p16^{INK4a}$ asociados a la DM1.

8. Los compuestos ahulkenoides no provocan toxicidad celular y, al igual que la metformina, rescatan varios de los rasgos del fenotipo de envejecimiento prematuro presentes en fibroblastos DM1, como son la producción de ATP por la vía mitocondrial, la producción de ROS y la proliferación celular, además de aumentar la supervivencia global del modelo de *Drosophila melanogaster*.

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Anexos

Anexo 1: Resolución del Comité de Ética de Investigación Clínica

Osakidetza

Donostia Ospitalea
Hospital Donostia

INFORME DEL COMITÉ ÉTICO DE INVESTIGACIÓN CLÍNICA

D. José Ignacio Emparanza Knörr, Presidente del Comité Ético de Investigación Clínica del Área Sanitaria de Gipuzkoa,

CERTIFICA:

Que este Comité, de acuerdo a la Ley 14/2007 de Investigación Biomédica, Principios éticos de la declaración de Helsinki y resto de principios éticos aplicables, ha evaluado el Estudio Observacional titulado: ***"Bases moleculares del riesgo oncológico en la distrofia miotónica".***

Y que este Comité reunido el día 22 de Septiembre ha decidido emitir **Informe Favorable** a que dicho estudio sea realizado por el siguiente investigador:

- Adolfo López de Munain – Neurología - OSI Donostialdea

Lo que firmo en San Sebastián, a 22 de Septiembre de 2015

Fdo.: Jose Ignacio Emparanza

Anexo 2: Consentimiento Informado

Osakidetza
Donostia Ospitalea
Hospital Donostia

biodonostia

Código Proyecto: ALM-DM1-2015 VERSIÓN 1.0 de 7 de Julio 2015

CONSENTIMIENTO PARA LA REALIZACIÓN DEL PROYECTO DE INVESTIGACIÓN

Investigador/Responsable clínico: Dr. Adolfo López de Munain,
(Servicio de Neurología, Hospital Universitario Donostia).

TÍTULO DEL PROYECTO: ***Bases moleculares del riesgo oncológico en la distrofia miotónica***

Yo.....con DNI.....
declaro que he leído la Hoja de Información al paciente, de la que se me ha entregado una copia. Se me han explicado las características del estudio, así como los posibles beneficios y riesgos que puedo esperar, los derechos que puedo ejercitar, y las previsiones sobre el tratamiento de datos y muestras. He recibido suficiente información sobre el estudio.

Sé que se mantendrá en secreto mi identidad y que se identificarán mis muestras SANGRE/PIEL/TUMOR con un sistema de codificación. Soy libre de revocar mi consentimiento en cualquier momento y por cualquier motivo, sin tener que dar explicación y sin que repercuta negativamente sobre cualquier tratamiento médico presente o futuro.

Yo doy mi consentimiento para que se utilicen mis muestras y los datos asociados como parte de **este proyecto de investigación.** Consiento en participar voluntariamente.

Por la presente afirmo haber sido advertido sobre la posibilidad de recibir información relativa a mi salud derivada de los análisis genéticos (cuando se realicen) que se realicen sobre mi muestra biológica.

Yo solicito información ☐
Yo no quiero recibir información ☐

una vez finalizada la investigación sobre los resultados del estudio.

Si hubiera excedente de la muestra, afirmo haber sido advertido sobre las opciones de destino al finalizar el proyecto de investigación.

En este sentido:

Solicito la destrucción de la muestra excedente ☐

Solicito la incorporación del excedente en el Biobanco Vasco ☐

Fecha .....

Firma del paciente .....

Fecha .....

Firma representante legal (si procede) .....

Nombre representante legal .....

Relación con el paciente .....

Constato que he explicado las características del proyecto de investigación y las condiciones de conservación que se aplicarán a la muestra y a los datos conservados.

Nombre del Investigador o la persona designada para proporcionar la información:

Fecha ..... Firma .....

CONSENTIMIENTO PARA LA DONACIÓN DE MUESTRAS AL BIOBANCO VASCO PARA LA INVESTIGACIÓN

Responsable clínico: Dr. Adolfo López de Munain

Yo: .....

He sido informado sobre la posibilidad de transferir y almacenar la muestra junto con la información clínica relacionada al Biobanco Vasco para la Investigación.

He sido informado sobre la finalidad de la **conservación,** el lugar de conservación, así como sobre las garantías de cumplimiento de la legalidad vigente y de la posibilidad de ceder las muestras para futuros proyectos de investigación. Se me ha informado que el presente consentimiento será custodiado en las instalaciones del Biobanco en el Hospital Donostia.

Yo **DOY** mi consentimiento para que el centro sanitario ..... transfiera mis muestras y los datos de salud relevantes (excepto los que me identifiquen) de la patología o proceso (distrofia miotónica), al Biobanco Vasco para la Investigación.

Se me ha advertido sobre la posibilidad de consentir en donar la muestra y los datos asociados de forma anonimizada:

DESEO QUE LAS MUESTRAS Y DATOS ESTÉN ANONIMIZADOS** ☐
DESEO QUE LAS MUESTRAS Y DATOS ESTÉN CODIFICADOS* ☐

Se me ha advertido sobre la posibilidad de recibir información relativa a mi salud derivada de futuros análisis genéticos que pudieran realizarse sobre mi muestra biológica (si la muestra se ha donado codificada y se han obtenido datos genéticos).

Yo solicito información ☐
Yo no quiero recibir información ☐

Fecha ..... Firma del paciente .....

Fecha ..... Firma representante legal (si procede) .....

Nombre representante legal .....

Relación con el paciente .....

Constato que he explicado las características de las condiciones de conservación y seguridad que se aplicarán a la muestra y a los datos clínicos conservados.

Nombre del clínico responsable:

Fecha ..... Firma .....

* muestra codificada: la muestra se identifica con un número que sólo su médico o el coordinador del Biobanco Vasco en su hospital podrá relacionarla con usted.

** muestra anonimizada es aquella no asociada con los datos identificativos.

Anexo aclaratorio

Se garantiza que la realización de este proyecto, el tratamiento, almacenamiento y

utilización de las muestras almacenadas en el biobanco cumplirán con la

normativa aplicable:

Ley Orgánica 15/1999, de 13 de diciembre, de Protección de Datos de Carácter Personal. En observancia a esta ley los datos de carácter personal recogidos en este estudio pasarán a formar parte de un fichero automatizado que reúne las medidas de seguridad de nivel alto.

Ley 41/2002, de 14 de noviembre, básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica

Ley 14/2007, de 3 de julio, de Investigación biomédica.

¿Qué es un biobanco?

Un **biobanco** es un centro de conservación, en condiciones adecuadas, de muestras,

tejidos, ADN y otros derivados, que representan un valioso instrumento con destino a la investigación de enfermedades y que puede permitir la obtención de conocimientos que sirvan para el desarrollo de nuevas estrategias y terapias aplicables a pacientes.

El Biobanco de BIOEF está constituido en nodos, uno de los cuales está ubicado en el Hospital ……, en donde se almacenará y conservará su muestra.

Los proyectos de investigación realizados con las muestras almacenadas en el Biobanco

serán aprobados por un Comité de Ética de la Investigación, y, si procede, autorizado

por la autoridad sanitaria pertinente, previo vio informe favorable de los comités ético y científico externos del Biobanco.

Tanto el Biobanco Vasco para la Investigación, como el investigador al que en un futuro

se puedan ceder las muestras, son responsables del manejo de los Datos, conforme a la

Ley orgánica 15/1999, de 13 de diciembre, sobre Protección de Datos de Carácter

Personal. El Hospital …… garantiza que en ningún caso saldrá del

centro dato alguno que le identifique personalmente.

Anexo 3: Hoja de información al paciente

Osakidetza
Donostia Ospitalea
Hospital Donostia

biodonostia

Código Proyecto: ALM-DM1-2015 VERSIÓN 1.0 de 7 de Julio 2015

HOJA DE INFORMACIÓN AL PACIENTE
(EXTRACCIÓN DE MUESTRA DE SANGRE, PIEL Y/O TUMOR)

Investigador/Responsable clínico: Dr. Adolfo López de Munain, (Servicio de Neurología, Hospital Universitario Donostia)

TÍTULO DEL PROYECTO: ***Bases moleculares del riesgo oncológico en la distrofia miotónica***

FINALIDAD DEL ESTUDIO

Como parte de las intervenciones y pruebas que le están realizando, se le va a extraer una muestra biológica de sangre/piel/tumor, parte de la cual será conservada para su análisis en el Instituto Biodonostia (área de Neurociencias/área de Oncología) y Biobanco Vasco de ADN y Tejidos para la Investigación.

Esta muestra es un valioso instrumento con destino a la investigación de la enfermedad que puede permitir la obtención de conocimientos que sirvan para la obtención y desarrollo de nuevas estrategias y terapias aplicables a pacientes.

Solicitamos su consentimiento para que el tejido que le ha sido retirado sea destinado a esta línea de investigación.

BENEFICIO Y ATENCIÓN MÉDICA

La cesión de muestra para la investigación es voluntaria y altruista. Su único beneficio es el que corresponde al avance de la medicina en beneficio de la sociedad, y el saber que ha colaborado en este proceso.

La muestra así recogida no podrá ser objeto directo de actividades con ánimo de lucro. No obstante, la información generada a partir de los estudios realizados sobre su muestra podría ser fuente de beneficios comerciales. En tal caso, están previstos mecanismos para que estos beneficios reviertan en la salud de la población, aunque no de forma individual en el donante.

Su participación en este estudio es completamente voluntaria y no tendrá ningún coste para usted.

RIESGOS Y MOLESTIAS

La única molestia y riesgo relacionado con la toma de muestras de sangre de su brazo que se realizará siguiendo el procedimiento habitual que le puede ocasionar es un pequeño hematoma o una leve infección, que desaparecen en pocos días o con tratamiento adecuado si lo precisase.

El riesgo físico y molestia más frecuente de una biopsia muscular es el dolor. La infección de la herida es muy poco frecuente al realizarse en condiciones estériles. Otra complicación muy poco frecuente es el hematoma en el sitio de la biopsia, apareciendo con mayor frecuencia en pacientes con enfermedades hematológicas o tratados con fármacos anticoagulantes, o el desvanecimiento.

No existen riesgos significativos en la biopsia de piel. Los riesgos poco frecuentes son leves: inflamación o infección de la zona de la muestra, que remiten con el tratamiento adecuado. Por el riesgo de sangrado local deberá comunicarse si existe alteración en la coagulación de la sangre, de forma directa o por la administración de fármacos anticoagulantes.

DESTINO DE LA MUESTRA Y CESIÓN A OTRAS LÍNEAS DE INVESTIGACIÓN

Se solicita su consentimiento para la utilización de las muestras biológicas sobrantes del proceso diagnóstico, con destino a futuras investigaciones de utilidad clínica.

El centro, Hospital Donostia, registrará los datos que puedan identificarle con las muestras a conservar, empleando procedimiento de encriptación y codificación de los datos personales. El centro, Hospital Donostia, garantiza que en ningún caso saldrá del centro dato alguno que le identifique personalmente.

Firmando el consentimiento, Vd. autoriza a que las muestras así conservadas se puedan ceder a terceros con destino a proyectos de investigación relacionados con la adquisición de conocimiento en el área de la DISTROFIA MIOTÓNICA que sean debidamente aprobados por la autoridad sanitaria y dictaminados favorablemente por un Comité de Ética de Investigación. Las muestras se conservarán en tanto sean necesarias para los fines que justificaron su recogida.

La utilización de la muestra biológica para una finalidad distinta a la expresada habrá de ser expresamente autorizada por Vd. en un nuevo documento de consentimiento, previa la información que fuere necesaria. Se le garantiza que, en su caso, el proyecto de investigación será dictaminado favorablemente por un Comité de Ética de Investigación y autorizado por la autoridad sanitaria.

REVOCACIÓN DEL CONSENTIMIENTO

En cualquier momento podrá Vd. revocar el consentimiento para utilizar las muestras obtenidas, pudiendo solicitar la destrucción o la anonimización de las mismas. No obstante, los efectos de la revocación no se extenderán a los datos resultantes de las investigaciones que se hayan llevado a cabo previamente a la misma.

En estos casos, se romperá irreversiblemente todo vínculo que permita relacionar las muestras y los datos almacenados con su persona.

DERECHOS Y GARANTÍAS

Los datos que se obtengan del estudio sólo le serán comunicados, en el caso de que dichos hallazgos tengan una implicación significativa para su salud y de que exista una posibilidad real de mejorar esa condición de salud. Sin embargo, en caso de estar interesado, usted tiene derecho a solicitarlos.

La información que se obtenga puede implicar a sus familiares, siendo únicamente Vd. el responsable de facilitar, en su caso, información a los mismos, o proporcionar a las personas responsables del estudio los datos precisos para su localización y contacto.

Los resultados del estudio podrán ser comunicados en reuniones científicas, congresos médicos o publicaciones científicas. Se le garantiza la absoluta confidencialidad en la información obtenida en este estudio máxime en lo concerniente al envío y manejo de los datos a terceros según la Ley Orgánica 15/1999 de Protección de Datos de Carácter Personal, la Ley 41/2002 básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica, y la Ley 14/2007 de Investigación biomédica, que garantiza el respeto a la calidad de los proyectos de investigación biomédica y el respeto a la dignidad de las personas durante su consecución.

Si usted precisa mayor información sobre el estudio (acceso, rectificación, cancelación y oposición) puede contactar con el investigador responsable del grupo clínico: Dr. LÓPEZ DE MUNAIN del HOSPITAL UNIVERSITARIO DONOSTIA.

Anexo 4: Muscular Impairment Rating Scale

Escala MIRS según Mathieu *et al*., 2001.

0 Sin afectación muscular

1 Signos mínimos: miotonía, debilidad mandibular y temporal, debilidad facial, debilidad de flexores del cuello, ptosis, habla nasal, debilidad aislada de flexores de dedos sin otra debilidad distal

2 Debilidad distal: no debilidad proximal, salvo extensores del codo

3 Debilidad proximal leve a moderada

4 Debilidad proximal grave: igual o menor a 3/5

Anexo 5: Listado de muestras de PBMC

Anexo 5. Tabla 1. Listado de muestras de PBMC de controles sanos usados para estudios de expresión de ARNm.

Código	Género	Edad (años)
Mujer-01	Mujer	40
Mujer-02	Mujer	42
Mujer-03	Mujer	38
Mujer-04	Mujer	44
Mujer-05	Mujer	59
Mujer-06	Mujer	43
Mujer-07	Mujer	51
Mujer-08	Mujer	57
Mujer-09	Mujer	55
Mujer-10	Mujer	44
Hombre-01	Hombre	40
Hombre-02	Hombre	53
Hombre-03	Hombre	42
Hombre-04	Hombre	45
Hombre-05	Hombre	43
Hombre-06	Hombre	42
Hombre-07	Hombre	53
Hombre-08	Hombre	41
Hombre-09	Hombre	46
Hombre-10	Hombre	31
Hombre-11	Hombre	34
Hombre-12	Hombre	54

Anexo 5. Tabla 2. Listado de muestras de PBMC de pacientes con DM1 usados para estudios de expresión de ARNm.

Código	Género	Edad (años)	CTG (n)	MIRS	Edad cáncer
DM1-Mujer-01	Mujer	46	667	3	No
DM1-Mujer-02	Mujer	47	1167	3	No
DM1-Mujer-03	Mujer	35	1167	4	No
DM1-Mujer-04	Mujer	53	1667	-	No
DM1-Mujer-05	Mujer	41	667	3	No
DM1-Mujer-06	Mujer	31	500	2	No
DM1-Mujer-07	Mujer	37	1333	3	No
DM1-Mujer-08	Mujer	18	667	1	No
DM1-Mujer-09	Mujer	36	1000	3	No
DM1-Mujer-10	Mujer	44	1000	4	No
DM1-Mujer-11	Mujer	56	667	4	No
DM1-Mujer-12	Mujer	29	467	2	No
DM1-Mujer-13	Mujer	50	600	3	No
DM1-Mujer-14	Mujer	37	1400	3	No
DM1-Mujer-15	Mujer	45	667	3	No
DM1-Mujer-16	Mujer	32	500	–	No
DM1-Mujer-17	Mujer	44	667	3	No
DM1-Hombre-01	Hombre	27	500	–	No
DM1-Hombre-02	Hombre	31	333	2	No
DM1-Hombre-03	Hombre	26	1000	–	No
DM1-Hombre-04	Hombre	24	467	3	No
DM1-Hombre-05	Hombre	51	1333	4	No
DM1-Hombre-06	Hombre	35	1000	3	No
DM1-Hombre-07	Hombre	50	667	3	No
DM1-Hombre-08	Hombre	39	1333	4	No
DM1-Hombre-09	Hombre	37	1000	2	No
DM1-Hombre-10	Hombre	37	1000	–	No
DM1-Hombre-11	Hombre	36	400	3	No
DM1-Hombre-12	Hombre	35	667	3	No

Anexo 5. Tabla 2 *(Continuación).* Listado de muestras de PBMC de pacientes con DM1 usados para estudios de expresión de ARNm.

Código	Género	Edad (años)	CTG (n)	MIRS	Edad cáncer
DM1-Hombre-13	Hombre	37	1000	2	No
DM1-Hombre-14	Hombre	58	833	3	No
DM1-Hombre-15	Hombre	56	1000	3	No
DM1-Hombre-16	Hombre	49	667	–	No
DM1-Hombre-17	Hombre	53	800	4	No
DM1-Hombre-18	Hombre	42	667	4	No
DM1-Hombre-19	Hombre	41	500	3	No
DM1-Hombre-20	Hombre	37	1000	3	No
DM1-Hombre-21	Hombre	34	667	3	No
DM1-Mujer-Cáncer-01	Mujer	65	1500	4	52
DM1-Mujer-Cáncer-02	Mujer	42	833	3	43
DM1-Mujer-Cáncer-03	Mujer	39	833	–	Desconocido
DM1-Mujer-Cáncer-04	Mujer	61	1167	4	39
DM1-Mujer-Cáncer-05	Mujer	41	1000	4	35
DM1-Mujer-Cáncer-06	Mujer	38	1000	4	37
DM1-Mujer-Cáncer-07	Mujer	41	500	4	38
DM1-Mujer-Cáncer-08	Mujer	65	333	4	65
DM1-Mujer-Cáncer-09	Mujer	51	1500	4	41 y 51
DM1-Mujer-Cáncer-10	Mujer	34	1650	4	17
DM1-Mujer-Cáncer-11	Mujer	48	1333	3	45
DM1-Mujer-Cáncer-12	Mujer	48	1333	2	43 y 47
DM1-Hombre-Cáncer-01	Hombre	48	333	3	31
DM1-Hombre-Cáncer-02	Hombre	52	1000	3	49
DM1-Hombre-Cáncer-03	Hombre	56	333	3	52
DM1-Hombre-Cáncer-04	Hombre	57	1333	3	50
DM1-Hombre-Cáncer-05	Hombre	55	267	–	Desconocido
DM1-Hombre-Cáncer-06	Hombre	54	333	4	51

Anexo 5. Tabla 3. Listado de muestras de PBMC tanto de controles como de pacientes con DM1 usadas en el array de expresión genética.

Código	Género	Edad (años)	CTG (n)	MIRS
Control-Mujer	Mujer	40	–	–
Control-Mujer	Mujer	42	–	–
Control-Mujer	Mujer	38	–	–
Control-Mujer	Mujer	44	–	–
Control-Mujer	Mujer	44	–	–
Control-Hombre	Hombre	42	–	–
Control-Hombre	Hombre	43	–	–
Control-Hombre	Hombre	42	–	–
Control-Hombre	Hombre	41	–	–
Control-Hombre	Hombre	34	–	–
DM1-Mujer	Mujer	50	600	3
DM1-Mujer	Mujer	44	667	3
DM1-Mujer	Mujer	42	833	3
DM1-Mujer	Mujer	44	1000	4
DM1-Mujer	Mujer	37	1333	3
DM1-Hombre	Hombre	50	233	2
DM1-Hombre	Hombre	41	500	3
DM1-Hombre	Hombre	49	667	–
DM1-Hombre	Hombre	42	667	4
DM1-Hombre	Hombre	35	1000	3

Anexo 5. Tabla 4. Listado de muestras de proteínas tanto de controles como de pacientes con DM1 usadas en el presente estudio.

Código	Género	Edad (años)	CTG (n)	
PROT Control-1	Hombre	26	–	–
PROT Control-2	Hombre	45	–	–
PROT Control-3	Mujer	25	–	–
PROT Control-4	Mujer	43	–	–
PROT Control-5	Mujer	48	–	–
PROT Control-6	Mujer	51	–	–
PROT Control-7	Mujer	53	–	–
PROT DM1-1	Mujer	47	667	3
PROT DM1-2	Mujer	35	333	1
PROT DM1-3	Hombre	75	167	3
PROT DM1-4	Hombre	41	1000	2
PROT DM1-5	Hombre	22	833	3

Publicaciones

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frontiers in Aging Neuroscience

REVIEW
published: 09 July 2015
doi: 10.3389/fnagi.2015.00125

Muscle wasting in myotonic dystrophies: a model of premature aging

*Alba Judith Mateos-Aierdi[1,2], Maria Goicoechea[1,2], Ana Aiastui[2,3], Roberto Fernández-Torrón[1,2,4], Mikel Garcia-Puga[5], Ander Matheu[5] and Adolfo López de Munain[1,2,4,6]**

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Myotonic dystrophy type 1 (DM1 or Steinert's disease) and type 2 (DM2) are multisystem disorders of genetic origin. Progressive muscular weakness, atrophy and myotonia are the most prominent neuromuscular features of these diseases, while other clinical manifestations such as cardiomyopathy, insulin resistance and cataracts are also common. From a clinical perspective, most DM symptoms are interpreted as a result of an accelerated aging (cataracts, muscular weakness and atrophy, cognitive decline, metabolic dysfunction, etc.), including an increased risk of developing tumors. From this point of view, DM1 could be described as a progeroid syndrome since a notable age-dependent dysfunction of all systems occurs. The underlying molecular disorder in DM1 consists of the existence of a pathological (CTG) triplet expansion in the 3' untranslated region (UTR) of the *Dystrophia Myotonica Protein Kinase* (*DMPK*) gene, whereas (CCTG)n repeats in the first intron of the *Cellular Nucleic acid Binding Protein/Zinc Finger Protein 9 (CNBP/ZNF9)* gene cause DM2. The expansions are transcribed into (CUG)n and (CCUG)n-containing RNA, respectively, which form secondary structures and sequester RNA-binding proteins, such as the splicing factor muscleblind-like protein (MBNL), forming nuclear aggregates known as foci. Other splicing factors, such as CUGBP, are also disrupted, leading to a spliceopathy of a large number of downstream genes linked to the clinical features of these diseases. Skeletal muscle regeneration relies on muscle progenitor cells, known as satellite cells, which are activated after muscle damage, and which proliferate and differentiate to muscle cells, thus regenerating the damaged tissue. Satellite cell dysfunction seems to be a common feature of both age-dependent muscle degeneration (sarcopenia) and muscle wasting in DM and other muscle degenerative diseases. This review aims to describe the cellular, molecular and macrostructural processes involved in the muscular degeneration seen in DM patients, highlighting the similarities found with muscle aging.

Keywords: myotonic dystrophy, aging, muscle wasting, satellite cells, sarcopenia

OPEN ACCESS

Edited by:
Jaime J. Carvajal, Centro Andaluz de Biología del Desarrollo, Spain

Reviewed by:
John Charles McDermott, York University, Canada
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Received: *27 March 2015*
Accepted: *22 June 2015*
Published: *09 July 2015*

Citation:
Mateos-Aierdi AJ, Goicoechea M, Aiastui A, Fernández-Torrón R, Garcia-Puga M, Matheu A and López de Munain A (2015) Muscle wasting in myotonic dystrophies: a model of premature aging. Front. Aging Neurosci. 7:125. doi: 10.3389/fnagi.2015.00125

Introduction

Myotonic dystrophy type 1 (DM1), also known as Steinert's disease (OMIM: 160900), is a dominantly inherited multisystem disease. DM1 is the most common form of adult-onset muscular dystrophy; it affects one out of 8000 people worldwide, with an even greater prevalence in some specific areas such as Quebec (Canada) and the Basque Country (Spain) (López de Munain et al., 1993; Mathieu and Prévost, 2012). The DM1 phenotype shows an extremely wide variability among affected patients, with some being asymptomatic while others have severe congenital forms. Patients are classified into four categories regarding the age of onset of symptoms: late-onset, adult-onset, childhood-onset and congenital forms (Harper, 2001; Arsenault et al., 2006).

The disease is caused by an unstable expansion of a trinucleotide (CTG) repeat motif located in the 3′ untranslated region (UTR) of the *Dystrophia Myotonica Protein Kinase (DMPK)* gene (Brook et al., 1992; **Figure 1**). Unaffected individuals carry less than 50 triplet repeats, whereas expansions ranging between 50 and 4000 CTG repeats have been found in affected individuals. Importantly, the length of CTG expansion is associated with the age of onset of the disease and its severity (Martorell et al., 1998).

DM1 patients experience a progressive dysfunction of multiple organs and tissues, including skeletal, cardiac and smooth muscles, the endocrine system, eyes, gonads, the central nervous system (CNS), and an increased risk of developing neoplasias (López de Munain et al., 1993; Harper et al., 2002; Gadalla et al., 2013). Thus, DM1 somewhat resembles a progeroid syndrome, defined as an accelerated aging and dysfunction of several systems.

A second form of myotonic dystrophy exists, DM type 2 (OMIM: 602668), initially named proximal myotonic myopathy due to the greater weakness of proximal as compared to distal muscles (Ricker et al., 1994). DM2 patients also develop a multisystem dysfunction but they generally experience a milder phenotype as compared to DM1 (**Table 1**). Consistent with this, congenital and childhood-onset forms of DM2 are absent, and the disease phenotype ranges from early adult-onset severe forms to very late-onset mild forms (Day et al., 2003). The prevalence of this disorder has yet to be clearly defined but it is estimated to be similar to DM1 in Northern European Countries (Udd and Krahe, 2012). However, very late-onset forms of this disease might often go undiagnosed due to its mild phenotype, which can be concealed by other age-related dysfunctions. Unlike what occurs in DM1, aging-like symptoms in DM2 might not be so evident. Regarding the muscle-specific phenotype, it is unknown why distal muscles are predominantly affected in DM1 patients whereas DM2 patients show a more proximal affectation.

TABLE 1 | Summary of main clinical features that differ between both DM forms.

Features	DM1	DM2
Age of onset	At any age	Adulthood
Congenital forms	Yes	No
Gene expansion	DMPK, (CTG)n	CNBP, (CCTG)n
Predominantly affected muscles	Distal	Proximal
Predominantly affected fibers	Type 1	Type 2

As is the case with the genetic origin of DM1, DM2 is caused by a tetranucleotide (CCTG) expansion in intron 1 of the *Nucleic Acid-binding Protein (CNBP or ZNF9)* gene (Liquori et al., 2001; **Figure 1**). Healthy individuals carry less than 30 tetranucleotide repeats, whereas repetition lengths between 55 and 11000 have been found in affected patients (Liquori et al., 2001).

Skeletal muscle, one of the most severely affected tissues in these diseases, may age prematurely in DM patients, mimicking sarcopenia, known as the age-related loss of muscle mass and function (Evans and Campbell, 1993; Fielding et al., 2011). This syndrome can refer to loss of muscle mass alone or in conjunction with fatty substitution (Fielding et al., 2011). Although the causes of sarcopenia have not as yet been accurately determined, several age-related factors, such as muscle disuse, nutritional deficiencies, hormonal changes and insulin resistance could notably contribute to its onset.

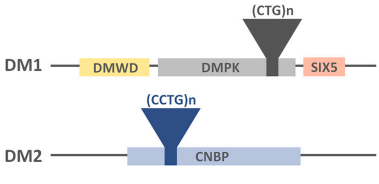

FIGURE 1 | Representation of the DM-causing genes, the location of the tandem repeats and their neighboring genes.

Multisystem Dysfunction in DM

The previously described genomic tandem repeats lead to the progressive degeneration of several tissues and organs, which is more prominent in DM1 and milder in DM2 patients (**Figure 2**).

DM patients may suffer a broad variety of symptoms affecting the three muscle types: cardiac, skeletal and smooth muscles. Cardiac failure is common in DM1 patients, often manifested as arrhythmias and conduction defects (Antonini et al., 2000; Mammarella et al., 2000; Pelargonio et al., 2002; Groh et al., 2008; Cudia et al., 2009; Petri et al., 2012). Although congestive heart failure is a rare complication, subclinical systolic dysfunction as shown by echocardiographic or magnetic resonance imaging is frequent (De Ambroggi et al., 1995; Tokgozoglu et al., 1995; Bhakta et al., 2004; Hermans et al., 2011; Petri et al., 2012). The frequency of heart failure correlates with age, male gender, length of the tandem repeat sequence and the degree of neuromuscular disability (Tokgozoglu et al., 1995; Antonini et al., 2000; Groh et al., 2008; Cudia et al., 2009; Kaminsky and Pruna, 2012). Symptoms involving the smooth muscle, such as dysphagia, constipation, intestinal

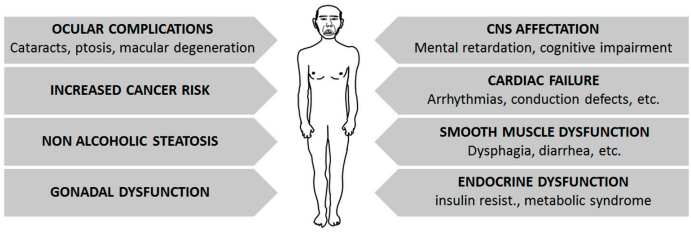

FIGURE 2 | Summary of main symptoms affecting DM patients, which constitute the multisystem affectation found on them.

pseudo-obstruction and diarrhea, are also relatively frequent in DM1 patients (Bujanda et al., 1997; Bellini et al., 2006; Ercolin et al., 2013). As for skeletal muscle involvement, both DM forms share common muscle histopathologic features, with a markedly increased variation of fiber diameter and prominent central nucleation, which is a feature of constantly regenerating muscles with immature fibers. Other signs, such as the presence of basophilic regenerating fibers, branched fibers, and adipose and fibrotic tissue can also be found to varying degrees, depending on the extent of muscle degeneration and the severity of the disease. Although DM1 affects mainly distal muscles and DM2 affects proximal muscles, only a few DM type-specific features have been described. Among these, ring fibers (peripheral myofibers that surround other fibers) and sarcoplasmic masses are more frequently seen in DM1 muscles. Nuclear clumps (condensed chromatin structures in the myonuclei, indicating cell death) are found in DM2 patients even when muscular weakness is not clinically evident, whereas they arise later in DM1 patients, mainly in end-stage muscles (Vihola et al., 2010). Moreover, it must be noted that DM1 muscles show a prominent loss of type 1 fibers, whereas type 2 fibers are predominantly affected in DM2 patients (Vihola et al., 2003; Schoser et al., 2004; Bassez et al., 2008; Pisani et al., 2008). Interestingly, the histology of DM muscles resembles that of aged muscles, with fiber size variability, centrally located nuclei with chromatin clumps and fiber atrophy. Muscle regeneration also seems to be decreased in both conditions, probably due to satellite cell dysfunction, which may fail to activate and/or differentiate to muscle upon myogenic stimuli (Huichalaf et al., 2010; Malatesta, 2012; Malatesta et al., 2014).

As part of the multisystem involvement, many DM1 patients show insulin resistance due to the aberrant splicing of the insulin receptor (IR) mRNA, which is highly expressed in skeletal muscle. Consequently, patients show a reduced responsiveness to insulin as compared to healthy individuals (Morrone et al., 1997; Savkur et al., 2001).

The CNS is also negatively affected in DM1 patients. The large majority of congenital and childhood-onset DM1 patients suffer mental retardation, whereas patients with the adult-onset forms may show varying degrees of cognitive dysfunction, where a positive correlation is observed between triplet expansion length and patients' age. Cognitive dysfunction is characterized by a dysexecutive syndrome with predominant frontoparietal involvement (Sistiaga et al., 2010). Moreover, DM1 patients go through behavioral-personality changes (e.g., reduced initiative, inactivity, apathic temperament and paranoid personality traits) and excessive daytime sleepiness. There is tentative data supporting an age-dependent decline of cognitive functioning in DM1 patients (Modoni et al., 2008), possibly associated with the degeneration of the diffuse (predominantly temporo-insular) subcortical white matter, and a reduction of the cerebral blood flow in frontal areas (Romeo et al., 2010).

DM1 patients also present hepatic involvement. Indeed, 66% of patients show abnormal hepatic enzyme levels and non-alcoholic steatosis (Achiron et al., 1998). Ocular complications, including ptosis, weakness of the ocular muscle and cataracts are also common in DM1 patients, and other less frequent features, such as retinal changes or macular degeneration, may also be present in these patients (Kimizuka et al., 1993; Krishnan and Lochhead, 2010).

Finally, DM patients may also suffer fertility dysfunction. Approximately two thirds of affected males have reduced sperm quality as a result of testicular atrophy (Pan et al., 2002). Affected female fertility is less well documented (Verpoest et al., 2008), but the length of triplet expansion does not seem to be correlated with this aspect of the disease. Importantly, the age of the pregnant patient and parity significantly affect the live birth delivery rate (Verpoest et al., 2010).

In order to decipher why some specific tissues are more severely affected than others in these diseases, it must be highlighted that some tissues and cell types possess a higher tendency to extend these tandem repeat sequences. This leads to the existence of cells with different repeat lengths within an organism, known as *somatic mosaicism*. The longest tandem repeats have been found in severely affected tissues; indeed, skeletal muscle cells possess considerably longer repeat sequences as compared to other cell types (Anvret et al., 1993; Thornton et al., 1994). Importantly, cells with longer tandem repeats tend to accumulate more repetitions than cells with shorter repeat sequences (Monckton et al., 1995), thus aggravating

the degenerative state of predominantly affected tissues in these patients, such as the CNS and cardiac and skeletal tissues.

In addition to somatic cells, germline cells are also prone to genomic instability and thus accumulate tandem repeats in DM. This leads to *anticipation*, which refers to the increase in disease severity and decrease in the age of onset in each generation of affected families (López de Munain et al., 1994).

Repeat Expansion Mechanisms in DM

As previously mentioned, somatic mosaicism is an important feature of myotonic dystrophies; tandem repeats increase with aging in an unsynchronized fashion, leading to cells with different repeat lengths in their genomes. Furthermore, somatic instability is prevalent in highly affected tissues, such as skeletal muscle (Morales et al., 2012).

Slippage of DNA polymerase during DNA replication was at first considered the main mechanism through which repeat sequences are expanded in myotonic dystrophies. Therefore, highly proliferative cells would have a higher tendency to expand DNA repeat sequences, which contradicts the fact that the longest tandem repeats are found in severely affected tissues that happen to be post-mytotic. In this regard, DNA repair mechanisms, which are also active in non-cycling cells, have been found to notably contribute to this phenomenon (van den Broek et al., 2002; Savouret et al., 2003; Kovtun et al., 2004; Seriola et al., 2011; Gomes-Pereira et al., 2014), which could explain the elongation of tandem repeats in non-proliferating cells in culture (Gomes-Pereira et al., 2014).

DNA tandem repeats in DM acquire secondary conformations, usually forming hairpin structures. DNA repair proteins recognize these structures and may abnormally repair them, varying the repeat length (McMurray, 2008). Knock-out animal models for components of the DNA repair machineries, as well as silencing these genes in patient-derived cultures, reduce or even abrogate the elongation of repeat expansion sequences. For example, it has been established that Msh2, a component of the mismatch repair (MMR) machinery, is required for triplet expansion in DM1-derived pluripotent stem cells (iPS) cells, as its silencing blocks the expansion of the triplet repeat sequence (Du et al., 2013). Knock-down and overexpression experiments of proteins involved in Base Excision Repair (BER) systems in yeast also indicate that these proteins might play a role in the expansion of repeat sequences (Refsland and Livingston, 2005; Subramanian et al., 2005). However, this fact has not been fully demonstrated in mice due to the lethality of knock-out mouse models for these proteins (Xanthoudakis et al., 1996; Tebbs et al., 1999).

The fact that skeletal muscle is composed of postmitotic cells suggests that these DNA repair systems may contribute in a major way to the expansion of repeat sequences in these tissues. Interestingly, a study performed by Vahidi Ferdousi et al. (2014) shows that double-strand breaks produced by irradiation are more rapidly repaired in muscle satellite cells than their progeny, thus indicating that satellite cells possess a more efficient DNA repair system, which usually acts through non-homologous end joining. Therefore, it could be hypothesized that triplet expansions are predominantly elongated in satellite cells due to the high activity of the DNA-repair system in these cells. This is consistent with the muscle degeneration seen in DM patients, as dysfunction of satellite cells would disrupt muscle regeneration.

Recent studies have shown that, in addition to DNA repair molecules, the 26S proteasome also participates in the expansion of tandem repeat sequences (Debacker et al., 2012; Concannon and Lahue, 2013). Importantly, the proteolytic activity of 26S, rather than the stress-induced activation of the ubiquitin-proteasome system (UPS), is implicated in this elongation process. Indeed, DNA repair mechanisms could interact with the proteasome to induce repeat expansions through Rad23, a protein involved in both nucleotide excision repair (NER) system and the carriage of ubiquitinated proteins to the proteasome (Concannon and Lahue, 2014).

Pathogenic Mechanisms in DM

It is remarkable that severely affected tissues in DM possess very low mitotic rates. Interestingly, *in vitro* experiments have shown that nuclear foci tend to predominantly accumulate in non-cycling cells. Cycling DM2 fibroblasts accumulate nuclear foci during interphase but these are cleared out during mitosis, leading to mitosis-dependent cycles of foci formation and disassembly. On the contrary, mitotically arrested fibroblasts accumulate more foci, which keep enlarging progressively (Giagnacovo et al., 2012). In this regard, muscle biopsies of DM2 patients have confirmed that the size of myonuclear foci increases in an age-dependent manner (Giagnacovo et al., 2012). Therefore, this could help clarify why tissues and cell types with low mitotic rates are preferentially affected in these diseases.

Several mouse models have been created to study the pathogenic mechanisms of DMs. These models can be categorized into 4 subgroups regarding the genetic modifications that have been introduced in order to mimic different molecular aspects of the diseases: (i) introduction of unstable CTG repeat sequences; (ii) overexpression of toxic CTG/CCTG repeats; (iii) inactivation of the genes located in the DM1 locus; and (iv) mice models of MBNL inactivation or CUGBP1/CELF overexpression (Gomes-Pereira et al., 2011).

Based on these studies, three pathogenic mechanisms that link the nucleotide expansion with the clinical manifestation of myotonic dystrophies have been proposed: (a) DMPK and CNBP haploinsufficiency; (b) *Cis* alteration of neighboring genes; and (c) RNA-induced toxicity (**Figure 3**).

Haploinsufficiency

Haploinsufficiency refers to the deleterious effect of having only one wild type copy of a gene in the phenotype of the organism. DM patients carry only one wild type copy of the *MDPK* or *CNBP* gene, as the other copy harbors the tandem repeats that form nuclear foci, therefore reducing the amount of DMPK or CNBP protein synthesized.

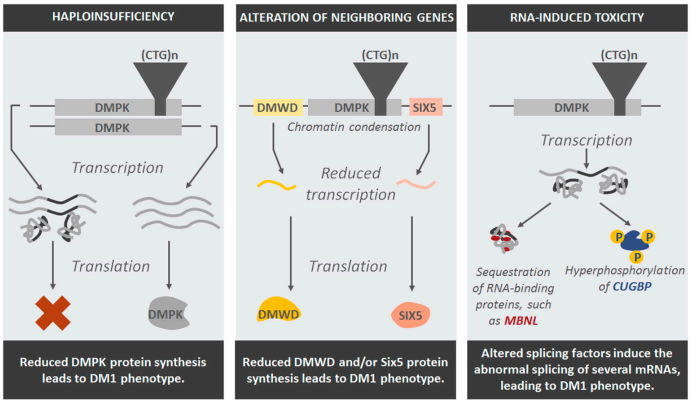

FIGURE 3 | Representation of potential pathogenic mechanisms that explain the effect of DNA expansions in DM1-affected cells and the phenotype seen in patients.

DMPKs is a serine-threonine kinase, whose function in skeletal muscle is not fully understood. It has been shown to localize in the nuclear membrane, where it interacts with lamin-A/C and its deficiency leads to nuclear envelope instability (Harmon et al., 2011). However, it has also been found in the cytoplasm and the cellular membrane during cell division and cardiomyocyte differentiation (Harmon et al., 2008). Depletion of DMPK reduces myogenin expression and prevents proper myoblast differentiation (Harmon et al., 2008). Other studies have also shown that DMPK can phosphorylate the myosin-binding subunit of myosin phosphatase (MYPT1), which inhibits myosin phosphatase (PP1c) activity (Murányi et al., 2001). DMPK also phosphorylates the SERCA2a inhibitor Phospholamban, thus regulating calcium uptake in cardiomyocytes (Kaliman et al., 2005), and Phospholemman, a regulator of Na, K-ATPase (Mounsey et al., 2000).

As for DM2, CNBP is a nucleic-acid binding protein that mainly binds single stranded DNA and RNA, and modulates the transcription of genes involved in Wnt signaling pathway (Margarit et al., 2014).

The proposed pathogenic mechanism hypothesizes that reduced DMPK and CNBP/Znf9 levels in DM1 and DM2, respectively, cause the onset of the diseases. Consistent with this, decreased amounts of DMPK transcripts and protein have been detected in DM1 patients, and this decrease inversely correlated with the CTG repeat length (Fu et al., 1993).

In this regard, DMPK knock-out mice fail to reproduce the multisystem phenotype of DM1 patients, and only develop late-onset myopathy (Jansen et al., 1996; Reddy et al., 1996; Berul et al., 1999), suggesting that haploinsufficiency of DMPK is not the primary mechanism that initiates this disease.

On the contrary, $Znf9^{+/-}$ mice (lacking one CNBP allele) develop a multisystem phenotype resembling DM, including muscle wasting, heart failure and cataracts (Chen et al., 2007), which suggests that CNBP insufficiency may have a role in the pathologic mechanisms of DM2 (Raheem et al., 2010). However, other studies have yielded contradictory results, indicating that aberrant CCTG expansions do not alter CNBP protein levels, and thus, CNBP deficiency would not contribute to the onset of the disease (Margolis et al., 2006).

Cis-Alteration of Neighboring Genes

Tandem repeats produce changes in chromatin structure. Indeed, nucleosomes tend to localize in these repeat sequences, inducing chromatin condensation (Wang et al., 1994; Volle and Delaney, 2012), which could have a notable impact on the transcriptional activity of DMPK and ZNF9 flanking genes (Klesert et al., 1997; Thornton et al., 1997; Westerlaken et al., 2003). Similar to the haploinsufficiency of the repeat-containing genes in myotonic dystrophies, the altered expression of genes located in the neighborhood of the disease-causing genes could also contribute to the molecular mechanisms leading to these diseases.

In line with this, mRNA level of SIX5 (also known as DMAHP), a transcription factor coding gene located downstream of DMPK, is reduced in DM1 patients (Klesert et al., 1997; Thornton et al., 1997; Westerlaken et al., 2003). Expression of DMWD, an upstream DMPK flanking gene, also seems to be somewhat reduced in the cytoplasm of DM cells, although nuclear levels remain unchanged (Alwazzan et al., 1999; Frisch et al., 2001).

In order to ascertain the contribution of reduced levels of SIX5 to the development of DM1, a knock-out mouse model of SIX5 was created. Besides showing an increased susceptibility to developing cataracts (Klesert et al., 2000; Sarkar et al., 2000),

reduced fertility in males (Sarkar et al., 2004) and altered cardiac function (Wakimoto et al., 2002), these mice do not show multisystem involvement and thus fail to recapitulate the dystrophic phenotype of DM1 patients.

RNA-Induced Toxicity

The third pathogenic mechanism proposes that repeat expansions, once translated into RNA, exert a gain-of function toxic effect in the cells. The expanded CUG-containing transcripts form secondary structures of a hairpin shape (Michalowski et al., 1999) and sequester specific RNA-binding proteins that participate in pre-mRNA transcription and maturation, such as MBNL (Muscleblind-like) (Miller et al., 2000), thus leading to splicing defects in both DM1 and DM2 patients (Du et al., 2010; Malatesta and Meola, 2010). Double-strand RNA structures also abnormally activate the RNA-dependent protein kinase R (PKR), which in turn hyperphosphorylates CUG-BP/CELF1 protein and alters its function (Tian et al., 2000). This protein is involved in the splicing of several genes directly implicated in the multisystem phenotype of DM patients, such as cardiac Troponin (cTNT), IR and chloride channel 1 (CLCN1; Osborne et al., 2009). Overactivation of PKR also inactivates its substrate eIF2α, inhibiting the translation of specific mRNAs, such as the DNA repair factor MRG15 (Huichalaf et al., 2010).

In order to assess the contribution of foci formation to the multisystem involvement seen in DM patients, several mouse models have been created carrying variable repeat length sequences and tissue specificity. In all these models the toxicity of foci has been tested, and such models have helped to elucidate how these aggregates play a key role in the onset of the disease. For example, HSA mice, which harbor 5 (short repeat length, HSA^{SR}) or 250 (long repeat length, HSA^{LR}) CTG repeats in the Human Skeletal Actin gene (HSA), thus inducing a muscle-specific expression of tandem repeat-containing RNAs, have clearly established the role of RNA-protein aggregates in the development of the disease, as mice harboring long repeats developed a DM-like phenotype of muscle degeneration (Mankodi et al., 2000). RNA toxicity has been confirmed with transgenic mice harboring long triplet repeats in the DMPK gene. Such mice develop multisystem abnormalities mimicking the human DM phenotype, with predominant involvement of muscles and the CNS, although the resulting phenotype is milder than in other mouse models of the disease (Seznec et al., 2001).

Therefore, all three mechanisms may participate to some extent in the onset of a range of symptoms in DM patients, although RNA toxicity seems to contribute most notably to the multisystem degeneration seen in DM patients.

Age-Related Genomic Events in DM

Cellular aging is accompanied by accumulating DNA damage (Moskalev et al., 2013), which includes point mutations, translocations, and double strand breaks, among others. These mutations can seriously affect the functionality of several cell types, such as the regenerative capacity of stem cells. Cells possess several mechanisms to cope with these abnormalities, either by activating DNA repair mechanisms or inducing cell death. Unfortunately, DNA damage increases with aging partially due to decreased efficiency of DNA repair systems (Gorbunova et al., 2007).

As previously mentioned, these DNA repair mechanisms participate in the expansion of tandem repeats, which cause not only DM but also other degenerative diseases such as Huntington's Disease and Friedreich's ataxia. Besides DNA repair mechanisms, telomere maintenance could also be implicated in muscle aging and DM.

Chromosomal ends, also known as telomeres, are composed of highly repetitive sequences that play an important role in the maintenance of chromosomal structure. Importantly, these DNA ends cannot be replicated by common DNA polymerases and thus, they continue to shorten in every cell division. Telomere shortening has been observed in most cell types during the aging of both human and mice tissues (Blasco, 2007) and has become a cellular marker of aging. However, some cell types, such as most adult stem cells, express telomerase, a specific enzyme able to replicate telomeric sequences, counteracting their shortening (Vaziri et al., 1994; Chiu et al., 1996; Morrison et al., 1996; Espejel et al., 2004; Ferrón et al., 2004; Flores et al., 2005).

Absence of telomere shortening in satellite cells of aged mice, together with the insignificant reduction of telomerase activity in aged muscle stem cells, indicates that satellite cells possess mechanisms to maintain telomeres, and thus, the age-related reduction of their regenerative potential is telomere independent. On the contrary, myogenic differentiation of these satellite cells abolishes their telomerase activity (O'Connor et al., 2009).

In vitro studies with human congenital DM1 samples show that despite DM1-affected satellite cells having a higher telomere shortening rate, these cells enter senescence prior to reaching critically short telomere lengths (Bigot et al., 2009; Thornell et al., 2009). In these cases, p16-dependent signaling seems to induce the senescence of satellite cells. Thus, telomere shortening, even though it is altered in DM1 patients, does not seem to play a role in the pathology of this disease. On the contrary, telomere shortening could influence the regenerative capacity of DM2-affected satellite cells, as will be discussed below Renna et al. (2014).

Epigenetic Modifications in DMs and Aging

Epigenetic modifications encompass post-translational modifications of DNA and histones that lead to chromatin remodeling processes. Different trends of these modifications have been associated with aging, such as increased histone H4K16 acetylation, H4K20 trimethylation and H3K4 trimethylation, and decreased H3K9 methylation or H3K27 trimethylation (Fraga and Esteller, 2007; Han and Brunet, 2012; López-Otín et al., 2013). Importantly, the family of histone deacetylases known as sirtuins have been shown to possess outstanding antiaging potential (Kaeberlein, 2008; Houtkooper et al., 2012; Imai and Guarente, 2014).

Epigenetic modifications also seem to correlate with the age-dependent loss of the regenerative potential of stem cells (Liu and Rando, 2011). This also applies to muscle satellite cells, in which H3K27 trimethylation seems to increase overall in their genome, along with reduced histone biosynthesis (Liu et al., 2013).

Although epigenetic modifications in DM-affected satellite cells have not yet been studied specifically, triplet expansions seem to induce different DNA methylation patterns. In this regard, the DMPK locus shows a variably methylated sequence upstream of the triplet expansion, whereas this sequence is unmethylated in healthy controls (Ghorbani et al., 2013). Interestingly, the expanded sequence and the downstream sequence are not methylated (López Castel et al., 2011). Methylation is more frequently found in tissues of congenital and young-onset patients, although it is not limited to cells with the largest expansions or to specific tissues (López Castel et al., 2011). In an attempt to elucidate the consequences of these methylations, disruption of CTCF binding to its DNA binding sites, which flank the DM1 locus, has been postulated to explain the reduced transcription of DMPK and Six5 genes (Filippova et al., 2001; He and Todd, 2011). However, CTCF binding to DNA seems to be unaffected in a mouse model of DM1, despite the methylation and heterochromatinization found in the DM1 locus (Brouwer et al., 2013). These authors alternatively propose proliferating cell nuclear antigen (PCNA), which also binds DNA repeat sequences, as a player in the triplet-expansion mechanism.

The epigenetic modifications resulting from triplet expansions require more precise study in order to determine their contribution to the development of the disorder. The study of these modifications in muscle satellite cells would contribute to unravel the role of epigenetic modifications in the regenerative capacity of DM-affected satellite cells.

Is Muscle Wasting in Myotonic Dystrophy a Matter of Premature Aging?

Satellite Cells and Muscle Regeneration in DM and Aging

Satellite cells play a key role in the maintenance of muscle structure and function both in homeostasis and after acute damage. The age-related dysfunction of satellite cells has been postulated to be a key cause of sarcopenia, apart from also being implicated in other muscle wasting conditions such as muscular dystrophies (Day et al., 2010).

Satellite cells maintain skeletal muscle structure and functionality by differentiating into myogenic cells that regenerate muscle tissue (Lepper et al., 2011; Murphy et al., 2011; Sambasivan et al., 2011). In adulthood, these cells are quiescent and locate in the periphery of muscle fibers, between the basal membrane and the sarcolemma (Mauro, 1961). When muscle regeneration is required, these cells activate the cell cycle and proliferate. Most of these activated satellite cells enter the myogenic program and differentiate into myoblasts that fuse to each other or to preexisting myotubes, regenerating the damaged tissue. However, a small subpopulation of these cells repopulates the satellite cell niche (Chargé and Rudnicki, 2004; Yin et al., 2013).

Age-dependent regenerative dysfunction of skeletal muscle mainly depends on the decreased amount and functionality of satellite cells (Shefer et al., 2006; Brack and Rando, 2007; Collins et al., 2007). However, it has been widely debated if this reduced regenerative potential is due to extrinsic factors, such as age-dependent alterations of the satellite cell niche and circulating factors (Brack and Rando, 2007; Gopinath and Rando, 2008; Urciuolo et al., 2013), or due to intrinsic defects of these stem cells (Bernet et al., 2014; Sousa-Victor et al., 2014).

With regard to extrinsic factors, satellite cells are responsive to a wide variety of circulating *biomolecules*, whose concentration is constantly being modified in an age-dependent manner. Experiments involving surgical sharing of the circulating system (also known as heterochronic parabiosis) between aged and young mice have revealed that aged serum harbors factors that reduce the regenerative capacity of young satellite cells, whereas young serum is able to partially revert the myogenic-to-fibrogenic transition of aged muscle stem cells, thus reducing the muscle fibrosis seen in old muscles (Conboy et al., 2005; Brack and Rando, 2007). *In vitro* experiments performed with human satellite cells have confirmed this fact. These findings show that age-dependent systemic factors act on the regenerative potential of satellite cells (Carlson and Conboy, 2007; Carlson et al., 2009).

The satellite cell *niche*, composed of cells and extracellular matrix located in the close vicinity of satellite cells, also affects satellite cell functionality, probably through secreted factors as well as cell-to-cell interactions (Murphy et al., 2011; Naito et al., 2012). Moreover, aging alters the pattern of secreted molecules and cellular interactions, thus affecting satellite cell biology. For example, age-dependent increases in basic fibroblast growth factor (bFGF) activate satellite cells in homeostasis, inducing their differentiation, and consequently, the satellite cell pool is depleted (Chakkalakal et al., 2012). On the other hand, matrix molecules, such as extracellular fibronectin, participate in the activation of Wnt signaling in satellite cells, promoting their symmetric division (Bentzinger et al., 2013).

Apart from environmental factors, satellite cell-intrinsic factors also play a central role in the maintenance of the regenerative capacity of these cells. Recently, Sousa-Victor et al. (2014) have demonstrated that the pronounced decline in the regenerative potential of satellite cells in very old (geriatric) mice is caused by the induction of $p16^{Ink4a}$, which drives cells from quiescence to irreversible senescence. p38 signaling also occupies a central role in the functionality of satellite cells. In this regard, activation of p38 signaling in dividing aged satellite cells induces the differentiation of both daughter cells, whereas in young animals, asymmetric localization of p38 enables satellite cells to divide asymmetrically, thus favoring the maintenance of the quiescent satellite cell pool (Bernet et al., 2014; Cosgrove et al., 2014).

Therefore, both satellite cell-extrinsic and -intrinsic factors affect the regenerative potential of muscle stem cells during aging and these factors may also play a role in DM-affected satellite cells. Indeed, it has been shown that cell-intrinsic events, such as nuclear foci accumulation, affect DM cell functionality (Malatesta, 2012), whereas the multisystem dysfunction seen in

these diseases may probably alter circulating factors, impairing the regenerative potential of these cells.

Several studies have aimed to study the involvement of satellite cells in DM. In this regard, histopathologic analysis of severely affected distal muscles vs. slightly affected proximal muscles of DM1 patients has shown a two-fold increase in the number of satellite cells in severely affected muscles. Interestingly, telomere length is not altered and the number of regenerative fibers is low in both distal and proximal muscles (Thornell et al., 2009). In this regard, *in vitro* proliferative capacity of DM1 satellite cells is considerably reduced, and their entry into senescence is telomere-independent (Thornell et al., 2009). Indeed, this has been corroborated with *in vitro* cultured satellite cells from congenital DM fetuses and newborns. These satellite cells show a considerably lower proliferative rate than age-matched controls, besides having activated senescence-associated beta galactosidase, high levels of cyclin D1 and hypophosphorylated Rb. Interestingly, these cells also enter senescence prior to reaching critically short telomere lengths and express $p16^{Ink4a}$, a Cdk4 inhibitor that induces cell cycle arrest (Bigot et al., 2009). It remains to be clarified which cellular events induce $p16^{Ink4a}$ expression, although disturbed DNA functioning due to the formation of unusual structures, as well as increased free radicals and oxidative stress might be involved in the activation of this gene.

Besides the involvement of the proliferative capacity of DM satellite cells, the myogenic program is also abnormal in these cells. Indeed, there is evidence of the defective differentiation and maturation of DM1 myogenic progenitors *in vitro*, resulting in smaller and thinner myotubes, with a 30% lower fusion index and the lack of expression of mature myosin forms (Furling et al., 2001). This lack of fiber maturation has also been confirmed in DM1 muscle biopsies, where late myogenic differentiation markers are not fully expressed (Vattemi et al., 2005). Moreover, DM1 myoblasts seem to have impaired cell cycle withdrawal, probably due to the inability to induce the expression of p21 (Timchenko et al., 2001).

Satellite cells in DM2 show an intermediate phenotype between DM1 and healthy satellite cells. Renna et al. (2014) suggest that DM2 satellite cells also show premature entry into senescence, although later than DM1 satellite cells. Importantly, senescence seems to correlate with telomere shortening rather than the induction of $p16^{Ink4a}$ (Renna et al., 2014). In contrast to DM1 progenitors, DM2 progenitors did not show any differentiation defects and CGUBP1 levels were also unchanged. However, DM2 cells had abnormal IR splicing (Pelletier et al., 2009). In this regard, the interaction of CUGBP1 with eIF2α and Cyclin D3-CDK4/6 seems to be crucial to achieve a correct myogenic differentiation. Reduction of these interactions in DM1 cells could explain the impaired differentiation of these cells (Salisbury et al., 2008).

This reduced differentiation and premature senescence of satellite cells resembles physiological satellite cell aging. Moreover, age-related cell-senescence features, such as cytoplasmic vacuolization, accumulation of heterochromatin and impaired pre-mRNA maturation (Malatesta and Meola, 2010) have also been found in DM cells (Malatesta et al., 2011a,b).

As previously mentioned, secreted factors also have an impact on muscle regeneration during aging, and could also play a role in DM. In this regard, congenital DM1 muscle progenitor cells with long triplet expansions seem to secrete prostaglandin, which in turn hampers myogenic differentiation, probably by lowering intracellular calcium levels (Beaulieu et al., 2012).

Loss of Proteostasis in Aging and DM

Proteostasis encompasses cellular mechanisms that preserve the stability and functionality of its proteome in order to prevent the accumulation of damaged proteins and ensure continuous renewal of intracellular proteins. Many studies have demonstrated that protein homeostasis collapses during aging, leading to the accumulation of unfolded, aggregated and misfolded proteins, a phenomenon that causes several age-related diseases (Powers et al., 2009). Cells have various mechanisms to tackle these protein failures. The three principal proteostatic systems are the UPS, the autophagy-lysosomal system and chaperones, the efficiency of all of which decreases with aging (Calderwood et al., 2009; Tomaru et al., 2012). Consequently, old cells carry more non-enzymatic posttranslational protein modifications and accumulate more cross-linked and aggregated proteins than young cells (Soskić et al., 2008).

The Ubiquitin-Proteasome System

The UPS actively participates in the regulation of protein synthesis and degradation. The age-dependent decay of UPS efficiency may be the result of the reduced expression of proteasome subunits, their inadequate assembly, and/or reduced ATP availability due to mitochondrial dysfunction (Chondrogianni et al., 2014).

The activity of UPS is reduced in several aged mammals, such as humans, mice, rats and sheep. However, contrary to what could be expected, some aged tissues such as muscle, show increased expression of UPS subunits (Ferrington et al., 2005), which could be a compensatory effect for reduced constitutive proteasomal activity (Husom et al., 2004). Maintenance of proteostasis in stem cells may also play an important role in organismal aging (Vilchez et al., 2014). Indeed, proteasome activation is a conserved mechanism that regulates aging and longevity (Chondrogianni et al., 2014).

The breakdown of proteostasis has been linked to several disorders, including myotonic dystrophy. UPS is increased in skeletal and cardiac muscle of transgenic DM mice with 550 CTG repeats, triggered by the up-regulation of Fbx032/Atrogin-1 and/or Trim63/Murf1. These mice develop progressive muscle weakness between 3 and 10 months of age (Vignaud et al., 2010). Overactivation of UPS has also been confirmed in the DMSXL mouse model, which exhibits more than 1.000 CTG repeats. UPS activity was considerably increased at 4 months of age in these mice, suggesting that this proteolytic pathway could play a role in the physiopathological remodeling of muscle (Huguet et al., 2012).

Proteomic analyses have also confirmed that protein degradation is altered in DM2 myotubes (Rusconi et al., 2010). Indeed, an overall reduction in ubiquitinated proteins as well as reduced proteasome subunits have been found in DM2 myotubes (Rusconi et al., 2010). DM2 myoblasts degrade faster a variety of short-lived proteins, such as c-myc and p21, due to increased UPS activity that results from RNA CCUG repeats that bind the 20S core complex (Salisbury et al., 2009). Skeletal muscles of DM2 patients also show a deregulated *neural precursor cell expressed developmentally down-regulated protein 4* (NEDD4) ubiquitin ligase-PTEN pathway, which could contribute to the increased risk of statin-adverse reactions in patients with DM2, due to PTEN accumulation in highly atrophic muscle fibers (Screen et al., 2014).

It is worthy of note that besides UPS overactivation in DM, the 26S proteasome itself is implicated in trinucleotide expansion, thus favoring the expansion of these pathogenic repeat sequences (Concannon and Lahue, 2014).

Autophagy

Autophagy mediates the degradation of cellular components in order to recycle them or to obtain energy. Indeed, autophagy is activated during starvation and induces the degradation of cellular components, providing the cell with energy and thus promoting cell survival during a period of low nutrient availability. In contrast to autophagy, mTOR signaling is activated by high nutrient availability, such as insulin and amino acids, and activates cell division and protein synthesis. Importantly, age-dependent decline of autophagy disrupts cellular proteostasis.

In skeletal muscle, autophagy seems to participate in the activation of quiescent satellite cells, probably providing the additional energy required for this process (Tang et al., 2014). Thus, age-related dysfunction of autophagy could undermine satellite cell activation with aging.

In vitro myoblast cultures of DM1 patients show that nearly half of the myoblasts undergo abnormal differentiation. Strikingly, cells that fail to differentiate show autophagic features after 6 days in culture, with increased cellular volume and a high density of autophagic vacuoles as compared to control and DM1 differentiated cells (Beffy et al., 2010). Senescence was ruled out as an activator of autophagy in this study, as the percentage of cells expressing senescence-associated beta galactosidase was similar in DM1 cells and controls.

Autophagy may also be abnormal in DM1-affected neurons. In this regard, autophagy has been found to be activated and mTOR signaling partially inhibited in DM1-hESC-derived neurons as compared to wild type-hESC-derived neurons. Phosphorylation of the mTOR downstream components GSK3α/β and rpS6 were decreased in these cells. Importantly, reduction of mTOR signaling was p53-independent, therefore suggesting that inhibition of mTOR is not induced by cellular stress (Denis et al., 2013). In line with this, GSK3β has been found to be overactivated in muscles of the DM1 mouse model HSA^{LR} prior to the onset of muscle wasting, and GSK3β blockers improved skeletal muscle strength and reduced myotonia in this mouse model, suggesting that these inhibitors could have a beneficial effect on the treatment of DM1 by alleviating muscle wasting (Jones et al., 2012).

It has been speculated that autophagy could be a mechanism either to avoid apoptosis or to protect cells against metabolic stress. However, transfection of C2C12 myoblasts with the human DMPK-A isoform not only shows increased autophagy, but also enhances apoptosis (Oude Ophuis et al., 2009). Therefore, autophagy in DM must be thoroughly studied in order to determine the causes and the effects of its overactivation in these patients.

Chaperones

Chaperones exert a key function in proteostasis by folding peptides, refolding incorrectly folded proteins and unfolding damaged proteins to facilitate their degradation. Heat Shock Proteins (HSPs) constitute a subgroup of chaperones that are specifically induced by different cell stressors, such as protein damage. As expected, chaperones play a central role in protecting cells from protein damage and cell death during aging (Calderwood et al., 2009). Moreover, experiments performed in *S. cereviasiae* have shown that overexpression of specific HSPs, such as HSP104, increases protein disaggregation, reduces protein accumulation and restores UPS in aged cells (Andersson et al., 2013).

The muscle-specific HSPs HSPB3 and HSPB2, the latter also known as Myotonic dystrophy Protein Kinase Binding Protein (MKBP), seem to occupy a central role in muscle regeneration. HSBP2 specifically binds and activates MDPK, which contributes to muscle maintenance (Suzuki et al., 1998; Prabhu et al., 2012). HSBP2 and HSBP3 are induced by MyoD during myogenic differentiation, which strongly suggests that they play central roles in muscle regeneration, probably through the interaction of MKBP with DMPK (Sugiyama et al., 2000). Other HSPs, such as HSP70, may also play muscle-specific roles, as it is induced in type 1 fibers (Locke et al., 1991).

Expression of HSPB2/MKBP is specifically up-regulated in the skeletal muscle of DM1 patients, probably in order to partially compensate for the reduced amount of DMPK (Sugiyama et al., 2000). Therefore, an increase in chaperone activity would potentially benefit the maintenance of skeletal muscle functionality in both DM-affected and aged muscles.

Mitochondrial Dysfunction

The Mitochondrial Free Radical Theory of Aging (MFRTA), which proposes that mitochondrial free radicals cause oxidative damage that gives rise to cellular aging, has been postulated as one of the main hypotheses to explain how cells age (Sanz and Stefanatos, 2008). Mitochondria play an important role in mediating and amplifying the oxidative stress that drives the aging process (Bratic and Larsson, 2013).

The largest isoform of myotonic dystrophy protein kinases, DMPK-A, supplies antioxidants and antiapoptotic signals needed for correct muscle fiber function and differentiation (van Herpen et al., 2005; Pantic et al., 2013). However, anchorage and accumulation of DMPK-A in the mitochondrial outer membrane can lead to mitochondrial fragmentation and the formation of

perinuclear clusters of morphologically altered mitochondria, finally inducing the activation of autophagy (Oude Ophuis et al., 2009).

DM-patients' muscles show a reduced expression of DMPK, mitochondrial accumulation in degenerated myofibrils and disorganization of the sarcoplasmic reticulum (Ueda et al., 1999). On the other hand, these muscles show reduced Coenzyme Q10 (CoQ10) levels, a component of the electron transport chain that participates in aerobic cellular respiration, generating energy as ATP (Siciliano et al., 2001). Blood samples have confirmed an inverse correlation between CoQ10 levels and CTG expansion length in DM patients (Tedeschi et al., 2000).

As for DM2, proteomic analyses of myotubes have detected abnormalities in two proteins involved in mitochondrial fatty acid degradation and another two proteins involved in the import, chaperonin and quality control functions of mitochondria (Rusconi et al., 2010). The elongation factors Tu and Ts, two posttranslational proteins that participate in the mitochondrial translational machinery, were also reduced in DM2 cultures. Mutations and/or reductions in these proteins are associated with muscle hypotonia and decline of motor skills (Valente et al., 2007). Therefore, mitochondrial dysfunction seems to be common to both muscular aging and DM.

Deficiencies in Nutrient Sensing

DM patients show several metabolic defects that are also common in aged individuals, such as glucose resistance, hyperinsulinemia and in some cases, the development of diabetes mellitus. Studies performed with large DM1 samples have revealed that there are metabolic dysfunctions associated with this disease, such as primary hyperparathyroidism, calcium metabolism, thyroid insufficiency, hypogonadism, hyperprolactinemia or diabetes. Some of these dysfunctions seem to correlate with the length of the repeat expansion (Ørngreen et al., 2012).

Hyperphosphorylation of CUGBP, which is a feature of DM, leads to abnormal splicing of the IR mRNA, lacking exon 11 (Osborne et al., 2009). The IR is mainly expressed in skeletal muscle and its binding to the ligands insulin and IGF-I activates metabolic pathways implicated in muscle hypertrophy, whereas binding to IGF-II induces mitosis. This binding affinity is isoform-dependent; the form that lacks exon 11 (immature form), is mainly expressed in embryonic tissues and shows high affinity towards IGF-II, compared to insulin and IGF-I. Due to the abnormal splicing, DM skeletal muscles are characterized by a predominant expression of the immature isoform, which leads to insulin insensitivity (Savkur et al., 2001, 2004). This splicing defect seems to be independent of muscle fiber type, as both fiber types show a reduced expression of the adult IR isoform (Santoro et al., 2013), despite DM muscle wasting being fiber-type dependent (Vihola et al., 2003; Pisani et al., 2008).

These splicing abnormalities have been observed in muscle tissue and myotube cultures of both DM1 and DM2 patients prior to the development of muscle histopathology, which indicates that DM1 and DM2 share common pathogenic mechanisms and that these splicing abnormalities appear before myofiber degeneration (Santoro et al., 2013).

Insulin secretion has also been found to be abnormal in DM patients, probably due to loss of calcium homeostasis that regulates insulin secretion by pancreatic beta-cells (Savkur et al., 2001).

Thus, deficiencies in nutrient sensing are shared by both physiological aging and DM patients, which leads to the use of common therapeutic approaches to treat both conditions.

Concluding Remarks

Myotonic dystrophies represent a new paradigm of how a genetically determined disease initiates a cascade of events that lead to a wide variety of symptoms (myotonia, cataracts, heart dysfunction, baldness, etc.) that resemble the multisystem involvement induced by aging.

Different pathogenic mechanisms exist to explain how these tandem expansions in the genome of affected patients lead to the DM phenotype, although it has not yet been clearly defined to what degree each mechanism contributes to the development of the disease.

An important feature of myotonic dystrophies and aging resides in their progressive nature. The described molecular events, such as genomic instability, alteration of autophagy or mitochondrial dysfunction, among others, induce cell damage that continues to accumulate throughout life (**Figure 4**).

Satellite cells have been hypothesized to be the main contributors to muscle regeneration. Numerous studies have established that both cell-intrinsic and environmental factors induce the age-related decline of their regenerative capacity in muscle aging and sarcopenia. However, it is still unclear how these cells behave in different muscle dystrophies and if their

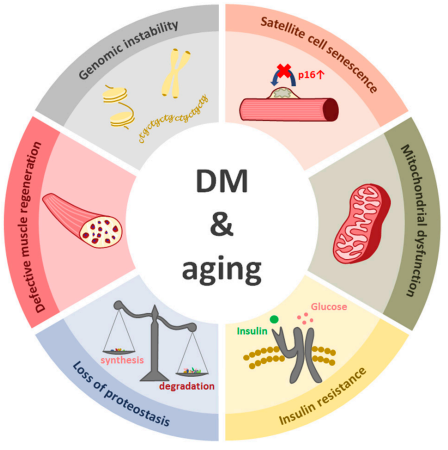

FIGURE 4 | The figure represents six cellular events that happen in both aging and myotonic dystrophy.

loss of regenerative potential is crucial in muscle wasting seen in affected patients.

The molecular pathologic mechanisms of DM, as well as the aging-related events reviewed above, strongly suggest that satellite cell dysfunction could be a major contributor to the development of muscle wasting in these patients and thus, that these cells could become potential targets for the treatment of both age-related and DM-induced muscle dysfunction. It is worth highlighting that skeletal muscle fibers also present proteostatic and mitochondrial abnormalities that resemble general aging processes. Thus, DM patients and animal models can be considered *bona fide* models of aging, and this should be kept in mind when designing treatments to treat both myotonic dystrophy patients and aging-derived disorders.

Author Contributions

All authors have contributed to the design, data collection and writing of the manuscript. Authors have critically revised the work and they have approved the submitted version of the review.

Acknowledgments

This work was supported by grants from the Spanish Ministry of Health (FIS PS 09–00660 and PI147436), Ilundain Foundation, and Isabel Gemio Foundation. MAJ is supported by the Basque Government's program of predoctoral fellowships.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Cancer risk in DM1 is sex-related and linked to miRNA-200/141 downregulation

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ABSTRACT

Objective: Describe the incidence of cancer in a large cohort of patients with myotonic dystrophy type 1 (DM1) and to unravel the underlying molecular mechanisms.

Methods: Standardized incidence ratios (SIRs) were calculated in the Gipuzkoa DM1 cohort (1985–2013), dividing observed numbers by expected numbers for all cancers combined and stratified by sex. An estimation of the expected incidence was achieved by multiplying the age- and sex-specific incidence rates from the Basque population cancer registry by the person-years observed in the study cohort. Large-scale gene expression of peripheral blood mononuclear cell samples derived from 10 individuals with DM1 (5 men, 5 women) and 10 healthy matched controls was analyzed by the Human Gene 1.0 ST Affymetrix microarray.

Results: During 18,796 person-years of follow-up, corresponding to 424 patients with DM1, we observed 70 cancers in 62 patients giving a 1.81-fold risk (95% confidence interval [CI] 1.37–2.36), which was stronger in women than in men. Ovary (SIR 8.33, 95% CI 1.72–24.31) and endometrium (SIR 6.86, 95% CI 2.23–16.02) in women and thyroid (SIR 23.33, 95% CI 9.38–48.08) and brain (SIR 9.80, 95% CI 3.18–22.88) in both sexes were tumor sites with significantly higher risks in DM1. There were differences in gene expression between healthy controls and patients with DM1 and between men and women with DM1; all patients with DM1 combined and female patients with DM1 displayed significant downregulation of the microRNA (miRNA)-200c/141 tumor suppressor family.

Conclusions: Oncologic risk is increased in DM1, especially in women and for gynecologic, brain, and thyroid cancer. Expression of the miRNA-200/miRNA-141 tumor suppressor family is decreased in women with DM1. ***Neurology*® 2016;87:1250–1257**

GLOSSARY

CI = confidence interval; **DM1** = myotonic dystrophy type 1; **DM2** = myotonic dystrophy type 2; **ICD** = *International Classification of Diseases*; **miRNA** = microRNA; **MIRS** = Muscular Impairment Rating Scale; **SIR** = standardized incidence ratio.

Myotonic muscular dystrophies are a group of autosomal dominant, multisystem diseases encompassed by 2 subtypes. Myotonic dystrophy type 1 (DM1), also known as Curschmann-Steinert disease (OMIM: #160900), is caused by the expansion of an unstable trinucleotide (CTG) repeat expansion in the 3′ untranslated region of a *DMPK* kinase gene located in chromosome 19. The type 2 (DM2) (OMIM #602668) is originated by a tetranucleotide (CCTG) repeat expansion in intron 1 of the *CNBP* gene. DM1 displays a more severe phenotype than DM2 and represents the most common adult muscular dystrophy, with an estimated prevalence ranging from 0.5 to 18 of 100,000 people,[1] although the disease prevalence is higher in some regions such the province of Gipuzkoa in the Spanish Basque Country.[2]

The DM1 phenotype is grossly related to the size of the CTG expansion and shows an extremely wide variability ranging from very mild forms with CTG repeats less than 50 and

Supplemental data at Neurology.org

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usually associated with cataracts developed in presenile age, to severe neonatal forms with more than CTG 1,000 repeats, associated with severe developmental delays. Life expectancy is reduced because of complications derived from muscle weakness, respiratory and cardiac involvement, neoplasms, and metabolic disturbances such as hypercholesterolemia or diabetes.[3,4]

In 1965, Cantwell and Reed[5] first reported an association between DM1 and pilomatricoma, a rare and benign cutaneous tumor. Since then, several case reports describing benign and malignant neoplasms in virtually any location have been published.[6] Furthermore, in recent years, large epidemiologic studies performed in population- or clinical-based cohorts provided evidence of increased risk of malignant tumors in patients with DM.[7–10] Excess risks of endometrium, ovarian, thyroid, skin, eye, and colon cancer were observed in 2 of the 3 studies, while brain cancer excess risk was observed in one study. A follow-up study including 911 patients with DM showed that females with DM1 were more likely to develop cancers.[11] Several studies have shown no association between the size of leukocyte repeat expansion and cancer risk in those patients[6] and the underlying causes, and the biological mechanisms of the susceptibility for developing tumors are still unknown.

In this study, we quantified cancer risk in the clinically and genetically well-characterized Gipuzkoa Myotonic Dystrophy Cohort, and used gene expression analysis to identify possible molecular mechanism of cancer susceptibility in those patients. The cohort includes all patients diagnosed with DM in the past 30 years in an area with one of the largest DM1 prevalences worldwide.

METHODS

Study population. Data from patients were retrospectively obtained from the medical records of the Gipuzkoa historical myotonic dystrophy cohort, established in 1985. We identified 503 patients with a molecularly confirmed DM diagnosis between 1985 and 2013. We excluded 4 patients with DM2 diagnosis and 75 patients with DM1 because of lack of data or they were lost to follow-up.

From patients' medical records, we extracted the following information: sex, age at the time of study enrollment and age at death, nucleotide expansion size (CTG triplets), calendar year of diagnosis, disease severity assessed using the Muscular Impairment Rating Scale (MIRS)[12] at the time of the last visit, age and calendar year of cancer diagnosis, and cancer anatomical site. Diagnoses of all types of malignant neoplasia were coded using ICD, Ninth Revision. Death causes were coded using ICD-10 (2014 version).[13]

Genetic analysis. The CTG repeat was measured at the time of DM1 diagnosis by conventional PCR and Southern blot. Conventional PCR was performed with 100 ng of genomic DNA using gene-specific primers flanking the *DMPK* CTG repeat. All normal homozygotes and expanded alleles were confirmed with Southern blot. We included patients with 40 or more CTG repeats because of the demonstrated instability of the fragment size from this threshold, independently of the presence or absence of clinical manifestations, assuming that if we eliminate the cases considered as premutations, we neglect a possible effect of genomic condition over cancer prone.

Statistical analysis. Follow-up started at the date of DM1 diagnosis and ended at the date of first cancer diagnosis, death, or last visit. We calculated standardized incidence ratios (SIRs) by dividing the observed numbers of cancer by the expected numbers for all cancers combined and cancer-specific anatomical sites, overall and stratified by sex. Expected numbers were calculated by multiplying the age- and sex-specific incidence rate from the Basque population cancer registry[14] by the person-years of the study cohort. SIRs were obtained for all types of cancers except for basal cell carcinoma of the skin because this type of cancer is not collected in the registry. Confidence intervals (CIs) were calculated using the Poisson distribution.

Mean repeat length was compared for patients with and without cancer using the Student t test. All tests were considered statistically significant if $p < 0.05$. Statistical analysis was performed using Stata/SE 12 (StataCorp LP, College Station, TX).

Transcriptomic analysis. This analysis included 10 patients with DM1 (5 women, 5 men; mean age: 43.4 ± 5.16 years; expansion size: 750 ± 306 CTG triplets) who had moderate or severe clinical manifestations (MIRS median score = 3, range 3–5) and no malignancy before or at the time of blood collection and 10 age- and sex-matched healthy controls. We extracted RNA from peripheral blood mononuclear cells using the LeukoLOCK Total RNA Isolation System (Life Technologies, Carlsbad, CA). For the RNA extraction, we use a 2-step protocol, first with the miRNesay Mini Kit (QIAGEN, Valencia, CA) followed by automated RNA extraction in the QIAcube. Large-scale gene expression was measured by the Human Gene 1.0 ST Affymetrix microarray (Affymetrix, Santa Clara, CA). RNA integrity was checked with an Agilent RNA 6000 Nano Kit (Agilent Technologies, Inc., Santa Clara, CA). Samples with an RNA integrity value above 7 were accepted to be processed. Three hundred nanograms of total RNA were used for microarray analysis following the manufacturer's instructions.

We analyzed gene expression differences in patients with DM1 and healthy controls, and in a second step, we studied the differences between patients with DM1 by sex. Results of the microarray data were extended by reverse transcription–PCR in an additional DM1 male (n = 16) and female (n = 25) subset of patients.

Western blot analysis. We did a Western blot of the protein products of the targets altered in the transcriptomic analysis. Immunoblots were performed as previously reported.[15] We used ab16123 (Abcam, Cambridge, UK) for $p16^{Ink4a}$ detection and A-5441 (Sigma, St. Louis, MO) for β-actin and horseradish peroxidase–linked anti-rabbit or anti-mouse (Santa Cruz Biotechnology, Dallas, TX) secondary antibody at a 1:2,000 dilution. Detection was accomplished by chemiluminescence using Novex ECL Chemi Substrate (ThermoFisher Scientific, Waltham, MA).

Standard protocol approvals, registrations, and patient consents. This study was approved by the Donostia Universitary

Hospital Ethical Board and conducted in accordance with the Declaration of Helsinki ethical standards.

RESULTS Cancer incidence rate in patients with DM1. The study included 424 patients with DM1; 214 (50.5%) of them were women. Mean CTG repeat expansion size at the time of DM1 diagnosis was 684 ± 535 CTG triplet expansion (range: 43–2,000 CTG repeats). One hundred thirty-seven patients were deceased at the time of analysis. Additional demographic and clinical features of the patients are shown in table 1. The most common causes of death were diseases of the respiratory system (38%), the circulatory system (24.1%), and neoplasms (15.3%); causes of death were unknown in 15.3% of the patients (table e-1 at Neurology.org).

During 18,796 person-years of follow-up, we observed 70 cancers in 62 patients with DM1 (32 women, 30 men; table e-2). We observed multiple primary cancers in 7 patients (6 patients had 2 cancers and 1 patient had 3 different cancers) (table e-3). Mean age at cancer diagnosis for all 62 patients was 46.6 ± 14.7 years. Mean CTG repeat expansion size in patients with DM1 and cancer was 629 ± 79 CTG repeats and there were no statistical differences between the length of the CTG expansion in DM1 patients with or without cancer ($p > 0.05$). Although we only had MIRS data available at the time of the study in 217 patients, there were no statistical differences regarding cancer status ($p > 0.05$). Digestive organs (24.8%), genitourinary system (21.4%), skin (12.8%), and thyroid gland (11.4%) were the most frequent sites for malignant tumors (table e-2).

When compared to the general population from Gipuzkoa, we detected an approximately 2-fold increase in risk of cancer (SIR 1.81, 95% CI 1.37–2.36). This risk increase was stronger in women (SIR 2.71, 95% CI 1.77–3.97) than in men (SIR 1.40, 95% CI 0.94–2.01). When we compared the anatomical site of the cancer in the DM1 population with the general population stratified by sex and age, we found a high risk of developing ovary (SIR 8.33, 95% CI 1.72–24.31) and endometrium (SIR 6.86, 95% CI 2.23–16.02) cancer in women and thyroid (SIR 23.33, 95% CI 9.38–48.08) and brain (SIR 9.80, 95% CI 3.18–22.88) cancer in both sexes (table 2). Besides these malignant tumors, only colorectal cancer nearly reached statistical significance (SIR 2.06, 95% CI 0.94–3.92).

Table 1 Clinical and molecular characteristics of the myotonic dystrophy type 1 cohort

No. of patients	424
Sex	214 women (50.5%), 210 men (49.5%)
Mean CTG repeat expansion size	684 ± 535.6 CTG repeats (43-2,000 CTG repeats)
Transmission	Paternal 65%
MIRS score (n = 217)	2.54 ± 1.21
Age at last visit, y	50.7 ± 15.61
Respiratory symptoms	86 patients (20.3%)
Cardiac disease	184 patients (43.4%)
Patients with cancer	62 (14.6%)
Death	137 patients
Mean age at death, y	60.25 ± 13.11
Follow-up days	18,313.6 ± 5,739.2

Abbreviation: MIRS = Muscular Impairment Rating Scale.

Transcriptomic analysis of patients with DM1. As previously described,[16] we observed differences in gene expression between healthy men and women (figure 1, A and B). Similar sex differences were detected in the transcriptome of patients with DM1 (figure 1, A–C). Compared to healthy controls, patients with DM1 had an upregulation of RNA5SP211, an unknown pseudogene, and downregulation of TAS2R13, a subtype of taste receptors.

When we analyzed the results according to sex, we found that EMR4P (a hormone receptor), CD24 (a glycoprotein expressed on mature granulocytes and B cells), PLA2G7 (a platelet activating factor), and caspase-5 (a gene implicated in apoptosis) were significantly upregulated in men (figure 1, B and C). Moreover, we identified 11 genes differentially expressed in women with DM1, including upregulation of a subtype of histone (HIST1H2AK) and pyruvate dehydrogenase kinase 4 (PDK4), and downregulation of 3 microRNA precursors (pre-miR-3978, pre-miR-141, and pre-miR-200c), a transcription factor (ZEB2), an olfactory receptor (OR52K2), an inhibitory receptor of myeloid cells (CLEC12B), myoferlin (a protein that has a role in calcium-mediated membrane fusion events, membrane regeneration and repair, and subsequently in muscle weakness), a death-associated protein kinase (DAPK1), and MS4A4E (figure 1, B and C). The same results were observed in an independent validation set of patients (figure 1, D and E). Specifically, the differentially expressed levels of 13 of 17 genes detected in the array were confirmed in the validation cohort.

Identification of *miR-200c/miR-141* cluster associated with the cancer susceptibility phenotype in DM1. The miR-200c/miR-144 and miR-3978 precursors were downregulated in the group of women with DM1 in the array and validated in the extended cohort (figure 2, A and B). Of note, the mature forms miR-200c-5p, miR-141-3p, miR-141-5p, and miR-3978 were downregulated in the cohort of women with DM1 (figure 2C). On the contrary, the expression was slightly elevated in men, revealing a sex differential expression of those miRs in DM1 (figure 2, C and D).

Table 2 SIRs by anatomical site

Cancer	Age at diagnosis of cancer, y	O	E	SIR	95% CI
Women					
Ovary	48 (16-63)	3	0.36	8.33	1.72-24.31[a]
Endometrium	53 (38-59)	5	0.73	6.86	2.23-16.02[a]
Breast	57 (56-69)	3	2.88	1.04	0.21-3.04
Men					
Prostate	75 (73-77)	2	4.33	0.46	0.06-1.67
Testes	15	1	0.07	14.25	0.35-79.6
Both sexes					
Thyroid	51 (40-67)	7	0.30	23.33	9.38-48.08[a]
Brain	53 (30-56)	5	0.51	9.80	3.18-22.88[a]
Kidney	53.5 (40-67)	2	0.79	2.53	0.31-9.15
Non-Hodgkin lymphoma	65.5 (61-70)	2	0.70	2.86	0.35-10.32
Liver	69 (66-72)	2	0.79	2.54	0.31-9.15
Colorectum	59 (41-86)	9	4.36	2.06	0.94-3.92
Stomach	56.5 (55-58)	2	1.27	1.57	0.19-5.69
Lung	59.5 (47-73)	4	3.40	1.18	0.32-3.01
Leukemia	57	1	0.26	3.80	0.09-21.43
Head and neck	43	1	1.07	0.94	0.02-5.21
Melanoma	34	1	0.58	1.72	0.04-9.61
Urinary bladder	64	1	1.56	0.64	0.02-3.57

Abbreviations: CI = confidence interval; E = expected cancer in the Basque general population adjusted for sex and age; O = observed cancer in myotonic dystrophy type 1 cohort; SIR = standardized incidence ratio.
[a] Indicates statistical significance, $p < 0.05$.

Because the *BMI1* and *ZEB* family member oncogenes are reported as miR-200 target genes,[17–22] we measured their expression finding that ZEB1 and ZEB2 were decreased while BMI1 levels were upregulated in the group of women with DM1 (figure 2E). Moreover, the expression of $p16^{Ink4a}$ tumor suppressor, a known target of BMI1 epigenetic silencing, was lower at both messenger RNA and protein level in the same group of women with DM1 (figure 2, F and G).

DISCUSSION There is recent accumulating evidence that patients with DM1 are at high risk of developing cancers. Confirming these findings in an independent study and in different DM populations is important for patient clinical management. Using data from a large molecularly confirmed cohort of patients with DM1, we showed a statistically significant excess risk of endometrium, ovary, thyroid, and brain cancer. In the largest epidemiologic cohort published so far accounting for more than 1,600 patients with DM1 and DM2, Gadalla et al.[7] found an apparent high risk of cancers of the endometrium, brain, ovary, and colon, and possibly thyroid and choroidal melanoma and nonmelanoma skin cancers. In smaller studies, Win et al.[8] reported a higher risk of developing thyroid cancer and choroidal melanoma whereas Mohamed et al.[9] concluded that there was a high risk of thymoma, gynecologic, and lung cancer. Recently, in a small published cohort, Bianchi et al.[10] found that skin, thyroid, ovary, and breast cancers were most frequent in patients with DM.

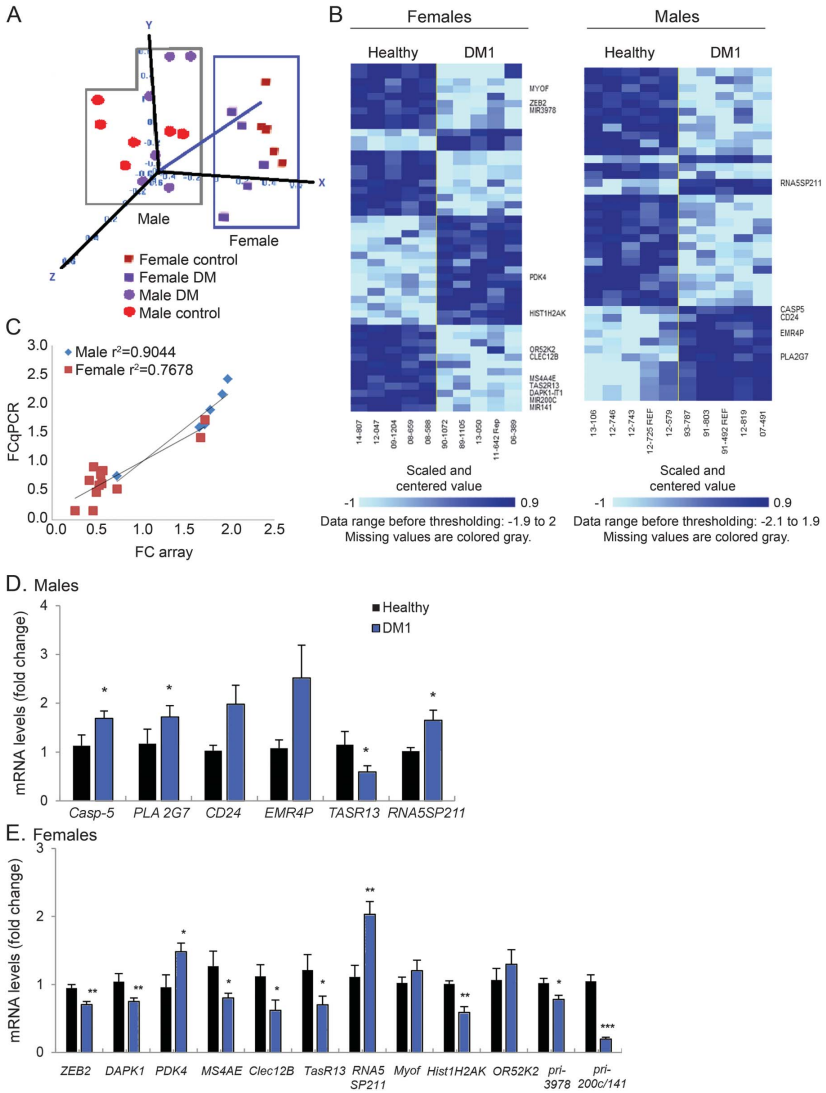
Our results are coincidental with those reported by Gadalla et al. in a population-based study of more than 1,600 patients with DM[7] with the exception of colorectal cancer. However, the CI (0.94–3.92) of this type of cancer was near to significance values and the smaller sample size in our cohort could explain this result. Of note, colorectal cancer was also the most frequent among those patients who had 2 or more cancers. The excess risks of thyroid,[8,10] endometrium, and ovary[9,10] were also found in smaller studies. Of importance, and as also indicated by Gadalla et al.,[4,7] we did not find an overrepresentation of screening-related cancers (i.e., breast and prostate), strongly suggesting that the results are genuine and not biased by the close medical surveillance in patients with DM. The fact that there are no incidence and mortality cancer differences between the Basque and other regions in Spain[23] led us to eliminate the notion that the excess cancer in this population could be explained by other genetic factors rather than the DM1 condition itself.

Our data show that malignancies developed at a mean age of 46.6 ± 14.7 years. In agreement with previous reports,[3,24] cancers represented the third leading cause of death after respiratory and circulatory diseases in our DM1 population. A progressive increase in cumulative incidence of cancer mortality has been previously reported (2% by age 50 to 6% by age 70).[4]

Another striking feature is the apparent absence of a correlation between cancer risk and nucleotide repeat length. This result could be potentially biased for methodologic reasons because the CTG repeat expansion size was measured years before cancer emergence and also because of the somatic instability that characterizes DM1. Based on the differences found between tumoral and healthy tissues in patients with DM1, some authors have suggested that there might be an underlying independent mechanism of somatic instability in the tumoral tissue.[25–27] However, the later contrast with the recent finding that MBLN1, a splicing factor sequestered in the nuclear foci of DM1 cells, which correlates with CTG expansion size, could be implicated in the physiopathology of the progression of certain cancers.[28]

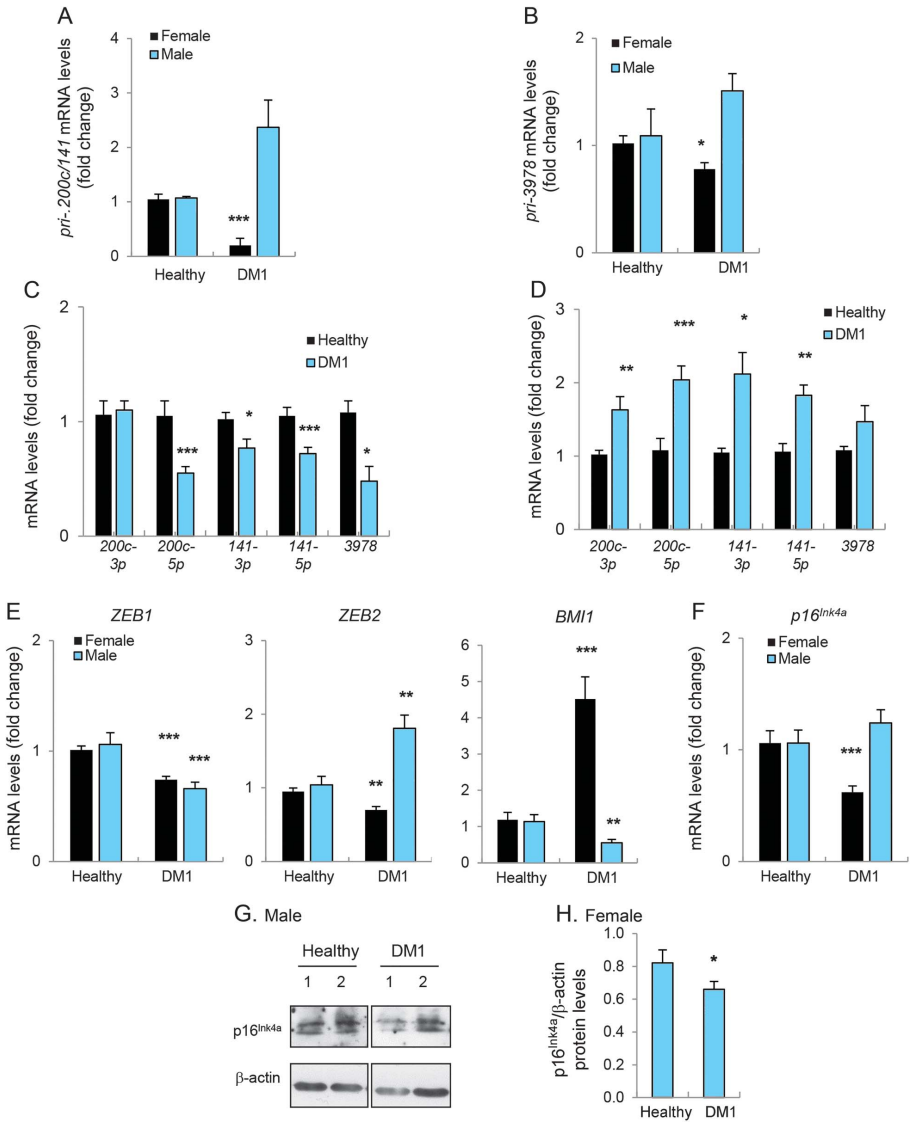
Our results showing a differential transcriptional profiling of several genes previously linked to cancer, such as *PDK4*,[29] *DAPK1*,[30] *CASP5*,[31] and *PLA2G7*[32] in patients with DM1 compared to healthy controls, open the door to a mechanistic explanation of this increased oncogenic risk in DM1. In addition, we observed a downregulation of miR-200c, miR-141, and miR-3978 mature and

Figure 1 Sex-dependent mRNA expression pattern in patients with DM1



(A) Dendrogram explaining the sex differences between the expression patterns. (B) Heatmap with the genes differentially expressed between patients with DM1 (n = 10) and healthy controls (n = 10) in women (left, n = 5) and men (right, n = 5). (C) Chart of the validation data (fold change from arrays vs fold change from reverse transcription–quantitative PCR) in women (red) and men (blue). (D) mRNA levels of genes altered in the male cohort (n = 16) compared with healthy controls (n = 5). (E) mRNA levels of genes altered in the female cohort (n = 25) compared with healthy controls (n = 5). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. DM1 = myotonic dystrophy type 1; mRNA = messenger RNA.

Figure 2 miR-200c/141 cluster and its targets in patients with DM1



(A, B) mRNA expression of 2 miRNA precursors, *miR-200c/141* and *miR-3978*, which are downregulated in women with DM1 (n = 25) and upregulated in men with DM1 (n = 16). (C, D) Mature forms of these miRNAs are downregulated in women with DM1 (left, n = 25) and upregulated in men with DM1 (right, n = 16). (E) In these groups of patients, *ZEB1* is downregulated in both sexes, while *ZEB2* is downregulated only in women with DM1. On the contrary, *BMI1* is upregulated in women and downregulated in men with DM1. (F) *p16^{Ink4a}* expression is decreased in women with DM1 (n = 25). (G) Representative immunoblots and quantification of p16^{Ink4a} derived from 2 different blood samples of female healthy controls and DM1 patients (n = 4). (H) p16^{Ink4a} protein quantification in the blots shown in G. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. DM1 = myotonic dystrophy type 1; miRNA = microRNA; mRNA = messenger RNA.

precursor forms in female patients with DM1. Of note, 2 of them, miR-200c and miR-141, belong to the same miR-200 tumor suppressor cluster. Lower levels of the miR-200c family members were detected in tumor tissues and blood-derived samples in a wide range of cancer types.[33] This decline was associated with tumor progression and poor prognosis including metastasis.[34,35]

The miR-200 family has several well-described oncogenes as downstream targets, likely the *ZEB* family and the polycomb group gene *BMI1* becoming crucial targets.[18–21,36] Our results show that these genes are altered in both sexes of patients with DM1 supporting the relevance of this molecular signaling on the disease. In particular, women with DM1 express elevated *BMI1* levels, while *ZEB1* and 2 are decreased. The existence of such alterations in patients with severe disease and no cancer prompted us to hypothesize that the downregulation of miR-200 cluster members have a role in cancer susceptibility, and the consequent upregulation of *BM1* instead of through *ZEB* may be required for its development. In support of this idea, (1) ZEB factors are epithelial–mesenchymal transition inducers and their role has been associated with metastasis rather than tumor formation; (2) the expression of $p16^{Ink4a}$ tumor suppressor, a critical target of BMI1, inversely correlates with BMI1 levels in patients with DM1; and (3) miR-200 and BMI1 play an important role in the maintenance of adult stem cells and tumor-initiating cells in many organs. Of note, many of the cancers detected in patients with DM1 emerge in tissues with a high rate of cellular replications.

Taken together, our findings support a model through a coordinated action between the miR-200 expression and its downstream targets ZEB1/2 and BMI1. In support of this notion, there is a close functional link between the miR-200 family and ZEB factors and BMI1 in a double-negative feedback loop, respectively. Thus, the activation of one of them affects the expression and activity of the others.[37,38]

Our study strength includes the population-based design (all patients with DM1 in the Gipuzkoa population were included), and therefore no selection bias exists. We are limited by the lack of information of known cancer risk factors such as smoking, diet, lifestyle, environmental influences, and alcohol intake among others. Patients with DM1 have more obesity and the prevalence of tobacco smoking is higher than in the general population,[39] so these risk factors might have had a confounding role in our results and should be considered when planning longitudinal studies. However, a recent study suggested that lifestyle factors in patients with DM1 do not explain the observed excess risk of cancers.[10]

We believe that our study provides independent cancer site–specific confirmation of recently reported excess cancer risks as part of the DM1 phenotype, especially for women, and suggests that this association could be more related to a transcriptomic regulation of oncogenic pathways than a direct consequence of the DM genomic signature.

AUTHOR CONTRIBUTIONS

Roberto Fernández-Torrón: had the original idea, collected clinical data from the patients, evaluated the patients, did the clinical and statistical analysis, and wrote the manuscript. Mikel García-Puga: performed the transcriptomic analysis and wrote the manuscript. José-Ignacio Emparanza: performed the statistical analysis, reviewed the ethics, and wrote the manuscript. Miren Maneiro: collected clinical data, evaluated the patients, and revised the manuscript. Ana-María Cobo: performed the molecular analysis and revised the manuscript. Miren Zulaika: collected clinical and molecular data, performed the transcriptomic analysis, and revised the manuscript. Juan-José Poza: collected clinical data, evaluated the patients, and revised the manuscript. Juan-Bautista Espinal: collected clinical data, evaluated the patients, and revised the manuscript. Irune Ruiz: performed the anatomopathologic analysis and revised the manuscript. Loreto Martorell: performed the molecular analysis and revised the manuscript. David Otaegui: performed the transcriptomic analysis and revised the manuscript. Ander Matheu: had the original idea, performed the transcriptomic analysis, and wrote the manuscript. Adolfo López de Munain: had the original idea, collected clinical data from the patients, followed and evaluated the patients, performed the clinical analysis, and wrote the manuscript.

ACKNOWLEDGMENT

The authors thank S. Gadalla (NIH) for reviewing the manuscript and for her helpful suggestions, L. Manterola for miRNA analysis, and all of the patients with myotonic dystrophy.

STUDY FUNDING

R.F.-T. was recipient of a Río Hortega from the Instituto de Salud Carlos III. A.M. was granted with an Ikerbasque Research Fellow position. M.G.-P. was recipient of a predoctoral fellowship from UPV/EHU (PIF 15/245). This work was supported by CIBERNED, and grants from the Isabel Gemio Foundation, Diputación Gipuzkoa (OF30/2014), Ministry of Economy and Competition and FEDER Funds to A.L.d.M. (FIS PS 09-00660 and PI147436) and A.M. (PI13/02277), and European Union (Marie Curie CIG 2012/712404).

DISCLOSURE

The authors report no disclosures relevant to the manuscript. Go to Neurology.org for full disclosures.

Received February 17, 2016. Accepted in final form June 8, 2016.

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ANNALS of Clinical and Translational Neurology
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BRIEF COMMUNICATION

Leukocyte telomere length in patients with myotonic dystrophy type I: a pilot study

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Funding Information
The study was supported by the Intramural Research Program of the Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health.

Received: 16 October 2019; Revised: 31 October 2019; Accepted: 31 October 2019

***Annals of Clinical and Translational Neurology* 2020; 7(1): 126–131**

doi: 10.1002/acn3.50954

Abstract

Myotonic dystrophy type I (DM1) is an autosomal dominant disease of which clinical manifestations resemble premature aging. We evaluated the contribution of telomere length in pathogenesis in 361 DM1 patients (12 with serial measurements) and 223 unaffected relative controls using qPCR assay. While no differences in baseline leukocyte relative telomere length (RTL) was noted, the data suggested an accelerated RTL attrition in DM1 (discovery cohort: T/S change/year = −0.013 in DM1 *vs.* −0.005 in controls, $P = 0.04$); similar trend was noted in validation cohort. Further investigations are needed to examine the role of TL in the pathophysiology of DM1.

Introduction

Myotonic dystrophy type 1 (DM1) is an autosomal dominant multisystem disorder resulting from an unstable trinucleotide $(CTG)_n$ repeat expansion in the 3' untranslated region of the *dystrophia myotonica-protein kinase* (*DMPK*) gene.[1] Clinical manifestations of DM1 include progressive muscle weakness and wasting, myotonia, premature cataracts, cardiac conduction defects, gastrointestinal/endocrine abnormalities, and increased risk of certain cancers.[2,3] It has been suggested that the DM1 phenotypes may resemble a premature aging syndrome.[4]

Telomeres are tandem hexanucleotide $(TTAGGG)_n$ repeats and protein complexes at the end of chromosomes, maintaining genomic stability.[5] They shorten with each cell division[6] and are marker of cellular aging. Few studies suggested a link for telomere length in the pathogenesis of Duchenne (DMD) or limb girdle muscular dystrophies.[7,8] Telomere length studies in DM1 were limited; an *in vitro* investigation using DM1 muscle precursor cells

from three fetuses with congenital DM1 showed abnormal accumulation of P16 and higher telomere attrition per cell division compared with controls.[9]

Here we compared leukocyte relative telomere length (RTL) in a large cohort of genetically- confirmed DM1 patients and DM1 mutation-free clinically healthy relative controls and evaluated telomere length attrition overtime in a subset of patients with serial blood samples.

Materials and Methods

Study participants

We used data and samples from 379 DM1 patients and 235 DM1-free relative controls from the Spanish Guipúzcoa historical myotonic dystrophy cohort.[10] Participants' demographics and clinical information were obtained through medical records review. We excluded 30 individuals (28 missing age, one twin, and one with failed quality control metrics for RTL assay). The final analysis included 361 DM1 patients and 223 controls. Twelve participants provided a second sample for RTL measurement, as convenience permitted.

The study was approved by the Donostia University Hospital Ethical Board and by the Office of Human Subjects Research Protections at the National Institutes of Health.

DNA extraction and telomere length measurement

Blood DNA was extracted using the salting out procedure or Qiagen Flexigen DNA kit (QIAGEN, Germantown, MD). We measured RTL after adapting the originally published quantitative polymerase chain reaction (qPCR) method.[11] Method details are available elsewhere.[12] Briefly, qPCR assay measures a ratio between telomere repeats amplification (T) to that of an autosomal single-copy gene (S; *36B4*). The T/S ratio then get normalized using internal QC calibrator samples to yield a standardized T/S ratio. All samples were assayed in triplicate. The coefficient of variation for internal control samples was 4.5%.

Statistical analysis

All analyses were stratified by DNA extraction method to account for known variable qPCR sensitivity to extraction method.[13] Samples extracted by salting out ($N = 223$ patients and 162 controls) served as a discovery cohort, and those extracted by Qiagen Flexigen DNA kit ($N = 138$ patients and 61 controls) served as a validation cohort. RTL data from serial samples extracted by the same method were available for 12 DM patients ($N = 7$ for discovery, $N = 5$ for validation cohort).

We used generalized linear models to compare the mean RTL between DM1 and controls, and in DM1 patients by CTG repeat size categories (40–50, 51–249, 250–499, ≥500). A random intercept linear mixed model was used for repeated measurement analysis; RTL annual attrition rate in DM1 patients with repeated samples (median time between samples = 9 years, range = 4-17 years in the discovery, and median time = 3 years, range = 2–3 years in the validation) was compared with that expected from regression models of RTL and age using control cross-sectional data using R package 'lme4'. Differences in RTL attrition rates between DM1 patients and controls was assessed by an interaction term between DM1-control status and age. Statistical analyses were conducted using SAS version 9.4 and R version 3.4.4.

Results

Characteristics of study participants

DM1 patients and controls showed similar ages (median age = 42.0 and 43.0 years in DM1 *vs.* controls, respectively) and sex distributions (proportion of males = 45.7% and 43.9% in DM1 *vs.* controls, respectively). No age or sex differences were noted between DM1 patients in the two study subsets, or between patients and relatives in each set ($P > 0.05$). Patients with DM1 in the discovery and validation cohorts also had similar CTG repeat size (median repeat size = 433 and 500 for discovery *vs.* validation cohorts, respectively, $P = 0.37$) (Table S1).

In DM1 patients, the size of CTG repeat was inversely correlated with age at genetic testing in both discovery ($r = -0.26$, $P = 0.0003$) and validation cohorts ($r = -0.21$, $P = 0.02$) (Fig. S1A-B).

The patients with a second blood sample showed similar age, and sex distribution. However, despite of lack of statistical significance, the data suggested that patients with subsequent sample may have a less severe disease as evident by shorter CTG repeat sizes (median = 167 *vs.* 500, in those with and without subsequent sample, respectively) and less Muscular Impairment Rating Scale (MIRS) (0% *vs.* 24% with MRIS > 3, respectively) (Table S2).

Telomere length in DM1 patients and controls

RTL was inversely correlated with age among both DM1 patients and controls (discovery cohort: $r = -0.39$, $P < 0.0001$ for DM1 and $r = -0.51$, $P < 0.0001$ for controls; validation cohort: $r = -0.27$, $P = 0.002$ for DM1 and $r = -0.23$, $P = 0.07$ for controls; Fig. S2A-B).

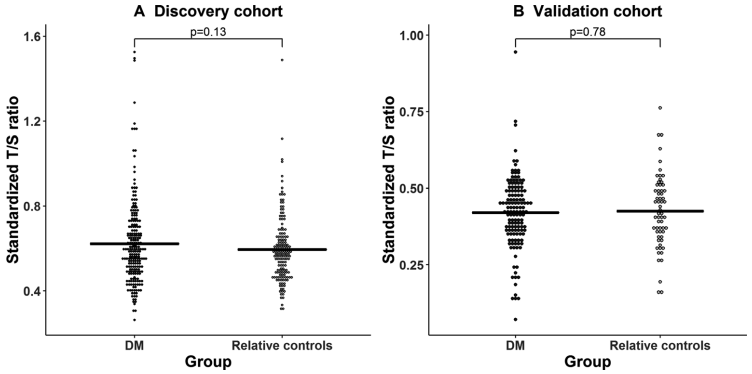

Figure 1. Comparison of relative telomere length between DM1 patients and their healthy relative controls. (A) Discovery cohort, (B) Validation cohort.

No differences were observed in RTL between DM1 patients and controls at baseline: discovery cohort: (mean T/S ± SD) = 0.62 ± 0.20 and 0.60 ± 0.16 for DM1 and controls, respectively, $P = 0.13$; or in the validation cohort: (mean T/S ± SD)=0.42 ± 0.12 and 0.43 ± 0.12, respectively, $P = 0.78$, Fig. 1A and B). Age- and sex-adjustment did not alter the results. In DM1 patients, RTL was not associated with CTG repeat expansion size, nor with MIRS in models adjusted for age and sex (Fig. 2A and B).

Telomere length attrition overtime in patients with DM1

Baseline RTL was similar in patients with and without repeated RTL measurements (median T/S = 0.58 *vs.* 0.52, $P = 0.27$).

In the repeated RTL measurement analyses, and when compared with expected based on RTL attrition from relatives' cross-sectional data, a more rapid RTL decline was noted in DM1 in the discovery cohort (T/S decline/year = −0.013 *vs.* −0.005 for DM1 and controls, respectively, $P = 0.04$). A similar (non-significant) trend was noted in the validation cohort (T/S decline/year = −0.005 *vs.* −0.002, $P = 0.33$) (Fig. 3A and B, Table S3).

Discussion

In this analysis of a large cohort of patients with genetically confirmed DM1 and clinically healthy relative controls, we observed no difference in leukocyte RTL measured at genetic testing between patients and controls, yet, a higher rate of annual telomere length attrition was noted in DM1 patients.

Prior DM1 telomere length studies have been limited to few small *in-vitro* investigations focusing on its possible role in muscle precursor cell dysfunction. In agreement with our results of similar leukocyte RTL in DM1 and controls, a study comparing telomere length in distal (with clinical and pathological alterations) and proximal muscles (relatively spared) from four DM1 patients showed no telomere length difference.[14] Yet, we showed a higher rate of RTL attrition overtime in DM1 patients than expected based on RTL attrition rate from controls. Our findings were also consistent with a study of muscle satellite cells in fetuses/infants with congenital DM1 which showed a faster telomeric DNA loss per cell division (171.9 ± 17 base pairs) when compared with control cells (108.1 ± 10 base pairs); the authors concluded that large CTG expansion repeats may interfere with telomere homeostasis in those patients.[9] Of note, we found no association between patients' leukocyte RTL and CTG repeat size; suggesting no direct role for repeat expansion size in the observed telomeric attrition. However, our data were limited by the unavailability of progenitor repeat size. Also, testing this hypothesis in blood cell (not known to be affected in DM1), may not be directly applicable to somatic changes in affected organs such as the muscles. Somatic mosaicism and instability in repeat expansion size are well-described in DM1.[15] It is possible that the accelerated telomere attrition observed in our DM1 patients is a consequence of the cellular exposure to oxidative stress. The triple-guanine sequence in telomeric DNA $(TTAGGG)_n$ is very sensitive to oxidative damage,[16] resulting in accelerated telomere shortening.[17] Previous studies have shown a significant increase in blood level of free radicals and oxidative

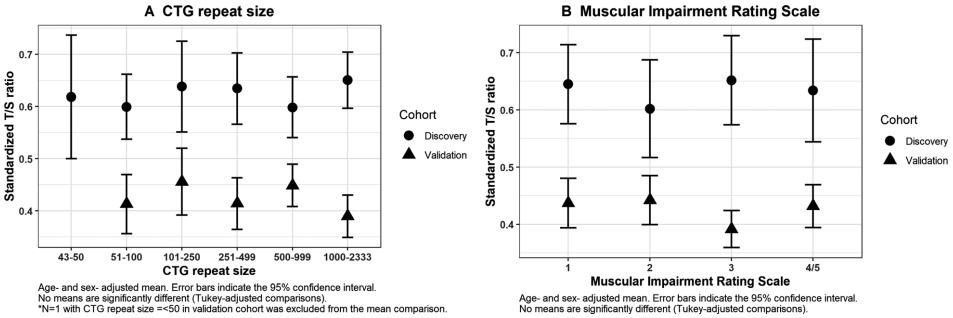

Figure 2. Relative telomere length in DM1 patients by CTG repeat size and by Muscular Impairment Rating Scale (MIRS) in age- and sex-adjusted models. (A) CTG repeat size, (B) Muscular Impairment Rating Scale.

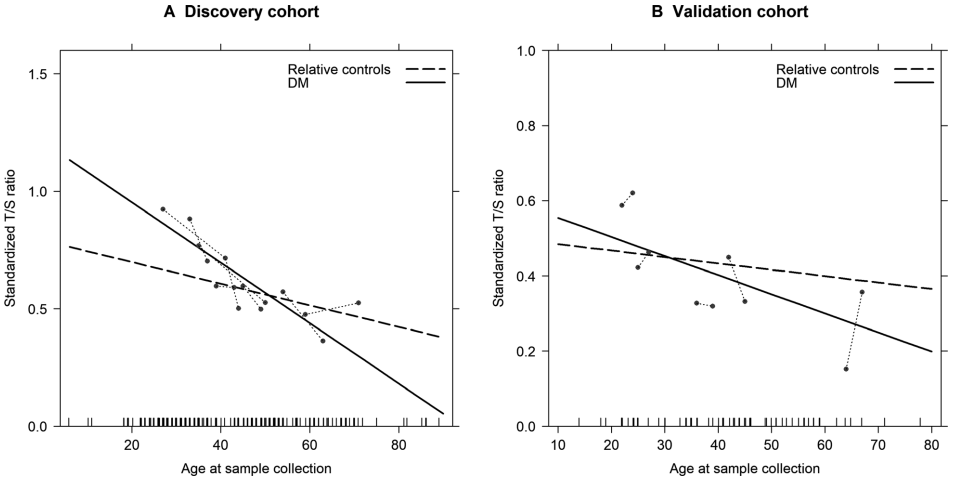

Figure 3. Comparison of annual relative telomere length attrition between DM1 patients and their healthy relative controls. (A) Discovery cohort, (B) Validation cohort.

stress biomarkers in DM1 patients,[18,19] suggesting a role for oxidative stress in disease progression.

The observed differences in the rate of TL attrition between the discovery and validation cohort could be affected by the difference in the time interval between samples (median interval = 9 years in the discovery, and 3 years in the validation). It has been shown that TL elongation in longitudinal studies is mostly artifact related to measurement errors in studies with short follow-up (≤5 years) where TL changes are usually small and hard to capture.[20]

Our study strengths include a relatively large sample size, the genetic diagnosis confirmation, the objectively healthy status of controls, and the availability of repeat expansion size. Limitations include the small sample size of patients with serial samples, who were not systematically sampled. Due to short time period between blood sampling, observed TL elongation in some patients could be artifactual.[20] Our control data were based on baseline measurement of RTL in relatives because of the unavailability of serial samples; a prior study has shown small differences in TL attrition rate calculated from cross-

sectional or longitudinal data in the general population (24.6 *vs.* 31 bp per year).[20] The qPCR assay is sensitive to pre-analytic processing, notably DNA extraction method. Consequently, we presented our results stratified by DNA extraction method. Although similar trends were observed in the two datasets, we cannot rule out the possibility that the difference in DNA extraction methods and/or time between serial samples contributed to the observed difference in the rate of TL attrition between the two sets.

In conclusion, our results suggest that telomere shortening does not play a direct role in DM1 etiology but may be a consequence of molecular alterations over time, possibly secondary to oxidative stress. It is possible that accelerated telomere shortening may mediate the age-related phenotypic presentation of DM1.

Acknowledgments

We thank the patients and their family members for their participation.

Conflict of Interest

The authors declare no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Correlation between CTG repeat size and age in DM1 patients. A. Discovery cohort, B. Validation cohort

Figure S2. Correlation between relative telomere length and age. A. Discovery cohort, B. Validation cohort
Table S1. Baseline characteristics of DM1 patients and their unaffected relative controls, stratified by DNA extraction method
Table S2. Comparison of baseline characteristics of DM1 patients with and without repeated relative telomere length measurement
Table S3. Telomere length attrition in DM1 patients and controls

www.aging-us.com AGING 2020, Vol. 12, No. 7

Research Paper

Myotonic Dystrophy type 1 cells display impaired metabolism and mitochondrial dysfunction that are reversed by metformin

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Keywords: myotonic dystrophy type 1, aging, metabolism, metformin, mitochondria, neuromuscular disease
Received: December 1, 2019 **Accepted:** March 3, 2020 **Published:** April 8, 2020

ABSTRACT

Myotonic dystrophy type 1 (DM1; MIM #160900) is an autosomal dominant disorder, clinically characterized by progressive muscular weakness and multisystem degeneration. The broad phenotypes observed in patients with DM1 resemble the appearance of a multisystem accelerated aging process. However, the molecular mechanisms underlying these phenotypes remain largely unknown. In this study, we characterized the impact of metabolism and mitochondria on fibroblasts and peripheral blood mononuclear cells (PBMCs) derived from patients with DM1 and healthy individuals. Our results revealed a decrease in oxidative phosphorylation system (OXPHOS) activity, oxygen consumption rate (OCR), ATP production, energy metabolism, and mitochondrial dynamics in DM1 fibroblasts, as well as increased accumulation of reactive oxygen species (ROS). PBMCs of DM1 patients also displayed reduced mitochondrial dynamics and energy metabolism. Moreover, treatment with metformin reversed the metabolic and mitochondrial defects as well as additional accelerated aging phenotypes, such as impaired proliferation, in DM1-derived fibroblasts. Our results identify impaired cell metabolism and mitochondrial dysfunction as important drivers of DM1 pathophysiology and, therefore, reveal the efficacy of metformin treatment in a pre-clinical setting.

INTRODUCTION

Myotonic dystrophy is the most common type of muscular dystrophy in adults and is inherited in an autosomal dominant manner [1]. There are two clinically similar but genetically distinct types: DM type 1 (DM1, also known as Steinert's disease; MIM #160900), caused by an unstable expansion of a CTG trinucleotide repeat in the noncoding region of the dystrophia myotonic-protein kinase gene (*DMPK*) [2], and DM type 2 (DM2; MIM #602668), caused by a tetra-nucleotide repeat CCTG expansion in the zinc finger 9 (*ZNF9*) gene [3]. CTG and CCTG expansions lead to formation of transcript aggregates in the nucleus, which interfere with proteins that play an important role in RNA metabolism, including members of the muscleblind (MBNL) and CUGBP RNA-Binding Protein Elav-Like Family Member 1

(CELF1) families of RNA-binding proteins [4]. Both diseases are characterized by missplicing of several downstream effector genes with negative effects on multiple tissues, thus contributing to the multisystem pathogenesis of DM [5]. DM1 is more common than DM2 and represents a more severe phenotype. In DM1, unaffected individuals carry less than 50 triplet repeats, whereas expansions ranging between 50 and 4000 CTG repeats have been found in affected individuals [6].

Patients with DM1 present a multisystem degenerative process that includes progressive muscular weakness and atrophy, myotonia, cardiomyopathy, insulin-resistance, cataracts, increased cancer incidence, neurodegeneration, metabolic syndrome, or premature death. This multisystem degenerative process strongly resembles an accelerated aging process [7, 8]. From a cellular point of view, different pathogenic mechanisms, such as alteration of autophagy, increased senescence, telomere shortening, or genomic instability, all of them hallmarks of aging [9], have been proposed to explain how the expansion in the CTG repeat of affected patients leads to the DM multisystem phenotypes [7]. However, detailed experimental validation of these mechanisms remains incomplete and has not yet been clarified.

It is well known the existence of several metabolic alterations, which accumulate over time, that affect longevity, aging and neurodegeneration [10, 11]. As a consequence, deregulated nutrient sensing and mitochondrial dysfunction have been proposed as hallmarks of aging [9] and metabolism is a pillar of aging [12]. In DM1, patients present several metabolic defects such as hyperinsulinemia, glucose resistance, and, in some cases, diabetes mellitus [7]. Moreover, muscle samples *in vitro* and blood samples *in vivo* show reduced Coenzyme Q10 (CoQ10) levels, a component of the electron transport chain that participates in aerobic cellular respiration [13, 14], which is indicative of mitochondrial dysfunction. However, the role of metabolism and mitochondria in the pathogenesis of DM1 has not been addressed in detail. In this work, we studied their contribution using human primary fibroblasts and peripheral blood mononuclear cells (PBMCs) derived from healthy donors and patients with DM1 as models. Our results indicated that DM1 fibroblasts showed impaired metabolism and mitochondrial dysfunction resulting in lower levels of ATP production and increased reactive oxygen species (ROS) production. PBMCs from DM1 patients also showed impaired mitochondrial dynamics and energy homeostasis. Interestingly, treatment with metformin resulted in the restoration of these phenotypes.

RESULTS

DM1-derived fibroblasts present impaired metabolism

To investigate the role of cellular metabolism in the pathogenesis of DM1, we first measured the oxygen consumption rate (OCR) in the fibroblasts of patients with DM1 and healthy donors. DM1 fibroblasts showed a 40% and 50% reduction in basal respiration and maximal respiration, respectively, compared to controls, which leads to a 50% reduction in ATP production via the Mitochondrial Oxidative Phosphorylation System (OXPHOS) activity (Figure 1A, 1B). Next, we hypothesized that the reduction in OXPHOS activity could be responsible for a reduction in the glycolysis pathway. To examine this hypothesis, we measured extracellular acidification (ECAR) as a measure of glycolysis [15]. We did not find any alteration in the glycolysis pathway (Supplementary Figure 1A, 1B), suggesting that all glucose taken by DM1 fibroblasts was coupled to pyruvate production.

The addition of carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) simulates an exacerbated physiological energy demand by stimulating the respiratory chain to operate at maximum capacity. DM1 cells were not able to respond to this stress as efficiently as controls, farther indicating impaired maximal respiration (Figure 1A, 1B). However, we did not find any difference in the proton-leak nor the coupling efficiency (Figure 1A–1C). Therefore, it seems that all the protons generated are coupled to ATP production. Moreover, DM1 fibroblasts have a more quiescent metabolism compared to healthy controls. In addition, after simulating a stress, DM1 fibroblasts could not switch to a more energetic metabolism (Figure 1D, 1E), resulting in a lower metabolic potential. Consistent with these results, DM1 fibroblasts presented lower AKT activation (measured as phosphorylated AKT) (Figure 1F), which is the central mediator of the PI3K pathway that serves a key role in multiple cellular processes, including glucose metabolism [16]. In summary, DM1-derived fibroblasts present decreased cellular metabolism.

Correlation between impaired metabolism and markers of disease pathophysiology

Next, we attempted to associate the impaired metabolism of DM1-derived fibroblasts with several pathophysiological characteristics of the disease. First, we found that the decrease in AKT phosphorylation in DM1-derived fibroblasts correlated with lower expression of DMPK and MBNL1, both at protein (Figure 2A) and mRNA (Figure 2B) levels. Moreover,

we examined whether there was a correlation between the severity of the metabolic alterations and both the number of CTG expansions and the Muscular Impairment Rating Scale (MIRS) score. We did not detect significant differences in basal and maximal respiration or in ATP production when fibroblasts were divided into those with less or more than 500 CTG repeats and 3 MIRS score (Figure 2C, 2D, Supplementary Figure 2). Moreover, there were no marked differences between cells obtained from DM1 patients of different ages, although the cells from a 71 year-old patient showed slightly higher impairment than others (Table 1, Supplementary Figure 3). Overall, metabolic dysfunction in fibroblasts derived from patients with DM1 seems not to be significantly altered by the repeat expansion of these patients.

DM1-derived fibroblasts display mitochondrial dysfunction but no changes in mitochondrial biogenesis

The results presented above indicate that the mitochondria of patients with DM1 could function normally, but with a reduced OXPHOS activity. We further investigated this by examining the biogenesis of mitochondria [17]. First, we evaluated the levels of two markers of mitochondrial content and biogenesis such as TOMM20 and PGC1-α. Immunofluorescence showed that expression of these two markers was not markedly altered in cells from DM1 compared to healthy controls (Figure 3A, 3B). In addition, flow cytometry was used to analyze another marker of mitochondrial content, MitoTracker, obtaining similar

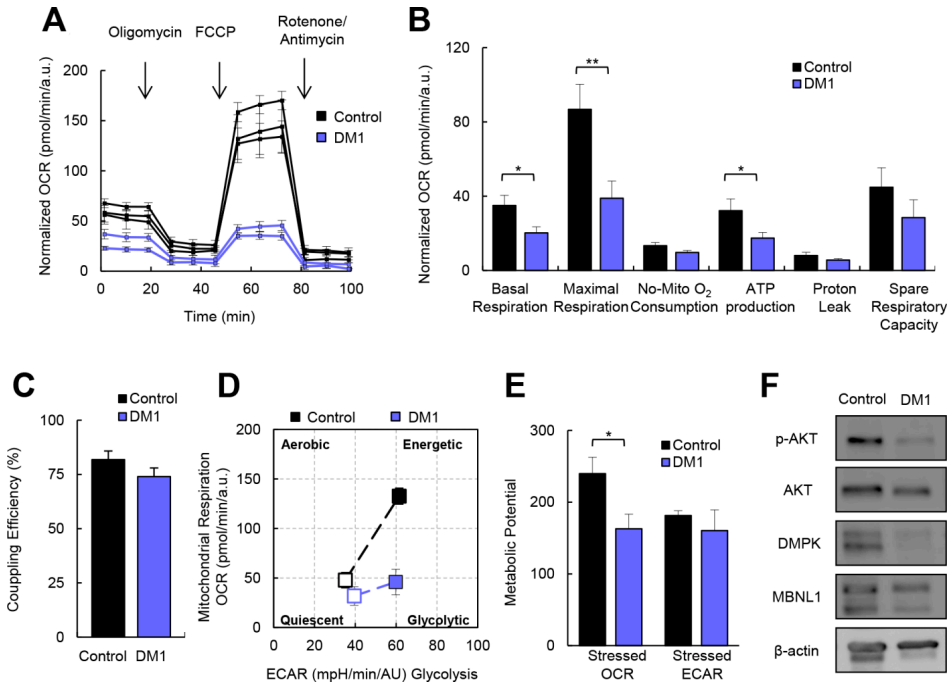

Figure 1. DM1-derived fibroblasts present impaired metabolism. (**A**) Kinetic normalized OCR response in DM1 and control fibroblasts in basal conditions and after consecutive addition of Oligomycin 1.5 μM, FCCP 1.5 μM and Antimycin-A/Rotenone 1.5 μM. A representative experiment out of 3 is shown with 3 independent control cultures and 2 DM1. (**B**, **C**) Quantification of mitochondrial respiratory functions and coupling efficiency in DM1 (n=7) and control fibroblasts (n=3). (**D**) Representative energy map and (**E**) Quantification of metabolic potential of DM1 and control fibroblasts. *Stressed* indicates the values of OCR and ECAR after the injection of oligomycin and FCCP simultaneously. Results are obtained from controls (n=3) and DM1 (n=5) cultures. (**F**) Representative immunoblots of phospho-AKT, AKT, DMPK and MBNL1 in DM1-derived fibroblasts and healthy controls (n=3).

results. Indeed, no differences were detected in the mitochondrial content in DM1 and control cells (Figure 3C). Moreover, the mitochondrial membrane potential remained elevated in DM1 cells (Figure 3D). However, the expression of the mitochondrial transcription factor A (*TFAM*) gene that participates in the regulation of the mitochondrial genome [18], was reduced by 50% in DM1 fibroblasts (Figure 3E). These results suggest that the impaired cellular bioenergetics were not related to substantial alterations in mitochondrial biogenesis and content.

Mitochondria are organelles with high dynamic plasticity to rapidly adapt in response to stress situations. Mitochondrial dynamic is regulated by a machinery of pro-fusion and -fission proteins, which constitutes an important part of the mitochondria quality control as it facilitates the elimination of damaged mitochondria by mitochondrial selective autophagy (mitophagy) [19]. We studied the expression of *OPA1*, *MFN1* and *MFN2* fusion related genes, *DRP1* fission related gene, and *PARKIN*, which is involved in mitophagy [20]. Interestingly, the levels of *OPA1*, *MFN2*, *DRP1,* and *PARKIN* were decreased in DM1-derived fibroblasts (Figure 3F). Overall, DM1-derived fibroblasts show mitochondrial dysfunction.

DM1-derived blood samples show mitochondria dysfunction

Next, we investigated whether these results could be translated to the clinical setting. Therefore, we measured the expression levels of several of the aforementioned genes in PBMCs from a cohort of patients with DM1 established in Guipuzcoa (Basque Country, Spain) [21]. Interestingly, we found lower expression levels of *SIRT1*, a key metabolic sensor that modulates a large variety of cellular processes such as energy metabolism stress response and aging [22], *OPA1* and *TFAM* (Figure 4), further supporting

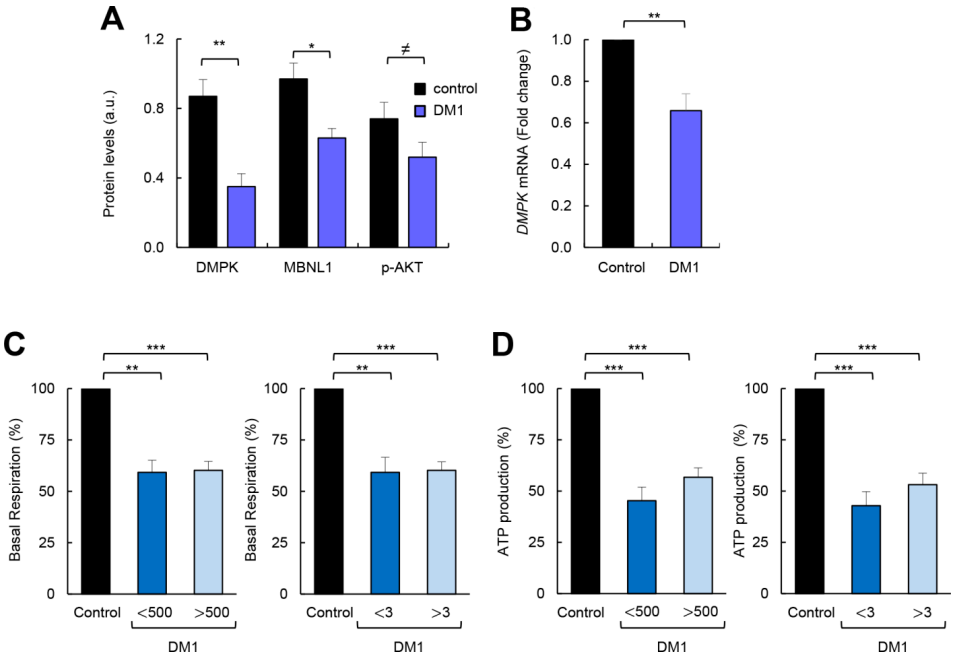

Figure 2. Correlation between impaired metabolism and markers of disease pathophysiology. (**A**) Quantification of protein levels shown in Figure 1F (n=4). (**B**) mRNA levels of *DMPK* in DM1 fibroblasts (n=7) and controls (n=3). (**C**) Basal respiration levels in controls (n=3) and DM1 fibroblasts stratified by CTG expansion in <500 (n=4) and >500 (n=3) (left) and MIRS scale in <3 (n=2) and >3 n=5 (right). (**D**) ATP production levels using the same stratification.

Table 1. Characteristics of human primary fibroblasts.

Fibroblasts	Status	Gender	Age biopsy (years)	MIRS	CTG (n) in blood	Age diagnosis (years)
C1	Control	M	49			
C2	Control	F	48			
C3	Control	Unknown	53			
DM1-1	DM1 patient	M	71	3	167	53
DM1-2	DM1 patient	F	45	2	333	41
DM1-3	DM1 patient	F	59	3	333	26
DM1-4	DM1 patient	F	44	2	833	27
DM1-5	DM1 patient	M	56	3	1333	20
DM1-6	DM1 patient	F	34	4	1650	12
DM1-7	DM1 patient	M	50	5	233	20

MIRS: Muscle impairment rating scale; M: male; F: female.

the relevance of the results obtained in cell culture and highlighting the importance of metabolism and mitochondria for the disease.

DM1-derived fibroblasts present accumulation of ROS and p38MAPK activation

Production of ROS is enhanced in several pathological conditions in which the respiratory chain is impaired [23]. Therefore, we measured ROS production and found a 50% increase in total ROS in DM1 cells compared to controls (Figure 5A). Similar results were obtained when specific ROS produced by the mitochondria were measured in DM1 fibroblasts and compared to controls (Figure 5B). Further, the expression of glutathione peroxidase 1 (*GPX1*) antioxidant gene was decreased by 50% in DM1 cells (Figure 5C). In agreement with metabolic studies, we

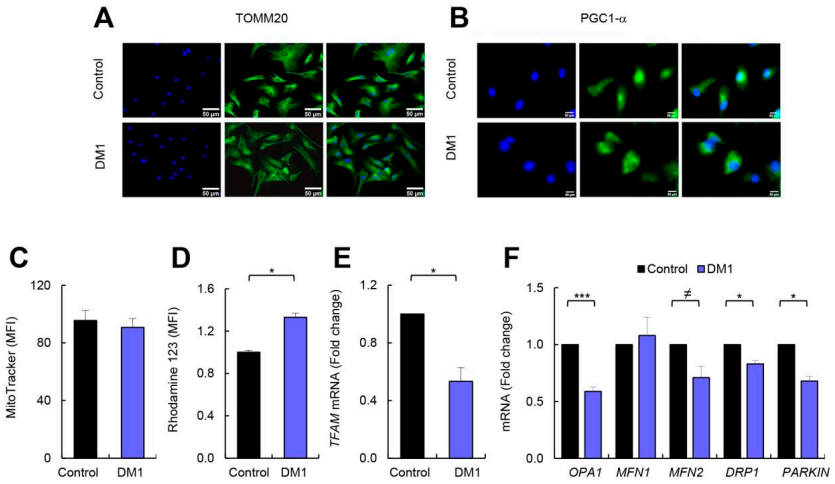

Figure 3. DM1-derived fibroblasts have no changes in mitochondria biogenesis. (**A**, **B**) Representative images of immunofluorescence of TOMM20, and PGC1-α in DM1 and control fibroblasts (n=3). (**C**) Medium fluorescence intensity of MitoTracker Red FM in control (n=3) and DM1 cells (n=5) and (**D**) of Rhodamine 123 in DM1 and control fibroblasts (n=3). (**E**) mRNA levels of *TFAM* transcription factor (n=3). (**F**) mRNA levels of *OPA1*, *MFN1*, *MFN2*, *DRP1* and *PARKIN* in DM1 and control fibroblasts (n≥2).

did not observe differences in ROS accumulation when fibroblasts were divided based on the number of CTG repeats and MIRS score (Figure 5D, 5E).

p38MAPK is a stress-activated protein kinase, which accumulates with aging and it is activated by the presence of ROS [24, 25]. Consequently, we measured the total levels of p38MAPK and its phosphorylated form (P-p38MAPK) and found an over 2-fold increase in the levels of P-p38MAPK in DM1-derived fibroblasts compared to control cells (Figure 5F, 5G). In summary, DM1 fibroblasts display increased ROS production, which is associated with an enhanced activation of p38MAPK.

Metformin restores metabolism and mitochondria activity

Metformin is a first-line anti-diabetic agent that functions mainly through the suppression of glucose production and alleviation of insulin resistance and has recently been shown to improve mitochondrial respiratory activity [26, 27]. We examined whether metformin could improve the impaired OXPHOS activity in patients with DM1. To test this idea, we treated DM1 and control fibroblasts with 1 μM of metformin for 72 hours and evaluated cellular metabolism and mitochondrial activity. Interestingly, metformin improved the basal oxygen consumption rate and maximal respiration of DM1 fibroblasts by more than twice (Figure 6A–6C), which resulted in an increased ATP production via OXPHOS (Figure 6A, 6D). Moreover, it increased the levels of *OPA1*, *MFN2*, *DRP1* and *TFAM* in DM1 cells by at least 1.5-fold (Figure 6E).

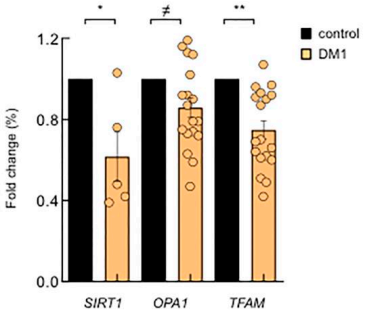

Figure 4. DM1-derived blood samples also show mitochondria dysfunction. mRNA levels of *SIRT1, OPA1* and *TFAM* in PBMCs derived from DM1 (n≥12) and controls (n=4).

Next, we measured ROS production and found that treatment with metformin significantly decreased ROS production in control as well as in DM1 fibroblasts (Figure 6F). In accordance, the levels of *GPX1* and *PARKIN* were elevated in the presence of metformin in DM1 cells (Figure 6G). The levels of *SIRT1*, a critical downstream target, were also induced in DM1 cells cultured in the presence of metformin validating the effect of metformin in metabolic pathways (Figure 6G). In summary, metformin restores the impaired metabolism and mitochondrial activity in DM1 fibroblasts.

Metformin restores additional DM1-associated phenotypes

Metformin exerts a potent anti-aging activity, in part by increasing proliferation and inhibiting senescence [28, 29]. It has been previously reported that DM1 fibroblasts display decreased cell proliferation and enhanced senescence accumulation [8, 30]. Next, we investigated the impact of metformin in the proliferative potential of DM1 fibroblasts. For this, we treated DM1 and control fibroblasts with 1 and 10 μM of metformin and measured cell viability. As expected, DM1 cells had lower viability than controls but, importantly, the treatment increased significantly the viability of DM1 fibroblasts, reaching almost the levels of control cells (Figure 7A and Supplementary Figure 4). Moreover, we measured the number of cells positive for phospho-Histone H3 (p-H3) and Ki-67, which are well-established markers of mitosis and cell division, respectively, and found reduced numbers in both markers in DM1 cells (Figure 7B–7E). Importantly, metformin increased the number of p-H3 and Ki-67 positive cells by almost 3-fold in DM1 cells (Figure 7C, 7E). These functional results were further validated at the molecular level. Metformin modulated the expression of critical genes involved in cell proliferation and cell cycle activity such as *BMI-1*, $p16^{INK4a}$ and $p21^{CIP}$. In particular, treatment for 72 h increased the levels of *BMI-1,* and partially decreased the levels of $p16^{INK4a}$ and $p21^{CIP}$ cell cycle inhibitors (Figure 7F). Finally, we also detected that metformin restored by 1.5-fold the levels of *DMPK* and *MBNL1* (Figure 7G). Thus, metformin rescues multiple phenotypes associated to DM1 cells.

DISCUSSION

We established primary cultures of fibroblasts derived from patients with DM1 and found that they display impaired metabolism and mitochondrial dysfunction. In particular, DM1 fibroblasts present lower production of ATP by OXPHOS, less efficient mitochondrial electron transport chain, impaired mitochondrial dynamics, and

higher production of ROS compared with healthy control-derived fibroblasts. Interestingly, some of these defects, such as energy homeostasis and mitochondrial dynamics, were also detected in PBMCs from patients with DM1, together revealing the impact of metabolism and mitochondrial function on the pathophysiology of DM1.

These results show that fibroblasts, which are a well-established model for cell aging studies *in vitro* [31], might be a good cellular model to characterize the pathophysiology of the disease, as they resemble multiple molecular and cellular phenotypes of the disease. However, we did not detect a correlation between the severity of the phenotypes and the number of CTG repeats. This result might be potentially biased by methodological reasons because CTG expansion was measured several years before isolation of skin fibroblasts and blood samples. In addition, some experiments were performed at early passage.

Our results reveal novel processes involved in the pathophysiology of the disease. Indeed, the role of mitochondria in DM1 remained practically unknown. A previous study observed an inverse correlation between the expression of CoQ10, an electron carrier in the mitochondrial respiratory chain, and lactate production with CTG expansion in PBMC samples [14], whereas mitochondrial dysfunction was suggested to occur in muscles of patients with DM1 as well [32]. The results of these studies are in line with our data and are indicative of mitochondrial dysfunction in DM1. The lower mitochondrial efficiency detected in our study could be due to the conversion of pyruvate, generated

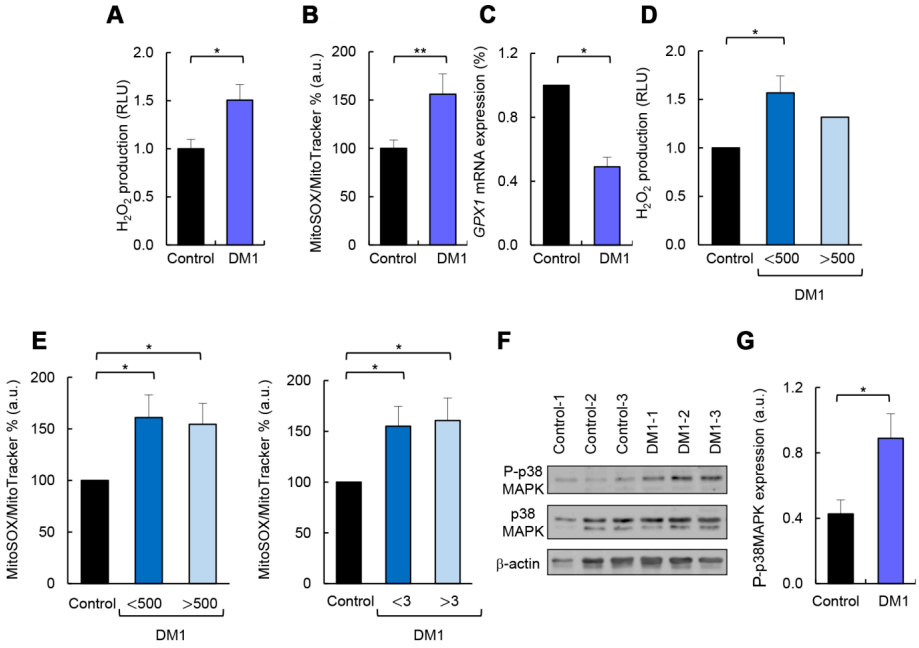

Figure 5. DM1-derived fibroblasts present accumulation of ROS and p38MAPK activation. (**A**) Luminescence signal proportional to H_2O_2 production in DM1 (n=4) and control fibroblasts (n=3). (**B**) Medium fluorescence intensity of MitoSOX+ values normalized to mean fluorescence of MitoTracker values in controls (n=3) and DM1 (n=5). (**C**) *GPX1* mRNA levels in DM1 and control fibroblasts (n≥2). (**D**) Luminescence signal proportional to H_2O_2 production in controls (n=3) and DM1 fibroblasts stratified by CTG expansion in <500 CTG (n=3) and >500 CTG (n=1). (**E**) Medium fluorescence intensity of MitoTracker Red FM in controls (n=3) and DM1 stratified by CTG expansion in <500 (n=3) and >500 (n=2) (left) and MIRS scale in <3 (n=2) and >3 (n=3) (right). (**F**, **G**) Representative immunoblot and quantification of P-p38MAPK and p38MAPK protein levels in DM1 and control fibroblasts (n=3).

during glycolysis, to lactate instead of acetyl CoA, which is transported to mitochondria and enters into the Krebs cycle. Moreover, our results show that mitochondrial biogenesis seems to be normal in DM1 cells, but they are not able to maintain the metabolic state as a consequence of unbalance remodeling of mitochondrial network morphology, which is not correctly controlled by the machinery of pro-fusion and fission proteins, and impaired elimination through mitophagy.

DM1 patients develop a large variety of symptoms in multiple systems that strongly resemble the clinical signs of accelerated aging, including some related to metabolism and mitochondria dysfunction such as insulin resistance, glucose intolerance, hyperinsulinemia, and increased risk of type 2 diabetes [33, 34]. Our results shed light in the underlying molecular mechanisms of these symptoms. Given that mitochondria is the main energy hub of the cell and the main intracellular source of ROS, our results might be extended to additional DM1 symptoms, particularly in the muscle, since a shift in energy production anticipates muscle atrophy with aging. Finally, our results further support the link between DM1 and accelerated aging [8], since cellular metabolism and mitochondrial dysfunction are critical mechanisms in aging.

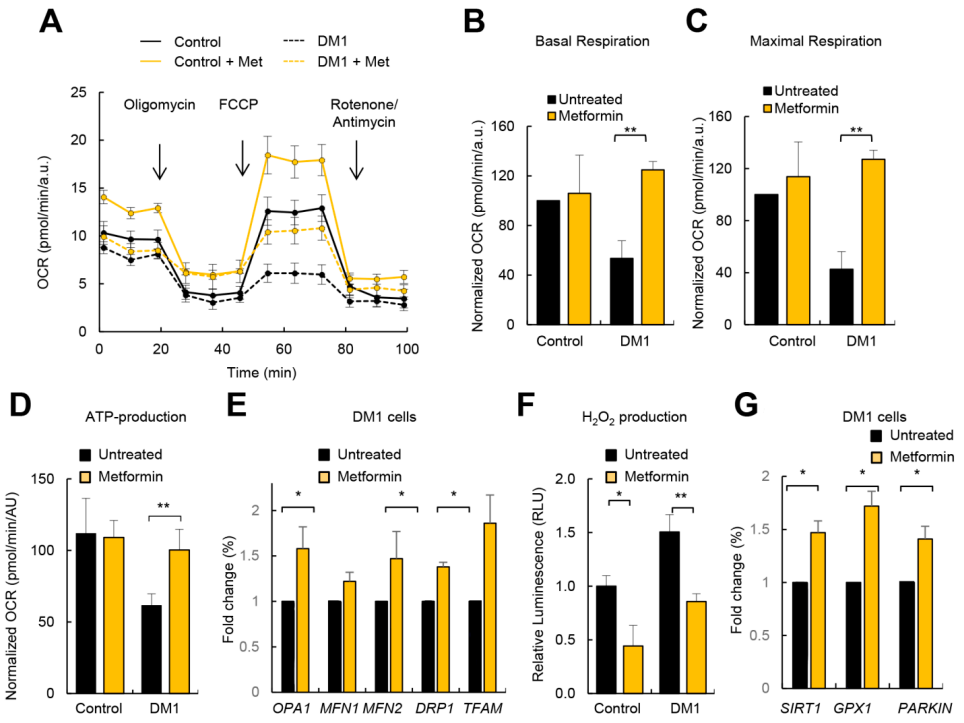

Figure 6. Metformin restores OXPHOS activity and ROS production in DM1 fibroblasts. (**A**) Representative kinetic normalized OCR response in DM1 (n=6) and control fibroblasts (n=3) after treatment with 1 μM of metformin for 72 h. DM1 and control fibroblasts were plated at 5.000 cells/well 24 hours prior to the assay. A representative experiment out of 3 is shown. (**B**–**D**) Quantification of mitochondrial basal respiration, maximal respiration, and ATP production respectively after treatment with 1 μM of metformin for 72 h of controls (n=3) and DM1 fibroblasts (n=6). (**E**) mRNA levels of *OPA1*, *MFN1*, *MFN2*, *DRP1* and *TFAM* after treatment with 1 μM of metformin for 72 h (n≥2). (**F**) H_2O_2 production after treatment with 1 μM of metformin for 72 h (n=3). (**G**) mRNA levels of *SIRT1*, *GPX1* and *PARKIN* in DM1 and control fibroblasts after treatment with 1 μM of metformin 72 h (n≥2).

DM1 is a rare, clinically variable disease with no currently available treatment to slow or stop disease progression. Supportive treatments, preventive measures and clinical surveillance are the only options available for patients with DM1 [35]. Metformin is a synthetic biguanide that is currently one of the most recommended medications for type 2 diabetes treatment around the world. Interestingly, studies in both vertebrates and invertebrates have shown that metformin delays aging and increases longevity [29]. Moreover, a meta-analysis has suggested that metformin reduces all-cause mortality and aging-related diseases in humans independent of its effect on diabetes [36]. We show here that metformin improves ATP production by OXPHOS and decreases the production of ROS in DM1 cells even at a much lower concentration compared to its current therapeutic dose (1 µM *vs* 75 µM). In line with our results, it has been recently shown that mitochondria might be a target of metformin [27]. We also found that metformin treatment reverses additional DM1-related phenotypes such as impaired proliferation, suggesting that its mechanism of action in DM1 is wider. In support, low doses of metformin may also correct several alternative splicing defects in DM1 myoblasts *in vitro* [37], the use of metformin reduced the risk of cancer in patients with DM1 having diabetes [38], and also improved mobility of DM1 patients in a small randomized clinical trial [39]. If the hypothesis of an accelerated aging in patients with DM1 is validated, our results could be added to the potential benefits of expanding metformin use in DM1, outside of the management of T2D, to include cancer prevention [38] and also phenotypes associated with aging. In summary, our results showed the efficacy of metformin in a pre-clinical setting and suggest that it warrants further assessment as a candidate drug for DM1 treatment.

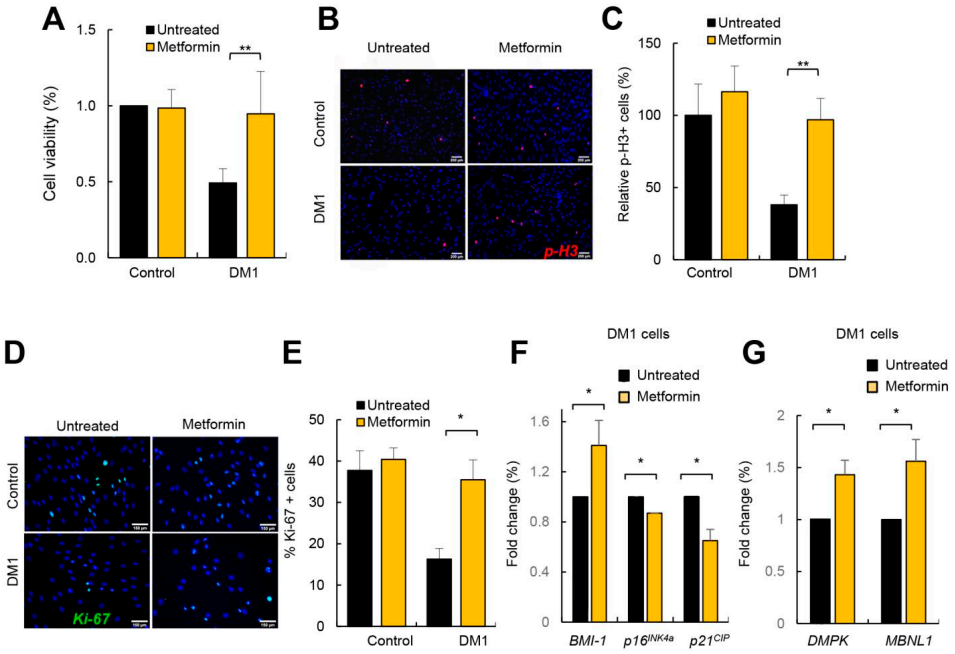

Figure 7. Metformin restores cell viability and proliferation in DM1 fibroblasts. (**A**) Cell viability of DM1 (n=5) and control (n=3) fibroblasts measured in MTT studies after treatment with 1 µM of metformin for 72 h. (**B**, **C**) Representative image and quantification of p-H3 (Ser10) staining in the same conditions in controls (n=3) and DM1 (n=7). (**D**, **E**) Representative image of Ki-67 staining and quantification in controls (n=2) and DM1 cells (n=3). (**F**) mRNA levels of *BMI-1*, *p16*^{INK4a} and *p21*^{CIP} in cells treated or not with 1 µM of metformin for 72 h (n≥2). (**G**) mRNA levels of *DMPK* and *MBNL1* in the same conditions (n=3).

MATERIALS AND METHODS

Study approval

This study was approved by the Donostia University Hospital Ethical Board (approval number 15-57) and was conducted in accordance with the Declaration of Helsinki's ethical standards. All subjects gave written informed consent before sample donation.

Reagents and cell culture

For the isolation of primary fibroblasts, punch skin biopsies were chopped into 2–3 mm^3 fragments and placed on a surface moistened with modified Eagle's medium containing 13% newborn calf serum, 0.4% penicillin/streptomycin (Gibco, Waltham, MA, USA) and 2 mM L-glutamine (Gibco). Flasks were incubated vertically for 3–6 hours at 37 °C in a 5% CO_2 atmosphere and then returned to the horizontal position. Human fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich, St Louis, MO, USA), 1% L-glutamine (Gibco) and 1% penicillin/streptomycin (Gibco). 7 independent cultures from different patients with DM1 and 3 from healthy controls were established (see Table 1 for patient characteristics). When indicated, fibroblasts were treated with metformin (Sigma-Aldrich) for 72 hours. Experiments were performed in early passage cultures (range of 5 to 10 passages).

Metabolic measurements

Measurement of OCR and ECAR were performed in XF96 plates with XF Extracellular Flux Analyzer (Seahorse Bioscience). Fibroblasts were seeded in collagen (BD Biosciences) coated XF 96-well plates (Seahorse/Agilent) in octuplicates at $1.2x10^4$ cells/well in 100 µl of growth medium. Mitochondrial activity was evaluated using the *Seahorse XF Cell Mito stress Test Kit,* according to manufacturer's instructions (Agilent). In the metformin treatment experiments, cells were plated at $5x10^3$ cells/well 24–28 hours prior to the assay. Oligomycin (75351, Sigma-Aldrich), FCCP (C2920, Sigma-Aldrich), and Rotenone/Antimycin A (R8875 and A8674, Sigma-Aldrich) were used at 1.5 µM concentration, after a titration experiment. Glycolytic activity was evaluated using the *XF Glycolysis Stress Test* according to manufacturer's instructions (Agilent). Glucose (G8769, Sigma-Aldrich) was used at 10 mM, oligomycin at 1 µM and 2-D-Deoxy-Glucose at 50 mM (D6134, Sigma-Aldrich). Cell content was normalized using crystal violet. The post-normalization values of OCR and ECAR reflect both the metabolic activities of the cells and the number of cells being measured. Data were further processed according to manufacturer's instructions.

Total ROS measurement

A total of $1x10^3$ fibroblasts were plated in 96-well plates and grown for 3 days. Afterwards, *ROS-Glo H_2O_2 Assay* (G8820, Promega) was performed according to the manufacturer's instructions. Briefly, a H_2O_2 substrate reacts directly with H_2O_2 to generate a luciferin precursor and, upon addition of a detection reagent, this precursor is converted to luciferin, which generates a luminescent signal that is proportional to the H_2O_2 concentration. White flat bottom plates (Corning) were used for final readout in a PHERAstar (BMG Labtech) luminometer plate reader.

Mitochondrial ROS production and mitochondrial content measurement

Mitochondrial ROS analysis was performed using the dye *MitoSOX* (M36008, Invitrogen). Mitochondrial content was assayed using the dye *MitoTracker FM* (M22425, Invitrogen), which passively diffuses across the plasma membrane and accumulates in active mitochondria.

$20x10^4$ fibroblasts per condition were grown for two days, reaching 70% confluence in p100 plates. Cells were detached using trypsin for 5 min at 37 °C. For MitoSOX staining, cells were washed once using warm HBSS, incubated with 5 µM of MitoSOX in HBSS for 30 min at 37 °C, washed 3x using warm HBSS and suspended in HBSS. For MitoTracker staining, cells were washed with PBS, incubated with 0.2 µM MitoTracker for 30 min at 37 °C, washed 3x using warm PBS and suspended in PBS. Cells were directly analyzed via flow cytometry. In FSC and SSC, we first gated the population; next, two gates were set on SSC-A vs. SSC-H and SSC-A vs. SSC-W to exclude doublets. Based on an unstained control, MitoSOX+ and MitoTracker+ gates were set. Mean fluorescence of MitoSOX+ was normalized as a mean fluorescence of MitoTracker values, which represents ROS production per mitochondria. Antimycin was used as a positive control and FCCP as a negative control.

Mitochondrial membrane potential measurement

$20x10^4$ fibroblasts per condition were grown for 2 days, reaching 70% confluence in p100 plates. Cells were detached using trypsin for 5 min at 37 °C. We used 1 µM of *Rhodamine 123* (Invitrogen) for 15 min at 37 °C to measure the mitochondrial membrane potential. This probe is readily sequestered by functioning

mitochondria and is easily washed out of cells once the mitochondria experience a loss in membrane potential.

Cell viability

Fibroblasts were seeded in 96-well plates followed by treatment with metformin for 72 h. Viable cells were quantified using the modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) assay in six replicates per condition.

mRNA expression analysis

Total RNA was extracted using TRIzol (Life Technologies). Reverse transcription was performed using random priming and the *Maxima First Strand cDNA Synthesis Kit* for RT-qPCR, with dsDNase (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's guidelines. Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 10 mM of each primer and 20 ng of cDNA, in a CFX384 thermocycler (Bio-Rad, Hercules, CA, USA). Primer sequences will be given upon request. Variations in RNA input were corrected by analyzing the expression of *GAPDH* as a housekeeping gene. The ΔΔCT method was used for relative quantification.

Western blot and immunofluorescence analysis

Immunoblot and immunofluorescence assays were performed following standard procedures, as previously described [40]. Primary antibodies were: phospho Histone H3 Ser10 (ab14955, Abcam), TOMM20 (11802-1-AP, Proteintech), PGC1-α (NBP1-04676, Novus Biologicals), phospho p38MAPK Thr180/Tyr182 (9211, Cell Signaling), p38MAPK (sc-7972, Santa Cruz Biotechnology) AKT1/2/3 (sc-8312, Santa Cruz Biotechnology), phospho AKT Ser 473 (9271, Cell Signaling), DMPK (sc-134319, Santa Cruz Biotechnology), MBNL1 (ab45899, Abcam) and β-actin (AC-15, Sigma-Aldrich). For western blot detection of primary antibodies, we used HRP-linked antibodies (Santa Cruz Biotechnology) and the detection was performed by chemiluminescence using *Novex ECL Chemi Substrate* (Thermo Fisher). For immunofluorescence, nuclear DNA was stained with Hoechst (33342, Sigma-Aldrich).

Statistics

Data are presented as mean values ± S.E.M., with the number of experiments (n) in parentheses. Unless otherwise indicated, statistical significance (p-values) was calculated using the Student´s t-test. Asterisks (*, **, and ***) indicate statistical significance ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively).

AUTHOR CONTRIBUTIONS

MG-P, AS-A, and RF-T performed all the experiments on fibroblasts. MG-P analyzed the results and completed the figures. RF-T and ALM collected clinical data and samples from the patients. AM and ALM directed the project, contributed to data analysis, and wrote the manuscript.

ACKNOWLEDGMENTS

MG-P and AS-A are recipients of predoctoral fellowships from the University of the Basque Country (PIF 15/245) and Carlos III Institute (FI17/00250), respectively. The Authors would like to thank Miren Zulaica for technical help with fibroblasts isolation and culture, and the Cell Culture Platform of the Biodonostia Health Research Institute, Monica Zufiria and Maddalen Otaegi-Ugartemendia for their help with SeaHorse Experiments.

CONFLICTS OF INTEREST

The authors declare no competing financial interests.

FUNDING

This work was supported by grants from the Instituto Salud Carlos III and FEDER funds (CP16/00039, PI16/01580, PI17/01841) and Health department from Basque Country (2017 and 2018-2017222021).

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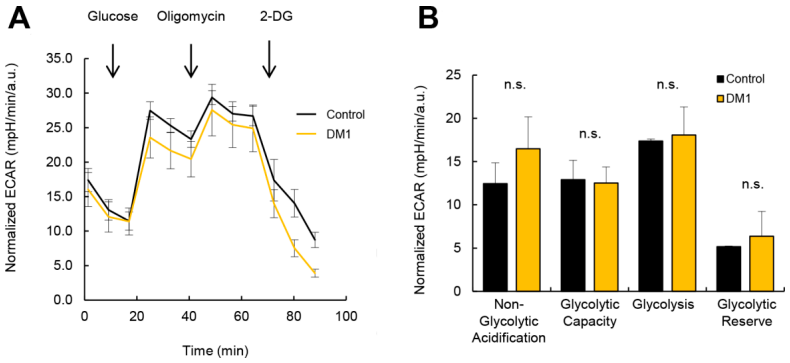
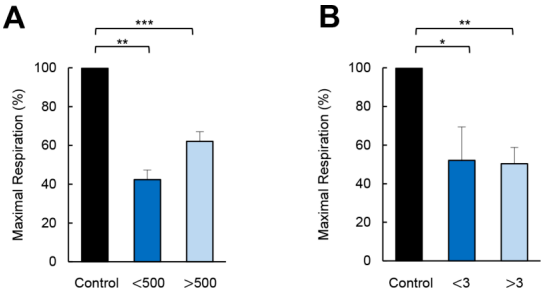
SUPPLEMENTARY MATERIAL

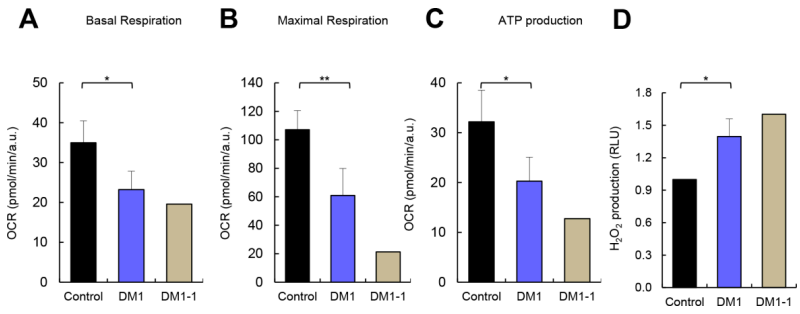
Supplementary Figures



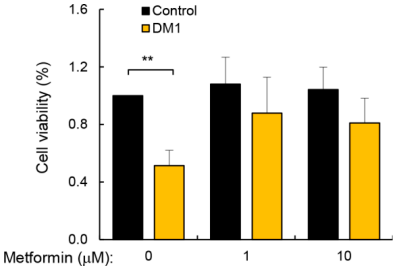
Supplementary Figure 1. DM1-derived fibroblasts have no changes in glycolysis. (**A**) Kinetic normalized ECAR response in DM1 and control fibroblasts obtained in basal conditions and after consecutive addition of Glucose 10 mM, Oligomycin 1 µM and 2-D-Deoxy-Glucose 50 mM. DM1 and control fibroblasts were plated at $5x10^3$ cells/well, in XF96 cell culture plates, 24–28 h prior to the assays. The assay medium was the substrate-free base medium supplemented with 2 mM glutamine. Upon completion of an assay, cells were normalized using violet crystal. A representative experiment is shown here (n=3 controls and n=4 DM1 patients). (**B**) Quantification of glycolytic functions in DM1 and control fibroblasts (n=3 controls and n=4 DM1 patients).



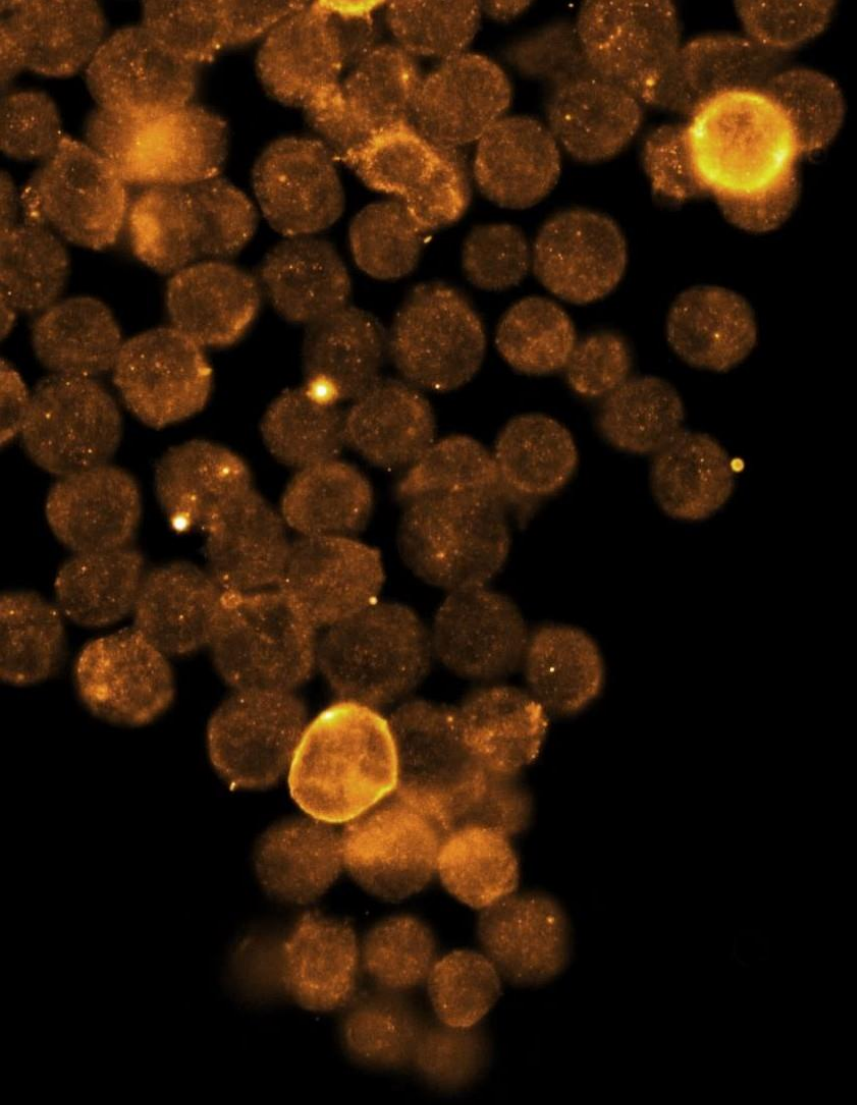
Supplementary Figure 2. No differences in maximal respiration of DM1 fibroblasts stratified by CTG amplification and MIRS scale. (**A**) Maximal respiration in fibroblasts derived from controls (n=3) and DM1 patients stratified by CTG expansion in <500 CTG (n=4) and >500 (n=3). (**B**) Maximal respiration in fibroblasts derived from controls (n=3) and DM1 patients stratified by MIRS scale in <3 (n=2) and >3 (n=5).



Supplementary Figure 3. Comparison of results in DM1 fibroblasts based on patient age. (**A**) Analysis of Basal respiration. (**B**) Maximal respiration, (**C**) ATP production and (**D**) H_2O_2 production in control fibroblasts (control; black) (n=3), in DM1-derived fibroblasts excluding a 71 years-old case (DM1; blue) (n=6), and cells derived from a 71 years-old patient (DM1; grey).



Supplementary Figure 4. Metformin restores cell viability in DM1-derived fibroblasts. Cell viability after treatment with 1 and 10 mM of metformin for 72 h. Figure shows results from controls (n=3) and DM1 (n=5) cells.



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