UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE MEDICINA

TESIS DOCTORAL

APLICACIÓN DE MEDICINA PERSONALIZADA EN PATOLOGÍA MITOCONDRIAL: TRATAMIENTO CON NUCLEÓSIDOS PIRIMIDÍNICOS EN EL DÉFICIT DE TIMIDINA QUINASA 2

PROGRAMA DE DOCTORADO EN INVESTIGACIÓN EN CIENCIAS MÉDICO-QUIRÚRGICAS

DEPARTAMENTO DE MEDICINA

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Madrid, a 1 de junio de 2020

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Aplicación de medicina personalizada en Medicina Mitocondrial: Tratamiento con nucleósidos pirimidínicos en el déficit de Timidina quinasa 2

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

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Aplicación de la medicina personalizada en patología mitocondrial:
Tratamiento con nucleósidos pirimidínicos en el déficit de timidina quinasa 2

y dirigida por: Miguel Ángel Martín Casanueva y Joaquín Arenas Barbero

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ÍNDICE

RESUMEN

Título: Aplicación de Medicina Personalizada en Patología Mitocondrial: tratamiento con nucleósidos pirimidínicos en el déficit de Timidina quinasa 2.

La timidina quinasa 2, codificada en el ADN nuclear, es una proteína implicada en la síntesis intramitocondrial de nucleótidos pirimidínicos (desoxitimidina monofosfato y desoxicitidina monofosfato), imprescindible en el mantenimiento del ADN mitocondrial (ADNmt). Mutaciones recesivas en el gen *TK2* causan depleción y deleciones múltiples del ADNmt que se manifiestan en forma de miopatía progresiva con edad de inicio y gravedad variable. Se desconocen las características clínicas y el pronóstico de las formas de inicio tardío (después de los 12 años), que son las menos frecuentes (con menos de 17 casos publicados en la literatura hasta el año 2019). No se dispone de ningún tratamiento para este trastorno y su mortalidad es muy alta; sólo un 27.3% de pacientes con la forma de inicio infantil sobreviven al menos 2 años después del comienzo de los síntomas.

Los objetivos de este trabajo, cuyos resultados se han publicado en los tres artículos incluidos en la tesis fueron: i) Describir la historia natural y gravedad de una serie de pacientes adultos con mutaciones en el gen *TK2*, ii) Evaluar la seguridad y eficacia de la administración de deoxinucleósidos como tratamiento del déficit de TK2 y iii) Analizar la utilidad de los niveles plasmáticos del factor de diferenciación de crecimiento 15 (GDF-15), una citoquina cuya expresión es

inducida por disfunción del sistema de fosforilación oxidativa mitocondrial, como posible biomarcador tanto del pronóstico como de la respuesta al tratamiento.

Los pacientes con déficit de TK2 de inicio tardío presentaban un fenotipo clínico homogéneo y reconocible caracterizado por una miopatía progresiva, con o sin ptosis y oftalmoparesia, con afectación selectiva, precoz, intensa y progresiva del músculo diafragma y por tanto con mal pronóstico clínico. El tratamiento con desoxinucleósidos consiguió una clara mejoría clínica que se mantiene con el tiempo, o al menos, una estabilización de la enfermedad, sin efectos tóxicos relevantes, en todas las formas clínicas. El beneficio obtenido es mayor en el grupo de pacientes más graves donde este tratamiento puede detener e incluso revertir la enfermedad. Bajo tratamiento, todos los pacientes sobrevivieron y en algunos casos recuperaron la capacidad para caminar e incluso a algunos se les pudo retirar la asistencia respiratoria. Aunque la respuesta es mayor cuando el tratamiento se inicia en la edad pediátrica, los pacientes adultos también experimentaron una mejoría significativa en las pruebas funcionales respiratorias y en las pruebas cronometradas de la marcha. Los niveles de GDF-15 mostraron correlación con la gravedad de la enfermedad; estaban incrementados 60 veces en las formas de inicio temprano y rápida progresión, y 6 veces en las formas de inicio tardío, respecto a controles. En todos los pacientes tratados, los niveles de GDF-15 se redujeron de manera sostenida a medida que los pacientes mejoraban, y llegaron a valores control tras varios meses de tratamiento.

En conclusión, los resultados de esta tesis han demostrado la eficacia y seguridad del tratamiento con desoxinucleósidos en todas las formas clínicas asociadas a déficit de TK2, y la utilidad de los niveles de GDF-15 para evaluar la gravedad de la enfermedad y monitorizar la respuesta al tratamiento. Basados

en estos resultados, una empresa farmacéutica ha diseñado un ensayo clínico actualmente en marcha, con el objetivo de conseguir la aprobación por parte de las agencias reguladoras del primer fármaco capaz de modificar la historia natural de una enfermedad mitocondrial.

ABSTRACT

Title: Personalized medicine in mitochondrial disorders: treating TK2 deficiency with pyrimidine deoxynucleosides.

Thymidine kinase 2, encoded by the nuclear gene TK2, is an enzyme involved in deoxynucleotides synthesis (deoxythymidine monophosphate and deoxycytidine monophosphate), which is required for mitochondrial DNA (mtDNA) maintenance. Autosomal recessive TK2 mutations cause both mtDNA depletion and multiple mtDNA deletions predominantly leading to progressive myopathy with variable age at onset and severity. Clinical characteristics and prognosis of the late-onset form of TK2 deficiency (age-at-onset after 12 years) are unknown, being the less frequent presentation with only 17 cases reported until 2019. Currently, treatment for this disorder is not available and the mortality rate is very high with only 27.3% of infantile forms cases surviving at least 2 years after onset.

The aims of the thesis, which results are reported in the three articles included in this thesis , were: i) To describe the natural history and severity by the evaluation of a series of adult patients with TK2 defect; ii) To evaluate the security and efficacy of deoxynucleosides as a treatment of TK2-deficiency, and iii) To assess the growth differentiation factor-15 (GDF-15) plasma levels, a cytokine which is induced by mitochondrial oxidative phosphorylation dysfunction, as a biomarker of both prognostic and response to treatment in TK2 patients.

Patients with late-onset TK2 deficiency showed a very homogeneous and recognizable phenotype characterized by progressive myopathy, with or without ptosis and ophthalmoparesis, with selective, early and severe diaphragmatic involvement and therefore with a poor prognosis. All the patients under treatment showed important beneficial effects that were maintained over time or, at least, showed a stabilization of the disease without relevant side effects. The response

to treatment was better in patients with early-onset and severe forms of the disorder, where deoxynucleosides could halt the progression or even reverse the disease. All the patients under treatment survive and some of them gained independent ambulation or even can be weaned off respiratory support. Although treatment was more beneficial for pediatric patients, late-onset TK2 patients had also significant improvement in respiratory parameters and 6MWT test. Plasma levels of GDF-15 correlated with disease severity; there were 60-fold increase over the value in the control group in early onset patients and 6-fold increase in late-onset TK2 deficiency. In all treated patients, GDF-15 decreased steadily with time in parallel of clinical improvements, reaching normal values after 12 months of treatment.

In conclusion, we demonstrate safety and efficacy of deoxynucleosides treatment in all clinical forms of TK2 deficiency. We also demonstrate utility of GDF-15 as a valuable biomarker of disease severity and response to treatment.

Based on these data, a clinical trial promoted by a pharmaceutical company has been designed to obtain approval for this new treatment which is capable for the first time of modifying the natural course of a mitochondrial disease.

INTRODUCCIÓN

Características generales de las enfermedades mitocondriales

Las **mitocondrias** son organelas esenciales, presentes en todas las células eucariotas, relacionadas fundamentalmente con el metabolismo energético. Su función más importante es la producción de energía en forma de adenosin trifosfato (ATP), a través de la cadena respiratoria (CR), por medio de la fosforilación oxidativa (OXPHOS). Están también implicadas en otras funciones biológicas como la apoptosis, la generación y detoxificación de las especies reactivas de oxígeno, la regulación del calcio intracelular y el metabolismo lipídico, entre otras (1).

Habitualmente, se consideran **enfermedades mitocondriales (EM)** sólo a aquellos trastornos que alteran la función de la CR o el sistema OXPHOS (2).

En su conjunto son unas de las enfermedades metabólicas hereditarias más comunes con una prevalencia estimada de 1/4300 individuos afectos (3).

La mitocondria tiene un origen endosimbiótico y retiene muchas de las características de la bacteria ancestral, incluyendo una doble membrana (interna y externa), material genético propio (ADN mitocondrial, ADNmt), su tamaño y su dinamismo (movimiento constante, y fusión y fisión mitocondrial (4)). En la membrana interna se localiza la CR que está formada por cuatro complejos enzimáticos (CI, CII, CIII y CIV) y dos transportadores móviles (coenzima Q_{10} y citocromo c), que catalizan la transferencia de electrones desde los equivalentes reductores (NADH y FADH2) que proceden del metabolismo intermediario hasta

el oxígeno molecular. Durante este proceso se genera en los CI, CIII y CIV un gradiente de protones cuya energía, llamada protón-motriz, es utilizada por el Complejo V o F_0-F_1-ATP sintasa para sintetizar ATP a partir de ADP y fosforo inorgánico en el proceso de fosforilación oxidativa.

El **ADNmt** humano es una molécula circular de 16,5 Kb multicopia, que codifica 13 subunidades estructurales de los complejos I, III, IV y V de la CR (el resto de subunidades es codificado por genes nucleares), y alguno de los genes necesarios para su traducción, es decir, 22 genes ARN de transferencia (ARNt) y 2 genes ribosómicos (ARNr). Todas las demás proteínas que se necesitan para el mantenimiento y la expresión del ADNmt están codificadas por el genoma nuclear (ADNn). Hasta la fecha se han descrito más de 250 genes nucleares mutados en enfermedades de la CR (5). Se han identificado genes con herencia autosómica dominante, autosómica recesiva y genes ligados al cromosoma X.

El ADNmt se hereda por vía materna, por tanto, la madre trasmite el genoma mitocondrial a todos sus hijos, pero solamente las hijas lo trasmiten a todos los miembros de sucesivas generaciones. La mayor parte de los tejidos contienen entre 1.000 y 10.000 mitocondrias por célula, con 2 a 10 moléculas de ADN por mitocondria (poliplasmia). Todas las moléculas de ADNmt son idénticas en tejidos sanos (homoplasmia); si aparecen dos poblaciones de ADNmt, normal y mutada (heteroplasmia), estas segregan al azar entre las células hijas durante la división celular (segregación mitótica) originando tres posibles genotipos diferentes: homoplásmico para el ADNmt normal, heteroplásmico y homoplásmico para el ADNmt mutado. Por ello, el fenotipo de una célula con heteroplasmia dependerá del porcentaje de ADNmt mutado que contenga y, por tanto, del grado de disfunción del sistema mitocondrial OXPHOS que produzca

(expresión umbral). Las manifestaciones clínicas dependerán de este nivel umbral crítico (6).

Cualquier órgano o tejido puede verse afectado por la disfunción de la CR, y en función de la carga mutacional en aquellos casos con mutaciones del ADNmt (porcentaje de heteroplasmia) y umbral patológico de cada uno, condicionar la aparición de síntomas clínicos. Los tejidos más dependientes de la fosforilación oxidativa y, por tanto, más sensibles a su disfunción serán los más frecuentemente afectados. De este modo, síntomas comunes en las EM serán, entre otros, ptosis, oftalmoplejia externa, retinitis pigmentosa, neuropatía óptica, hipoacusia, crisis epilépticas, ataxia, intolerancia al ejercicio o debilidad, diabetes, miocardiopatía, nefropatía, alteración de la conducción cardíaca y pseudoobstrucción intestinal.

El músculo es por tanto uno de los tejidos que se afecta con más frecuencia en las EM debido en parte a la mayor carga de heteroplasmia mutante que soporta y a sus altas demandas de energía (7).

Históricamente se han descrito diversos síndromes mitocondriales, definidos por una combinación más o menos constante de una serie de síntomas y signos clínicos como MELAS (encefalomiopatía mitocondrial con acidosis láctica y episodios similares a ictus), MERRF (epilepsia mioclónica con fibras rojo rasgadas) o SANDO (neuropatía sensorial atáxica, disartria y oftalmoparesia), entre muchos otros. Es bien conocida, sin embargo, la existencia de un gran solapamiento entre los distintos síndromes y la presencia de EM cuyos síntomas no permiten adscribirlos a un síndrome en particular (3). Además, existe gran heterogeneidad genética de forma que el mismo síndrome puede estar asociado con mutaciones en distintos genes. Por todo ello, en los

últimos años se prefiere describir y nombrar las EM en función de su defecto molecular subyacente.

Un ejemplo ilustrativo es la *oftalmoplejia externa crónica progresiva* (CPEO), uno de los trastornos más comunes asociado a alteraciones mitocondriales. Se caracteriza por ptosis palpebral y parálisis progresiva de los músculos oculares, fija, simétrica o asimétrica, puede presentarse formando parte de un síndrome mitocondrial (MNGIE o encefalomiopatía mitocondrial neurogastrointestinal, KSS o síndrome de Kearns-Sayre, entre otros), o bien ser la manifestación fundamental (CPEO pura), o estar asociada a otros síntomas (CPEO plus) como intolerancia al ejercicio, debilidad muscular, disfagia o disfonía, entre otros. Este defecto puede tener como base genética: a) deleción única de gran tamaño del mtDNA, que es un trastorno esporádico, b) mutaciones puntuales en el ADNmt de herencia materna, o c) mutaciones en genes nucleares de herencia mendeliana (dominante o recesiva), fundamentalmente *POLG,* que codifica la polimerasa gamma mitocondrial y *TWKN* que codifica la helicasa mitocondrial (8).

Clasificación de las enfermedades mitocondriales.

Por tanto, el correcto funcionamiento de la fosforilación oxidativa mitocondrial tiene un control genético dual, estando regulado tanto por el genoma mitocondrial como por el genoma nuclear. De este modo, el mecanismo a través del cual puede desarrollarse una disfunción primaria de la CR es múltiple y complejo, clasificándose las EM en función de éste en los siguientes grupos:

1) Trastornos por a mutaciones en el ADNmt, pudiendo ser de herencia materna o trastornos esporádicos.
2) Enfermedades debidas a mutaciones en el ADNn (con alteración directa o indirecta de la CR), de herencia mendeliana.
3) Defectos en genes nucleares implicados en el mantenimiento del ADNmt (alteración en la comunicación intergenómica).

Los trastornos asociados a mutaciones en el ADNn pueden dividirse a su vez en:

1) Mutaciones en genes que codifican subunidades de la cadena respiratoria.
2) Mutaciones en genes que codifican proteínas de ensamblaje.
3) Mutaciones en genes que regulan la composición lipídica de la membrana mitocondrial interna.
4) Mutaciones en genes relacionados con la dinámica mitocondrial.

Trastornos de la alteración en el mantenimiento y reparación del ADNmt.

Un importante subgrupo de enfermedades mitocondriales es el relacionado con defectos en el mantenimiento y reparación del ADNmt**.**

Desde el punto de vista molecular, se caracterizan por una disminución del número de copias de ADNmt (depleción del ADNmt) y/o por el acúmulo de mutaciones puntuales y deleciones múltiples en el ADNmt (9).

Por tanto, aunque el defecto genético ocurra sobre genes codificados por el ADNn, las manifestaciones clínicas son secundarias a su efecto sobre el ADNmt (depleción o deleciones múltiples).

La primera vez en la que se describió una reducción drástica en el número de copias del ADNmt como mecanismo responsable de una EM fue en el año 1991, por Moraes et al (10). En este trabajo describieron 4 pacientes con depleción de ADNmt en distintos tejidos y lo correlacionaron con la disfunción de la cadena respiratoria y la disminución de la síntesis de proteínas mitocondriales. El mecanismo molecular subyacente no fue descubierto hasta una década después cuando el grupo del Dr. Hirano identificó por primera vez mutaciones en el gen *TYMP* , que codifica para la enzima timidina fosforilasa, en pacientes con síndrome MNGIE (neuro-gastrointestinal-encefalo-miopatía) que también presentan depleción parcial de ADNmt así como deleciones múltiples en ADNmt (11). En este trabajo acuñan el término de alteración en la 'comunicación intergenómica' para definir trastornos cuyo origen son mutaciones en el ADNn que secundariamente afectan al ADNmt, bien disminuyendo su síntesis sin presentar alteraciones en su secuencia, o bien provocando el acúmulo de múltiples deleciones por fallo en su replicación. Posteriormente, en 2001, se identificaron mutaciones en el gen *TK2*, que codifica para la enzima timidina quinasa mitocondrial, implicada en la fosforilación de los nucleósidos pirimidínicos (desoxicitidina, dC y timidina, dT) y por tanto relacionada con el adecuado mantenimiento del "pool" de nucleótidos necesarios para la síntesis del ADNmt (12).

Además de los ya mencionados, se han identificado varios genes nucleares causantes de síndromes de depleción y/o deleciones múltiples en el ADNmt. Algunos de ellos codifican proteínas involucradas directamente en la replicación del ADNmt (*POLG, POLG2, TWINKLE, DNA2, MGME1 y RNasaH1*), otros intervienen en la homeostasis de los desoxiribonucleósidos trifosfato (dNTPs)

necesarios para la síntesis del ADNmt (*DGUOK, RRM2B;* además de *TYMP y TK2*), y un grupo de genes cuyo mecanismo patogénico es aún desconocido (*SUCLA2, SUCLG1, PEO2, MPV17, MFN2, OPA1, FBXL4, SPG7 y AFG3L2*)(13, 14).

Por tanto, un aporte constante y equilibrado de desoxinucleótidos trifosfato (dNTPs) es crucial para el mantenimiento de la integridad del ADNmt. La distribución de las enzimas implicadas en su metabolismo, específicas de tejido, explican la diversidad de las manifestaciones clínicas observadas (15).

La replicación del ADNmt tiene lugar de manera independiente a la replicación del ADNn y continúa tras la diferenciación de los tejidos, de manera independiente al ciclo celular (a diferencia de la replicación del ADNn que está limitada a la fase S de este)(16). Los precursores necesarios para la síntesis del ADNmt se obtienen a través de la *vía de rescate* intramitocondrial desde los desoxinucleósidos o bien a través de la importación de desoxinucleótidos sintetizados en el citoplasma en la vía de síntesis *de novo.* Esta vía implica la conversión de ribonucleótidos difosfato en desoxinucleótidos difosfato por la ribonucleótido reductasa (17). Cuando las células se están replicando, la elevada producción de dNTPs en el citosol a través de su síntesis *de novo* es también la principal fuente de dNTPs para la síntesis de ADNmt en las mitocondrias (18). En las células quiescentes, la síntesis *de novo* está muy reducida y sólo se mantiene en un grado muy limitado a expensas de la isoforma R1-p53R2 de la ribonucleótido reductasa, que está presente a lo largo de todo el ciclo celular (19). Debido a este discreto aporte de dNTPs desde el citoplasma, en las células postmitóticas la síntesis del ADNmt depende en gran medida de las *vías de*

rescate intramitocondriales de desoxiribonucleósidos. Estas vías *de rescate* se basan en la fosforilación secuencial de los desoxinucleósidos precursores (dNs) hasta los dNTPs (20), a través de las enzimas mitocondriales timidina quinasa 2 (TK2) y desoxiguanosina quinasa (dGK).

La **timidina quinasa 2** es una enzima localizada en la matriz mitocondrial codificada por el gen *TK2* (localizado en el brazo largo del cromosoma 16, OMIM *188250). Es la enzima limitante en la vía de rescate de los nucleótidos pirimidínicos en la matriz mitocondrial. Fosforila los nucleósidos desoxicitidina (dC) y desoxitimidina (dT) para generar desoxicitidina monofosfato (dCMP) y desoxitimidina monofosfato (dTMP), que al ser fosforilados nuevamente de forma secuencial, se convierten en nucleótidos trifosfato (dNTP) necesarios para la replicación y mantenimiento del ADNmt (21, 22). Las mutaciones en el gen *TK2,* que afectan a la actividad de la enzima timidina quinasa mitocondrial, producen un descenso de dCMP y dTMP, y en consecuencia un desequilibrio en el pool de desoxinucleótidos, que finalmente conducen a la depleción o deleciones múltiples en el ADNmt. Esta pérdida de integridad del ADNmt provoca como consecuencia una alteración en los niveles de expresión de las enzimas implicadas en la fosforilación oxidativa mitocondrial.

La enzima limitante de la síntesis de los nucleótidos purínicos es la **deoxiguanosina quinasa** (dGK), codificada por el gen *DGUOK,* que se encarga de fosforilar los nucleósidos desoxiguanina y desoxiadenina. Alteraciones en este gen también son responsables de un síndrome de depleción de ADNmt por alteración en el metabolismo de los dNTPs. Otros síndromes de depleción de ADNmt que comparten este mecanismo patogénico son los originados por

mutaciones en el gen *RRM2B* que codifica la ribonucleótido reductasa controlada por p53 (p53R2) (23).

Desde el punto de vista clínico, los síndromes de depleción/deleciones múltiples del ADNmt, son un grupo muy heterogéneo de trastornos de gravedad variable. La mayoría de estos trastornos son letales a edad temprana, aunque se han descrito formas clínicas más leves en jóvenes y adultos (9).

Algunas de las presentaciones clínicas más frecuentes son:

1. Oftalmoplejia crónica externa progresiva de inicio en la edad adulta, de transmisión dominante o recesiva, caracterizada desde el punto de vista molecular por la presencia de deleciones múltiples en el ADNmt.
2. Síndrome de MNGIE, definido por la asociación de oftalmoplejia crónica externa progresiva, polineuropatía y afectación intestinal grave, de herencia recesiva, con depleción parcial y deleciones múltiples en ADNmt.
3. Cuadros multisistémicos de edad de inicio variable y amplio espectro clínico de afectación. Incluye el síndrome SANDO (Ataxia sensitiva, neuropatía, disartria y oftalmoparesia), entre otros.
4. Cuadros fatales de inicio muy temprano y depleción profunda de ADNmt. Se distinguen tres grupos en función del tejido afectado; forma miopática, forma hepatocerebral y forma encefalomiopática.

Enfermedades mitocondriales debidas a mutaciones en el gen que codifica la timidina quinasa mitocondrial.

Mutaciones autosómicas recesivas en el gen *TK2* son responsables de la forma miopática del síndrome de depleción mitocondrial (**OMIM# 609560)**.

Las manifestaciones clínicas del déficit de timidina quinasa son fundamentalmente debilidad muscular, de curso y gravedad variable, que generalmente incluye afectación precoz de la musculatura respiratoria y deglutoria, lo que condiciona una muerte prematura. Fue descrito por primera vez por Saada et. al en el año 2001 (12), en cuatro niños con miopatía grave, elevación de la creatinquinasa (CK), déficit múltiple de actividad de los complejos de la CR y depleción del ADNmt.

Sin embargo, se han descrito formas menos graves, de inicio juvenil y progresión más lenta, con supervivencia más prolongada. Los síntomas cardinales son los mismos que en las formas graves (miopatía progresiva con insuficiencia respiratoria y disfagia), asociados a depleción con o sin presencia de deleciones múltiples en ADNmt (24-26).

Más recientemente se han descrito casos más leves de presentación tardía (a partir de la tercera, cuarta o quinta década de la vida) de curso clínicamente variable, asociados en este caso exclusivamente con deleciones múltiples en ADNmt (27-30).

En 2018, Garone C. et al (31) publicaron la revisión clínica de 92 casos (67 procedentes de la literatura médica y 25 casos nuevos) con el fin de estudiar y describir la historia natural de la enfermedad. Basándose en sus características

clínicas y moleculares, diferenciaron tres fenotipos, en función de la edad de inicio:

1) Miopatía de inicio durante el primer año de vida (42.4% de los casos): se caracteriza por la presencia de depleción grave del ADNmt, frecuente afectación de sistema nervioso central y curso rápidamente progresivo con supervivencia < a los 3 años de vida. El 94% de los pacientes pierden la capacidad de andar de forma independiente durante los primeros tres años de vida. La causa de fallecimiento en todos ellos es la insuficiencia respiratoria.
2) Miopatía de inicio entre el año de vida y los 12 años (40,2%): cursan con depleción de ADNmt, progresión clínica variable, (el 37% de los publicados en esta serie no pierde la capacidad de andar de manera independiente), y su supervivencia media es de 13 años, siendo la causa de muerte en los fallecidos la insuficiencia respiratoria.
3) Miopatía de inicio tardío (a partir de los 12 años) (17.4%): con debilidad muscular lentamente progresiva y evolución hacia la insuficiencia respiratoria, con supervivencia superior a los 23 años de media. Todos los pacientes de este grupo publicados en esta serie son ambulantes, pero hasta un 44.4% requieren ventilación mecánica no invasiva en el momento de la recogida de los datos.

Además de los síntomas musculares, se describen casos infantiles muy graves con afectación de sistema nervioso central (16%) y otras manifestaciones extramusculares poco frecuentes en todos los grupos de edad (cardiomiopatía, polineuropatía axonal sensitiva o hipoacusia en menos del 10% del total de casos). También se describe con relativa frecuencia la existencia de disfagia con

repercusión ponderal llegando a precisar, sobre todo en las formas de inicio más temprano, una sonda nasogástrica o una gastrostomía percutánea para asegurar una nutrición adecuada.

En otra publicación posterior, firmada por Wang et al. (32), cuyo objetivo también era ampliar el conocimiento sobre la historia natural de la enfermedad, además de los casos descritos en la literatura y ya incluidos en el trabajo de Garone, se describen 11 casos nuevos, de los cuáles sólo 3 son formas de inicio tardío, llegando a las mismas conclusiones que la publicación previa.

Por tanto, el déficit de timidina quinasa 2 que no se manifiesta hasta, por lo menos, después de los 12 años, es la forma clínica menos frecuente y conocida.

Hasta el año 2019, se habían descrito en la literatura únicamente 14 casos clínicos de estas características. En el año 2012, Tyynismaa et al (28) publica los primeros dos casos de inicio a lo largo de la quinta década de la vida. En ambos casos el síntoma que motiva la atención médica es una CPEO, asociada a debilidad muscular de predominio proximal y disfagia. Estos casos se consideran CPEOs plus, comparándolos con los cuadros clínicos producidos por otros genes relacionados con el mantenimiento del ADNmt como *POLG* o *TWINKLE*, donde las manifestaciones oculares pueden ser el síntoma fundamental (8), y son considerados fenotipos muy leves asociados a mutaciones en *TK2*. A partir de este momento se considera que el gen *TK2* puede manifestarse como CPEO y queda así categorizado como un fenotipo posible entre los relacionados con el déficit de la timidina quinasa 2, y lo diferencian de aquellos de inicio más temprano que se manifiestan fundamentalmente como miopatía progresiva, sin alteraciones oculares y que

son considerados mucho más graves. Sin embargo, llama la atención que ambos pacientes descritos en el articulo fallecen tempranamente y en ninguno consta la realización de estudios respiratorios. A pesar de ello se considera que la causa de muerte es ajena a las mutaciones identificadas en TK2 y que su fenotipo en relación con éstas es leve. En el año siguiente, Alston et al (29) publica el caso de una mujer de 74 años con una CPEO asociada a debilidad muscular que fallece por insuficiencia respiratoria secundaria y en este caso, la causa de la muerte sí es atribuida a la miopatía *TK2,* con un fenotipo similar al de formas de presentación más temprana.

Ese mismo año (2013), Paradas et al (26) publican otro caso de miopatía de inicio tardío y curso lentamente progresivo, que también se asocia a afectación de los músculos respiratorios, poniendo en evidencia que las formas menos graves de este trastorno están posiblemente infradiagnosticadas. En este caso, se alcanzó el diagnóstico sólo tras un estudio de secuenciación de exoma dado que el fenotipo clínico no estaba aún caracterizado con detalle para permitir un estudio genético dirigido.

A partir de esa fecha se continúa identificando nuevos casos en adultos con formas menos graves de la enfermedad, y se inicia el estudio de los factores que pueden condicionar la heterogeneidad del fenotipo. En este sentido, Cámara et. al (30) describen en 2015 siete nuevos casos con miopatía de presentación tardía y curso lentamente progresivo, donde analizan los niveles de ADNmt, la presencia de deleciones múltiples y, en dos de ellos, la actividad enzimática residual de la timidina quinasa, con el fin de determinar si alguno de estos factores puede explicar las diferencias clínicas observadas. Destaca que ningún paciente de este estudio presenta alteración significativa de los niveles de

ADNmt, es decir en todos ellos la cantidad de ADNmt es superior al 30% respecto a controles apareados por edad y tejido - 30% es el umbral por debajo del cual se considera que la reducción del número de copias es significativa (33) -, pero en todos ellos se demuestra la presencia de deleciones múltiples en el ADNmt, reflejando la alteración de la replicación del ADNmt. En los dos casos donde se midió la actividad enzimática de la timidina quinasa mitocondrial en cultivos de fibroblastos, se observaron actividades residuales del 3% y un 6% respectivamente, que son equiparables a los demostrados en casos con formas infantiles graves. Por tanto, demuestran que no existe una correlación entre los niveles de actividad enzimática y la gravedad o edad de inicio de la enfermedad.

El gen *TK2* está constituido por 10 exones y se han identificado más de 30 mutaciones patogénicas diferentes a todo lo largo del mismo. La mayoría (70%) son mutaciones *missense* aunque también se han descrito *nonsense*, mutaciones en sitios reguladores del splicing, deleciones e inserciones e incluso una deleción de 5.8 kb que incluye a los exones 1 y 2 del gen.

Hasta la fecha, no ha podido establecerse una correlación genotipo-fenotipo para la mayoría de las mutaciones identificadas. Se ha sugerido sin embargo que la mutación p.Arg130Trp parece asociarse al fenotipo más grave, con afectación del SNC (34) y que la mutación p.Lys202del en homocigosis se asocia a las formas más leves de presentación tardía, puesto que se ha encontrado sólo en pacientes con la forma adulta de la enfermedad (32).

Modelos preclínicos del síndrome de depleción secundario a mutaciones en gen TK2.

Se han desarrollado dos modelos de ratón para investigar el mecanismo molecular de la enfermedad, un modelo "knock-in" con una mutación frecuente en homocigosis (p.His121Asn) y un modelo "knock-out".

En el año 2008 se describe el primer modelo de ratón de la enfermedad (ratón "knock-in" con la mutación H126N en homocigosis del gen *TK2*) que manifiesta la forma clásica del déficit de timidina quinasa 2 (35). El ratón homocigoto mutante ($TK2^{-/-}$), tras nacer normal, comienza a presentar a partir del día 10 post nacimiento, retraso en el crecimiento, reducción de los movimientos espontáneos, temblor y alteración de la marcha con posterior desarrollo de debilidad rápidamente progresiva y muerte con dos semanas de vida. Los ratones $TK2^{-/-}$ presentan actividad enzimática TK2 reducida en todos los tejidos, siendo el más afectado el cerebro, con una actividad del 1.7% respecto a controles sanos. También es en cerebro donde demuestran la mayor reducción en el número de copias de ADNmt, con niveles residuales de ADNmt del 12.5% respecto a los controles sanos. Por tanto, aunque el modelo reproduzca los cambios moleculares y bioquímicos del déficit de TK2, en el ratón el fenotipo es fundamentalmente una encefalomielopatía a diferencia de la enfermedad en humanos donde el músculo esquelético es el tejido más afectado.

Ese mismo año se desarrolló un modelo "knock-out" de ratón por el grupo de Karlsson et. al (36), caracterizado por la ausencia completa de actividad TK2 y depleción de ADNmt en todos los tejidos. Su fenotipo, al igual que el del ratón

“knock-in”, se caracteriza por una grave alteración del sistema nervioso central que lleva a la muerte del ratón tras 2 a 4 semanas de vida.

Existe en el citoplasma celular una proteína con la misma actividad que la TK mitocondrial, conocida como TK1. El estudio del modelo “knock-in” de ratón H126N $TK2^{-/-}$ permitió identificar un descenso paulatino en su actividad entre los días 8 y 13 tras el nacimiento. El periodo de reducción de la actividad TK1 coincide con el inicio de la detección de depleción de ADNmt en cerebro y tejido cardiaco y el inicio del desarrollo de los primeros síntomas en este modelo (37). La disminución de la actividad TK1 parece por tanto desenmascarar en el ratón el déficit de TK2. Esta reducida actividad TK2 en los tejidos provoca una alteración en el “pool” de los trinucleótidos por disminución de dTMP y dCMP, dependientes de la fosforilación de dT y dC por TK2, y a consecuencia del descenso de los niveles de ADNmt provocados por esta alteración, un déficit de los complejos I, III, IV y V de la cadena respiratoria mitocondrial, que contienen proteínas codificadas por el ADNmt (35, 37).

Basándose en estos hallazgos se postula si la suplementación oral con dTMP y dCMP en el ratón conseguirá balancear el pool de los trinucleótidos y corregir por tanto la capacidad de síntesis del ADNmt. También se especula si las diferencias en la expresión de TK1 en los diferentes tejidos y la progresiva reducción de su actividad en tejidos postmitóticos podría explicar en parte la expresión fenotípica del déficit de timidina quinasa mitocondrial.

Tratamiento con nucleótidos y nucleósidos en modelos experimentales.

El tratamiento con los nucleótidos pirimidínicos (dCMP y dTMP) consigue rescatar el fenotipo retrasando el inicio de la enfermedad y prolongando la supervivencia del ratón.

El grupo de Dr. Hirano demostró en 2014 (38) que la administración de los desoxinucleotidos dCMP+dTMP por vía oral en un modelo "knock-in" de ratón retrasa el inicio de los síntomas, disminuye la gravedad del fenotipo y prolonga la supervivencia de manera dosis dependiente y sin efectos tóxicos aparentes. Además, observó que, sin tratamiento, el ratón fallece a los 13.2 ± 2.5 días, y que en tratamiento con 200 mg/kg/día de dCMP+dTMP sobrevive hasta el día 34.6 ± 3.2 y con dosis de 400 mg/kg/día hasta el día 44.3 ± 9.1 ($p= 0.0071$; $n=7$).

El tratamiento con dCMP+dTMP demuestra atravesar la barrera hematoencefálica, aumentar los niveles de dTTP en cerebro e hígado en el día 13, y corregir los niveles de ADNmt en distintos tejidos, así como la actividad de los complejos de la CR.

Por otro lado, en el ratón tratado con dCMP+dTMP se retrasa la disminución de la actividad TK1 demostrada previamente, sugiriendo que la administración de estos nucleótidos podría estar aumentando la actividad timidina quinasa citoplasmática, compensando parcialmente el déficit de TK2.

El aumento de la síntesis de dTTP y dCTP en el citosol, gracias al incremento en la actividad TK1, a partir de los precursores administrados por vía oral en el ratón, serían transportados a la mitocondria a través de los receptores PNC1 (39).

Sin embargo, el análisis de los niveles plasmáticos de dTMP+dCMP y sus metabolitos muestra que 30 minutos después de su administración oral, no se consiguen detectar niveles plasmáticos de dTMP ni dCMP y sí niveles elevados de dT y desoxiuridina (dU) (procedente de la desaminación de la dC en el hígado). Esto plantea la posibilidad de que las moléculas activas sean en realidad los nucleósidos dC y dT , y no los nucleótidos monofosfato.

Para comprobar esta hipótesis, realizaron un estudio sobre mitocondrias aisladas de corazón de rata tratadas con dT y dTMP marcados radiactivamente, demostrando que la mayoría de la síntesis del TTP mitocondrial se producía a partir del dT y no del dTMP (40).

Por otro lado, el grupo de Cámara et. al demuestra en modelos *in vitro* (cultivo de células con déficit de desoxiguanosina quinasa y cultivo de células con depleción de ADNmt inducido por timidina) que los suplementos con los desoxinucleósidos involucrados en el déficit bioquímico subyacente previene la reducción del número de copias de ADNmt (41). En estos experimentos se obtiene el mismo efecto aumentando la biodisponibilidad de los nucleósidos al inhibir las enzimas implicadas en su catabolismo (tetrahidrouridina como inhibidor de la citidina deaminasa e inmunocilina H como inhibidor de la fosforilasa de nucleósidos de purina).

En un estudio in vivo se demuestra que son los nucleósidos, y no los nucleótidos monofosfato, las moléculas con el efecto biológico, siendo estos profármacos de los primeros. (42).

Para demostrar in vivo que el efecto bioquímico y clínico observado en los ratones knock-in H126N TK2$^{-/-}$ es debido a la acción de los nucleósidos y no de los nucleótidos, Lopez-Gomez C. et al administraron dT+dC a dosis equimolares de las administradas de dTMP+dCMP en los experimentos anteriores, obteniendo resultados equivalentes (42). Sin embargo, observaron que el tratamiento con los nucleótidos monofosfatos es discretamente más eficiente puesto que se requieren mayores dosis de dT+dC para obtener la misma prolongación de la supervivencia que con el tratamiento con dTMP+dCMP. Así, el tratamiento con dosis de 260mg/kg/día de dT+dC consigue prolongar la supervivencia el mismo número de días que el tratamiento con 200mg/kg/día de dTMP+dCMP mientras que son necesarias dosis de 520mg/kg/día de dT+dC para igualar el efecto obtenido con el tratamiento con dosis de 400mg/kg/día de dTMP+dCMP. La causa que explique estas diferencias no está clara. Una posibilidad es que el catabolismo de los nucleósidos disminuya su disponibilidad en los tejidos diana. Sin embargo, se estudió si la administración conjunta en el ratón de dT+dC junto con inhibidores de su catabolismo mejoraba su eficiencia (igual que se había demostrado en los estudios previos *in vitro* de otros modelos de depleción de ADNmt (41)), obteniéndose resultados negativos en forma de una menor supervivencia del ratón al añadir tetrahidrouridina al tratamiento (la tetrahidouridina inhibe la citidina deaminasa y parece exacerbar la alteración en el equilibrio del “pool” de trinucleótidos en este modelo).

Tras estos experimentos permanece la incógnita de por qué el efecto beneficioso obtenido muestra diferencias en distintos órganos, siendo incapaz de rescatar los niveles de ADNmt en el cerebro del ratón a pesar de demostrar un aumento en ese tejido de los niveles de los nucleósidos administrados.

Tratamiento compasivo con nucleósidos (dT y dC) en pacientes con formas graves de miopatía debida a déficit de TK2.

Tras demostrarse el gran beneficio clínico de los nucleósidos en ausencia de efectos tóxicos en el modelo de ratón, y tras discutirse entre expertos internacionales en la materia en una reunión organizada por los Dr. Martí y Dr. Hirano en un Workshop en el Eurpean Neuromuscular Centre en Junio de 2017 (www.enmc.org; 232nd ENMC International Workshop: Recommendations for Diagnosis and Nucleoside Treatments of Mitochondrial DNA Maintenance Disorders), los primeros pacientes con formas graves de la enfermedad comenzaron a recibir el tratamiento de forma compasiva tras autorización previa por la Agencia Española del Medicamento para su uso en humanos (tratamiento "off label"). Previamente, algunos pacientes con las formas más graves de la enfermedad habían iniciado el tratamiento sin supervisión médica, administrado por sus padres, ante la gravedad, pronóstico sombrío y ausencia de tratamientos alternativos. La ausencia de toxicidad y los indicios de clara eficacia en algunos de estos niños a los que se administró el tratamiento durante periodos prolongados, animó a solicitar de manera acelerada la autorización para su uso "off label", sin seguirse los pasos habituales en el desarrollo de un nuevo fármaco.

Desde abril de 2017 los nucleósidos están designados como medicamentos huérfanos para el tratamiento de los síndromes de depleción mitocondrial secundarios a mutaciones en el gen *TK2* ("orphan designation (EU/3/17/1870) was granted by the European Commission to Vall d'Hebron Institute of Research, Spain, for thymidine and deoxycytidine for treatment of mitochondrial DNA depletion syndrome, myopathic form").

Búsqueda de un marcador para evaluar la gravedad y respuesta al tratamiento en enfermedades mitocondriales.

El diagnóstico de las EM es un proceso complejo dada la gran heterogeneidad clínica y genética de estos trastornos. Una prueba clásicamente utilizada para establecer su diagnóstico, particularmente en los casos de miopatía mitocondrial, sigue siendo la biopsia muscular, que permite identificar la presencia de signos histológicos de disfunción mitocondrial, realizar la medida espectofotométrica de las actividades enzimáticas de los complejos de la CR sobre el tejido muscular y extraer ADN para el estudio de alteraciones del ADNmt en un tejido post-mitótico y con elevado requerimiento energético.
Los hallazgos histológicos que sugieren la existencia de patología mitocondrial son: i) en la tinción con hematoxilina-eosina (HE) pueden identificarse fibras con acúmulos granulares basófilos como reflejo de la proliferación mitocondrial (esta proliferación es un mecanismo celular que trata de compensar su disfunción). ii) la tinción con succinato deshidrogenasa (SDH) refleja la actividad del complejo II de la CR, el único complejo que está totalmente codificado por ADNn; en consecuencia, esta tinción no se afecta en presencia de mutaciones del ADNmt y proporciona un excelente marcador de la *proliferación mitocondrial.* Por el contrario, las tres subunidades catalíticas de la citocromo c oxidasa (COX) son codificadas por el ADNmt y por tanto la tinción con COX es un buen marcador de la *función mitocondrial.* iii) Con la tinción con tricrómico de Gomori la proliferación mitocondrial da lugar a las características fibras “rojo rotas” (FRR), aunque esta técnica es menos sensible que la tinción con SDH (que da lugar a las llamadas fibras “azul-rotas”). El resto del estudio

morfológico no suele mostrar alteraciones significativas; la presencia de fibras necróticas o el reemplazamiento endomisial por tejido conectivo o adiposo no es frecuente en las EM. Sí puede existir un aumento secundario del contenido de lípidos.

Esta prueba, aún siendo extremadamente útil en el diagnóstico, no puede ser utilizada para el seguimiento evolutivo y valoración de respuesta al tratamiento al ser un procedimiento invasivo.

En cuanto a marcadores plasmáticos, con frecuencia se identifica un incremento del ácido láctico en sangre puesto que, a consecuencia del bloqueo del metabolismo aeróbico producido por la disfunción de la CR, el consumo de glucosa para producir ATP se deriva hacia una metabolización anaeróbica citoplasmática. Por tanto, la presencia de un aumento de lactato plasmático apoya el diagnóstico de sospecha de EM aunque su normalidad no permite excluirla (43). En consecuencia, aunque el incremento del lactato pueda ser un indicador útil para sugerir un trastorno mitocondrial, su sensibilidad y especificidad es muy baja.

Recientemente, un análisis del perfil de expresión génica utilizando microarrays de ADN complementario sobre muestras de tejido muscular en pacientes con miopatía por déficit de TK2, permitió al grupo de Kalko et. al identificar el factor de diferenciación de crecimiento 15 (GDF-15) como un potencial biomarcador para el diagnóstico de EM (44). GDF-15 es una citoquina relacionada de manera distante con la superfamilia del factor de crecimiento transformante beta. Su síntesis se induce ante distintos estímulos como el estrés oxidativo, la inflamación y la hipoxia (45, 46). Se ha relacionado con enfermedades cardiovasculares, ictus, diabetes mellitus, síndrome metabólico, enfermedades

hepáticas, enfermedades renales, sepsis y tumores, entre otras (47, 48). Tras identificar una sobreexpresión de GDF-15 en el tejido muscular de 4 pacientes con déficit de TK2 (su expresión estaba incrementada x200 en relación con el tejido muscular sano), el grupo de Kalko et. al analizó sus niveles séricos en un pequeño grupo de 13 pacientes con enfermedad mitocondrial confirmada genéticamente, y los comparó con un grupo control de 37 niños sanos y 6 niños con distrofia muscular. Los valores de GDF-15 en el grupo de niños con patología mitocondrial fue 9 veces mayor que los niveles en controles apareados por edad y todos los pacientes con distrofia muscular tuvieron niveles en suero comparables a los controles sanos (44). Con estos hallazgos proponen por primera vez que el GDF-15 pueda ser un marcador sensible para el diagnóstico de EM.

Estos resultados se confirmaron posteriormente en varias series de pacientes, tanto pediátricos como adultos, tanto en EM debidas a mutación en el ADNmt como a las secundarias a mutación en ADNn (49-53), apoyando que los niveles de GDF-15 son un marcador sensible y específico para el diagnóstico de EM.

Previamente, el grupo de Suomalainen et. al había identificado al factor de crecimiento de fibroblastos 21 (FGF-21) como un marcador útil en el diagnóstico de EM, particularmente de aquellas que cursan con afectación muscular (54). FGF-21 tiene un papel regulador en el metabolismo lipídico y en la respuesta a la inanición y sus concentraciones se elevan en el músculo y suero de ratones con alteración de la cadena respiratoria mitocondrial. Estudios comparando la utilidad de GDF-15 con FGF-21, y éstos con biomarcadores convencionales (niveles de creatin quinasa, niveles de lactato, piruvato y ratio lactato/piruvato), revelaron que el marcador más sensible y

específico para el diagnóstico de las EM son los niveles de GDF-15, independientemente del fenotipo de la enfermedad (49, 51).

El potencial uso de GDF-15 y FGF-21 como marcadores para evaluar la respuesta a tratamientos ya ha sido explorada en algunos estudios como en el reciente trabajo de Koga et. al, donde realizaron un ensayo piloto sobre el tratamiento con piruvato sódico para la acidosis láctica y demostraron una correlación del GDF-15 con la gravedad del fenotipo y con la respuesta al tratamiento (55). Esta correlación no pudo ser demostrada sin embargo con los niveles de FGF-21 sugiriendo que el mecanismo fisiopatológico por el que se induce la expresión de ambos factores en los casos de disfunción del sistema OXPHOS es diferente. La posible asociación de los niveles de GDF-15 con la gravedad del fenotipo ya fue sugerida en los estudios de Yatsuga et. al (51), donde los niveles elevados de GDF-15 mostraban correlación con los niveles de gravedad valorados por escalas clínicas diseñadas para el estudio de pacientes con EM (NMDAS, Newcastle Mitochondrial Disease Scale for Adults).

HIPÓTESIS Y OBJETIVOS

HIPÓTESIS

El tratamiento por vía oral con nucleósidos pirimidínicos (desoxicitidina -dC- y desoxitimidina -dT-) en pacientes con déficit de timidina quinasa mitocondrial es eficaz y seguro en todas sus formas clínicas.

OBJETIVOS

1. Profundizar en el conocimiento de la historia natural de la miopatía por déficit de timidina quinasa 2 de inicio tardío a través de la descripción de una serie de pacientes con este diagnóstico.

2. Analizar el efecto y seguridad del tratamiento con dC y dT en pacientes con miopatía debida a mutaciones recesivas en el gen *TK2*, que lo reciben de forma compasiva.

3. Analizar la utilidad de los niveles plasmáticos de GDF-15 y FGF-21 como marcadores para evaluar la gravedad de la enfermedad y la respuesta al tratamiento con nucleósidos.

MÉTODOS Y RESULTADOS

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Orphanet Journal of Rare Diseases

LATE-ONSET THYMIDINE KINASE 2 DEFICIENCY: A REVIEW OF 18 CASES

Orphanet Journal of Rare Diseses. 2019 May 6;14(1):200. Doi:10.1186/s13023-019-1071-z. PIMD: 31060578

Domínguez-González et al. Orphanet Journal of Rare Diseases (2019) 14:100
https://doi.org/10.1186/s13023-019-1071-z

Orphanet Journal of Rare Diseases

RESEARCH Open Access

Late-onset thymidine kinase 2 deficiency: a review of 18 cases

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Abstract

Background: TK2 gene encodes for mitochondrial thymidine kinase, which phosphorylates the pyrimidine nucleosides thymidine and deoxycytidine. Recessive mutations in the TK2 gene are responsible for the 'myopathic form' of the mitochondrial depletion/multiple deletions syndrome, with a wide spectrum of severity.

Methods: We describe 18 patients with mitochondrial myopathy due to mutations in the TK2 gene with absence of clinical symptoms until the age of 12.

Results: The mean age of onset was 31 years. The first symptom was muscle limb weakness in 10/18, eyelid ptosis in 6/18, and respiratory insufficiency in 2/18. All patients developed variable muscle weakness during the evolution of the disease. Half of patients presented difficulty in swallowing. All patients showed evidence of respiratory muscle weakness, with need for non-invasive Mechanical Ventilation in 12/18. Four patients had deceased, all of them due to respiratory insufficiency. We identified common radiological features in muscle magnetic resonance, where the most severely affected muscles were the gluteus maximus, semitendinosus and sartorius. On muscle biopsies typical signs of mitochondrial dysfunction were associated with dystrophic changes. All mutations identified were previously reported, being the most frequent the in-frame deletion p.Lys202del. All cases showed multiple mtDNA deletions but mtDNA depletion was present only in two patients.

Conclusions: The late-onset is the less frequent form of presentation of the TK2 deficiency and its natural history is not well known. Patients with late onset TK2 deficiency have a consistent and recognizable clinical phenotype and a poor prognosis, due to the high risk of early and progressive respiratory insufficiency.

Keywords: TK2 deficiency, Mitochondrial myopathy, Multiple deletions

Background

Defects in the maintenance and repair of mitochondrial DNA (mtDNA) result in an emerging and heterogeneous group of mitochondrial disorders, caused by alterations of the nuclear genes involved in mtDNA replication [1–3]. This group includes defects in enzymes involved in the maintenance of the balanced pool of deoxynucleotides of the mitochondria, which are crucial in the biosynthesis of the mitochondrial genome and have therapeutic implications [4, 5]. The disrupted synthesis of mtDNA results in qualitative (multiple deletions) and/or quantitative (a drastic decrease in the number of copies or depletion) defects of the mtDNA. In particular, one of the 'myopathic forms' of the mitochondrial depletion/multiple deletions syndromes is caused by mutations in the *TK2* gene which encodes for mitochondrial thymidine kinase, which phosphorylates the

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pyrimidine nucleosides thymidine (dT) and deoxycytidine (dC) [1, 6].

Recessive mutations in the *TK2* gene (MIM# 609560) are responsible for diverse clinical presentations mainly characterized by progressive muscle weakness, dysphagia and respiratory involvement with a wide spectrum severity and of age of onset. TK2 deficiency was initially described by Saada, et al. in 2001 [6] in four children with a severe myopathy associated with depletion of the mtDNA. Since then, a number of cases have been reported depicting a heterogeneous clinical presentation with a continuum spectrum of the disease, which includes early-onset extremely severe and rapidly progressive forms with survival of less than two years, to less severe forms with late or very late onset, and a variably slower rate of progression [7, 8]. In 2012, Tyynismaa, et al. reported the first two cases with mutations in the *TK2* gene with onset in the fifth decade of life, manifesting chronic progressive external ophthalmoplegia (CPEO) associated with limb muscle weakness and dysphagia [9]. A recent publication that included 92 patients describing the natural history of this disorder proposed the classification of three clinical forms according to ages-at-onset: infantile (< 1 year-old), childhood (1–12) and late (> 12 years) onset. Nearly 40% of the reported TK2 cases presented with the symptoms prior to the age of 1, in another 41% the onset occurred between the ages of one and 12, and only in 19% of patients did the symptoms appeared after the age of 12 [7]. A subsequent retrospective review, with similar frequencies for those three subgroups, included eleven new cases of which only three were classified as late-onset [8]. So far, the natural history of patients with late onset TK2 deficiency has not been defined in detail.

Here, we report on the clinical features and assessments in a large series of 18 patients with late-onset TK2 deficiency, the less known and poorest defined form of this disease, to further characterize this patient subgroup. Expanding the natural history and prognosis of late-onset TK2 deficiency will facilitate earlier diagnosis and identification for treatment with therapies under clinical development.

Methods

Patients

We describe the phenotypic features of 16 Spanish and 2 US patients with mitochondrial myopathy due to mutations in the *TK2* gene with the absence of clinical symptoms until the age of 12. The series include three pairs of siblings (P3-P4, P6-P10 and P14-P15). Partial data from five patients have been previously published elsewhere (P1, P5, P9 [7], P3 and P12 [10]).

Clinical evaluation

The electronic records were reviewed to collect information about the age of onset, initial symptoms, severity, distribution and progression of the muscle weakness and extra-muscular symptoms. We gathered information from the latest neurological examination registered including, when available, the Muscle Research Council (MRC) scale to assess the muscle strength and the 6 min walk test (6MWT) for functional evaluation.

Respiratory assessment

The latest value of the forced vital capacity (FVC) in seated and supine position, maximum inspiratory pressure (MIP), blood gas analysis, nocturnal ventilation (assessed with nocturnal pulse oximetry and/or capnography [11] and the need for mechanical ventilation (MV) type and hours of use were recorded.

Laboratory tests

CK (creatine kinase) and lactate levels were quantified in serum in basal conditions, at diagnosis. GDF-15 (growth/differentiation factor-15) levels were quantified in plasma samples using human GDF-15 quantitative ELISA kit (R&D Biosystems) according to the manufacturer's instructions.

Muscle MRI

Muscle MRI was performed in 8 of the 18 patients. All of them were scanned in a 1.5 T MR scanner (Siemens). Lower limb axial T1-weighted sequences were used for morphological analysis and short-tau inversion recovery (STIR) sequences were examined to detect muscle edema. The muscle MRI studies were evaluated by the same neurologist (R F-T) with wide experience in neuromuscular disorders. The evaluator was blind regarding the clinical manifestations. He scored pelvic, thigh and lower leg muscles in axial T1-sequences with the semiquantitative Mercuri visual scale (MVS) modified by Fisher [12]: 0: Normal appearance; 1: Mild involvement, less than 30% of individual muscle volume; 2: Moderate involvement, 30–60% of individual muscle volumes; 3: Severe involvement, > 60% of individual muscle; 4: End stage, all the muscle is severely affected, replaced by increased density of connective tissue and fat, with only a rim of fascia and neurovascular structures distinguishable. We compared median value of muscle fatty replacement using the Wilcoxon-Mann-Whitney test. Statistical analyses were performed using IBM SPSS Statistics, V.22 (IBM, Armonk, New York, USA).

Aerobic exercise testing

Exercise testing was performed in 5 patients on a cycle ergometer, following a ramp-like protocol (workload increases of 1 W every 6 s [averaging 10 $W \cdot min^{-1}$] starting from an initial load of 0 W, with a pedal cadence of 60–70 rpm throughout the test). Gas-exchange variables were collected breath-by-breath with an automated metabolic cart (Quark CPET, COSMED, Rome, Italy).

The peak oxygen uptake (VO_2 peak) was computed as the highest value obtained for any 10-s period during the tests [13].

Muscle biopsy

Muscle samples were obtained by open biopsy and processed following the standard procedures: Hematoxylin and eosin (H&E), modified Gomori trichrome, ATPase (adenosine triphosphatase), NADH (nicotinamide adenine dehydrogenase), SDH (succinate dehydrogenase), COX (cytochrome C oxidase), and COX-SDH stains were performed in all available samples. Respiratory chain enzyme activity levels were recorded when available.

Genetic studies

Molecular diagnosis was performed either by direct Sanger sequencing of exons and intron/exon boundaries of the *TK2* gene, or by customized next generation sequencing (NGS) panels. Patient's skeletal muscle mtDNA deletions were investigated by long-range PCR (polymerase chain reaction) and/or Southern blot, and mtDNA copy number was assessed by quantitative PCR as previously described [10, 14].

The study was approved by the institutional review board of every centre and all patients signed an informed consent for the anonymous publication of this data.

Results

Clinical manifestations (Table 1)

We included 18 patients (6 male, 12 female). The mean age-at-onset was 31 years (range 12 to 60 years) with a mean age at diagnosis of 48.5 years (range 23 to 73 years) resulting in an average of 17.4 years between the onset of the disease until reaching a genetic diagnosis (range 1 to 44 years). The mean duration of the disease was 19.8 years (range 6 to 44 years). Four patients from the series were deceased, all of them due to respiratory insufficiency a mean of two decades after the onset.

The first symptom was muscle limb weakness in 10/18 (55.6%), eyelid ptosis in 6/18 (33%) (two patients also presented ophthalmoparesis), and respiratory insufficiency in 2/18 (11.1%). All patients developed muscle weakness during the evolution of the disease, 17/18 showing proximal and distal limb muscle weakness, 1/18 with only distal limb weakness, and 16/18 axial involvement. It is noteworthy that neck flexor weakness was clearly more severe than limb weakness (mean, 2.14 on the MRC scale).

The following muscle groups were the most frequently affected, in a symmetrical manner: shoulder abductor (mean, 4 on the MRC scale), hip flexor (mean, 3.75 on the MRC scale) and hip extensor (mean, 3.87 for both on the MRC scale) and finger extensor muscles (mean, 4.14 on the MRC scale). Four patients (22%) lost the ability to walk without support. Facial musculature was symmetrically affected in 17 patients (94.4%), with predominance of the orbicular oculis muscle. 16/18 of the patients (88.9%) also had symmetrical eyelid ptosis of variable severity, with this being the first symptom in 6 patients (33.3%). Six of them required surgical blepharoplasty due to vision impairment. Nine patients had CPEO.

The majority (11/18) had difficulty in swallowing, which resulted in severe weight loss and/or detriment to the safety of oral feeding in 6 cases, requiring percutaneous gastrostomy tube in 5 cases (27.8%) on average 19.6 years after the onset of the disease (ranging from 12 to 28 years).

Other clinical manifestations included sensory axonal polyneuropathy (7/18;38.9%), neurosensory hearing loss (3/18;16.6%) and dysphonia due to vocal cord palsy (2/18;11.1%). No patient had cardiomyopathy.

Respiratory function

FVC at diagnosis from the total cohort was 55.4% (ranging from 17 to 103) with a mean decrease of FVC in supine position of 8% (ranging from 0 to 14), and a mean MIP of 36.8% (ranging from 20 to 101%), independent of the associated muscle symptoms. From a respiratory perspective, the high frequency of complications should be noted, with need for non-invasive MV in 12/18 patients (66.6%). The mean use of the MV was 11.6 h per day (ranging from 8 to 24 h). Eight out of the 12 patients with MV (66.6%) presented with acute respiratory insufficiency following a routine upper respiratory infection as the first manifestation of the disease. None of these cases had any prior respiratory symptoms; however, once detected, they required MV due to hypercapnia secondary to alveolar hypoventilation. Although limb muscle weakness and/or eyelid ptosis were already present at the onset of respiratory insufficiency, those neuromuscular symptoms had not prompted a neurology consultation in any of the eight patients. Thus, the respiratory involvement resulted in the diagnosis of an underlying myopathy in these patients; the mean FVC was of 40.8% (range from 28 to 58) at the time of diagnosis. Of the six patients who did not needed MV, all showed evidence of respiratory muscle weakness on functional tests, although only one of them (P8) reported respiratory symptoms (orthopnoea), suggesting diaphragmatic weakness. This patient displayed ptosis and CPEO at the age of 50 associated with moderate axial and proximal limb muscle weakness (4 on the MRC scale). Strikingly, although the functional respiratory tests and nocturnal pulse oximetry were normal (FVC seated 103%, FVC decubitus 100% and MIP 101%) nocturnal transcutaneous capnography revealed high mean levels of carbon dioxide (CO_2, mean of 48 mmHg, with a maximum peak of 54 mmHg).

Four patients died of respiratory insufficiency at mean age of 56 years (ranging from 40 to 68), and a mean of

Table 1 Clinical manifestations summary

ID	Age At Onset	Gender	Current Age	Clinical Assessments										Respiratory Assessments			Other manifestations
				Ptosis	CPEO	Facial Weakness	Dysphagia	Neck flexor weakness	Limb Weakness	Wheelchair-bound	6MWT (Meters)	PEG (AT AGE)	Orthopnea	BMI	Sitting FVC (%)	FVC Sitting/ Supine (%)	Hours on MV (AT AGE)	
1	12	F	32	+	–	+	Yes	+++	+	No	530	No	No	13.86	46	–8	8 (32)	–
2	20	F	33	–	–	+	Yes	+++	+	No	390	Yes (32)	Yes	17.9	26	–22	8–9 (28)	Hepatopathy
3	30	F	61	+	–	+	Yes	++	+	No	386	No	Yes	27.12	71	–14	8 (61)	PNP-S
4	60	F	73	+	+	++	Yes	++	+	No	345	No	Yes	26	69	NA	8 (73)	Hearing loss PNP-S
5	50	M	50	++	–	+	No	+	+	No	475	No	Yes	27.6	47	NA	12 (50)	PNP-S
6	14	F	58	++	++	++	No	+++	+	No	450	No	Yes	24.5	51	–28	8 (56)	–
7	40	M	Death at 68	++	++	+	Yes	+++	++	Yes	NA	Yes (68)	Yes	23.7	48	NA	10–12 (49)	Hearing loss PNP-S Vocal cord palsy
8	50	F	63	+	+	++	No	+	+	No	417	No	Yes	28.7	103	–3	–	PNP-S
9	23	F	Death at 40	–	–	+	Yes	+++	++	Yes	NA	Yes (39)	Yes	15	28	NA	22 (30)	–
10	14	M	Death at 49	+	–	–	Yes	++	+++	Yes	NA	Yes (42)	Yes	17	32	NA	24 (42)	–
11	40	M	51	+	–	+	No	–	+	No	600	No	No	26.3	70	–10	–	–
12	30	M	60	++	–	+	Yes	NA	+	No	NA	No	No	23	75	NA	–	Vocal cord palsy
13	48	F	Death at 67	+	+	+	No	+++	+++	Yes	NA	No	Yes	NA	43	NA	12 (58)	–
14	15	M	26	+	+	+	Yes	+	+	No	413	No	No	NA	72	–10	–	Hearing loss PNP-S
15	12	F	31	+	+	+	No	+	+	No	428	No	No	NA	75	–12	–	PNP-S
16	30	F	43	++	+	+	No	+	+	No	425	No	Yes	NA	69	2	8 (43)	–
17	45	F	51	++	+	++	Yes	+	+	No	NA	No	No	NA	NA	NA	–	NA
18	25	F	58	++	++	++	Yes	++	++	No	228	Yes (39)	Yes	NA	17	NA	11 (59)	NA

NA Not available. Ptosis: ++ History of eyelid surgery, + mild, – no ptosis. *CPEO* chronic progressive external ophthalmoplegia: ++ complete, + partial, – no ocular weakness. Facial weakness: ++ severe, + mild, – no facial weakness. Neck flexor weakness; +++ unable to lift head in supine position, ++ 3 on Medical Research Council (MRC) scale, +4 on MRC scale, – no neck flexor weakness. Limb weakness: +++ 1–2 on MRC scale, ++3 on MRC scale, + 4 on MRC scale, – no weakness. *6MWT* six-minute walking test, *FVC* forced vital capacity, *MV* Mechanical ventilation, *PNP-S* sensory polyneuropathy, *BMI* body mass index

24 years after the onset of their initial symptoms (ranging from 17 to 35).

CK and lactate levels (Table 2)

94.4% of patients had increased variable serum CK levels ranging from 190 to 2435 UI/l (normal levels < 170 UI/l)), and 16.7% showed levels 10-fold above the upper normal limit. Serum lactate levels were measured in basal conditions in 12 of the 18 cases. Of these, only three (25%) displayed slightly increased levels (1.4-2x above the upper normal limit).

GDF-15 levels

GDF-15, a biomarker identified in the analysis of transcriptomic profiling of TK2 deficient human skeletal muscle [15], has been proven useful in the diagnosis of mitochondrial myopathies [16], being especially increased in patients with mitochondrial TK2 deficiency [17]. Serum levels of GDF-15 were increased in 5 out of 5 cases analysed (100%), ranging from 1529 to 2438 pg/mL (2113 pg/mL ± 462, mean ± standard deviation, upper limit of normal =550 pg/mL) [16].

Muscle MRI findings

It was performed in 8 patients. Mean age at muscle MRI was 46.4 years old (range: 23–73). Mean disease duration at the time of the scan was 18 years (range 10–31). The most severely affected muscles in axial T1-weighted sequences were the gluteus maximus, semitendinosus, sartorius and gastrocnemius medialis (median MVS: 3). Of these, only the gluteus maximus and sartorius were affected in all patients. Apart from the later, gluteus medius, adductor magnus and semitendinosus were also moderately affected in the thighs and gastrocnemius lateralis in the legs (median MVS: 2). No muscle fat infiltration was observed in obturator, quadratus femoris, extensoris digitorum and tibialis posterior (Fig. 1). The

Table 2 Biochemical and molecular characteristics

ID	Mutation		Muscle Biopsy	Multiple Deletions	Residual mtDNA (%)	Respiratory Chain Enzyme Activity	CK (UI/l)	GDF-15 (pg/mL)	Lactate (mmol/l)
	Allele 1	Allele 2							
1	c.323C > T (p.Thr108Met)	c.323C > T (p.Thr108Met)	Yes	Yes	17	CI, CIII and CIV deficit	2435	2423	1.95
2	c.323C > T (p.Thr108Met)	c.323C > T (p.Thr108Met)	Yes	Yes	39	Normal	303	2439	2.3
3	c.604–606 AAGdel (p.Lys202del)	c.604–606 AAGdel (p.Lys202del)	Yes	Yes	60	CIII deficit	294	1695	2.6
4	c.604–606 AAGdel (p.Lys202del)	c.604–606 AAGdel (p.Lys202del)	ND	ND	NA	ND	647	2483	2.2
5	c.604–606 AAGdel (p.Lys202del)	c.604–606 AAGdel (p.Lys202del)	Yes	Yes	66	Normal	357	1529	1.5
6	c.323C > T (p.Thr108Met)	c.323C > T (p.Thr108Met)	Yes	Yes	19	Normal	425	NA	1.6
7	c.604–606 AAGdel (p.Lys202del)	c.604–606 AAGdel (p.Lys202del)	Yes	Yes	33	NA	568	NA	2.6
8	c.604–606 AAGdel (p.Lys202del)	c.604–606 AAGdel (p.Lys202del)	Yes	Yes	NA	NA	405	NA	2.4
9	c.323C > T (p.Thr108Met)	c.323C > T (p.Thr108Met)	Yes	Yes	35	CI, CIII and CIV deficit	190	NA	NA
10	c.323C > T (p.Thr108Met)	c.323C > T (p.Thr108Met)	Yes	NA	NA	Normal	405	NA	3
11	c.604–606 AAGdel (p.Lys202del)	c.604–606 AAGdel (p.Lys202del)	Yes	Yes	NA	ND	266	NA	NA
12	c.604–606 AAGdel (p.Lys202del)	c.604–606 AAGdel (p.Lys202del)	Yes	Yes	NA	ND	350	NA	NA
13	c.388C > T (p.Arg130Trp)	c.415G > A (p.Ala139Thr)	Yes	Yes	NA	CI, CIII and CIV deficit	170	NA	4.14
14	c.623A > G (p.Tyr208Cys)	c.623A > G (p.Tyr208Cys)	Yes	Yes	NA	CI, CIII and CIV deficit	1739	NA	NA
15	c.623A > G (p.Tyr208Cys)	c.623A > G (p.Tyr208Cys)	ND	NA	NA	ND	381	NA	NA
16	c.323C > T (p.Thr108Met)	c.323C > T (p.Thr108Met)	Yes	Yes	53	Normal	233	NA	1.77
17	c.469_470insTGGG (p.Asp157Valfs*11)	c.156 + 6 T > G	Yes	Yes	50	NA	537	NA	NA
18	c.604–606 AAGdel (p.Lys202del)	c.604–606 AAGdel (p.Lys202del)	NA	NA	NA	NA	1348	NA	NA

NA not available, *ND* not done, *CK* creatine kinase, *GDF-15* Growth differentiation factor-15

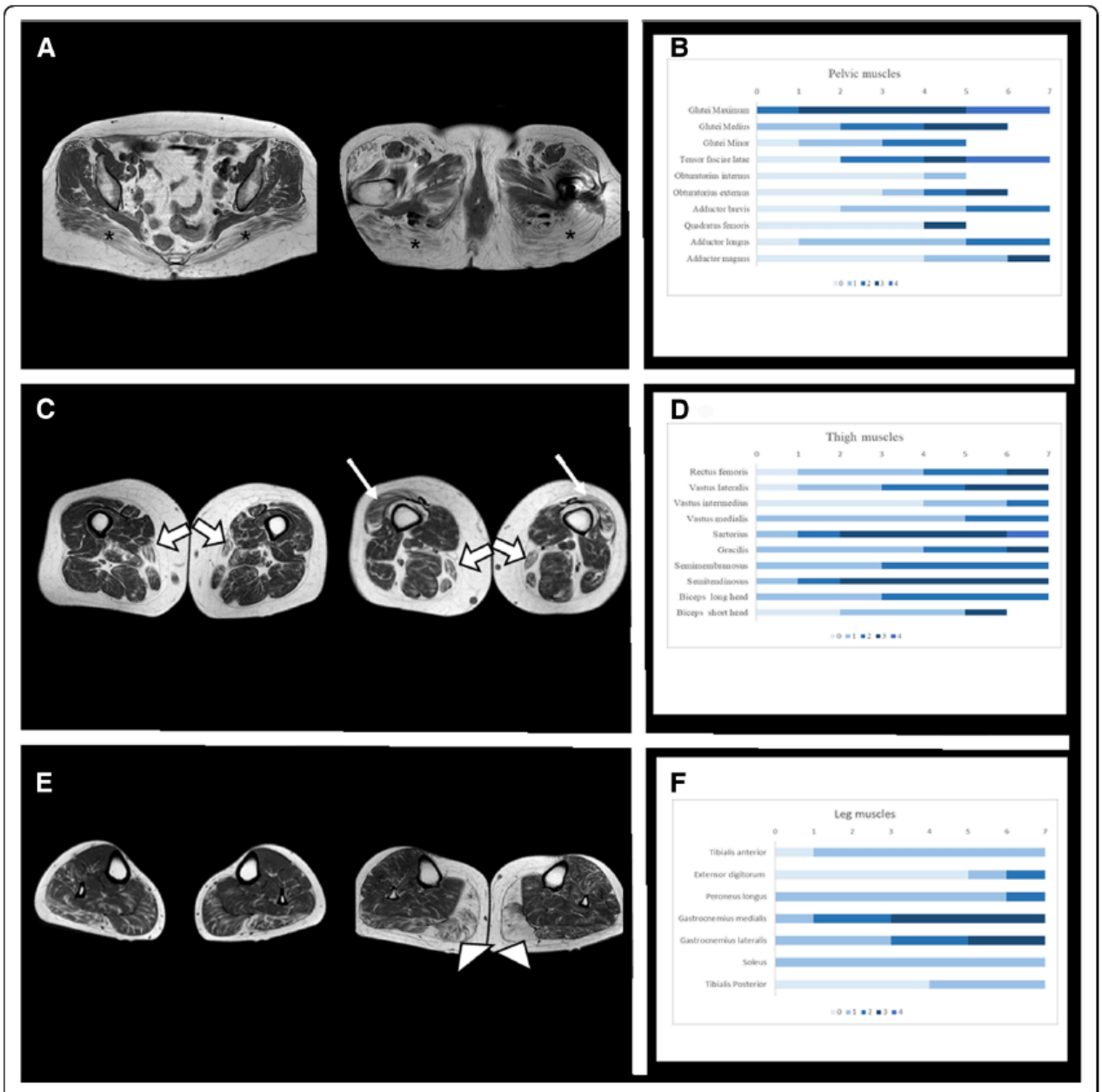

Fig. 1 Axial T1 muscle MRI and bar charts with Mercuri Visual Scale (MVS) distribution for 7 patients and per anatomical region. **a**, Axial T1 muscle MRI in pelvis: These two consecutive slices from different patients are showing that the gluteus maximus (marked with asterisk) is the most affected muscle. Tensor fascia latae is affected while obturator and quatratus femoris are less affected. **b**, Bar chart MVS fat replacement in pelvis: MVS (0: no fat replacement, 4: the muscle is completely replaced) for all patients. Gluteus maximus is the most affected muscle, followed by tensor fascia latae. **c**, Axial T1 muscle MRI in thighs: These two slices from two different patients are showing the fat replacement of sartorius (wide white arrow) and vastus lateralis (thin white arrow). Other muscles like semitendinosus, semimembranosus and gracilis are also moderately affected. **d**, Bar chart MVS fat replacement in thighs: MVS for all patients. Sartorius, semimembranosus, semitendinosus, gracilis and vastus lateralis are the most affected muscles. Sartorius and gracilis are affected in all patients. **e**, Axial T1 muscle MRI in legs: These two slices from two different patients are showing the fat replacement of gastrocnemius medialis (white arrow head). Gastrocnemius lateralis and soleus are also moderately affected. Tibialis anterior and tibialis posterior are the least affected. **f**, Bar chart MVS fat replacement in legs: MVS for all patients. Gastrocnemius medialis and lateralis are the most affected muscles in legs. Tibialis anterior, extensoris digitorum and tibialis posterior are the least affected muscles

fat replacement followed a diffuse pattern and no focal areas of fat infiltration were detected. We did not observe statistical differences regarding asymmetric involvement. STIR sequence was normal in all patients.

Aerobic exercise testing

In addition to weakness, one of the most frequent clinical manifestations in the mitochondrial myopathies is poor exercise capacity [18].The latter is reflected by low levels of VO_2peak or by poor muscle-oxygen extraction (as assessed with near-infrared spectroscopy) during graded cycle-ergometer/treadmill testing [19]. Aerobic exercise testing was performed on a cycle ergometer in five patients. The mean ± SD VO_2 peak obtained was 14.8 ± 3.2 mL/kg^{-1}/min^{-1}, with normal consumption values of 40.0 ± 9.5 mL/kg^{-1}/min^{-1} [20].

Muscle biopsies

Muscle biopsies were performed in 16 patients, 11 were available to re-analysis. The morphological study revealed numerous ragged-red fibers in 100% of the biopsies, which were hyper-reactive with SDH reaction and usually COX-deficient. COX-deficient fibers accounted for approximately 5–15% of all fibers. Frequently these muscles also showed dystrophic features with frequent necrotic fibers, some with phagocytosis, and increased endomysial connective tissue (present in 7 out of 11 biopsies revised). Marked type I fiber predominance was also observed in 2 patients (Fig. 2). These findings differ from the usual pattern displayed in other mitochondrial myopathies, where the typical signs of mitochondrial proliferation and dysfunction are not associated with other relevant changes in muscle histology structure [21]. We have results of the analysis of the enzymatic activity of respiratory chain complexes of 10 patients. Only in half of them a reduction in the activity of one or more enzymatic complexes were identified (Table 2).

Genetic studies

All patients harbored biallelic mutations in the *TK2* gene (Ref.Seq. NM_004614.4) (Table 2). Most patients (16/18;88.9%) were homozygous. All mutations were previously reported [7, 8], with the in-frame deletion p.Lys202del (c.604_606AAGdel) being the most frequent (16/36 alleles; 44.4%), followed by the missense mutation p.Thr108Met (c.323C > T) (12/36;27.8%). Additionally, three missense mutations were identified in 3 patients: p.Arg130Trp (c.388C > T), p.Ala139Thr (c.415G > A), and p.Tyr208Cys (c.623A > G). Finally, one patient harbored a frameshift mutation p.Asp157Valfs*11(c.469-470insTGGG) in compound heterozygosis with a splice site mutation c.156 + 6 T > G. Genetic data from patients P1, P2, P5, P9 and P12, were previously reported [7, 10]. Muscle mtDNA copy-number was studied in 9 patients and severe mtDNA depletion was detected in only two (17% of residual mtDNA in P1 and 19% of residual mtDNA in P6). Fourteen out of 14 patients (100%) showed the presence of multiple mtDNA deletions in muscle.

Discussion

The late-onset presentation of TK2 deficiency is the least frequent clinical mode of presentation known. These patients are considered to have a milder presentation than those with infancy and childhood onset disease, however, few cases have been described to date and those reported were not extensively explored. So far, 17 patients with late-onset were reported to harbour *TK2* biallelic mutations [7–10, 22]. However clinical details were scarce, heterogeneous, and reports did not clearly define the phenotype or rate of progression of the disease. In some cases, clinical presentation is similar to that described in the childhood onset patients, with progressive limb, facial, extraocular, oropharyngeal and respiratory muscle weakness, but with a slower progression, whereas in other cases, CPEO is the main manifestation [9]. Respiratory insufficiency has been mentioned as a potential cause of death although comprehensive data about the respiratory involvement is not available for all the previously published patients: severe respiratory insufficiency is described in 41% of the reported cases but in the remaining 59% this data is unavailable or superficially described [7, 8, 22].

We identified 16 Spanish and two North American patients, from 13 different families, with TK2 mutations and a late-onset presentation. Exhaustive clinical description is here provided to facilitate earlier and accurate diagnosis and to improve the knowledge of the natural history of this rare, and probably underdiagnosed disorder.

The clinical features and results of the diagnostic tests described in our series show a homogeneous phenotypic pattern in late-onset TK2 deficiency consisting of progressive proximal limb, axial neck flexor and facial muscle weakness frequently associated with ptosis, ophthalmoparesis and bulbar weakness, along with an early and severe, although unrecognized, respiratory involvement. Diaphragmatic weakness is very characteristic, occurring in all of our cases, showing an early onset but slow progression; 12/18 (66.6%) required MV during the evolution of the disease and in 8/18 (44.4%) was the cause for the first medical consultation. This pattern of respiratory involvement was found even in patients who only had an apparently isolated CPEO phenotype. Therefore, it is critical to identify signs of nocturnal hypoventilation during clinical evaluation of these patients, regardless of the severity of the skeletal myopathy. This discrepancy between diaphragmatic and limb weakness was also reflected in some patients with virtually

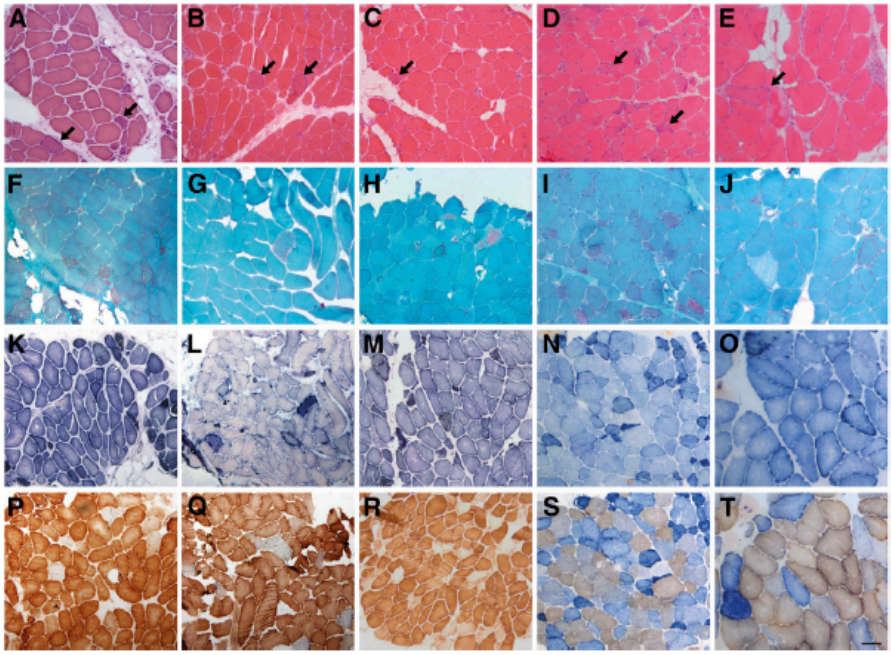

Fig. 2 Morphological alterations in muscle biopsies from patients P1 (**a**, **f**, **k**, **p**), P5 (**b**, **g**, **l**, **q**), P9 (**c**, **h**, **m**, **r**), P14 (D, **i**, **n**, **s**) and P16 (**e**, **j**, **o**, **t**). **a-e** H&E shows dystrophic features in all cases with mild endomysal fibrosis, adipose tissue replacement, atrophy and necrotic fibers. Ragged-red fibers are frequently identified in all muscle samples (arrows). **f-j** Gomori trichrome showed the characteristic ragged-red fibers in all the biopsies. **k-o** Succinate dehydrogenase (SDH) reveals an increase of the oxidative staining in numerous fibers. **p-t** Frequent cytochrome C oxidase (COX) deficient fibers are present in variable proportion in the different cases (**p** and **r**, COX staining; **o**, **s** and **t**, COX-SDH combined staining). Scale bar =100 μm

normal 6MWT results, despite using MV (see Table 1). In our series, the capnography was the most sensitive test for detecting the respiratory dysfunction, since it was abnormal even before basal FVC and MIP revealed alterations.

Muscle biopsies showed the typical findings of mitochondrial dysfunction described in most mitochondrial myopathies. However, as in other TK2 deficiency forms, they also revealed dystrophic features which are distinct from the majority of other mitochondrial myopathies. Thus, our data support that the association of both mitochondrial and dystrophic pattern strongly suggest mutations in the *TK2* gene as the underlying cause.

All previously published late-onset patients showed multiple mtDNA deletions, while mtDNA depletion was found only in one of the five cases in whom the mtDNA copy-number was quantified. Our findings corroborate the previous results indicating the presence of multiple mtDNA deletions is more frequent than mtDNA depletion in the late-onset TK2 deficient patients. Previous reports showed that mtDNA depletion is found in the most of early onset patients [7], but our data support that it cannot be considered a valid prognostic marker since it can also be found in late-onset cases.

In muscle MRI the fat muscle replacement was diffuse, resembling many muscular dystrophies and congenital myopathies. Muscle degeneration in MRI was described in five MERRF patients with the m.8344A > G mutation [23], and more recently fatty infiltration has been communicated in patients with single, large-scale deletions of mitochondrial DNA [24]. However, no extensive studies have been published trying to define muscle MRI patterns in different mitochondrial myopathies. So, there is no specific MRI pattern for any mitochondrial myopathy described so far. In our series of TK2 patients although no clear pattern of fat infiltration was detected, we have identified some radiological common features, as the involvement of the sartorius muscle in all cases. This muscle is usually spared until late stages in many genetic muscle diseases (is only affected early in some myofibrillar myopathies, in the Laing distal myopathy and in RYR1-related myopathies (encodes for ryanodine receptor 1 protein) [12, 25–27]), so this finding could be helpful for differential diagnosis.

Serum GDF-15 levels have recently been revealed as a sensitive and specific biomarker for the diagnosis of mitochondrial myopathies [16, 17]. In our series, it proved to be very high in all analysed cases, so it could orientate the molecular diagnosis in a proper clinical context, before the muscle biopsy was performed.

As in other mitochondrial myopathies [19], in our series the cardiopulmonary exercise testing identified a

very reduced consumption of oxygen, even in patients with CPEO as a predominant clinical manifestation (P8). This indicates that, although the weakness may not be severe in late-onset TK2 deficiency patients, the exercise capacity is abnormally low, ultimately impairing physical activity.

Noticeably, the p.Lys202del was the most frequent mutation in the *TK2* gene in our series of late-onset patients, which is consistent with the finding that this mutation appears to be restricted to adult-onset cases, since it has not been reported in any infantile-onset patients who have not even harbouring this mutation in a single allele [8]. Nevertheless, it was reported in one patient with childhood-onset, who was compound heterozygous for this mutation and a frameshift mutation, and began showing symptoms at 2.5 years but survived until 8.5 years-old [28]. The eight cases with this mutation in our series were all homozygous supporting the idea that this mutation is associated with a milder effect (age at onset ranging from 25 to 60 years). Interestingly, this mutation has only been identified in 13 unrelated Spanish patients ((11, 13, 26, 27, and this study), 2 related patients from Hispanic ethnic background [10], and one patient from Venezuela (this study) suggesting that it could be a private mutation and that Spanish/Hispanic candidate patients may be amenable for a rapid genetic screening of this mutation. However, haplotype analysis would be required to confirm the possible founder effect of this mutation. The p.Thr108Met mutation was the second most common mutation in this study, however it has been found in infantile and childhood onset cases [6, 7] of different geographic origin.

TK2 deficiency is a severe disorder causing premature death. In recent pre-clinical studies, it has been demonstrated that treatment with pyrimidine nucleosides (dC + dT) in the H126N knock-in mouse model of TK2 deficiency, leads to a prolonged life span in the animals and a restored mtDNA copy number, without significant toxicity [4]. This opens the door to a potential therapeutic intervention in humans with this metabolic hereditary disorder, making it necessary to define sensitive and objective outcomes to assess an eventual response to treatment. Our findings suggest that functional respiratory tests, serum GDF-15 level and the stress cyclometer evaluation are potentially good candidates for monitoring the progression of disease.

Conclusion

In summary, our study shows that late-onset patients with mitochondrial TK2 deficiency have a consistent and recognizable clinical phenotype, characterized by a progressive myopathy with predominant facial and axial neck flexor weakness, and respiratory involvement, often associated to CPEO. Their prognosis is poor, due to the high risk of early and progressive respiratory insufficiency. Yet, some patients may present with a severe acute respiratory failure. Early detection of respiratory involvement requires an active search in the clinics, even in asymptomatic patients. A small number of rationally designed treatments are being developed for mitochondrial disorders [29], including nucleoside substrate enhancement therapy designed specifically for TK2 deficiency [4]. Therefore, early diagnosis of TK2 deficiency is important as patients could benefit from the existence of a potential therapy.

Abbreviations

6MWT: 6-min walking test; ATPase: Adenosine triphosphatase; BMI: Body mass index; CK: Creatine kinase; CO_2: Carbon dioxide; COX: Cytochrome C oxidase; CPEO: Chronic progressive external ophthalmoplegia; dC: Deoxycytidine; dT: Thymidine; FVC: Forced vital capacity; GDF-15: Growth differentiation factor 15; H&E: Hematoxylin and eosin; MIP: Maximal inspiratory pressure; MRC: Muscle Research Council; MRI: Magnetic resonance imaging; mtDNA: Mitochondrial DNA; MV: Mechanical ventilation; MVS: Mercury visual scale; NADH: Nicotinamide adenine dehydrogenase; NGS: Next generation sequencing; PCR: Polymerase chain reaction; SDH: Succinate dehydrogenase; STIR: Shor tau inversion recovery; VO_2peak: Peak oxygen uptake

Acknowledgements

Not applicable.

Funding

This work was supported by research grants of Plan Nacional de I + D + I and Instituto de Salud Carlos III (ISCIII), Subdirección General de Evaluación y Fomento de la Investigación Sanitaria", project PI16–01843 (CP), PI16/00579 and CP09/00011 for CJM and the European Regional Development Fund (FEDER a way to achieve Europe). MAM has received funding from the Spanish ISCIII (grant PI 15/00431). A multicentric grant funded by the ISCIII (PMP15/00025 to MAM, RM, MO, CP).

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

CDG, AHV, JSC, JG, GM, MO, FM, JDM, CC, JBM, JE, MH and CP handled patients and recollected clinical data for the manuscript. RT collected and analysed the MRI data of the patients. EG and ABE performed molecular analysis; CB and CJ provided GDF-15 analysis. AH and ER collected and review musde biopsy data. CD and CP coordinated all the study. CD, MAM and CP wrote the initial manuscript. RM, JA, MAM, CP, and MH provided critical discussion of the research. All authors contributed to the final version of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from patients for publication of anonymised clinical data.

Consent for publication

Not applicable.

Competing interests

MH and RM are co-inventors on patent applications filed by Columbia University Medical Center (CUMC) for deoxynucleoside therapy for mitochondrial DNA depletion syndromes including TK2 deficiency. The patent applications and other intellectual property have been licensed by CUMC to Meves Pharmaceuticals, Inc. CUMC may be eligible to receive payments related to the development and commercialization of the technology. Any potential licensing fees earned will be paid to CUMC and are shared with inventors through CUMC distribution policy. MH and RM are paid consultants to Meves Pharmaceutical, Inc. The rest of authors declare that they have no conflict of interest.

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Received: 5 February 2019 Accepted: 17 April 2019
Published online: 06 May 2019

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Annals of NEUROLOGY

DEOXYNUCLEOSIDE THERAPY FOR THYMIDINE KINASE 2-DEFICIENT MYOPATHY

Ann Neurol. 2019 May 24. doi: 10.1002/ana.25506. PubMed PMID: 31125140.

Deoxynucleoside Therapy for Thymidine Kinase 2–Deficient Myopathy

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Objective: Thymidine kinase 2, encoded by the nuclear gene *TK2*, is required for mitochondrial DNA maintenance. Autosomal recessive *TK2* mutations cause depletion and multiple deletions of mtDNA that manifest predominantly as a myopathy usually beginning in childhood and progressing relentlessly. We investigated the safety and efficacy of deoxynucleoside monophosphate and deoxynucleoside therapies.
Methods: We administered deoxynucleoside monophosphates and deoxynucleoside to 16 TK2-deficient patients under a compassionate use program.
Results: In 5 patients with early onset and severe disease, survival and motor functions were better than historically untreated patients. In 11 childhood and adult onset patients, clinical measures stabilized or improved. Three of 8 patients who were nonambulatory at baseline gained the ability to walk on therapy; 4 of 5 patients who required enteric nutrition were able to discontinue feeding tube use; and 1 of 9 patients who required mechanical ventilation became able to breathe

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.25506

Received Dec 31, 2018, and in revised form May 21, 2019. Accepted for publication May 22, 2019.

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independently. In motor functional scales, improvements were observed in the 6-minute walk test performance in 7 of 8 subjects, Egen Klassifikation in 2 of 3, and North Star Ambulatory Assessment in all 5 tested. Baseline elevated serum growth differentiation factor 15 levels decreased with treatment in all 7 patients tested. A side effect observed in 8 of the 16 patients was dose-dependent diarrhea, which did not require withdrawal of treatment. Among 12 other TK2 patients treated with deoxynucleoside, 2 adults developed elevated liver enzymes that normalized following discontinuation of therapy.
Interpretation: This open-label study indicates favorable side effect profiles and clinical efficacy of deoxynucleoside monophosphate and deoxynucleoside therapies for TK2 deficiency.

ANN NEUROL 2019;00:1–11

Encoded by the nuclear gene *TK2*, thymidine kinase 2 (TK2) is the first enzyme in the deoxypyrimidine salvage pathway within mitochondria. TK2 phosphorylates the nucleosides deoxycytidine (dC) and deoxythymidine (dT) to generate deoxycytidine monophosphate (dCMP) and deoxythymidine monophosphate (dTMP). These pyrimidine deoxynucleoside monophosphates are subsequently converted to deoxynucleoside triphosphates required for mitochondrial DNA (mtDNA) replication and maintenance.[1,2] Autosomal recessive *TK2* mutations cause mtDNA depletion, multiple deletions, or both.[3–5]

Although the phenotypic spectrum of TK2 deficiency includes late onset cases of mild chronic progressive external ophthalmoplegia,[3,6–8] the most frequent clinical presentations are infantile onset and childhood onset progressive limb and bulbar myopathy with restrictive lung disease.[3,4,9–13] The infantile onset form manifests rapidly progressive myopathy weakness with motor regression and respiratory insufficiency occasionally accompanied by central nervous system involvement and virtually uniform early fatality with postonset survival of 1 year.[4,5,10] The majority of patients with early onset before age 2 years also show severe and rapid progression. The less severe forms begin in childhood through adulthood and exhibit slower progression; however, bulbar, proximal limb, and respiratory weakness can be severe, causing inability to walk or breathe independently.[3,4,6,12]

We reported that, in the H126N knockin mouse model of TK2 deficiency, oral administration of the TK2 products dCMP and dTMP act as molecular bypass therapy and prolong median life span by 2- to 3-fold.[14,15] However, we subsequently observed that after administration, dCMP and dTMP are rapidly catabolized to the nucleosides dC and dT, suggesting that nucleosides, rather than nucleotides, are the major active therapeutic agents. We demonstrated that dC and dT treatment of Tk2-deficient mice delayed disease onset, prolonged life span, and restored mtDNA copy number.[16] In this situation, deoxynucleosides function as a substrate enhancement therapy.

Based on our promising preclinical results for this often-lethal disease, we obtained approval to initiate compassionate use of oral dC + dT, dTMP+dCMP, or both sequentially in TK2-deficient patients. Here, we report the outcomes of 16 TK2-deficient patients who received these pharmacological treatments.

Patients and Methods

Study Design

The objective of the study was to evaluate safety and efficacy of oral dC + dT, dCMP+dTMP, or both for TK2 deficiency on a compassionate use basis. In 10 academic medical centers from 5 countries (Spain, USA, Chile, Guatemala, and Italy), treatments were initiated under compassionate use (expanded access as defined by the US Food and Drug Administration [FDA][17]) protocols. Compounds were obtained under investigator-sponsored Investigational New Drugs in the United States and under compassionate-use exemptions in other countries. All patients or legal guardians signed informed consent forms for treatment. The FDA, Spanish Drug Agency (Spanish Agency of Medicines and Health Products), and Italian Medicines Agency as well as the Columbia University Medical Center Institutional Review Board and the local pharmacy committees of each center approved the compassionate use treatment. We also obtained written informed consent for publication of the patients' images and videos.

Patients

We analyzed outcomes of 16 genetically confirmed TK2-deficient patients (P1–P16) who had received treatment for at least 1 year prior to September 1, 2017. Twelve cases were from Spain and 1 from Italy, and 3 were followed in the USA (1 each from the USA, Chile, and Guatemala).

Treatment

In 6 patients, treatment was initiated with oral dCMP+dTMP at 1-to-1 (weight:weight) ratios until 2015, when cell and mouse studies indicated that deoxynucleotides were precursors for deoxynucleosides[16,18] (mean duration of treatment with nucleotides = 21.4 months, range = 11–47 months) and therapy was converted to oral dC + dT at 1-to-1 (weight:weight) ratios in all but 1 patient (P11), who opted not to change to nucleosides. The duration of nucleoside treatment varied from 6 to 36 months, with an average of 15.5 months as of August 31, 2017. All 16 patients are continuing treatment.

Doses administered to patients were based on the dosages used in preclinical studies of H126N Tk2 mutant mice.[15,16] Doses were titrated up to 400mg/kg/day depending on tolerance. Doses differed and were adjusted

based upon frequency of stools, which was observed to increase in proportion to the dose. All patients are currently maintained on doses between 300 and 400mg/kg/day of each nucleoside or nucleotide.

Outcome Measures

Survival Analysis. Survival was assessed in all patients.

Motor Assessment. All patients underwent periodic motor assessments using at least 1 of the following scales: 6-minute walk test (6MWT),[19] North Star Ambulatory Assessment (NSAA; evaluates motor goals with a score range of 0–34 as values of minimum and maximum motor skills, respectively and expressed in linearized data logit transformed 0–100 scores),[20,21] and Egen Klassifikation (EK; evaluates functional capacity in nonambulatory patients with a score range of 30–0 as values of minimum and maximum functional capacity, respectively).[22]

Respiratory Evaluation. We measured forced vital capacity (FVC) and maximal inspiratory pressure (MIP) in an upright position in compliant patients. In individuals on mechanical ventilation (MV), daily ventilatory support requirements were recorded.

Nutritional Status. We collected data on the evolution of weight (percentile) or body mass index (BMI) data. Underweight is defined as BMI < 18.5 or weight percentile <10. Requirements for nasogastric tube and percutaneous endoscopic gastrostomy (PEG) were recorded.

Creatinine Kinase. Creatine kinase (CK) was measured in serum, before and after treatment.

Serum Growth Differentiation Factor 15. Serum growth differentiation factor 15 (GDF-15), a proposed biomarker of mitochondrial myopathy, was measured before and during treatment as described.[23]

Safety Assessments

Serial blood tests and electrocardiograms were performed to assess baseline hematological, renal, hepatic, and cardiac functions as well as possible toxicity of the treatment. All Common Terminology Criteria for Adverse Events 4.03 grade 2 or higher adverse events or new clinical events were recorded.

Statistical Analyses

Kaplan–Meier survival analysis (SAS 9.4 for analysis and R 3.5.1 for replication and graphical output) was used to determine the survival rate of a historical control group of 44 early onset severe myopathy patients (onset before 24 months and rapid progression) from the literature.[5,6,9–13,24–30] For other outcomes, 95% confidence intervals (CIs) and associated 2-sided p values were obtained for the mean values of each outcome at every visit and for the change between visits. We evaluated the change in the outcome measures after 6 months of treatment and after ≥12 months of treatment, relative to baseline. The value after ≥12 months was the mean of all values in that period.

Role of the Funding Sources

The sponsors of this study did not contribute to the study design; collection, analysis, and interpretation of data; writing of this paper; and decision to submit this work for publication.

Results

Patients

The phenotype of the 16 TK2-deficient patients (P1–P16) spanned the clinical spectrum of the disease (Tables 1 and 2).[4,5,10,12]

Five patients (P1–P5) had early onset severe myopathy defined by (1) onset before 24 months; and (2) inability to walk, use of MV, or both within 1 year of onset. Four of these 5 patients required mechanical ventilation and enteric feeding. Two of these patients were never able to walk, and the other 3 had lost the ability to walk prior to treatment initiation. All were underweight before the treatment. They did not manifest encephalopathy or systemic involvement other than myopathy. These individuals were compared to a matched historical control group of 44 reported TK2 patients who fulfilled the same criteria.[4–6,9–13,24–30]

The other 11 patients showed slower progression. Three (27.3%) lost the ability to walk during the course of the disease, whereas 8 (72.7%) were still walking at treatment onset. All had dysphagia; P12 and P14 required enteric feeding. The majority of the patients (60%) had marked weight loss. Of the 11 patients, 5 (45.5%) required MV for an average of 11 hours per day. In the 4 late onset cases (P13–P16; onset ≥12 years old), respiratory symptoms overshadowed limb weakness, and 2 individuals (P14 and P15) required MV at night with only minimal limb weakness.

Survival and Function of Early Onset Severe Myopathy Subjects

Compared to a published cohort,[4] our treated early onset severe myopathy patients differed significantly in postonset survival ($p = 0.0078$; Fig 1). Only 27.3% of historical controls survived at least 2 years after onset (95% CI = 0.17–0.45),[4] compared to all 5 treated patients (range = 2.1–6.3 years, mean ± standard deviation = 3.93 ± 1.66). All 5 treated

TABLE 1. Baseline Characteristics of the Treated TK2-Deficient Patients

Patient	Age, yr/Sex	Country of Origin	Age at Onset	*TK2* Mutations	Muscle mtDNA Depletion (relative to normal)	Muscle mtDNA Multiple Deletions	Weight, BMI or P	CK, U/l
1	6/M	Spain	15 mo	p.His121Asn; p.Arg192Lys	13%	No	<1 P	284
2	6/M	Spain	17 mo	p.Tyr208Cys; p.Arg130Trp	15%	No	3 P	148
3	3/F	Spain	10 mo	p.Thr108Met; p.Pro227Serfs*9	30%	No	BMI = 14.3	179
4	7/M	USA	12 mo	p.Lys50Ilefs*99; p.Thr108Met	11%	No	<3 P	NA
5	4/F	Spain	23 mo	p.Lys202del; p.Asp177Tyr	25%	No	<3 P	1,183
6	30/M	Spain	24 mo	Homozygous p.Thr108Met	45%	Yes	BMI = 26.9	757
7	8/F	Spain	6 mo	Homozygous p.Thr108Met	18%	No	<3 P	1,009
8	6/M	Spain	18 mo	Homozygous p.Thr108Met	NA	NA	P50	393
9	12/M	Spain	30 mo	Homozygous p.His121Asn	50%	Yes	<3 P	538
10	8/F	Chile	11 mo	p.Thr108Met; p.His121Asn	NA	NA	BMI = 11.8	179
11	13/M	Italy	24 mo	p.Arg183Gln; p.Lys202del	10%	NA	NA	NA
12	28/M	Guatemala	18 mo	Homozygous p.His121Asn	NA	No	NA	200–5,500
13	32/F	Spain	12 yr	Homozygous p.Thr108Met	17%	Yes	BMI = 13.8	2,500
14	33/F	Spain	16 yr	Homozygous p.Thr108Met	39%	Yes	BMI = 17.7	303
15	60/M	Spain	50 yr	Homozygous p.Lys202del	NA	Yes	BMI = 27.6	400
16	61/F	Spain	30 yr	Homozygous p.Lys202del	60%	Yes	BMI = 27.2	294

Patients 1–5 had early onset severe myopathy patients; patients 6–16 had slower progression.
BMI = body mass index; CK = creatine kinase; F = female; M = male; NA = not available; P = percentile.

early onset patients achieved clinically meaningful improvements in motor functions (Supplementary Videos 1–5).

Motor Evaluation

6-Minute Walk Test. 6MWT was performed in the 8 patients who were able to ambulate prior to starting treatment and in 1 patient (P11) who regained the ability to walk after 1 year of treatment (Table 3). The distance walked improved in all but 1 (7/8 [87.5%]; P7 lacked baseline 6MWT). Six patients, who were on prolonged treatment (18–36 months), showed protracted improvement (Fig 2). The mean increase was 56m (95% CI = −21.7 to 113.7) after 6 months of treatment and 88.5m (95% CI = −5.47 to 171.5) at last follow-up, which ranged from 12 to 36 months of treatment; the mean increases appear to be clinically meaningful based upon estimates in Duchenne muscular dystrophy (DMD) indicating that mean minimal clinically important differences are 28.5 to 31.7m.[19] The subgroup of patients with low baseline 6MWT performance (<300m, range = 0–175m) displayed the greatest improvements; after 6 months of treatment, mean increase over baseline was 146m (95% CI = 133.3–158.7), and after 12 to 36 months, the average increase was 171.9m (95% CI = 84.5–259.2). In contrast, patients with high baseline 6MWT distances (≥300m, range = 386–530m) showed stable or slightly improved performance on therapy. Two patients with early onset severe myopathy (P3 and P5; Table 4, Supplementary Videos 3 and 5) and 1 childhood onset patient (P11) who had lost the ability to walk prior to treatment gained independent ambulation on treatment.

There were no statistically significant differences in the motor evaluation outcomes.

Egen Klassifikation. In the early onset severe myopathy group, EK score was available in 3 of 5 patients, revealing average improvements of 6 points (95% CI = −13.7 to 25.7) after 6 months of treatment, 17.3 points (95% CI = −3.8 to 38.5) after 12 months, and 23 points (95% CI = 7.9–38.1) after 18 to 36 months (see Fig 2C, Table 4). Among patients with disease onset at >2 years old, we obtained the EK score

TABLE 2. Baseline Functional Characteristics and Therapeutic Regimen of the Treated TK2-Deficient Patients

	Independent Gait			Ventilatory Support			Treatment (until August 2017)				
								dTMP+dCMP		dT+dC	
Patient	Yes/No	Age When Lost	Weight, BMI or P	Age at Initiation	h/day	Type of Feeding (oral or enteral)	Age at Treatment Onset	Duration	Daily Dose, mg/kg	Duration	Daily Dose, mg/kg
1	No	16 mo	<1 P	16 mo	14	Oral	31 mo	11 mo	400	27 mo	400
2	No	22 mo	3 P	29 mo	11	NGT	30 mo	11 mo	400	27 mo	400
3	No	Never	BMI = 14.3	12 mo	24	NGT	16 mo	–	–	25 mo	400
4	No	Never	<3 P	19 mo	24	NGT	19 mo	47 mo	200	21 mo	400
5	No	28 mo	<3 P	–	–	Oral	28 mo	–	–	20 mo	400
6	No	21 yr	BMI = 26.9	21 yr	8	Oral	28 yr	–	–	16 mo	400
7	Yes	–	<3 P	–	–	Oral	6 yr	–	–	24 mo	400
8	Yes	–	50 P	–	–	Oral	4 yr	–	–	16 mo	400
9	No	10 yr	<3 P	7 yr	NA	NGT	10 yr	–	–	13 mo	400
10	Yes	–	BMI = 11.8	–	–	Oral	4 yr	27 mo	200	20 mo	400
11	No	–	NA	–	NA	Oral	8 yr	65 mo	300	–	–
12	Yes	–	NA	15 yr	24	Oral	26 yr	11 mo	–	23 mo	350
13	Yes	–	BMI = 13.8	–	–	Oral	30 yr	–	–	26 mo	300
14	Yes	–	BMI = 17.7	28 yr	8	PEG	32 yr	–	–	15 mo	400
15	Yes	–	BMI = 27.6	50 yr	8	Oral	59 yr	–	–	20 mo	400
16	Yes	–	BMI = 27.2	–	–	Oral	60 yr	–	–	16 mo	400

Patients 1–5 had early onset severe myopathy patients; patients 6–16 had slower progression.
BMI = body mass index; NA = not available; NGT = nasogastric tube; P = percentile; PEG = percutaneous gastrostomy.

at baseline and after 6 months of treatment in 2 of 3 non-ambulant patients, who showed improvements of 3 and 17 points. These changes are not statistically significant; however, they are clinically meaningful based on an estimated 2.39-point change corresponding to functional and global state changes in patient with spinal muscular atrophy and DMD.[31]

North Star Ambulatory Assessment. In the sole early onset severe myopathy patient who was able to perform the NSAA, mean score improved by 16 points after 12 months of treatment (see Table 3). In the group of 11 patients with later onset, 5 patients were assessed by the NSAA. Their scores improved at every assessment relative to baseline, with mean improvements of 1.6 (95% CI = −0.6 to 3.8) after 6 months and 6 points (95% CI = −2.1 to 14.1) after 12 to 36 months of treatment (see Fig 2D). This improvement is not statistically significant. The mean NSAA score change is clinically meaningful after 12 to 36 months using a logit transformed scale of 0 to 100, which demonstrates an 11.5-point increase (baseline mean = 60 points and post-treatment = 74.5 points), with estimated minimal important differences of 6.9 to 8.8 points in patients with DMD.[21]

Respiratory Evaluations

Mechanical Ventilation. Nine patients required MV at baseline (see Tables 2 and 4). One (P3) was weaned off MV within 18 months of treatment initiation. The other patients who required nocturnal MV remained stable or improved partially after 12 months of treatment. None of the patients increased their time on MV, no patients initiated MV while on therapy, and none had respiratory complications including pneumonias.

FVC and MIP. Of 8 patients whose respiratory function could be evaluated, 7 showed low baseline FVC (<80% of predicted value), with restrictive lung disease patterns (see Fig 2E). After 6 months of treatment, FVC revealed an average increase of 7.2% (95% CI = −3.2 to 17.5). After 12 to 36 months of treatment, FVC showed a slight decline, but overall mean was 3.3% (95% CI = −0.4 to 7.0) higher than baseline (see Table 3). MIP measurements in 5 adult patients showed a mean increase of 5.6%

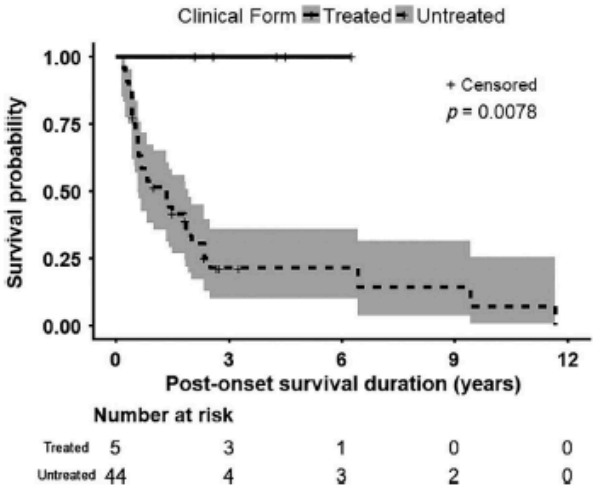

FIGURE 1: Kaplan–Meier survival curve of the 5 TK2-deficient patients with early onset severe myopathy (onset before 24 months and rapid progression defined by never acquiring ability to walk or loss of ability to walk, to breathe independently, or both within 1 year of onset) showed 100% survival for at least 2 years after treatment (range = 2.1–6.3 years; 3.93 ± 1.66 years, mean ± standard deviation). In contrast, the untreated historical control group with TK2-deficient early onset severe myopathy revealed that only 27.3% of patients survived at least 2 years after onset.[4–6,9–13,24–30] Shading indicates upper and lower 95% confidence interval. Comparison of treated versus untreated early onset severe myopathy patients demonstrated a significant difference in postonset survival ($p = 0.0078$).

(95% CI = −18.3 to 29.5) after 6 months of treatment that persisted after 12 to 36 months (see Fig 2F, Table 3). None of these relationships is statistically significant.

Nutrition/Dysphagia

In the early onset severe TK2-deficient patients, BMI and weight percentile increased progressively with prolonged treatment, eventually reaching normal values in 4 of 5 patients (80%; see Table 4 and Supplementary Table 1). Of the 3 patients in this group requiring enteric feeding at baseline, 2 (P1 and P3) were able to permanently discontinue enteric feeding after 18 and 36 months of treatment.

In the later onset group of 11 patients with slower progression, we obtained body weights in 10. Of six who were underweight at baseline, 5 (83%) gained weight and 4 (67%) normalized after 6 to 36 months of treatment (Supplementary Table 2). Both patients (P9 and P14) who required enteric feeding prior to treatment became able to feed exclusively by mouth after 6 months of therapy.

Creatine Kinase

In 4 of 16 (25%) patients, baseline CK levels were 5- to 10-fold above normal (see Table 2); in all 4, levels normalized after 6 months of treatment (Supplementary Tables 1 and 2). The remaining 12 cases showed normal or moderately altered CK levels (2–3-fold above normal) without changes on treatment.

Growth Differentiation Factor 15

Baseline serum GDF-15 levels were elevated above the upper limit of normal (550pg/ml) in all 7 patients tested (see Fig 2G, 2H, and Table 5). The highest levels were in P2 and P5 with the early onset severe presentation, followed by P12 with childhood onset and late onset patients with more moderate increases. In all cases, levels of GDF-15 declined between 4 and 33 months of treatment. Normal values were reached in 3 of 7 patients. In the remaining 4 patients, GDF-15 levels decreased between 1.5- and 12-fold.

Adverse Effects

No treatment-related serious adverse effects were observed in any patient. Safety blood tests and electrocardiogram were normal in all cases. The only drug-related adverse effect observed in 8 patients was diarrhea, which was dose-dependent, transient in most cases, and did not prompt suspension of treatment in any patients. Doses or administration schedules were modified to eliminate diarrhea. Diarrhea prevented 2 patients (P12 and P13) from reaching the recommended dose of 400mg/kg/day. One patient experienced mild and transient abdominal pain. Prior to treatment, P14 had elevated transaminases (approximately 10-fold above upper limit of normal) that were attributed to TK2 deficiency and normalized after 1 year of treatment with dC + dT.

In addition to the 16 patients reported here, 12 TK2-deficient patients initiated dT + dC (each at up to 400mg/kg/day; Supplementary Table 3). Two adults (P10, 58 years old; P11, 63 years old) after 3 to 4 months of treatment developed increased transaminases (alanine aminotransferase 8–13-fold and aspartate aminotransferase 3–6-fold above normal) and γ-glutamyl transferase (GGT; 2.1–3.7-fold above normal) with normal bilirubin and alkaline phosphatase levels. In both cases, 3 months after discontinuing therapy, transaminases returned to normal. P10 had a prior episode of spontaneous transient elevated transaminases several years before starting therapy. Twenty days after restarting dT + dC (each at 200mg/kg/day), P11 had recurrent elevated transaminases (3.0–4.1-fold above normal), which again returned to normal after stopping treatment.

Discussion

We have administered pyrimidine deoxynucleoside and deoxynucleotides as novel pharmacological therapies in 16 patients with mitochondrial myopathy due to TK2 deficiency. Although we initially used dTMP and dCMP, after identification of dT and dC as the active agents

TABLE 3. Assessments of Ambulation and Respiratory Functions at Baseline and after Treatment of TK2-Deficient Patients with Slower Progression

Assessment	After 6 Months of Treatment	After 12-36 Months of Treatment
6MWT overall		
n	6	7
Mean before treatment, m	351.5 (188.7 to 514.3)	304.4 (127.3 to 481.6)
Mean at visit, m	407.5 (304.1 to 510.9)	392.9 (283.2 to 502.6)
Change, m	56 (−21.70 to 113.7)	88.46 (5.471 to 171.5)
6MWT < 300m		
n	2	2
Mean before treatment, m	164 (24.23 to 303.8)	87.5 (−1024 to 1199)
Mean at visit, m	310 (182.9 to 437.1)	259.4 (−756.1 to 1284)
Change, m	146 (133.3 to 158.7)	171.9 (84.5 to 259.2)
6MWT > 300m		
n	4	5
Mean before treatment, m	445.3 (334.1 to 556.4)	419.2 (314.9 to 523.5)
Mean at visit, m	456.3 (326.8 to 585.7)	446.3 (355.3 to 537.3)
Change, m	11 (−40.23 to 62.23)	27.1 (−32.8 to 87)
NSAA		
n	5	4
Mean before treatment	19.6 (12.2 to 27)	21.5 (13.9 to 29.1)
Mean at visit	21.2 (13.7 to 28.7)	27.5 (22.4 to 32.6)
Change	1.6 (−0.6 to 3.8)	6 (−2.1 to 14.1)
FVC		
n	8	6
Mean before treatment, %	48.7 (25.4 to 72.0)	46.9 (22.8 to 71.0)
Mean at visit, %	55.9 (27.4 to 84.4)	50.2 (23.8 to 76.7)
Change, %	7.2 (−3.2 to 17.5)	3.3 (−0.4 to 7.0)
MIP		
n	4	5
Mean before treatment, %	29.2 (−0.3 to 58.3)	27.6 (7.1 to 48.1)
Mean at visit, %	34.9 ± (27.0 to 42.7)	33.6 (18.9 to 48.5)
Change, %	5.6 (−18.3 to 29.5)	6.1 (−4.8 to 16.9)

Mean and change in score were estimated by 95% confidence interval.
6MWT = 6-minute walk test; FVC = forced vital capacity; MIP = maximal inspiratory pressure; NSAA = North Star Ambulatory Assessment (evaluates motor goals with a score range of 0–34 as values of minimum and maximum motor skills, respectively).

in vitro and in vivo,[16,18] we administered dC and dT to all but 1 patient (P11), who continues dTMP and dCMP treatment. The therapies exerted striking effects on survival in the early onset severe myopathy patients through amelioration of muscle weakness, which enabled reductions or discontinuation of mechanical ventilation

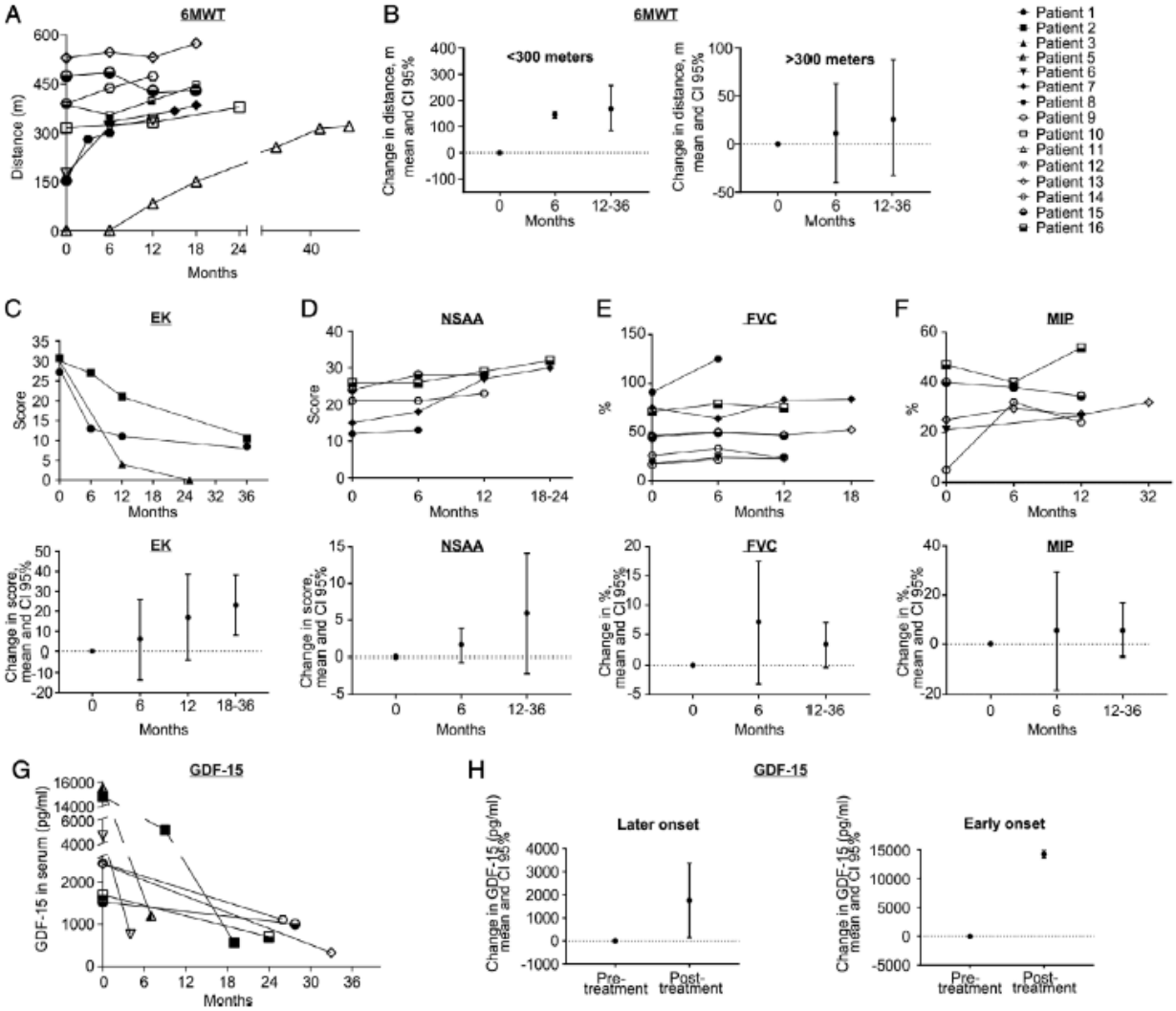

FIGURE 2: Changes from baseline in different outcome measures. (A) The graph shows individual values of distance walked in the 6-minute walk test (6MWT) at baseline and during the time on treatment, showing continuous improvements in 8 of 9 patients who were able to be evaluated by this test at any point in the treatment (note that P11 was nonambulant at baseline but regained independent gait after 12 months of treatment). (B) The group of patients with low performance (<300m) at baseline in the 6MWT showed more pronounced improvement after ≥12 months of treatment (mean 171.9m, 95% confidence interval [CI] = 84.5–259.2) than patients with higher basal performance (>300m, mean = 27.1m, 95% CI = −32.8 to 87). (C) Individual scores in the Egen Klassifikation (EK) at baseline and after treatment show improvement in every visit compared to the previous one in the 3 early onset severe myopathy patients evaluated by EK. The mean change in the score after 6 months of treatment and after 12 to 36 months, above baseline, showed progressive improvement. (D–F) North Star Ambulatory Assessment (NSAA) scores (D) showed improvement in all the patients evaluated in the group with slower progression, with a trend to improvement in forced vital capacity (FVC; E), and maximal inspiratory pressure (MIP; F) showed mild improvement or stabilization with slight fluctuations between visits, as reflected in the individual graphics. Both FVC and MIP showed a trend to mild improvement, with stabilization of the values during the treatment. Change in score (absolute values) in EK, NSAA, FVC, and MIP were estimated by 95% CI at every period of treatment relative to baseline. There were no statistical differences in any of the outcome measures. Serum levels of growth differentiation factor 15 (GDF-15) in individual subjects (G) and aggregated in later onset and early onset groups (H).

and gastrostomy feeding as well as gaining ability to walk in the majority of these severe cases. Oral deoxynucleosides and deoxynucleotides produced no major side effects during this long-term treatment. The beneficial effects of the therapy were verified by functional tests, including 6MWT, EK, and NSAA, with mean changes that appear to be clinically meaningful. In addition, serum levels of GDF-15, a sensitive diagnostic biomarker for mitochondrial myopathy,[23,32,33] were highly elevated at baseline and markedly declined in all 7 patients tested. Thus, GDF-15 assessment provides objective biomarker data supporting therapeutic response to deoxynucleosides and deoxynucleotides.

Our data indicate that nucleoside treatment can (1) reverse early onset tetraplegia and enable termination of

TABLE 4. Weight, Oral Intake Status, Ventilator Use, and Ability to Walk at Baseline and With Treatment in the TK2-Deficient Patients

Patients with Early Onset Severe Myopathy

Characteristic	Before Treatment	After 6 Months of Treatment	After 12 Months of Treatment	After 18–36 Months of Treatment
Underweight, n (%)	5 (100%)	3 (60%)	3 (60%)	1 (20%)
Enteric feeding, n (%)	3 (60%)	3 (60%)	3 (60%)	1 (20%)
MV, n (%)	4 (80%)	4 (80%)	4 (80%)	3 (60%)
Ambulatory, n (%)	0	1 (20%), P5	1 (20%), P5	2 (40%), P3 + P5
EK mean (95% CI)	29.3 (26.5–32.2), n = 3	20 (−68.9 to 108.9), n = 2	12 (−9.2 to 33.2, n = 3)	6.3 (−7.8 to 20.46, n = 3)
Change in EK relative to BT baseline (95% CI)		6 (−13.7 to 25.7)	17.3 (−3.8 to 38.5)	23 (7.9–38.1)

Patients with Later Onset Slower Progressive Myopathy

Characteristic	Before Treatment	After 6 Months of Treatment	After 12–36 Months of Treatment
Underweight, n (%)	6 (60%)	4 (40%)	3 (30%)
Enteric feeding, n (%)	2 (20%)	0 (0%)	0 (0%)
MV, n (%)	5 (45.5%)	5 (45.5%)	5 (45.5%)
Ambulatory, n (%)	8 (72.7%)	8 (72.7%)	9 (81.8 %)

CI = confidence interval; EK = Egen Klassifikation (evaluates functional capacity in nonambulatory patients with a score range of 30–0 as values of minimum and maximum functional capacity, respectively); MV = mechanical ventilation; P = Patient; BT = before treatment.

TABLE 5. GDF-15 Levels at Pretreatment Baseline and on Deoxynucleoside Treatment

Patient	Baseline GDF-15, pg/ml	GDF-15 Levels on Treatment, pg/ml									
		4 mo	7 mo	8 mo	9 mo	17 mo	19 mo	24 mo	26 mo	28 mo	33 mo
P1	–	–	–	2,011	–	255	–	–	–	–	–
P2	14,756	–	–	–	5,093	–	564	–	–	–	–
P5	15,490	–	1,200	–	–	–	–	–	–	–	–
P12	4,608	762	–	–	–	–	–	–	–	–	–
P13	2,422	–	–	–	–	–	–	–	–	–	330
P14	2,439	–	–	–	–	–	–	–	1,110	–	–
P15	1,529	–	–	–	–	–	–	–	–	1,003	–
P16	1,695	–	–	–	–	–	–	704	–	–	–

GDF-15 = growth differentiation factor 15.

mechanical ventilation and PEG (P3; Supplementary Video 3), (2) halt early onset disease progression and improve muscle weakness (P1–P5; Supplementary Videos 1–5), (3) produce considerable functional improvements in childhood onset patients (P9, P11), and (4) stabilize weakness in late onset patients after an initial mild improvement (P14, P16). Four treated patients were weaned off invasive respiratory support (P3), gastrostomy feeding (P1, P3, P9, and P14), or both (P3),

and 3 gained independent ambulation on treatment (P3, P5, and P11). Furthermore, the 6MWT showed improvements in treated patients with low baseline performance (164m, 95% CI = 24.3–303.8), with clear increases in the distance walked (mean increase 171.9m, 95% CI = 84.5–259.2) at last follow-up (12–36 months of treatment).

In contrast to the early onset patients, in 4 late onset patients, therapy produced smaller beneficial effects, with stabilization or mild improvements in motor and respiratory functions. Stabilization of respiratory function is an important clinical outcome, because it may reduce morbidity and mortality in late onset patients. Nevertheless, 2 adult patients who started dT + dC therapy after the initial cohort developed elevated transaminases and GGT, which normalized after discontinuing treatment; these findings raise the possibility of hepatic toxicity of the therapy in some older adults. Our observations require confirmation in a larger number of patients with long-term follow-up.

A cytokine member of the transforming growth factor β family, GDF-15, is induced and secreted by muscle cells in response to mitochondrial damage,[23] and has been identified as a biomarker for mitochondrial diseases via an unbiased global gene expression screening of muscle from patients with TK2 deficiency.[32] Since then, elevated GDF-15 levels have been confirmed to have high sensitivity (67.8–97.9%) and specificity (87.7–96.2%) for mitochondrial diseases, indicating potential utility as a first-line diagnostic test for these diverse disorders.[33–35] Although it has been proposed as a potential biomarker for therapeutic efficacy in mitochondrial diseases,[36] this is the first report to link GDF-15 levels with clinical improvements in a clinical trial for a mitochondrial disorder. Our data demonstrate that in patients with TK2 deficiency, baseline circulating GDF-15 levels were elevated before deoxynucleoside therapy and decreased significantly with treatment in patients. Furthermore, these results indicate that GDF-15 may be a valuable biomarker in future clinical trials for mitochondrial diseases.

In conclusion, treatment with oral dT + dC, the substrates of TK2, in 15 TK2-deficient patients and dTMP +dCMP in 1 patient provided clinically notable benefit, especially in the infantile and childhood onset forms of the disease. The improvement or stabilization of respiratory function in the late onset patients suggests that this subgroup may also benefit from nucleoside supplementation, although longer longitudinal studies are needed to establish this point. Further studies are ongoing to support potential regulatory approvals.

Acknowledgment

This work was supported in part by grants from the Spanish Carlos III Health Institute (PMP15/00025 for C.P., F.Ma., and R.M.; PI16/00579 and CP09/00011 for C.J.-M.), Muscular Dystrophy Association (577391), Arturo Estopinan TK2 Research Fund, Generalitat de Catalunya PERIS program (SLT002/16/00370 for J.T-T.), and European Regional Development Fund.

We thank the patients and their families for collaborating with this study. We are especially grateful to patients J.J.M.P. and A.E. and their families for their exceptional contributions to multiple aspects of this research.

Author Contributions

C.D.-G., R.M., C.P., and M.H. were responsible for the conception and design of this study. All authors acquired and analyzed data. C.D.-G., C.J.-M., F.Ma., R.M., C.P., and M.H. drafted the manuscript and figures.

Potential Conflicts of Interest

C.G., R.M., and M.H. are paid consultants to Modis Therapeutics. R.M. has equity in Modis Therapeutics. These relationships are de minimus for the United Kingdom Medical Research Council (C.G.), Vall d'Hebron Research Institute (R.M.), and Columbia University Medical Center (M.H.). Columbia University has submitted a patent, which has been licensed to Modis Therapeutics; this relationship is monitored by an unconflicted external academic researcher. The other authors declare no conflicts of interest.

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Scientific Reports (2020) 10:10111 | doi.org/10.1038/s41598-020-66940-8

SCIENTIFIC REPORTS
nature research

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Growth Differentiation Factor 15 is a potential biomarker of therapeutic response for TK2 deficient myopathy

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GDF-15 is a biomarker for mitochondrial diseases. We investigated the application of GDF-15 as biomarker of disease severity and response to deoxynucleoside treatment in patients with thymidine kinase 2 (TK2) deficiency and compared it to FGF-21. GDF-15 and FGF-21 were measured in serum from 24 patients with TK2 deficiency treated 1–49 months with oral deoxynucleosides. Patients were grouped according to age at treatment and biomarkers were analyzed at baseline and various time points after treatment initiation. GDF-15 was elevated on average 30-fold in children and 6-fold in adults before the start of treatment. There was a significant correlation between basal GDF-15 and severity based on pretreatment distance walked (6MWT) and weight (BMI). During treatment, GDF-15 significantly declined, and the decrease was accompanied by relevant clinical improvements. The decline was greater in the paediatric group, which included the most severe patients and showed the greatest clinical benefit, than in the adult patients. The decline of FGF-21 was less prominent and consistent. GDF-15 is a potential biomarker of severity and of therapeutic response for patients with TK2 deficiency. In addition, we show evidence of clinical benefit of deoxynucleoside treatment, especially when treatment is initiated at an early age.

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Mitochondrial DNA depletion and deletion syndrome 2 (MTDPS2) is caused by mutations in the nuclear gene *TK2* that encodes thymidine kinase 2 (*TK2*) which is necessary for mitochondrial DNA replication and maintenance[1]. TK2 deficiency manifests predominantly as a myopathy and includes extremely severe and rapidly progressive early-onset forms with survival of less than two years, to milder forms with late or very late-onset, and a slower rate of progression, but with almost invariable respiratory involvement than shortens life expectancy[2–4].

A new therapy for TK2 deficiency is under investigation, based on administration of oral deoxynucleosides. The rationale is to induce the synthesis of pyrimidine deoxynucleotides (dTTP and dCTP) via alternative enzyme pathways by means of supplementation with their precursors. Although some patients started with taking oral deoxynucleotides (dTMP and dCMP), after the demonstration *in vitro* and *in vivo*[5,6] that deoxynucleosides are the active agents, the therapy was changed to deoxynucleosides (dThd and dCtd). The efficacy of oral deoxynucleoside treatment was demonstrated in pre-clinical studies[7,8] and in a group of 16 patients treated under a compassionate use program[9]. Without any major side effect, the therapy had striking effects on early-onset severe myopathy patients, such as improvement in muscle strength, reduction or discontinuation of mechanical ventilation and gastrostomy feeding, and regaining the ability to walk. The patients also showed considerable functional improvements according to motor outcome measures in childhood-onset cases and at least stabilization in late-onset ones[9].

Biomarkers are playing an increasingly important role in drug development and treatment implementation[10]. In addition to contributing to a better understanding of diseases, biomarkers provide more sensitive and specific means of diagnosing and ways to determine responses to new treatments. In this way, they help to streamline clinical trial efficacy and reduce uncertainty in regulatory decision-making, thus accelerating drug approval and drug access for patients.

According to BEST (The Biomarkers, EndpointS and other Tools, FDA-NIH Biomarker Working Group, https://www.ncbi.nlm.nih.gov/books/NBK326791/) a monitoring biomarker is a biomarker which is measured serially for assessing the status of a disease or for evidence of an effect of a medical product. A pharmacodynamics/response biomarker is used to show that a biological response has occurred in an individual who has been exposed to a medical product.

Growth Differentiation Factor 15 (GDF-15) is a cytokine that is induced in response to various stimuli and in different pathological situations where it is associated with disease progression and negative prognosis[11–13]. We previously demonstrated that GDF-15 is a valuable circulating diagnostic biomarker for mitochondrial diseases including mitochondrial DNA depletion and deletion syndromes, MELAS and KSS[14–16]. Fibroblast Growth Factor 21 (FGF-21) has also been reported to be elevated in a range of mitochondrial diseases, particularly in those with muscle involvement[17]. Determination of circulating GDF-15 and FGF-21 has greatly aided the diagnosis of mitochondrial pathologies since their sensitivity is significantly higher than other conventional biomarkers such as venous lactate level[16,18].

The objective of this study was to explore the application of circulating GDF-15 as a biomarker of disease severity and prognosis as well as to monitor response to deoxynucleosides treatment in patients with TK2 deficiency, and compare it with FGF-21. Furthermore, since GDF-15 has been linked to inflammation in some diseases[13,19] we also investigated circulating levels of inflammatory cytokines.

Results

Patients. The 26 patients included span the spectrum of phenotypes associated with TK2 deficiency[4]. Pre-treatment data are summarized in Table 1. Patients P25 and P26 had to stop treatment soon after initiation because of an increase in liver enzymes. They were not further analyzed. Follow-up data of P1 to P24 are available in Tables 2 and 3.

The 24 treated patients were grouped according to age at treatment initiation: group 1 (the paediatric group) started treatment before 16 years of age (P1 to P15), and group 2 (the adult group) started treatment after this age (P16 to P24). This grouping allowed us to compare GDF-15 and FGF-21 serum levels with aged-matched controls and takes into account the observation that age-related metabolic changes contribute to the efficacy of deoxynucleoside therapy[8].

Group 1 consisted of 15 children (five female and ten male), six of them with early-onset and very rapid progression (P1, P3, P4, P5, P6, and P7) and nine with childhood-onset and an intermediate disease course (P2 and P8-P15). Group 2 consisted of nine patients (seven female and two male) and included one patient with childhood-onset (P22) and eight patients with late-onset forms (P16 to P21 and P23 to P24). To assess disease progression, patients were compared with other patients with similar clinical phenotypes based on previous publications[2,4,9]. The follow-up time ranged from 1 to 49 months for patients in group 1 and from 1 to 46 months for patients in group 2. A summary of the age of onset and treatment initiation for each group is provided in Table 4.

Change of serum GDF-15 and FGF-21 over time in treated patients. We previously described the baseline levels of GDF-15 in a healthy pediatric population[15]. For the purposes of this study, we analyzed GDF-15 serum levels in 13 adult healthy controls (age range 23–56 years) and found that the mean GDF-15 concentration was 323 pg/mL (SE 19.7; range 232–460 pg/mL) which is comparable to the mean values in the pediatric (age range 1 month to 18 years) control population (350 pg/mL; SE 20.7, range 155–584) and overlaps with values reported elsewhere[20]. Baseline levels were available from 12 participants in group 1 and nine in group 2. The mean values and ranges for GDF-15 and FGF-21 for both groups of patients are summarised in Table 4. GDF-15 levels were increased on average by 30-fold in group 1 (mean 10716 pg/mL; SE 2579; range 2641–27066), and by six-fold in group 2 (mean 1955 pg/mL; SE 149; range 1261–2483), relative to the mean values in the control groups. If we only take into account the five patients from group 1 with the most severe disease form for whom we have basal measurements, the average GDF-15 levels were 60-fold increased over the value in the paediatric control group (mean 20246 pg/mL; SE 2371; range 14756–27066).

CLINICAL FORM	ID	SEX	AGE AT ONSET	BMI	WALK INDEPENDENTLY (Y/N)	MECHANICAL VENTILATION (Y/N)	PEG (Y/N)	GENOTYPE (Allele 1/Allele 2)	DEPLETION (relative to normal)	MULTIPLE DELETIONS (Y/N)	CK (UI/l)	BASAL GDF-15 (pg/mL)	BASAL FGF-21 (pg/mL)
GROUP 1 (started treatment <16)	1	F	23 m	<P3	N	N	N	p.Lys202del/p.Asp177Tyr	25%	N	1183	15.000	1373
	2	M	30 m	<P3	N	Y	NGT	p.His121Asn/p.His12Asn	50%	Y	538	4608	402
	3	M	17 m	P3	N	Y	NGT	p.Tyr208Cys/p.Arg130Trp	15%	N	148	14756	3013
	4	M	15 m	<P3	N	Y	N	p.His121Asn/p.Arg192Lys	13%	N	284	ND	ND
	5	M	13 m	<P3	N	Y	NGT	p.Thr108Met/Leu215Pro	ND	ND	872	21641	4088
	6	M	13 m	<P3	N	Y	NGT	p.Thr108Met/Leu215Pro	ND	ND	949	22767	3244
	7	M	13 m	<P3	N	N	N	p.Lys85*/p.Pro154Leu	ND	ND	921	27066	7531
	8	M	24 m	ND	ND	ND	ND	p.Thr108Met/p.Thr108Met	ND	ND	285	4597	ND
	9	M	31 m	ND	ND	ND	ND	p.Thr108Met/p.Thr108Met	8%	ND	626	6825	ND
	10	F	3 y	P3	Y	Y	NGT	p.Asn58Ser/p.Asn58Ser	ND	ND	1789	4797	809
	11	M	3 y	P50	Y	N	N	p.Asn58Ser/p.Asn58Ser	ND	ND	1036	2641	398
	12	F	2 y	P50	Y	N	N	p.His121Asn/p.Arg183Trp	ND	ND	460	3686	786
	13	F	2 y	P50	Y	N	N	p.His121Asn/p.Arg183Trp	ND	ND	945	3841	471
	14	M	3 y	P3	N	Y	N	p.His121Asn/p.Arg183Trp	ND	ND	184	5006	1057
	15	F	6 y	<P3	Y	N	N	p.Asn58Ser/p.Asn58Ser	ND	ND	742	2781	595
GROUP 2 (started treatment >16)	16	F	> 12 y	13.86	Y	Y	N	p.Thr108Met/p.Thr108Met	17%	Y	2435	2423	666
	17	M	50 y	27.7	Y	Y	N	p.Lys202del/p.Lys202del	66%	Y	357	1529	252
	18	F	30 y	27.12	Y	Y	N	p.Lys202del/p.Lys202del	60%	Y	294	1695	353
	19	F	20 y	17.79	Y	Y	Y	p.Thr108Met/p.Thr108Met	39%	Y	303	2439	197
	20	F	60 y	26.01	Y	Y	N	p.Lys202del/p.Lys202del	ND	ND	647	2483	897
	21	M	30 y	23.7	Y	N	N	p.Lys202del/p.Lys202del	ND	Y	350	1640	185
	22	F	5 y	26.17	Y	Y	N	p.Thr108Met/p.Thr108Met	ND	Y	548	2149	943
	23	F	30 y	ND	Y	Y	N	p.Thr108Met/p.Thr108Met	53%	Y	233	1261	190
	24	F	20 y	20,5	Y	Y	GT	p.Lys202del/p.Ala420Val	ND	ND	1142	1979	179
	25	F	50 Y	28,7	Y	Y	N	p.Lys202del/p.Lys202del	ND	Y	405	ND	ND
	26	F	14 Y	24,5	Y	Y	N	p.Thr108Met/p.Thr108Met	19%	Y	425	ND	ND

Table 1. Clinical, biochemical and molecular characteristics of patients, before treatment. BMI, body-mass index; CK, creatine kinase; F, female; GDF-15, differentiation growth factor 15; M, male; m, months; ND, not determined; NGT, nasogastric tube; N, No; P, percentile; PEG, percutaneous endoscopic gastrostomy; y, years; Y, yes.

For FGF-21, average baseline levels were elevated by 25-fold in group 1 (mean 1981 pg/mL; SE 702; range 402–731) and by six-fold in group 2 (mean 429 pg/mL; SE 106; range 179–943), relative to control values[16]. Similarly to GDF-15, the average basal FGF-21 value almost doubled if we only took into account the most severe patients from group 1 (Table 4).

Thus, both GDF-15 and FGF-21 basal serum levels are associated with the severity of the phenotype. This was, however, not the case for CK since its basal levels were comparable regardless of the severity of the phenotype (Fig. 1).

The development of serum GDF-15 values at different times after the initiation of treatment is represented in Fig. 1. We applied a linear mixed model using the log-transformed GDF-15 concentration levels and found a significant decrease over time in patients in both group 1 ($p < 0.0001$) and group 2 ($p = 0.0082$) (Fig. 2A,B). FGF-21 followed a similar trend, although not significant, for group 1 ($p = 0.062$) but this trend was not present in group 2 (Fig. 2C,D).

Because the time points at which GDF-15 levels were measured are not uniform across patients and to homogenize the data we grouped the measurements at regular intervals corresponding to the follow-up intervals for each group (Supp. Fig. 1). We observed that in group 1 GDF-15 values decreased steadily over time, particularly between 0 and 24 months and that the difference between intervals was statistically significant (Kruskal-Wallis chi-squared $p < 0.001$). After 12 months of treatment, most values were very close or below the normal threshold (550 pg/mL) with the exception of P2.

In patient P2 the GDF-15 concentration already decreased to almost normal levels after only four months of treatment. For this patient the next measurement was performed after two years of treatment and a few days after the nucleoside treatment dose was reduced to 300 mg/Kg (and remained at this dose) because of diarrhea, and at this point GDF-15 was increased (to half the baseline level). Three months later, GDF-15 had almost returned to normal levels in P2. We do not know when exactly the increase in GDF-15 occurred and whether it happened before or during the few days interval between the change of dose and the measurement.

When we analysed FGF-21 levels, we found significant differences between the intervals ($p = 0.01$) although after 12 months of treatment half of the patients still had levels above the normal threshold (Supp. Fig. 1).

ID		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
AGE OF ONSET		23 m	30 m	17 m	15 m	13 m	13 m	13 m	24 m	31 m	36 m	36 m	24 m	24 m	36 m	6 y
AGE AT INITIATION OF TREATMENT		27 m	10 y	30 m	2.5 y	15 m	15 m	14 m	33 m	6 y	14 y	12 y	3 y	3 y	7 y	9 y
CURRENT AGE		5 y	13 y	7 y	7 y	21 m	21 m	24 m	3 y	7 y	16 y	13 y	5 y	5 y	9 y	10 y
TREATMENT DOSE (mg/kg/d)		400	300	400	400	400	400	400	400	400	400	400	400	400	400	400
CK	BASAL	1183	538	148	284	872	940	921	285	626	1789	759	460	945	184	742
	0–6 Months	ND	316	ND	ND	649	561	275	133	175	235	243	606	857	164	269
	6–12 Months	114	ND	68	132	ND	ND	164	130	165	273	290	173	87	111	195
	12–24 Months	55	712	ND	116	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	>24 Months	87	285	ND	61	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
GDF-15 (pg/mL)	BASAL	15000	4608	14756	ND	21641	22767	27066	ND	ND	4797	2641	3686	3841	5006	2781
	0–6 Months	1200	762	ND	ND	6628	2174	588	4597	6825	4753	3105	ND	2635	5180	2249
	6–12 Months	497	ND	5093	2021	ND	ND	ND	396	369	ND	ND	341	330	440	345
	12–24 Months	392	2084	594	255	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	>24 Months	288	631	ND	284	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
FGF-21 (pg/mL)	BASAL	1373	402	3013	ND	4088	3245	7531	ND	ND	809	398	786	471	1057	595
	0–6 Months	72	228	ND	ND	1445	776	208	583	743	769	1090	ND	351	1077	417
	6–12 Months	31	ND	2170	415	ND	ND	ND	ND	ND	ND	ND	42	<LOQ	<LOQ	34
	12–24 Months	30	689	603	51	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	>24 Months	37	134	ND	100	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
BMI	BASAL	<P3	<P3	P3	P1	13,2	13,5	12,7	ND	ND	15,3	20,2	13,5	16	11,5	10,5
	0–6 Months	ND	9.6	ND	ND	12,8	13,2	12,9	ND	ND	16,1	19,05	15,02	15,08	11	10,7
	6–12 Months	17.2	ND	16.02	12.75	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	12–24 Months	16.3	20	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	>24 Months	14.9	25.3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
6MWT	BASAL	0	ND	ND	ND	ND	ND	ND	ND	420	75	342	250	272	0	307
	0–6 Months	150	ND	ND	ND	ND	ND	ND	ND	531	350	354	ND	ND	ND	364
	6–12 Months	250	ND	ND	ND	ND	ND	ND	ND	512	379	375	394	368	15	ND
	12–24 Months	391	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	>24 Months	477	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
EK2	BASAL	ND	26	30	28	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	0–6 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	6–12 Months	ND	9	21	14	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	12–24 Months	ND	ND	ND	12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	>24 Months	ND	13	ND	11	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NSAA	BASAL	4	ND	ND	ND	ND	ND	ND	20	27	ND	ND	ND	ND	ND	ND
	0–6 Months	6	ND	ND	ND	ND	ND	ND	ND	33	ND	ND	ND	ND	ND	ND
	6–12 Months	20	ND	ND	ND	ND	ND	ND	31	33	ND	ND	ND	ND	ND	ND
	12–24 Months	26	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	>24 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HMFS	BASAL	ND	ND	ND	ND	ND	ND	ND	ND	ND	21	35	38	38	17	38
	0–6 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	30	33	ND	ND	ND	39
	6–12 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	36	37	39	39	23	ND
	12–24 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	>24 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
HMFSE	BASAL	ND	ND	ND	ND	ND	ND	ND	ND	ND	26	51	51	53	19	62
	0–6 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	48	49	ND	ND	ND	65
	6–12 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	57	55	65	65	27	ND
	12–24 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	>24 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CHOP INTEND	BASAL	ND	ND	ND	ND	42	45	17	ND	ND	ND	ND	ND	ND	ND	ND
	0–6 Months	ND	ND	ND	ND	45	54	64	ND	ND	ND	ND	ND	ND	ND	ND
	6–12 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	12–24 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	>24 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Continued

ID		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
FVC (%)	BASAL	ND	13	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	38
	0–6 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	19	50	ND	ND	ND	66
	6–12 Months	ND	22	ND	ND	ND	ND	ND	ND	ND	35	70	ND	ND	71	ND
	12–24 Months	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	>24 Months	ND	26	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 2. Outcome measures, Group 1 (age at treatment initiation <16 years). CK, creatine kinase; EK, Egen Klassifikation; HMFS, Hammersmith Motor Functional Scale; HMFSE, Hammersmith Motor Functional Scale Expanded; 6MWT, 6-minute walk test; m, months; ND, not determined; NSAA, North Star Ambulatory Assessment; y, years.

In the group that started treatment at an older age (>16 years-old), consisting mainly of patients with a late-onset phenotype, biomarker determinations spanned a longer period of time (0 to >36 months). Although the differences in GDF-15 levels between the different intervals were significant (Kruskal-Wallis chi-squared $p = 0.003$), levels appeared to decrease mainly during the first 12 months of treatment and then remained stable (Supp. Fig. 1). In contrast, there was no clear trend for FGF-21 and in some patients levels even increased with treatment. In this case, the difference between intervals was not significant (Supp. Fig. 1).

We calculated the rate of decline of GDF-15 and FGF-21 levels relative to the basal levels in the same time intervals in the paediatric and adult patients for whom we had measurements before treatment (% change at time T = log GDF-15 at T-log GDF-15 at T0/log GDF-15 T0 x 100). The rate of decline increased with time in group 1 for GDF-15 (−12.5% between 0 and 6 months, −26.7% between 6 and 12 months, −26.9% between 12 and 24 months and 32.3% after 24 months) and FGF-21 (−11.5%, −36.3%, −21.3% and −34.2% in the same intervals) whereas it was rather uniform in group 2 for GDF-15 (−10.3% between 0 and 6 months, −12.8% between 6 and 12 months, −12.5% between 12 and 36 months, and −12.6% after 36 months) and for FGF-21 (−15.4%, −8.4%, −14% and −9.1% for the same time intervals).

Change of serum GDF-15 over time in patients that stopped treatment. Patients P25 and P26 stopped treatment because their liver enzymes had increased. P25 received treatment for three months, then stopped for three months, and then took nucleosides for one more month before stopping treatment completely. We do not have baseline levels for P25. The first GDF-15 measurement, performed after 6 months without treatment, was elevated (1833 pg/mL). One month later, GDF-15 levels had decreased to 1489 pg/mL, two months later (9 months after stopping the treatment completely) GDF-15 went up to 1675 pg/mL. Then they remained elevated (1657 pg/mL) even after 18 months of stopping the treatment when the last measurement was taken.

P26 received treatment for three months, stopped for four months, and then restarted treatment for one more month before stopping completely. GDF-15 levels were measured nearly three months after treatment interruption and were then mildly elevated (688 pg/ml). Then, in this patient, they remained stable over the next four months (including one month with treatment) to 639 pg/mL. Three months later (10 months after the first treatment interruption) it had risen to 818 pg/ml. From then onwards, levels continued to rise (1301 pg/mL after 13 months of treatment interruption) to reach 1656 pg/mL at the last measurement, 16 months after treatment interruption.

Thus, the trend of GDF-15 in the absence of treatment appears to reflect the disease course in these two patients, P25 has remained clinically stable whilst P26 has a more progressive form of the disease.

The comparison between GDF-15 concentrations in treated adult patients and these two patients that stopped over a similar time frame (0 to 28 months) is shown in Fig. 3. While in the treated patients GDF-15 levels declined, in the two patients that stopped they went up or remained stable but did not lower in either case.

Clinical outcome measures and correlation analysis. We analyzed several clinical outcomes in both patients groups to determine whether the decline in GDF-15 was associated with clinical improvement following treatment. Tables 2 and 3 and Fig. 4 summarise this information.

Patients P1, P3, and P4 are the patients P5, P2, and P1 in our previous report[9]. They are in the early-onset and severe myopathy group in which the response to treatment was striking. This group of patients was defined by (1) onset before 24 months and (2) inability to walk, use of mechanical ventilation, or both within one year of onset. Without treatment, only 27.3% of patients described in the literature with such characteristics survived at least two years after onset (95% CI = 0.17–0.45)[9]. In contrast, with treatment, P1, P3, and P4 are still alive, four, six, and six years after disease onset respectively. One of these patients was weaned of ventilatory support and the two others regained the ability to walk without assistance. Patients P5, P6, and P7, not reported previously, also represent the most severe phenotype with early-onset and very rapid progression course. After treatment, they were evaluated with the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND) assessments, which showed a mean improvement of 13.7 points after only three months of follow-up, and one (P7) even reached the maximum score on the scale three months later, which indicates complete motor recovery after only six months of treatment.

In childhood-onset cases, when we compared the latest post-treatment value (around one year after the start of the treatment) to the pre-treatment value for the more frequent outcome measures used, we found a significant improvement in the distance walked on the 6-minutes walking test (6MWT), with a mean increase of 105.8 meters ($p = 0.01$) (Fig. 4A). We also found a significant improvement in the scores of the Hammersmith Motor Function

ID		16	17	18	19	20	21	22	23	24
AGE OF ONSET		>12 y	50 y	30 y	20 y	60 y	36 y	5 y	30 y	20 y
AGE AT INITIATION OF TREATMENT		30 y	58 y	59 y	31 y	74 y	60 y	35 y	46 y	57 y
CURRENT AGE		34 y	62 y	62 y	34 y	75 y	60 y	36 y	46 y	60 y
TREATMENT DOSE (mg/kg/d)		320	300	400	400	400	400	350	300	400
CK (UI/l)	BASAL	2435	525	294	303	647	402	548	188	1142
	0–6 Months	ND	ND	123	322	ND	264	107	ND	1195
	6–12 Months	393	ND	258	292	105	ND	142	ND	1348
	12–36 Months	330	351	108	110	ND	ND	ND	ND	ND
	>36 Months	561	459	185	467	89	ND	ND	ND	ND
GDF-15 (pg/mL)	BASAL	2423	1529	1695	2439	2483	1640	2149	1261	1980
	0–6 Months	ND	ND	ND	ND	1364	1357	473	486	1052
	6–12 Months	ND	ND	ND	ND	1320	ND	413	ND	1011
	12–36 Months	464	782	704	1110	1099	ND	ND	ND	ND
	>36 Months	480	1137	753	783	ND	ND	ND	ND	ND
FGF-21 (pg/mL)	BASAL	666	252	353	197	897	185	943	190	179
	0–6 Months	ND	ND	ND	ND	732	135	47	ND	113
	6–12 Months	ND	ND	ND	ND	344	ND	ND	ND	155
	12–36 Months	1222	944	245	915	ND	ND	ND	ND	ND
	>36 Months	632	1025	421	ND	ND	ND	ND	ND	ND
BMI	BASAL	13.86	27.7	27.12	17.79	26.01	23.7	26.17	ND	20,8
	0–6 Months	ND	ND	ND	19,06	25	23.6	25.4	ND	21
	6–12 Months	ND	ND	ND	21,09	27,6	ND	ND	ND	20,4
	12–36 Months	16.06	ND	26.3	21.23	ND	ND	ND	ND	ND
	>36 Months	ND	ND	26.9	18,63	ND	ND	ND	ND	ND
6MWT	BASAL	532	475	386	390	345	ND	368	413	225
	0–6 Months	ND	500	355	437	ND	ND	435	515	251
	6–12 Months	ND	ND	400	474	ND	ND	450	ND	228
	12–36 Months	550	435	354	369	ND	ND	ND	ND	ND
	>36 Months	600	442	450	476	ND	ND	ND	ND	ND
NSAA	BASAL	ND	ND	26	21	29	ND	16	30	ND
	0–6 Months	30	24	26	21	ND	ND	25	31	ND
	6–12 Months	ND	ND	29	23	ND	ND	26	ND	ND
	12–36 Months	29	27	32	28	ND	ND	ND	ND	ND
	>36 Months	30	ND	31	29	ND	ND	ND	ND	ND
PIM (%)	BASAL	25	40	49.9	5	59	ND	37	31	ND
	0–6 Months	ND	ND	40.2	ND	33,9	ND	37,8	ND	ND
	6–12 Months	29.5	ND	53	24	47.3	ND	ND	ND	ND
	12–36 Months	21.4	38.6	49.9	ND	39,8	ND	ND	ND	ND
	>36 Months	ND	28	53	27	ND	ND	ND	ND	ND
FVC (%)	BASAL	46.5	45	71.8	26	69	78	53.2	63.7	ND
	0–6 Months	ND	51.4	79.5	33	66,5	82.6	62	64	16
	6–12 Months	50.3	ND	75	23,7	78,9	ND	ND	ND	17
	12–36 Months	46.7	46.7	77.7	32,2	83,4	ND	ND	ND	ND
	>36 Months	50.5	43.6	72	34,8	ND	ND	ND	ND	ND

Table 3. Outcome measures, Group 2 (age at treatment initiation >16 years). ND, not determined; CK, creatine kinase; y, years; m, months; NA, not available, GDF-15, growth differentiation factor 15; FGF-21, fibroblast growth factor 21; BMI, body mass index; 6MWT, six-minute walk test; NSAA, north star ambulatory assessment; PIM, maximal inspiratory pressure; FVC, forced vital capacity.

Scale (HMFS) and the Hammersmith Motor Function Scale-Expanded HMFSE scales (Fig. 4B,C), with an average improvement of 4.3 points and 12 points respectively between the pre-treatment and last post-treatment determinations ($p = 0.03$ in both cases) (Fig. 4A). Other clinical outcome measures (Egen Klassifikation Scale 2, EK2; North Star Ambulatory Assessment, NSAA) also reflected improvements, although without reaching statistical significance, when pre-treatment and post-treatment scores were compared. We did not have enough basal data regarding respiratory function (Forced Vital Capacity, FVC) to perform specific analyses in this group of patients (Fig. 4D).

Group 1	Mean	SE	Range
Age onset	27.1 m	3.8	13–72 m
Age treatment start	62.1 m	12.6	14–168 m
Basal GDF-15 (pg/mL)	10716	2579	2641–27066
Basal FGF-21 (pg/mL)	1981	702	402–7531
Group 2			
Age onset	29.2 y	5.3	5–60 y
Age treatment start	50 y	4.3	30–74 y
Basal GDF-15 (pg/mL)	1955	149	1261–2483
Basal FGF-21 (pg/mL)	429	106	179–943

Table 4. Summary of descriptive statistics. m, months; y, years; SE, standard error of the mean.

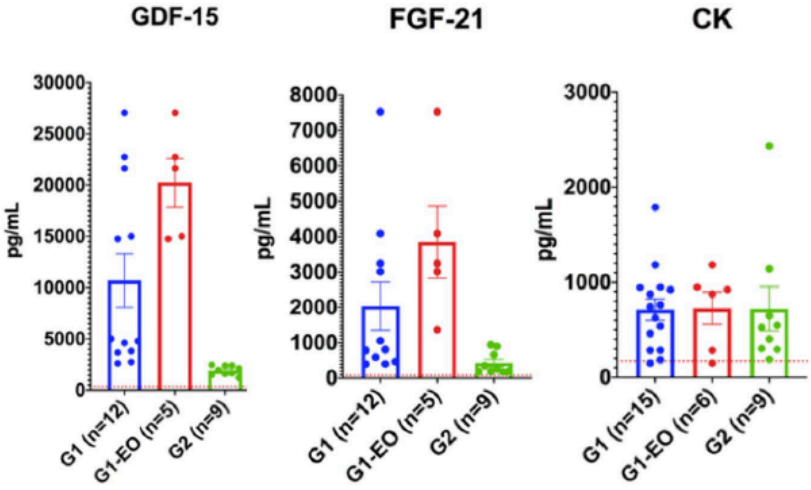

Figure 1. Pre-treatment GDF-15 and FGF-21 serum levels are associated with disease severity. Histograms representing the basal (before treatment) serum levels (mean and SEM) of GDF-15, FGF-21 and creatine kinase (CK) in patients from Group 1 (G1), those patients from Group 1 with the early onset and the most severe phenotype (G1-EO) and patients in Group 2 (G2). Dotted lines represent control values.

In the adult group, consisting mainly of patients with late-onset disease, the 6MWT and the FVC showed a significant improvement when we compared pre- and post-treatment values ($p = 0.05$ and $p = 0.04$ respectively). This difference became more striking when we considered the changes in FVC during the first 12 months of treatment ($p = 0.02$). In particular, the 6MWT showed an improvement of an average of 53.2 meters after treatment (Fig. 4E), while the NSAA increased a mean of four points (Fig. 4F) and, more importantly, the mean FVC after treatment was 5% greater (Fig. 4G).

We had previously shown that mRNA and protein levels of GDF-15 and FGF-21 correlate strongly and significantly in children with a range of mitochondrial diseases and in myogenic cell lines in which mitochondrial damage was induced experimentally[12]. In keeping with this previous result, baseline and longitudinal concentrations of both factors also correlated with each other significantly in treated patients ($p < 0.001$; Spearman $r = 0.843$), (Fig. 4H).

When analysing group 1 and group 2 together, basal circulating GDF-15 and FGF-21 concentrations correlated significantly and negatively with age-at-treatment initiation ($p = 0.006$, $r = -0.7$ and $p = 0.004$, $r = -0.7$ respectively) and with weight expressed as BMI ($p < 0.001$, $r = -0.8$ and $p = 0.024$ and $r = -0.5$ respectively). In addition, basal GDF-15 concentrations correlated with the meters walked in the 6MWT ($p = 0.003$, $r = -0.75$).

Inflammatory cytokines profile in patients with TK2 deficiency. Mitochondrial diseases and, in particular, TK2 deficiency are often associated with local (skeletal muscle) inflammatory responses which may be related to muscle cell damage or necrosis[12]. Given that GDF-15 can be induced by various stimuli related to inflammation, we assessed the circulating levels of the inflammatory cytokines IL-1, IL-6, IL-8, IL-15 and TNF (before and after treatment) in the eight patients from group 2 with late-onset phenotype that were followed in hospitals in Spain. We did not observe a significant increase in the average basal levels of any of the cytokines in either group of patients relative to the levels in age-matched controls nor a consistent pattern of change with time in treated patients (Supp. Fig. 2).

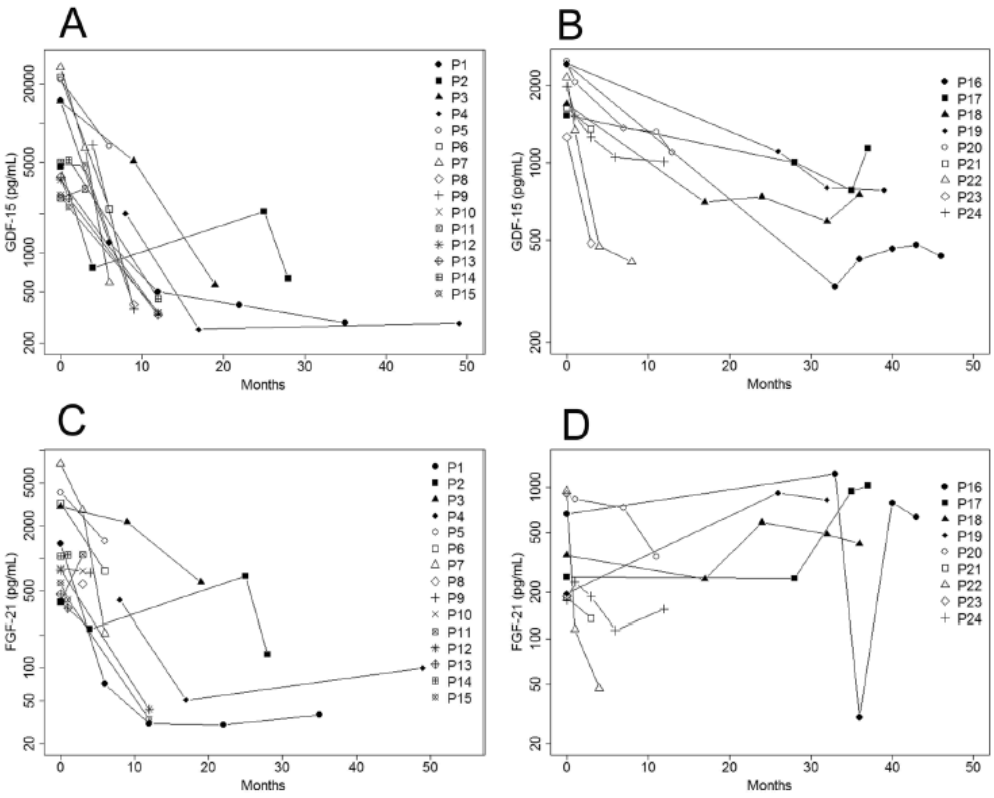

Figure 2. GDF-15 levels decreased during treatment with deoxynucleosides. Development of GDF-15 (**A**,**B**) and FGF-21 (**C**,**D**) circulating concentrations (pg/mL) over time in group 1 (left) and group 2 (right) patients.

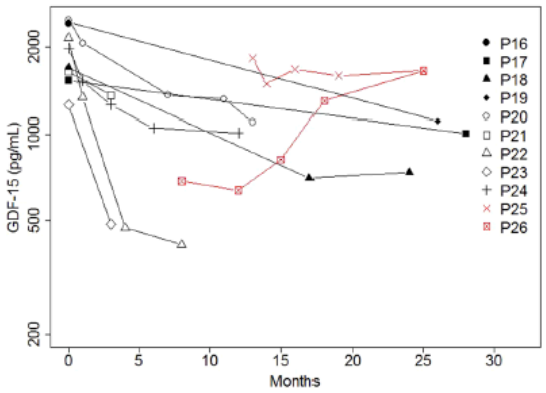

Figure 3. GDF-15 levels do not decrease over time in the absence of treatment. Development of serum GDF-15 over 28 months in treated patients (black lines) from group 2 versus untreated patients (P25 and P26, red lines).

Discussion

In previous work, our group described the global gene expression profile of skeletal muscle from patients with TK2 deficiency and identified the main molecular pathways implicated in the disease including the induction of GDF-15 expression partly under the control of p53. In addition, we demonstrated that GDF-15 outperformed other diagnostic biomarkers for mitochondrial diseases and postulated that it may also serve to monitor response to treatment based on preliminary data of one patient with TK2 deficiency that was treated with deoxynucleotides replacement therapy[14,15]. Since then, an expanded access program has shown a favorable side-effect profile and clinical efficacy of dNMP and deoxynucleoside therapies in patients with TK2 deficiency, some of them included in this study. Without any major side effects, the therapy had striking effects on early-onset severe myopathy patients, considerable functional improvements in childhood-onset cases and at least stabilization in late-onset ones[4].

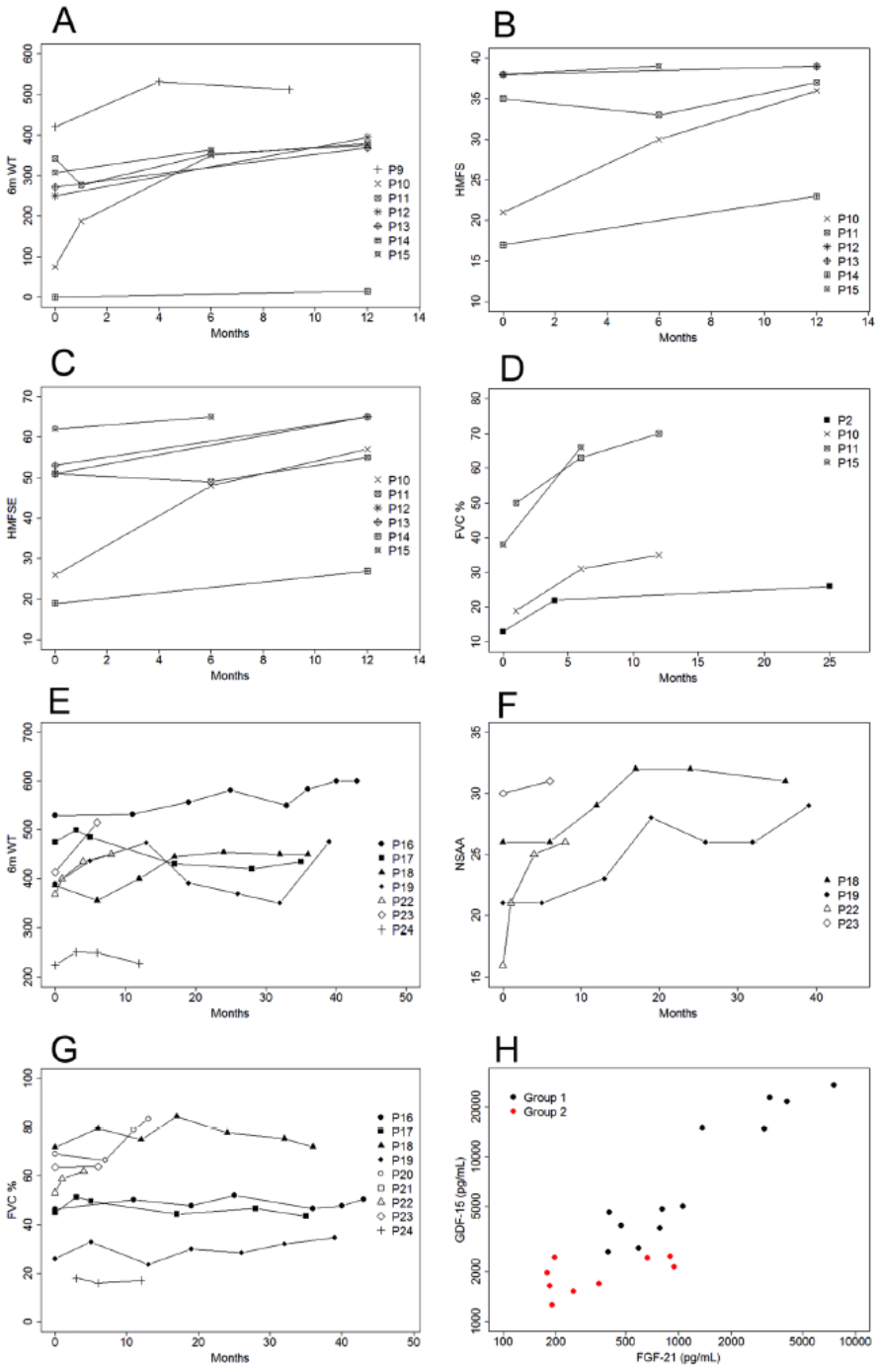

Figure 4. GDF-15 decrease was accompanied by improvement of several clinical outcome measures in treated patients. Development of selected functional tests for patients in group 1: 6MWT (**A**), HMFS (**B**), HMFSE (**C**), and % FVC (**D**) and for patients in group 2: 6MWT (**E**), NSAA (**F**), and % FVC (**G**) during treatment with deoxynucleosides. Correlation between basal levels of GDF-15 and FGF-21 in serum for group 1 (black) and group 2 (red) patients together (**H**).

In the present work, we have assessed GDF-15 and FGF-21 in 24 patients with TK2 deficiency that started deoxynucleoside therapy either in their earlier years or as adults. The objective of this study was to ascertain the value of GDF-15 as a prognostic and treatment response biomarker for TK2 deficiency and compare it to FGF-21.

Together, the two groups of patients we analyzed represent the majority of patients with TK2 deficiency around the world that are treated. In addition, they encompass the known spectrum of the disease, from patients with

early-onset and very rapid progression that is fatal in a few years without treatment, to childhood or late-onset cases with variable rates of disease progression, but also with bad prognosis because of respiratory involvement.

We confirm our previous findings that GDF-15 is markedly elevated in children and adults with TK2 deficiency[14,15]. GDF-15 levels before the start of treatment were elevated above the normal threshold value in all patients and on average six times higher in the paediatric patients compared to the adult patients, even more so in the children with early-onset and the most severe form of the disease. Similar results were obtained for FGF-21. Thus, both GDF-15 and FGF-21 are useful indicators of disease severity.

This was reflected in a significant and negative correlation between the basal concentration of both factors and the age of onset and/or treatment initiation so that the younger the children the higher the elevation of GDF-15 and FGF-21. Furthermore, pre-treatment GDF-15 levels were negatively associated with the meters walked on the 6MWT, one of the main indicators of motor function and also with body weight, expressed as the BMI. Thus, the higher the levels of GDF-15 (and FGF-21) the lower the BMI. Considering the evidence of the role of GDF-15 in the regulation of body weight and appetite[21], one may speculate that the dramatically increased levels of circulating GDF-15 observed in these patients may be directly contributing to the rapid loss of body weight which is an important component of the disease but this requires additional studies.

Our results demonstrate that in patients treated with deoxynucleosides, GDF-15 concentrations decrease following a significant dependence over time and therefore GDF-15 fulfills the criteria for a treatment response biomarker. Although a similar trend was obtained for FGF-21 the decrease was not as consistent or as marked, particularly for patients in group 2. In contrast to treated patients, in TK2 deficient patients without treatment levels of GDF-15 tended to increase over short periods of time although more natural history data on untreated patients are necessary. All the patients under treatment showed important beneficial effects that are maintained over time or, at least, the stabilization of the disease. The response to treatment was greatest in group 1 (the paediatric group), but the mild motor improvements and respiratory stability shown in group 2 (the adult group) are a relevant clinical result as well since they show that treatment can reduce morbidity and mortality also in patients who started treatment in adulthood. In keeping with the extent of the clinical benefit, the rate of GDF-15 decrease was faster in children than in adults, in particular, in the most severe patients who are the ones that respond more markedly to the treatment in the form of extended survival, recovery from ventilatory support dependency, and regaining motor abilities. It has been recently reported that age-related metabolic changes in mice (namely increased deoxynucleoside catabolism and decreased anabolism with age) account, at least in part, for the limited efficacy of the treatment in this model[8].

Both GDF-15 and FGF-21 are broad action cytokine/hormone that are altered at the same time in many disease states where they are routinely employed as diagnostic and prognostic biomarkers[12,20–25]. In particular, they are increasingly used individually or in combination as diagnostic biomarkers of mitochondrial diseases[9,16,18].

In this study, we found that both GDF-15 and FGF-21 were elevated in all patients before deoxynucleoside treatment but that GDF-15 was more robust than FGF-21 to monitor changes over time in treated patients. Furthermore, GDF-15 correlated with motor function (6MWT) although this needs to be confirmed in a larger group of treated patients.

Our previous transcriptomic data of skeletal muscle indicates that p53 is the key regulator in a network of genes which are coordinately activated in response to TK2 deficiency leading to inflammation, activation of muscle cell death by apoptosis and induction of GDF-15 in muscle and serum. p53 plays a fundamental role in the control of the cell cycle, DNA repair and apoptosis in the nucleus and in mitochondria. Under certain conditions such as increased oxidative stress, p53 translocates to mitochondria to repair and/or promote replication of mtDNA[14]. Thus, our hypothesis is that mitochondrial DNA depletion due to TK2 mutations lead to activation of p53 which binds to the promoter region of GDF-15 inducing its expression and release by affected tissues.

Both GDF-15 and FGF-21 have a role in inflammation, for example, in pancreas, heart and adipose tissue[13,21–23]. Our data show that the profile of inflammatory cytokines was not consistently altered at baseline (not even in the children with the most severe early-onset phenotype) indicating that the elevated levels of GDF-15 and FGF-21 are not secondary to systemic inflammation. Similarly, treatment with deoxynucleosides did not result in a consistent pattern of change on the baseline levels of the inflammatory cytokines. However, we cannot exclude a contribution from local, tissue-specific, inflammation since we have previously observed over-expression of inflammatory markers in muscle biopsies from patients with TK2 deficiency and other mitochondrial DNA depletion syndromes[14].

Limitations of the current study were that biochemical and clinical evaluations were performed at different time intervals depending on where the patients were being followed and that functional scales were not uniform across the cohort. This was difficult to overcome since in some cases samples had been collected retrospectively and not as part of a controlled study and some patients started treatment several years ago. Another important limitation is that we did not have the possibility to follow the levels of GDF-15 and FGF-21 in a group of untreated patients with TK2 deficiency in order to find out how they would change over time. TK2 deficiency is a very rare disease and given the severity of the phenotype and the effectiveness of deoxynucleoside treatment the vast majority of patients worldwide are being treated.

In conclusion, we propose that GDF-15 may be a potential biomarker of disease severity and response to treatment with deoxynucleosides in patients with TK2 deficiency and potentially also in other forms of mitochondrial DNA depletion and deletion syndromes where this treatment may also be beneficial.

Materials and Methods

Study design. We determined circulating GDF-15 and FGF-21 at baseline and at various time points in a group of 26 patients with mutations in TK2 treated with deoxynucleosides. We examined the rate of change over time, and correlated its basal and follow-up levels with various clinical parameters related to disease severity and response to treatment.

Standard protocol approvals, registrations, and patient consents. This study was conducted in accordance with the Declaration of Helsinki and legal regulations and was approved by the Ethics and Research Committee of the Fundación Sant Joan de Déu. Written informed consent was obtained from all patients or their parents/guardians prior to enrolment.

Study population and setting. Patients were included if they had two mutations in the TK2 gene and were receiving treatment with deoxynucleosides. Several of these patients have been described elsewhere[9] and their basal characteristics are detailed in Table 1 and Supplementary Table S1. Patients were followed in five Hospitals in Spain (Hospital 12 de Octubre, Madrid; Hospital Sant Joan de Déu, Barcelona; Hospital de la Santa Creu I Sant Pau, Barcelona; Hospital Virgen del Rocio, Seville and Hospital Universitario Donostia, San Sebastian) and in the Columbia University Medical Center (New York, USA).

Oral administration of deoxynucleosides. All patients were treated with oral doses between 300–400 mg/Kg/day of each nucleoside (thymidine, dThd, and deoxycytidine, dCtd). Two patients (P3 and P4) started with nucleotides (dTMP and dCMP) and later switched to nucleosides (in a one-to-one ratio of height:weight), once it became clear that these are the active agents[5,6]. The majority of patients (n = 24) are currently being treated with dThd and dCtd. Two adult-onset individuals (P25 and P26) had to stop treatment because of liver enzymes elevation and were excluded from the main analyses.

Analytical methods. Serum samples were collected, allowed to clot, centrifuged at 1300 g for 10 minutes, aliquoted, and stored at −80 °C until the moment of the analysis. Long-term storage of samples at −80 °C does not affect GDF-15 or FGF-21 stability according to our experience and published data[20].

Previously, we had compared serum (collected in serum separator tubes) and plasma samples (collected in EDTA, heparin lithium or heparin sodium tubes) from the same individuals and found comparable GDF-15 and FGF-21 levels regardless of the type of sample and collection method. We also compared different times of sample collection (fasting versus postprandial) and found no significant differences for either GDF-15 or FGF-21 (data not shown).

Quantitative sandwich ELISAs were performed using the Quantikine Human GDF-15 Immunoassay kit (R&D Systems, Minneapolis, USA) and the FGF-21 ELISA KIT (R&D Systems, Minneapolis, USA or Millipore, Massachusetts, USA) as previously described[9,15]. Sensitivity, intra-assay and inter-assay variances (expressed as CV%) are 4.39 pg/mL and 8.69 pg/mL, 2,26% and 3,43% and 5,43 and 7,5% for GDF-15 and FGF-21 respectively.

Briefly, serum samples were either diluted ¼ in the sample diluent buffer provided with the kit as recommended by the manufacturer (GDF-15) or used undiluted (FGF-21). A volume of 50ul of diluted serum sample was added to each well and assays were performed in duplicate. A standard curve was prepared using a dilution series (in pg/mL) of a recombinant human GDF-15 or FGF-21 standard diluted in the sample diluent buffer provided with the kit. To determine the optical density of the preparations we used a microplate reader (Molecular Probes) set to 450 nm subtracting readings at 540 nm for wavelength correction. The values from each sample were extrapolated from a standard four-parameter logistic curve using the SoftMax software of the microplate reader. Final concentrations were corrected by the dilution factor.

Interleukin (IL)-1B, IL-6, IL-8, IL-15, tumor necrosis factor (TNF) and monocyte chemoattractant protein-1 (MCP1) were quantified using a multiplex analysis system based on fluorescently labeled microspheres linked to specific antibodies (HCYTOMAG-60K-06, Millipore Linco Research/Millipore, Massachusetts, USA) using a Luminex100ISv2 instrument.

Outcome measures. Motor assessment: Patients underwent periodic motor assessments with at least one of the following: 6-minute walk test (6MWT); North Star Ambulatory Assessment (NSAA), which evaluates motor goals with a score range of 0–34 as values of minimum and maximum motor skills, respectively; Egen Klassifikation (EK2), which evaluates functional capacity in nonambulatory patients with a score range of 30–0 as values of minimum and maximum functional capacity, respectively; the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP INTEND) used to evaluate the motor skills of infants with SMA with a score range from 0 to 64, with higher scores indicating better motor function; Hammersmith Motor Functional Scale (HFMS), which assesses the physical abilities of children with non-ambulant Spinal Muscular Atrophy, with a total score achievable of 40, with higher scores reflecting better physical abilities; or the Hammersmith Motor Functional Scale Expanded (HFMSE), which contains additional items for the ambulant population, with a total score achievable of 66.

Respiratory evaluation: We measured forced vital capacity (FVC) and maximal inspiratory pressure (MIP) in an upright position in compliant patients.

Statistical analysis. Descriptive statistics and correlation analyses were performed using R version 3.2 and GraphPad PRISM version 8.1.2 A. A p-value of < 0.05 was considered statistically significant. Spearman's correlation was used to determine the relationship between numerical variables, and a paired non-parametric test was used to compare variables at two times (pre- versus post-treatment) for the same patient. Repeated measures mixed linear models were used to determine whether numerical variables changed over time if data was available at multiple time points.

Data availability

The raw data and protocols used for this study are available from the corresponding author.

Received: 22 January 2020; Accepted: 13 May 2020;
Published online: 22 June 2020

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Acknowledgements

We wish to thank the patients and their families for their collaboration in this study. We are grateful to the Biobank of the Hospital Sant Joan de Déu (Spanish Biobank Network, ISCIII). We are grateful to Esther van de Vosse from EVScienceConsultant for editing the Manuscript. This work was supported by research grants of Plan Nacional de I + D + I and Instituto de Salud Carlos III (ISCIII), Subdirección General de Evaluación y Fomento de la Investigación Sanitaria, projects PI16/01843 (CP), PI16/00579 and CP09/00011 (CJM), PI15/00431 (MAM), PI17/00109 (RA) and PI18/01574 (RM), co-funded with European Regional Development Fund (ERDF) "A way to achieve Europe"; multicentric grant funded by the ISCIII (PMP15/00025, MAM, RM, CP), Grants of the Ministerio de Ciencia, Innovación y Universidades SAF2017-85722 (FV) and SAF2017-87506-R (YC), the Muscular Dystrophy Association grant 577391 (MH), and the Arturo Estopinan TK2 Research Fund (MH).

Author contributions

C.D.G., A.B., M.H., A.N., C.J.M. contributed to the conception and design of the work, acquisition, analysis and interpretation of data and drafting the work or substantively revised it. C.B., M.M.G., I.M., C.P., C.O., J.D.M., J.A.P., S.T., D.C., S.G.K., C.B.B., Y.C., R.M., F.M.S., M.A.M., J.M., E.R.P., J.V., R.M., F.V., R.A. contributed to the analysis and interpretation of data and revising the work. All authors have approved the submitted version of this M.S.

Competing interests

MH, RM, Columbia University Medical Center (CUMC), and the Vall d'Hebron Research Institute (VHIR) have filed patent applications for deoxynucleoside and deoxynucleotide therapies for human mitochondrial DNA depletion and deletions syndrome including TK2 deficiency. RM, YC, CB, VHIR, and The Biomedical Network Research Centre on Rare Diseases (CIBERER) have filed patent applications covering potential use of deoxynucleoside treatment for POLG deficiency and other mtDNA replication defects in humans. CUMC, VHIR, and CIBERER have licensed pending patent applications related to these technologies to Modis Therapeutics, Inc. CUMC, VHIR, and CIBERER may be eligible to receive payments related to the development and commercialization of the technologies. Any potential licensing fees earned will be paid to CUMC, VHIR,

and CIBERER and are shared with all inventors mentioned above through VHIR and CIBERER policies on distribution and objectivity in research. MH and RM serve as paid consultants to Modis Therapeutics, Inc. and RM has equity in this company. RM, YC, CB, VHIR, and The Biomedical Network Research Centre on Rare Diseases (CIBERER) have filed patent applications covering the potential use of deoxynucleoside treatment as a way to increase mtDNA copy number.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41598-020-66940-8.

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DISCUSIÓN

La proteína timidina quinasa 2 es una proteína mitocondrial codificada por el genoma nuclear que está implicada en la síntesis de los nucleótidos pirimidínicos, desoxitimidina monofosfato (dTMP) y desoxicitidina monofosfato (dCMP), en los tejidos postmitóticos. Mutaciones recesivas en el gen que la codifica (*TK2*) conducen a una disminución del "pool" de dTTP y dCTP disponibles para la síntesis del ADNmt en las células en fase de quiescencia y, en consecuencia, a un trastorno en su replicación. Los defectos en la síntesis de ADNmt pueden llevar a un descenso en el número total de copias de ADNmt (depleción) o bien a un acúmulo de mutaciones puntuales en el mismo (12). Esta alteración en el ADNmt es responsable de una disfunción del sistema de fosforilación oxidativa mitocondrial que es la vía final común del metabolismo aeróbico y la fuente principal de ATP celular.

El síndrome de depleción de ADNmt secundario a un déficit de TK2 se describió por primera vez en el año 2001 (12), en 4 niños afectados por una miopatía grave rápidamente progresiva. Posteriormente se identificaron nuevos casos con un fenotipo homogéneo, pero gravedad y edad de inicio variable, abarcando un amplio espectro de afectación clínica. En el año 2012 se reconoce por primera vez la existencia de pacientes sin síntomas de la enfermedad hasta edades avanzadas y se les atribuye un fenotipo leve consistente en oftalmoparesia externa progresiva (28). En años posteriores se van sumando nuevos casos de pacientes adultos con síntomas similares a las formas de inicio infantil, con cuadros de miopatía progresiva que pueden

conducir a la muerte por insuficiencia respiratoria secundaria (26, 29). En el año 2018, estudios de revisión de casos ideados para analizar la historia natural de la enfermedad (31, 32), clasifican el trastorno en función de la edad de inicio en tres formas clínicas: 1) de inicio durante el primer año de vida, con rápida progresión y supervivencia inferior a los 3 años. 2) de inicio entre el año y los 12 años, con supervivencia media de 13 años tras el inicio de la enfermedad y 3) de inicio a partir de los 12 años, con progresión más lenta y supervivencia media de 23 años tras su debut. En todos ellos el trastorno fundamental es una miopatía progresiva que puede involucrar la musculatura respiratoria y conducir a una muerte prematura por insuficiencia respiratoria. En los casos más graves, generalmente de inicio neonatal, puede existir afectación del sistema nervioso central pero las manifestaciones extramusculares son en general poco frecuentes (56).

Las formas clínicas de inicio a partir de los 12 años son las menos frecuentes (< 20% en las grandes series publicadas (31, 32)) y a la vez las menos conocidas. Hasta el año 2019 se habían descrito únicamente 17 casos en la literatura y en la mayoría sin aportarse suficientes detalles como para conocer su historia natural y pronóstico clínico (28-32).

Con el objetivo de conocer las características clínicas de este subgrupo de pacientes, analizamos una serie de 16 pacientes españoles y 2 pacientes procedentes de Estados Unidos, pertenecientes a 13 familias diferentes, con mutaciones bialélicas en el gen TK2 e inicio tardío de la enfermedad. Se recopilaron datos clínicos relativos a la edad y síntomas de presentación, grado

y distribución de la debilidad muscular y presencia de manifestaciones extramusculares. Se recogieron y analizaron las pruebas complementarias realizadas durante el proceso diagnóstico, (estudios funcionales respiratorios, biopsia de músculo, resonancia muscular magnética, marcadores plasmáticos), y los datos genético-moleculares.

El análisis de los datos ha permitido identificar y definir un fenotipo homogéneo y reconocible caracterizado por debilidad muscular lentamente progresiva con o sin ptosis y oftalmoparesia y con predominio de la afectación de la musculatura facial, cervical y axial sobre la de extremidades. Describimos por primera vez la afectación precoz, selectiva y universal del músculo diafragma siendo su debilidad responsable de una insuficiencia respiratoria grave que incluso puede ser el síntoma de debut. En el momento del diagnóstico, la capacidad vital forzada (CVF) media de los pacientes de nuestra serie era de 55.4% (normal > 70-80%), con una caída significativa durante la exploración en decúbito, lo que refleja debilidad del músculo diafragma (57). El 66.6% de los pacientes de la serie (12/18) necesitaban ventilación mecánica en el momento de recoger los datos, y en 8/18 (44.4%) fue la causa que motivó la primera consulta médica. También los pacientes que no necesitaban ventilación mecánica tenían datos de compromiso respiratorio en las pruebas funcionales, incluso aquellos con CPEO como única manifestación clínica aparente de la enfermedad. La prueba más sensible para detectar datos incipientes de compromiso de la musculatura respiratoria es la capnografía, consistente en determinar los niveles de CO_2 vía transcutánea durante el descanso nocturno (58). En nuestra serie de casos se ha identificado la hipercapnia, compatible con hipoventilación alveolar nocturna

por debilidad diafragmática, en pacientes con pruebas funcionales respiratorias dentro del rango de la normalidad. Cuatro de los pacientes de la serie fallecieron por insuficiencia respiratoria a una edad media de 56 años (rango de 40-68), una media de 24 años después del desarrollo de los primeros síntomas (rango 17 a 35).

Además de por un fenotipo reconocible, los pacientes con déficit de TK2 de inicio tardío, pueden ser identificados por los resultados característicos de pruebas que forman parte de la rutina diagnóstica en pacientes con sospecha de enfermedad muscular. En este sentido, algunas características de la biopsia de músculo permiten diferenciar este grupo de pacientes de otros con miopatía mitocondrial de otro origen. Los pacientes con miopatía mitocondrial tienen en común la presencia de signos de proliferación y disfunción mitocondrial en forma de fibras rojo-rasgadas y negatividad de la tinción con citocromo oxidasa, sobre un tejido muscular sin alteración en su arquitectura ni otros cambios morfológicos asociados (59). Sin embargo, en pacientes con déficit de TK2 las biopsias se caracterizan por la presencia de cambios distróficos (fibrosis y sustitución adiposa) asociados a los signos comunes de disfunción mitocondrial.

El marcador bioquímico de los trastornos en el mantenimiento del ADNmt es la presencia de depleción o deleciones múltiples del ADNmt (9, 60, 61). En todos los casos de nuestra serie en los que se realizó este análisis (13/18), se pudo demostrar la presencia de deleciones múltiples, asociadas a depleción sólo en uno. Por tanto, en pacientes con miopatía mitocondrial, deben realizarse

estudios dirigidos a descartar la presencia de deleciones múltiples en ADNmt para dirigir el estudio diagnóstico en función de los resultados.
En 8/18 pacientes se realizó una resonancia magnética para estudiar el grado y distribución de la degeneración muscular a nivel de miembros inferiores. Al contrario que en otras miopatías, no hay ningún patrón de afectación radiológico conocido en las de origen mitocondrial. Sin embargo, a pesar de lo reducido de la muestra en nuestro estudio, se deduce una afectación preferente y precoz de los músculos glúteo mayor, sartorio y gemelo medial. La consistencia de este patrón de resonancia debe ser estudiada en series más amplias para confirmar su utilidad en el diagnóstico diferencial de pacientes con debilidad muscular progresiva.

Desde el punto de vista genético, la mutación más frecuente identificada en esta serie de pacientes es la p.Lys202del, presente en homocigosis en 8/18 de los casos. Previamente, se había descrito su presencia asociada a fenotipos más leves de la enfermedad, lo que nuestro estudio parece confirmar. Todos los pacientes con esta mutación son de origen español o hispano, lo que sugiere un posible efecto fundador. La segunda mutación más frecuente identificada en nuestra serie es la p.Thr108Met, que es la mutación encontrada con más frecuencia en el gen TK2 y para la que no ha podido demostrarse ninguna relación genotipo/fenotipo hasta la fecha.

Finalmente, analizamos de manera exploratoria la posible utilidad de los niveles de GDF-15 como marcador para el diagnóstico y seguimiento de los pacientes con esta entidad, así como la utilidad de la determinación del

consumo de oxígeno durante una prueba de esfuerzo con un cicloergómetro para monitorizar su progresión.

Se detectaron niveles alterados de GDF-15 en los 5 pacientes donde fue analizado, obteniendo valores medios de 2113 pg/mL ± 462, siendo 550 pg/mL el límite superior de la normalidad (50). Por tanto, su determinación en pacientes con debilidad muscular con la distribución clínica y radiológica descrita, apoya el diagnóstico de miopatía mitocondrial y ayuda a dirigir el estudio genético sin necesidad de realizar una biopsia muscular que es un procedimiento invasivo.

Además de la debilidad, uno de los síntomas más limitantes de la enfermedad es la fatiga, que es intensa y muy desproporcionada a la actividad realizada. Los pacientes aquejan incapacidad para realizar actividades del día a día, debido a la imposibilidad de mantener esfuerzos de manera prolongada. Este síntoma es difícil de capturar, evaluar y monitorizar de manera objetiva, y no suele tenerse en cuenta a la hora de graduar la gravedad de un paciente. Evaluamos la capacidad aeróbica de 5 pacientes mediante una prueba de esfuerzo en un cicloergómetro (62) obteniendo valores muy reducidos. El consumo de oxígeno medio fue de 14.8 ± 3.2 $mL/kg^{-1}/min^{-1}$, siendo los valores considerados normales en torno a 40 ± 9.5 $mL/kg^{-1}/min^{-1}$ (63). Por tanto, aún manteniendo la capacidad para caminar de manera autónoma, la presencia de una capacidad aeróbica tan reducida se traduce en una fatiga y falta de resistencia extrema que interfiere gravemente en el normal desarrollo de la vida de los pacientes (64).

En conclusión, nuestro estudio mostró que los pacientes con déficit de TK2 de inicio tardío tienen un fenotipo homogéneo y reconocible, fácil de identificar y diagnosticar si se conoce, y cuyo pronóstico es grave debido a una afectación respiratoria intensa y precoz que puede ser subestimada si no se analiza activamente. Esta entidad se debe sospechar ante cuadros de debilidad muscular progresiva, con afectación facial y axial intensa, que se acompañen o no de ptosis y oftalmoparesia. Los niveles de GDF-15 serán elevados y una resonancia magnética mostrará signos de sustitución grasa difusa con afectación más intensa y precoz de los músculos glúteo mayor, sartorio y gemelos mediales en miembros inferiores. En el caso de realizar una biopsia de músculo, además de los signos habituales de disfunción mitocondrial, podrán identificarse cambios distróficos muy inhabituales en miopatías mitocondriales de otro origen. Ante todos estos hallazgos y, sobre todo si una biopsia ha permitido extraer ADNmt del tejido muscular y se ha demostrado la presencia de deleciones múltiples en él, el estudio dirigido sobre el gen TK2 permitirá realizar el diagnóstico definitivo. Ante cualquier paciente con sospecha de déficit de TK2, independientemente de su edad de presentación, es imprescindible realizar estudios sensibles dirigidos a descartar hipoventilación alveolar nocturna, dado que se ha demostrado una afectación preferente, predominante y muy precoz del diafragma en esta entidad, incluso en pacientes sin debilidad aparente de extremidades. Finalmente, es la insuficiencia respiratoria la que marca su pronóstico, siendo la responsable de una muerte prematura en todos los casos. Este mal pronóstico obliga a incluir también a las formas tardías de la enfermedad entre los candidatos a recibir tempranamente tratamientos en vías de desarrollo.

En el año 2014, se publicó que en el modelo “knock-in” de ratón, con la mutación H126N en homocigosis (35), el tratamiento por vía oral con los productos de TK2, desoxitimidina mononfosfato (dTMP) y desoxicitidina monofosfato (dCMP), conseguía duplicar la supervivencia media del ratón sin efectos tóxicos aparentes (38). Posteriormente, tras observar que dTMP y dCMP son catabolizados inmediatamente tras su ingesta hacia desoxitimidina (dT) y desoxicitidina (dC), se sugirió que eran éstos los responsables del efecto biológico observado, demostrándolo posteriormente en estudios in vivo en el mismo modelo de ratón (42). En este modelo se consiguió además de prolongar la supervivencia media, restaurar el equilibrio del “pool” de trinucleótidos y los niveles normales de ADNmt.

Se postuló que el beneficio obtenido al administrar los sustratos de TK2, era secundario a la estimulación de la actividad residual de la enzima deficitaria y/o a mecanismos alternativos como la activación de las enzimas citosólicas TK1 y dCK, que fosforilan dT a dTMP y dC a dCMP respectivamente en el citoplasma (42). Sin embargo, también se demostró el mismo efecto beneficioso al tratar con dT+dC el ratón “knock-out” (TK^{KO}), que no tiene actividad residual TK2 en ningún tejido, y por tanto todo el efecto en este caso debía ser secundario a la activación de vías alternativas de síntesis de los dNTPs (65). Esto hizo suponer que el tratamiento sería válido para pacientes con cualquier mutación en TK2, independientemente de la actividad residual de ésta.

Con estos resultados tan prometedores, ante la gravedad de la enfermedad y la ausencia de un tratamiento alternativo, se solicitó autorización para el uso de dTMP+dCMP y dT+dC en humanos a la Agencia Española del Medicamento, y a las comisiones de farmacia hospitalaria su uso de forma compasiva en pacientes con miopatía TK2 con diagnóstico genético confirmado.

En el segundo trabajo que presentamos como parte de esta tesis, analizamos la seguridad y eficacia de este tratamiento en los primeros 16 pacientes tratados en todo el mundo, recopilando datos de los pacientes tratados por lo menos durante 12 meses hasta septiembre de 2017. Doce pacientes eran españoles, 1 italiano, y 3 seguidos en EEUU (1 natural de EEUU, 1 de Chile y 1 de Guatemala). En conjunto representaban todo el espectro clínico de la enfermedad. Cinco tenían la forma más grave de la misma, definida por un inicio de los síntomas durante los primeros 2 años e incapacidad para caminar de forma independiente y/o necesidad de ventilación mecánica (VM) durante el primer año de vida. Los pacientes con esta forma clínica de inicio temprano y curso rápidamente progresivo publicados en la literatura tenían una supervivencia media inferior a los 3 años. Cuatro de estos 5 pacientes necesitaban VM y eran alimentados por vía enteral por bajo peso y disfagia. La progresión en el resto de los pacientes era más lenta y 4 de ellos no habían presentado síntomas hasta después de los 12 años (formas de inicio tardío). Tres de estos 11 pacientes de evolución más lenta habían perdido la capacidad para caminar de manera independiente a lo largo de la evolución de la enfermedad, 2 necesitaban alimentación enteral (uno a través sonda

nasogástrica y el otro tenía hecha una gastrostomía percutánea) y 5 ventilación mecánica una media de 11 horas diarias.

Seis de estos pacientes primero iniciaron tratamiento con dTMP+dCMP cambiando posteriormente a dT y dC, tras demostrarse que estos eran los productos activos (42). Sólo uno de los pacientes prefirió no hacer el cambio y mantuvo el tratamiento con dTMP+dCMP. Los otros 5 tomaron desde el principio dT+dC. La media de tratamiento en todos ellos fue de 15.5 meses (hasta agosto de 2017) y la dosis administrada se basó en la utilizada en los estudios preclínicos, modificándose en función de su tolerancia (todos los pacientes recibieron tratamiento a dosis entre 300 y 400 mg/kg/d de cada nucleósido). Se evaluó la supervivencia, estado funcional motor a través de distintas escalas (test de los 6 minutos (6MWT), North Star Ambulatory Assessment (NSAA) y Egen Klassifikation (EK)), la función respiratoria, el estado nutricional, los niveles de CK y los niveles de GDF-15.

Sin un efecto tóxico relevante, el tratamiento mostró un espectacular beneficio en las formas más graves de inicio temprano y rápida progresión, mejoría clínica significativa en las formas intermedias de evolución más lenta y al menos estabilización clínica de las formas de inicio tardío. El único efecto tóxico identificado fue diarrea bien tolerada dosis dependiente en un 50% de los pacientes. Sólo en dos de ellos la diarrea impidió alcanzar la dosis máxima recomendada.

El tratamiento demostró:

1. Prolongar la supervivencia de los 5 pacientes del grupo de inicio temprano y rápida progresión. En las cohortes históricas publicadas sólo un 27.3% sobrevivía al menos 2 años después del inicio de la enfermedad (31). Todos los pacientes tratados de nuestra serie seguían vivos tras una media de 3.93 ± 1.66 años desde el debut de la enfermedad (p= 0.0078).

2. En dos pacientes que requerían asistencia respiratoria se pudo retirar la ventilación mecánica y 3 que necesitaban alimentación enteral retomaron la alimentación por vía oral.

3. Cuatro pacientes volvieron a caminar, dos del grupo de inicio temprano y rápida progresión, y dos de los pacientes de inicio infantil. En los pacientes con capacidad para caminar de forma independiente, la evaluación del número de metros recorridos en 6 minutos (6MWT) mejoró una media de 88.5 metros, mejorando todos salvo uno en este parámetro. En otras enfermedades musculares más comunes para las que se están ensayando nuevas terapias, se considera un cambio clínicamente relevante mejorías superiores a los 30 metros en esta misma prueba (66). Si analizamos el subgrupo de pacientes con peores resultados en esta prueba antes del inicio del tratamiento (6MWT < 300 metros), la mejoría alcanzada en la última evaluación anotada es de un

incremento de 171.9 metros de media (Intervalo de confianza del 95% = 84.5-259.2).

4. La capacidad funcional de los pacientes se evaluó utilizando la escala EK para los no ambulantes y la NSAA para los ambulantes. Con la EK se puntúa de 0 a 30 siendo 30 el valor de máxima discapacidad (67). Al contrario, en la NSAA la puntuación máxima (34 puntos) se corresponde con la de mayor capacidad funcional (68). En el grupo de inicio temprano y rápida progresión se demostró una mejoría media de 23 puntos en la EK (n=3) y en el grupo de progresión más lenta una mejoría media de 6 puntos en la NSAA (n=5). En ambos casos, las diferencias se consideran clínicamente relevantes (69, 70) pero no alcanzaron la significación estadística.

5. En los pacientes con un inicio tardío de la enfermedad, el tratamiento demostró al menos una estabilización de las pruebas funcionales respiratorias. Este dato es clínicamente relevante al ser la insuficiencia respiratoria de curso progresivo y el principal factor de mal pronóstico en este grupo.

6. En 7 de los pacientes incluidos se pudieron analizar los niveles de GDF-15. En todos ellos los niveles estaban elevados y se redujeron de manera significativa con el tratamiento.

En conclusión, demostramos por primera vez que el tratamiento con dT+dC es eficaz y seguro en pacientes con miopatía secundaria al déficit de TK2. El beneficio mostrado era mayor en las formas más graves de inicio temprano y rápida progresión, donde además de prolongar la supervivencia podía llegar a revertir el fenotipo permitiendo la retirada de la ventilación mecánica y la sonda nasogástrica y la recuperación de la capacidad para caminar de manera independiente. La estabilización de las pruebas funcionales respiratorias en las formas de inicio tardío es también un beneficio relevante, puesto que podría implicar una mayor supervivencia también en los pacientes adultos donde la insuficiencia respiratoria es la principal causa de muerte.

Son necesarios más estudios para conocer los factores implicados en la heterogeneidad de la respuesta clínica observada y probar la eficacia en los casos de inicio en la edad adulta. La publicación de estos datos iniciales, que demuestran la capacidad de los nucleósidos para modificar la historia natural de la enfermedad, atrajo el interés de la industria farmacéutica y motivó la creación de una compañía (Modis Therapeutics) que compró la patente para el uso de dT+dC en el tratamiento de los síndromes de depleción mitocondrial y, con el nombre de MT1621, ha iniciado los ensayos clínicos destinados a su aprobación por las agencias reguladoras (clinicaltrial.gov NCT03845712). La patente pertenecía los investigadores M. Hirano y R. Martí, que fueron quienes demostraron por primera vez in vitro e in vivo en modelos preclínicos su eficacia en distintos síndromes de depleción mitocondrial.

Con la existencia de un fármaco en investigación de resultados tan prometedores, cobraba relevancia la identificación de biomarcadores capaces de facilitar un diagnóstico precoz, dar información sobre la gravedad y el pronostico de la enfermedad, y ayudar en la monitorización de la respuesta al tratamiento. En el último trabajo integrado en esta tesis, tratamos de identificar un biomarcador que cumpliera todos estos requisitos.

Las citoquinas GDF-15 y FGF-21 son marcadores sensibles en el diagnóstico de EM, especialmente para aquellas como el déficit de TK2 donde los síntomas son fundamentalmente musculares (44, 49-51, 54). Ya habíamos analizado previamente GDF-15 en unos pocos pacientes sometidos a tratamiento y comprobado que, partiendo de niveles basales alterados, el tratamiento podía llegar a normalizarlos varios meses más tarde. Para profundizar en este hallazgo y establecer si GDF-15 y/o FGF-21 eran biomarcadores útiles para establecer la gravedad de la miopatía TK2 y monitorizar la respuesta al tratamiento con nucleósidos, analizamos en una muestra amplia de pacientes, que incluía todo el espectro conocido de la enfermedad, sus valores antes y después del inicio del tratamiento. Por otra parte, dado que GDF-15 se ha relacionado con procesos inflamatorios (71, 72), analizamos los niveles circulantes de citoquinas inflamatorias en algunas de las muestras.

Estudiamos muestras de 24 pacientes con diagnóstico genético de déficit de TK2 en tratamiento con nucleósidos dT+dC y analizamos sus niveles de GDF-15 y FGF-21 antes y durante el seguimiento, recogiendo variables clínicas para tratar de conocer si los niveles de estas citoquinas se correlacionaban con la

gravedad del fenotipo y su respuesta al tratamiento. Se analizaron también los niveles de GDF-15 y FGF-21 en dos pacientes que tuvieron que suspender el tratamiento al presentar alteración de perfil hepático para comparar sus niveles durante el seguimiento clínico con los de los pacientes tratados.

Los pacientes fueron agrupados en pediátricos y adultos, en función de la edad a la que iniciaron el tratamiento. Esta clasificación obedece a la necesidad de comparar los niveles de las citoquinas con controles apareados por edad y a la demostración reciente de la importancia de los cambios metabólicos que suceden con la edad en la respuesta al tratamiento (65). Y es que, a pesar del tratamiento precoz con dT+dC, el ratón sigue muriéndose de forma prematura (42). Por ello, el grupo de Martí et. al, utilizando el modelo “knock-out” de ratón ($TK2^{KO}$), que reproduce el fenotipo de las formas clínicas más graves en los humanos (36), estudió los mecanismos implicados en el metabolismo de los dNTPs para indagar sobre las causas responsables de esta pérdida de eficacia con el paso del tiempo. Demostraron una disminución de la biodisponibilidad de dC y dT en los ratones adultos debido a un incremento de la actividad en el intestino de los enzimas que los degradan (citidina deaminasa y timidina fosforilasa) así como una reducción de la actividad de las desoxinucleósido quinasas citosólicas dCK y TK1 con la edad, especialmente en el cerebro y el hígado del ratón. Estos hallazgos sugieren que la edad a la que se inicia el tratamiento podría ser un factor relevante en la respuesta clínica obtenida.

De los 24 pacientes tratados en nuestro estudio, 15 eran pediátricos; 6 con la forma de inicio temprano y rápida progresión y 9 con formas de evolución más

lenta (grupo 1). El grupo 2 estaba constituido por 9 pacientes que iniciaron el tratamiento después de los 16 años, la mayoría (8/9) con déficit de TK2 de inicio tardío y sólo uno con síntomas desde los 5 años.

Dentro del grupo pediátrico disponíamos de valores basales de GDF-15 y FGF-21 de 12 pacientes. De media, tenían valores aumentados 30 veces de GDF-15 y 25 veces FGF-21 respecto a la población control. Tomando los datos sólo de los pacientes con las formas clínicas más graves, los valores de GDF-15 y FGF-21 estaban incrementados 60 veces y 50 veces respecto a los controles, respectivamente. Disponíamos de niveles basales de GDF-15 y FGF-21 de todos los pacientes del grupo 2. En ellos, tanto los niveles de GDF-15 como los de FGF-21 estaban aumentados 6 veces respecto a los controles sanos apareados por edad.

Tras el inicio del tratamiento, en el grupo 1 los niveles tanto de GDF-15 como de FGF-21 medidos en distintos momentos durante el seguimiento, se iban reduciendo progresivamente siendo las diferencias de sus concentraciones entre los distintos intervalos de tiempo estadísticamente significativas ($p<0.001$ en el caso de GDF-15 y $p=0.01$ en el caso de FGF-21). Los niveles de GDF-15 tras 12 meses de seguimiento se encontraban ya por debajo del límite de la normalidad.

En el grupo 2, los niveles de GDF-15 se redujeron de forma significativa sobre todo durante los primeros 12 meses, permaneciendo posteriormente estables durante el resto del seguimiento (hasta 36 meses). En el caso de FGF-21, sin

embargo, no se observaron diferencias significativas entre los distintos intervalos e incluso aumentó en algunos pacientes. Al contrario, los niveles de GDF-15 de los dos pacientes adultos que tuvieron que suspender el tratamiento, se incrementaron de manera paulatina durante su seguimiento.

Los niveles de GDF-15 se correlacionaros negativamente de manera estadísticamente significativa con la edad de inicio del tratamiento, con el índice de masa corporal y con los metros recorridos en la prueba 6MWT ($p=0.006$, $r=-0.7$; $p=0.0024$, $r=-0.5$ y $p=0.003$, $r=-0.75$ respectivamente). Por tanto, los niveles de esta citoquina se correlacionaban positivamente con la gravedad del fenotipo y sus valores al diagnóstico pueden ser útiles para conocer su pronóstico (así, niveles incrementados más de 60 veces los correspondientes a los controles, indicarían una forma rápidamente progresiva y por tanto de peor pronóstico).

Para comprobar que la reducción demostrada en los niveles de GDF-15 y FGF-21 se acompañaban de mejoría clínica, se analizaron los resultados de escalas funcionales, la prueba de 6MWT y parámetros respiratorios (FVC), tanto basalmente como durante el seguimiento.
Los principales resultados obtenidos se pueden resumir en:

1. Ninguno de los pacientes pertenecientes al grupo de los más graves, de inicio temprano y rápida progresión falleció durante el seguimiento, teniendo dos de ellos más de 6 años, a pesar de que la supervivencia sin tratamiento en este subgrupo en las cohortes históricas es inferior a

los 2 años en más del 70% de los casos. Se identificaron mejorías funcionales relevantes evaluadas con la escala CHOP INTEND (Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders, diseñada para evaluar la capacidad funcional de pacientes con atrofia muscular espinal tipo 1 (73, 74)). Esta escala puntúa de 0 a 64, siendo 64 la capacidad funcional máxima. En los pacientes evaluados con esta escala se obtuvo una mejoría de 13.7 puntos de media en tan sólo tres meses.

2. En los pacientes con síntomas de inicio a partir del año y con progresión más lenta, se observó una mejoría media de 105.8 metros en la prueba 6MWT en 12 meses, una diferencia que resulta estadísticamente significativa con p=0.01.

3. En el grupo de pacientes de inicio tardío, se observaron diferencias estadísticamente significativas en la prueba de 6MWT, con una media de incremento de 53.2 metros tras el tratamiento y en la FVC, con un incremento medio del 5% (p=0.05 y p=0.04 respectivamente).

Se confirmó por tanto que los descensos significativos de los niveles de GDF-15 y FGF-21 post tratamiento se acompañaba de mejorías clínicas relevantes. El estudio del perfil de citoquinas inflamatorias, analizado en todos los pacientes pertenecientes al grupo 2 y que incluye IL1, IL6, IL8, IL15 y TNF-alfa no mostró ninguna alteración ni en las muestras basales ni en las muestras post tratamiento.

Por tanto, este estudio demostró la utilidad del GDF-15 como biomarcador para monitorizar la respuesta al tratamiento con nucleósidos en los pacientes con déficit de TK2, en todas sus formas clínicas, no siendo así para FGF-21. Aunque los niveles de ambas citoquinas suelen mostrar correlación en las distintas series en las que se ha analizado su uso como marcadores diagnósticos en EM, el mecanismo por el que están incrementados en pacientes con disfunción del sistema OXPHOS es diferente. Así, el tratamiento de células musculares con defectos de la cadena respiratoria con antioxidantes consigue bloquear el aumento de FGF-21 pero no el de GDF-15 (50).

Este estudio también confirmó la eficacia y seguridad del tratamiento, sobre todo para las formas de inicio temprano. Sin embargo, en los pacientes con formas de inicio tardío en las que el tratamiento se inicia ya en la edad adulta, el tratamiento también mostró beneficios estadística y clínicamente significativos.

Las limitaciones en los estudios aquí detallados radican en la ausencia de criterios homogéneos para la evaluación clínica de los pacientes antes y durante el seguimiento tras el inicio del tratamiento. Esto es debido a la baja prevalencia de la enfermedad y al reclutamiento de pacientes de distintos centros en todo el mundo, así como al carácter retrospectivo de la recogida de parte de los datos aquí analizados. Este trabajo aporta información relevante y sienta las bases para el desarrollo de un ensayo clínico prospectivo, necesario para conseguir la aprobación de la combinación de nucleósidos dT+dC como el

primer fármaco capaz de modificar la historia natural de una enfermedad mitocondrial.

Se presenta además una aproximación diferente en el desarrollo de un nuevo fármaco para una enfermedad ultra-rara, que ha permitido que pacientes con una enfermedad muy grave y pronóstico sombrío reciban bajo fórmulas especiales de uso compasivo moléculas en experimentación que han mostrado eficacia y ausencia de toxicidad en modelos preclínicos. Esto se ha conseguido gracias a una estrecha colaboración entre investigadores básicos e investigadores clínicos con la participación de los pacientes y sus familias en todo el proceso. Sin la involucración de los pacientes y sus familias no hubiera sido posible una traslación tan rápida de los resultados obtenidos en el laboratorio a las consultas. Este trabajo constituye también un ejemplo de medicina de precisión donde el conocimiento profundo de las bases fisiopatológicas del trastorno ha permitido modificar su curso clínico con la corrección del defecto bioquímico subyacente.

CONCLUSIONES

1. El déficit de timidina quinasa 2 mitocondrial es una enfermedad rara de herencia autosómica recesiva que puede debutar a cualquier edad, con evolución clínica variable.
2. La principal manifestación clínica de la enfermedad es una miopatía de curso progresivo cuyo pronóstico viene determinado por el compromiso precoz de la musculatura respiratoria.
3. Las formas de inicio tardío, con síntomas después de los 12 años de vida, son las menos frecuentes y conocidas. Tienen un fenotipo homogéneo y reconocible, y mal pronóstico clínico determinado por una afectación selectiva, precoz y progresiva del músculo diafragma.
4. El diagnóstico de sospecha de una miopatía por déficit de TK2 en un adulto se basa en su fenotipo con una distribución característica de la debilidad, con predominio de la afectación axial y respiratoria sobre la de extremidades, la presencia de cambios distróficos asociados a los signos de disfunción mitocondrial en la biopsia de músculo, y el hallazgo de deleciones múltiples en el ADNmt.
5. En la resonancia magnética de los músculos de las extremidades inferiores se identifica una afectación precoz de los glúteos mayores, sartorio y gemelos mediales que permite diferenciarlo de otras miopatías mitocondriales.
6. Las formas clínicas de inicio más tardío pueden debutar como oftalmoparesia externa progresiva pero todos ellos evolucionan a una miopatía difusa con insuficiencia respiratoria. En pacientes de origen

español o hispánico con este fenotipo, la mutación más frecuentemente identificada es la p.Lys202del.

7. El tratamiento con desoxinucleósidos dT+dC a dosis de 400mg/kg/día vía oral es eficaz y seguro en todas los pacientes con mutación bialélica en el gen TK2. El beneficio obtenido es mayor en los pacientes con formas de inicio temprano y curso rápidamente progresivo, donde un tratamiento precoz es capaz de detener la progresión de la enfermedad e incluso revertir el fenotipo. Ningún paciente bajo tratamiento en todo el mundo ha fallecido hasta el momento, y el único efecto tóxico identificado es diarrea dosis-dependiente.
8. Los pacientes con formas clínicas de inicio tardío también se benefician del tratamiento, al ser capaz de detener o reducir la progresión de la insuficiencia respiratoria, que es la responsable en todos los casos de una muerte prematura.
9. El marcador más sensible para el diagnóstico de una enfermedad mitocondrial es el GDF-15. Sus niveles se correlacionan con la gravedad de la enfermedad y son un marcador útil para monitorizar la respuesta al tratamiento. Cuando el tratamiento se inicia en la edad pediátrica, los niveles de GDF-15 se normalizan durante el primer año post tratamiento de manera paralela a la mejoría clínica observada.
10. La colaboración entre los investigadores básicos, los investigadores clínicos y los pacientes y/o sus familias es fundamental para asegurar el éxito en la traslación rápida de los hallazgos científicos a los enfermos, sobre todo en el caso de pacientes con enfermedades ultra-raras.

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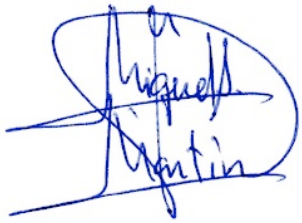

Fdo. Dr. Miguel Ángel Martín Casanueva

Madrid, 23 de junio de 2020.