UNIVERSITAT DE BARCELONA

Deficiencia de 2-metil-3-hidroxibutiril-CoA deshidrogenasa (MHBD o HSD10) e implicaciones en la enfermedad de Alzheimer

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DEFICIENCIA DE 2-METIL-3-HIDROXIBUTIRIL-COA DESHIDROGENASA (MHBD o HSD10) E IMPLICACIONES EN LA ENFERMEDAD DE ALZHEIMER

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Hay una fuerza motriz más poderosa que el vapor, la electricidad y la energía atómica: la voluntad.

Albert Einstein (1879-1955)

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ABREVIATURAS

2M3HBA	Ácido 2-metil-3-hidroxibutírico
2M3HBCoA	2-Metil-3-hidroxibutiril-CoA
3α,5α-THDOC	Alotetrahidrodeoxicorticosterona
4-HNE	Aldehído 4-hidroxi-2-noneneal
5α-DHT	5α-Dihidrotestosterona
aa	Aminoácidos
ABAD	Alcohol deshidrogenasa de unión al péptido β-amyloide
AβPP	Proteína precursora de péptido β-amiloide
ATP	Adenosina trifosfato
C5:1	Tiglilcarnitina
C5-OH	2-metil-3-hidroxibutirilcarnitina
CS	Citrato sintasa
EA	Enfermedad de Alzheimer
EHA	Ácido etilhidracrílico
EMH	Enfermedad Metabólica Hereditaria
ERAB	Proteína de unión al péptido β-amiloide asociada a retículo endoplasmático
ERα	Receptor α de estrógenos
GABA	Ácido gamma-amino-butírico
HADH2	L-3-hidroxiacil-CoA deshidrogenasa de tipo II
HSD10 o 17βHSD10	17β-hidroxiesteroide deshidrogenasa tipo 10
KO	“Knock-out”
MHBD	2-metil-3-hidroxibutiril-CoA deshidrogenasa
MPT	Poro de permeabilidad mitocondrial
MPTP	1-Metil-4-fenil-1,2,2,6-tetrahidropiridina
Mut	Mutado
NA	Ácido nicotínico
NAM	Nicotinamida
NMN	Mononucleótido de nicotinamida
NR	Ribósido nicotinamida
pβA	Péptido β-amiloide
qRT-PCR	PCR cuantitativa a tiempo real
PSD	Complejo de densidad postsináptica
ROS	Especies reactivas de oxígeno o radicales libres

RQ	Cuantificación relativa
SIRT1	Sirtuina 1
SCHAD	3-hidroxiacil-CoA deshidrogenasa de cadena corta
SDR	Familia de deshidrogenasas/reductasas de cadena corta
TG	Tiglilglicina
TRMT10C	tRNA metiltransferasa 10C
Wt	“Wild-type”

RESUMEN

Esta tesis se basa en el análisis de los metabolitos clave y de la proteína deficiente, 17β-hidroxiesteroide deshidrogenasa tipo 10 (HSD10), para el diagnóstico de una enfermedad minoritaria. Este estudio nos llevó al desarrollo de nuevas herramientas que podrían ser de utilidad en enfermedades más comunes como la Enfermedad de Alzheimer (EA)**.**

En el transcurso de este trabajo hemos realizado el diagnóstico de 6 familias españolas con deficiencia de HSD10, 6 pacientes y 8 portadoras. Se trata de una enfermedad ultrarara o infradiagnosticada de herencia ligada al cromosoma X. Una medida de la rareza de esta enfermedad se apoya en el hecho de que los pacientes aquí diagnosticados representan el 37% de los pacientes descritos en la literatura. Para llegar al diagnóstico completo, tuvimos que desarrollar un método cuantitativo de los metabolitos clave en orina mediante cromatografía de gases- espectrometría de masas, además de un método de análisis enzimático de la proteína HSD10 en fibroblastos cultivados. Este método espectrofotométrico resultó ser muy robusto, aunque para ello se tuvo que proceder a la síntesis del sustrato (Dr. Messeguer, CSIC, Barcelona) ya que éste no era asequible comercialmente. Se procedió también al análisis de los niveles de HSD10 mediante "SDS-PAGE", así como al análisis molecular mediante secuenciación "Sanger" y a los estudios patogenicidad de las mutaciones encontradas (**artículos 1 y 2).** Dado que los biomarcadores para esta enfermedad no eran cien por cien informativos, vimos la necesidad de buscar nuevas herramientas diagnósticas. Para ello realizamos un estudio mediante espectrometría de masas en tándem, de todas las muestras biológicas procedentes de los pacientes y de las portadoras de esta enfermedad. Encontramos que la elevación de tiglilcarnitina en orina se podía considerar como un nuevo biomarcador, ya que estaba presente en todos los pacientes independientemente del estado clínico y de la dieta (**artículo 3)**.

La herencia de esta enfermedad se halla ligada al cromosoma X. Sin embargo, el gen *HSD17B10,* que codifica para la proteína HSD10, fue descrito como uno de los pocos genes que se escapan de la inactivación del cromosoma X, pero la sintomatología clínica y el comportamiento bioquímico de nuestros pacientes no apoyaban este hallazgo. Para comprobar esta hipótesis decidimos realizar estudios de expresión de *HSD17B10* en varones y hembras, sanos y afectos, mediante PCR cuantitativa a tiempo real. Demostramos que, efectivamente, se cumplia nuestra hipótesis, es decir *HSD17B10* no escapaba a la inactivación del cromosoma X (**artículo 4).**

HSD10 es una proteína mitocondrial multifuncional que además de su papel en el metabolismo de la isoleucina y de los ácidos grasos, desempeña otras funciones en el metabolismo de hormonas esteroideas sexuales, esteroides neuroactivos, en la detoxificación de aldehídos citotóxicos y en la metabolización de cardiolipina peroxidada. La clínica de los pacientes con deficiencia de HSD10 es neurológica, a diferencia de las otras deficiencias de la vía metabólica

de la isoleucina. Por ello, se postula que la fisiopatología de esta enfermedad podría ser debida a la alteración del resto de funciones no implicadas en el metabolismo de la isoleucina. Para intentar explicar la fisiopatología de la enfermedad nos propusimos realizar estudios de microarrays de expresión. Obtuvimos una expresión significativamente diferente en 31 genes. Para confirmar estos resultados analizamos la expresión del 22% de los mismos por PCR cuantitativa. En todos los casos, excepto en uno, se confirmó la expresión diferencial; Entre los 14 genes que presentaban un patrón de expresión diferente y que podrían estar relacionados con los síntomas neurológicos, destacamos la sobre-expresión de *BCHE* y la infra-expresión de *MAOA* and *MAOB,* ya que la misma alteración en la expresión de los mismos se ha observado en la EA y podrían estar implicados en la neurodegeneración que presentan ambas entidades. Encontramos también una expresión diferencial en cinco genes, que podría explicar la ceguera que presentan algunos de los pacientes, y otros cuatro genes que podrían se podrían relacionar con la cardiomiopatía **(artículo 5).**

La proteína HSD10 parece estar implicada en la EA, debido a su interacción con el péptido β-amiloide (pßA), habiéndose sugerido que dicha interacción es una de las causas de la disfunción mitocondrial en la EA. Para determinar la implicación de HSD10 en la EA valoramos su actividad enzimática y realizamos análisis "western-blot" en muestras de cerebro de pacientes con EA. Se realizaron estudios de inhibición "in vitro" de la proteína recombinante de la isoforma 1 de HSD10 por el pßA y se estudió si la disrupción de la interacción pβA-HSD10- podría ser utilizada como diana terapéutica para la EA.

En cuanto a la actividad enzimática, encontramos disminución de la misma en homogenados de corteza parietal y occipital de pacientes con EA. Se observó también que la actividad HSD10 disminuye con la progresión de la enfermedad, por ello creemos que debe ser tenida en cuenta en el análisis de la disfunción mitocondrial en la EA.

Por otro lado, comprobamos que la incubación de homogenados de cerebro control con pßA provocaba una inhibición de la actividad HSD10, que se rescataba al incubar con elevadas concentraciones de NAD^+. Esta observación también fue corroborada en la EA. Este hecho se podía explicar por una interacción competitiva entre NAD^+ y pßA, ya que ambos interaccionan con HSD10 en la misma posición aminoacídica. Por ello, al incubar con elevadas concentraciones NAD^+ se inhibe la interacción pßA-HSD10. Por tanto, la medición de la actividad HSD10 podría ser utilizada como diana terapéutica para la EA. Con esta finalidad, obtuvimos la proteína recombinante HDSD10 isoforma 1, que es la que expresa mayoritariamente en cerebro humano, y medimos su actividad "in vitro" antes y después de la incubación con pßA, con y sin adición de NAD^+. Los resultados obtenidos fueron los mismos

que al utilizar homogenados de cerebro humano. Por lo tanto, quedaba validado un sistema para cribar moléculas terapéuticas que pudieran inhibir la interacción pßA-HSD10 y restablecer la actividad HSD10. Se probaron 117 compuestos de una librería peptídica, Coenzima Q10 y otros antioxidantes, pero no se consiguió rescatar la actividad HSD10. La actividad enzimática sólo se consiguió rescatar con NAD^+. Así, proponemos que los precursores de NAD^+ podrían ser considerados una terapia coadyuvante en la EA. Estudios recientes apoyan nuestros resultados, ya que la administración de precursores de NAD^+ a ratones modelo para la EA, mejoran la disfunción mitocondrial así como la cognición **(artículo 6).**

INTRODUCCIÓN GENERAL

1.-ENFERMEDADES METABOLICAS HEREDITARIAS

El concepto de Enfermedad Metabólica Hereditaria (EMH) fue introducido por Archibald Garrod a principios del siglo XX a raíz de sus estudios sobre la Alcaptonuria. Garrod observó que los pacientes con esta enfermedad excretaban niveles elevados de ácido homogentísico y que la herencia de la enfermedad se podía explicar según las leyes de Mendel (Garrod, 1902). Medio siglo después se comprobó la hipótesis de Garrod demostrándose el defecto de la homogentísico dioxigenasa (La Du et al., 1958). Las EMH son enfermedades monogénicas, en su mayoría de herencia autosómica recesiva, o bien ligadas al cromosoma X o bien de herencia materna en el caso de mutaciones en el DNA mitocondrial. Existe un número muy reducido de EMH con herencia autosómica dominante.

El 80% de las EMH debutan en la edad pediátrica. La primera aproximación diagnóstica se inicia a través de la clínica y de los análisis de metabolitos en líquidos biológicos (sangre, orina y, si es necesario, líquido cefalorraquídeo), que podrán orientar hacia la vía metabólica afectada. Posteriormente, la confirmación del defecto puede realizarse con el estudio de la proteína afectada y/o con los estudios genéticos. Durante los últimos años, la aparición de técnicas de secuenciación masiva ha permitido el descubrimiento de nuevos genes causantes de EMH, ofreciendo una mayor comprensión de las bases moleculares y de los mecanismos fisiopatológicos subyacentes, y aportando conocimiento para el desarrollo de nuevas terapias para estas enfermedades.

Clásicamente las EMH se dividen en los siguientes grupos, según el orgánulo afectado: enfermedades lisosomales, peroxisomales, mitocondriales, trastornos de la glicosilación de las proteínas y trastornos del metabolismo intermediario. Dentro de este último grupo se incluyen las acidurias orgánicas que en general presentan un cuadro clínico de toxicidad, en el período neonatal y de la lactancia, con descompensaciones metabólicas frecuentes. Una excepción a este tipo de presentación es la aciduria glutárica tipo I, la 4-hidroxibutírica, la 2-hidroxiglutárica, la enfermedad de Canavan y la aciduria 2-metil-3-hidroxibutírica o deficiencia de 2-metil-3-hidroxibutiril-CoA deshidrogenasa, enfermedad objeto de este estudio.

2. DEFICIENCIA DE 2-METIL-3-HIDROXIBUTIRIL-COA DESHIDROGENASA (MHBD o HSD10).

La deficiencia de 2-metil-3-hidroxibutiril-CoA deshidrogenasa o también llamada 17β-hidroxiesteroide deshidrogenasa tipo 10 (HSD10) es una EMH del metabolismo de la isoleucina y de los ácidos grasos de cadena ramificada (OMIM 300438). Esta enfermedad fue descrita por

primera vez en el año 2000 por Zschocke et al. Clínicamente se caracteriza por neurodegeneración progresiva, que en general se inicia entre los 6-18 meses de vida. Bioquímicamente destaca una elevada excreción de ácido 2-metil-3-hidroxibutírico (2M3HBA) y tiglilglicina (TG). Esta entidad es la única enfermedad del metabolismo de los aminoácidos ramificados que se hereda ligada al cromosoma X (Ofman et al., 2003).

En la literatura sólo se han descrito 14 familias (15 hemicigotos y 6 heterocigotas) con esta deficiencia (Zschocke et al., 2000; Ensenauer et al., 2002; Olpin et al., 2002; Poll The et al., 2004; Sass et al., 2004; Sutton et al., 2003; Rauschenberger et al., 2010; Seaver et al., 2011; Zschocke, 2012; Fukao et al., 2014; Chatfield et al., 2015).

HSD10, además de su papel en el metabolismo de la isoleucina, desempeña otras funciones y parece estar implicada en la patogénesis de la enfermedad de Alzheimer (Yang el al., 2005).

2.1. Síntomas clínicos

La mayoría de los pacientes presentan un desarrollo normal hasta los 6-18 meses de edad, seguido de una pérdida progresiva de las habilidades mentales y motoras (Poll The et al., 2004; Ensenauer et al., 2002; Sutton et al., 2003; Sass et al., 2004; Ruschenberger et al., 2010).

Las manifestaciones clínicas son heterogéneas, pudiéndose destacar: neurodegeneración progresiva (Zschocke et al., 2000; Ensenauer et al., 2002; Olpin et al., 2002; Sass et al., 2004; Sutton et al., 2003; Rauschenberger et al., 2010; Zschocke, 2012), cardiomiopatía, hipotonía (Zschocke et al., 2000; Ensenauer et al., 2002; Sass et al., 2004; Sutton et al., 2003; Rauschenberger et al., 2010; Zschocke, 2012, Chatfield et al., 2015), retraso en el lenguaje o dificultades en el aprendizaje (Zschocke et al., 2000; Ensenauer et al., 2002; Olpin et al., 2002; Sutton et al., 2003; Poll The et al., 2004; Seaver et al, 2011), epilepsia (Zschocke et al., 2000; Ensenauer et al., 2002; Sass et al., 2004; Seaver et al., 2011) y retinopatía, con pérdida de reacción a estímulos visuales y ceguera cortical (Zschocke et al., 2000; Ensenauer et al., 2002; Sass et al., 2004; Sutton et al., 2003; Zschocke, 2012). Otros síntomas menos frecuentes son: sordera (Poll The et al., 2004; Olpin et al., 2002; Sass et al., 2004), distonía (Ensenauer et al., 2002; Olpin et al., 2002), convulsiones, microcefalia (Sass et al., 2004), leve dismorfia (Poll The et al., 2004) o acidosis metabólica en contexto de una infección (Fukao et al., 2014).

En el año 2012 se clasificaron tres formas e presentación clínica (Zschocke, 2012):

Infantil o clásica (la mayoría de los pacientes descritos): letargia, acidosis metabólica, hiperlactacidemia, hipoglucemia e hiperamonemia en el período neonatal, pero después de este cuadro clínico los pacientes presentan un desarrollo normal hasta los 6-18 meses de vida. Posteriormente, se manifiesta una neurodegeneración y cardiomiopatía progresivas, a menudo

después de una enfermedad leve o después de una vacunación. Prosigue una pérdida de las habilidades motoras, de la visión y del lenguaje, junto con una ataxia, corea, epilepsia rebelde al tratamiento y en algún caso sordera y microcefalia. Suelen fallecer entre los 2 y 4 años de edad.

Neonatal (2 pacientes descritos): acidosis metabólica severa con importante acidosis láctica, desarrollo psicomotor pobre, convulsiones y cardiomiopatía hipertrófica progresiva. Éxitus en los primeros meses de vida.

Juvenil (2 pacientes descritos): desarrollo normal hasta los 6 años. A partir de esta edad y concomitantemente con una infección intercurrente se observa deterioro del lenguaje y de las habilidades motoras. Se observan también la aparición de otros síntomas como disartria, rigidez, temblor, distonía, epilepsia.

Portadoras: con una clínica más leve que los varones, caracterizada por retraso mental leve o dificultades en el aprendizaje, pero sin neurodegeneración progresiva. Aunque también pueden ser asintomáticas.

Presentación atípica (3 familias descritas): 3 pacientes de una misma familia, 2 de ellos con retraso del crecimiento y microcefalia y otro asintomático. En este grupo también incluye una familia con un paciente de 10 años que presenta cetoacidosis tras una gastroenteritis pero sin presentar regresión neurológica y una familia en la que se encontró una mutación silente en el gen que codifica para la 2-metil-3-hidroxibutiril-CoA deshidrogenasa y que clínicamente presentaban un defecto intelectual, coreatetosis y comportamiento anormal.

A nivel neuroradiológico en la mayoría de los pacientes se observa atrofia cerebral, principalmente fronto-temporal (Zschocke et al., 2000; Ensenauer et al., 2002; Sass et al., 2004).

A diferencia de otras deficiencias del metabolismo de la isoleucina que se presentan con episodios de cetosis, acidosis metabólica e hiperamonemia, la deficiencia de HSD10 se caracteriza fundamentalmente por neurodegeneración progresiva; por tanto desde el punto de vista clínico esta entidad es completamente distinta a las otras enfermedades de su misma vía metabólica.

2.2. Proteína 2-metil-3-hidroxibutiril-CoA deshidrogenasa (MHBD or HSD10)

2.2.1. Funciones de la proteína HSD10

Estudios realizados durante los últimos años han demostrado que HSD10 es una proteína multifuncional (“moonlighting”), que ha recibido diferentes nombres según las funciones que se le han ido atribuyendo: ERAB, HADH2, SCHAD, ABAD, SDR5C1, 17βHSD10 ó HSD10. A continuación se describen dichas funciones.

En 1974 Conrad et al., purificaron la proteína: 2-metil-3hidroxibutiril-CoA (2M3HBCoA) deshidrogenasa de *Pseudomonas putida*, la describieron como una enzima NAD^+-dependiente que tenía como sustratos 2M3HBCoA y 3-hidroxibutiril-CoA.

En 1995 Luo et al., purificaron a partir de hígado de rata la proteína L-3-hidroxi-2-metilacil-CoA deshidrogenasa de cadena corta, la describieron como una enzima mitocondrial soluble que catalizaba la deshidrogenación de 3-hidroxi-2-metilbutiril-CoA a 2-metilacetoacetil-CoA en la vía metabólica de la isoleucina. En 1996 Kobayashi et al., identificaron la misma proteína bovina en mitocondria que recibió el nombre de L-3-hidroxiacil-CoA deshidrogenasa tipo II (HADH2).

En humanos se hace referencia a esta proteína a partir de 1997, así Yan et al., la describieron como una proteína de unión al péptido β-amiloide (pβA) asociada a retículo endoplasmático (ERAB). pβA es un péptido neurotóxico implicado en la enfermedad de Alzheimer.

Furuta et al., 1997, clonaron el cDNA de la proteína HADH2 bovina, que resultó ser el cDNA homólogo de la proteína ERAB humana. Posteriormente, He et al., 1998, clonaron el gen de una 3-hidroxiacil-CoA deshidrogenasa en cerebro humano, denominada L-3-hidroxiacil-CoA deshidrogenasa de tipo II (HADH II o SCHAD), esta proteína era un homotetrámero con un peso molecular de 108 KDa y con una secuencia aminoacídica idéntica a la de la proteína ERAB.

Yan et al., 1999, He et al., 1999, observaron que ERAB ejercía la función de alcohol deshidrogenasa (ABAD), oxidando alcoholes secundarios como el 2-propanol.

Yan et al., 1999; He et al., 1999, 2000, 2001 encontraron que ERAB además tenía actividad 3α y 17β-hidroxiesteroide deshidrogenasa (17βHSD10 o HSD10). Esta función estaría implicada en el metabolismo de las hormonas sexuales, por un lado oxidando 17β-estradiol (principal estrógeno) e inactivándolo y por otro lado generando 5α-dihidrotestosterona (5α-DHT -principal andrógeno-) y androstenediona, siendo esta última una vía alternativa de síntesis de andrógenos. Además, en situaciones en que 3-hidroxibutirato es el único sustrato energético, HSD10 contribuye potencialmente a su metabolización para mantener la homeostasis celular (Yan et al., 2000 (a)).

Shafqat et al., 2003, encontraron una nueva actividad de HSD10 relacionada con la epimerización de los ácidos biliares. También se especuló que podría intervenir en el control de los niveles de progesterona y glucocorticoides con actividad 20β-hidroxi y 21-hidroxi deshidrogenasa.

Ofman et al., en el año 2003 purificaron HSD10 de hígado bovino y encontraron, que esta proteína era homóloga a la proteína bovina HADH2 e idéntica a ERAB humana.

En el año 2005 He et al., (a) y (b), describieron que la actividad 17βHSD10 era esencial para el funcionamiento de las neuronas gabaérgicas, ya que oxidaba alopregnanolona y

alotetrahidrodeoxicorticosterona (3α,5α-THDOC), moduladores alostéricos de los receptores del ácido gamma-amino-butírico (GABA).

Se ha especulado también que HSD10 está implicada en el mantenimiento de las funciones sinápticas (Laumonnier et al., 2007). Por otro lado Murakami et al., 2009 describieron que HSD10 detoxificaba el aldehído 4-hidroxi-2-noneneal (4-HNE) el cual proviene de la peroxidación lipídica, ejerciendo así un efecto citoprotector.

En 2008 Holzmann et al., identificaron a la proteína HSD10 como una de las tres proteínas que formaban el complejo mitocondrial RNasaP humano, el cual interviene en el proceso de maduración de tRNAs y se le atribuyó el nombre de SDR5C1. En 2012 Vilardo et al., identificaron HSD10 como componente del complejo mitocondrial tRNA metiltransferasa.

Recientemente, Boynton and Shimkets, 2016 han demostrado que HSD10 es capaz de metabolizar la cardiolipina peroxidada, evitando así la inducción de la apoptosis. Por otro lado, HSD10 interacciona con ciclofilina-D (Yan et al., 2005), proteína relacionada con el poro de permeabilidad mitocondrial (MPT). En respuesta a estrés, HSD10 se transloca a la membrana mitocondrial interna donde se ensambla el MPT, una alteración en esta función podría ser una de las causas de la neurodegeneración asociada con esta enfermedad (Rauschenberger et al., 2010).

Jazbutyle et al., 2009 detectaron que el receptor α de estrógenos (ERα) interaccionaba con HSD10 en la mitocondria de forma dinámica, ya que la unión sólo ocurría a concentraciones bajas de estrógenos provocando así la inhibición de HSD10.

En el año 2007 Korman SH and Yang SY, publicaron que ABAD era más eficiente oxidando hidroxiestereoides que alcoholes y se aceptó internacionalmente el nombre del gen *HADH2* sería reemplazado por el nombre *HSD17B10* y que el producto del gen se denominaría HSD10. Por tanto para no crear confusión utilizaremos esta nomenclatura en el resto del texto.

2.2.2. Localización tisular e intracelular de HSD10

HSD10 se expresa de forma ubicua. Se ha descrito su localización en corazón, hígado, riñón y cerebro (Yan et al., 1997, He et al., 2001), en gónadas, próstata, ovario, médula ósea, timo (He et al., 2000), en epidermis, músculo cardíaco, células de la musculatura lisa arterial, macrófagos de pulmón, neuronas, células glía y tejido conectivo (Frackowiak et al., 2001). En cerebro se expresa en varias regiones (He et al.(b), 2005; Yang et al., 2011).

Yan et al., 1997, 1999 encontraron que HSD10 era una proteína de retículo endoplasmático y mitocondrial. Posteriormente surgieron algunas discrepancias (Sambamurti et al., 1998; Oppermann et al., 1999). Finalmente los estudios de microscopia electrónica y confocal (He et al., 1999, 2000(a,b), 2001(a,b); Yang et al., 2001, 2005(a,b), así como todos los estudios publicados

posteriormente (Frackowiak et al., 2001; Lustbader et al., 2004; Yan et al., 2005; Holzmann et al., 2008; Jazbutyte et al., 2009; Rauschenberger et al., 2010) demostraron que HSD10 se localiza en la mitocondria y no en retículo endoplasmático.

2.2.3. Estructura molecular de la proteína HSD10

En primer lugar se cristalizó la proteína HSD10 de rata, la cual presentaba un 88% de homología con la humana (Powell et al., 2000). Posteriormente se cristalizó la proteína humana (Kissinger et al., 2004), figura 1. Además de su capacidad para unirse al pβA, se encontró que HSD10 tenía especificidad para muchos sustratos, desde derivados 2-hidroxiacil-CoA, hidroxiesteroides y alcoholes, hasta 3-hidroxibutirato.

HSD10 es un enzima de matriz mitocondrial que pertenece a la familia de las deshidrogenasas/reductasas de cadena corta (SDR) NAD^+-dependientes (He et al., 1998).

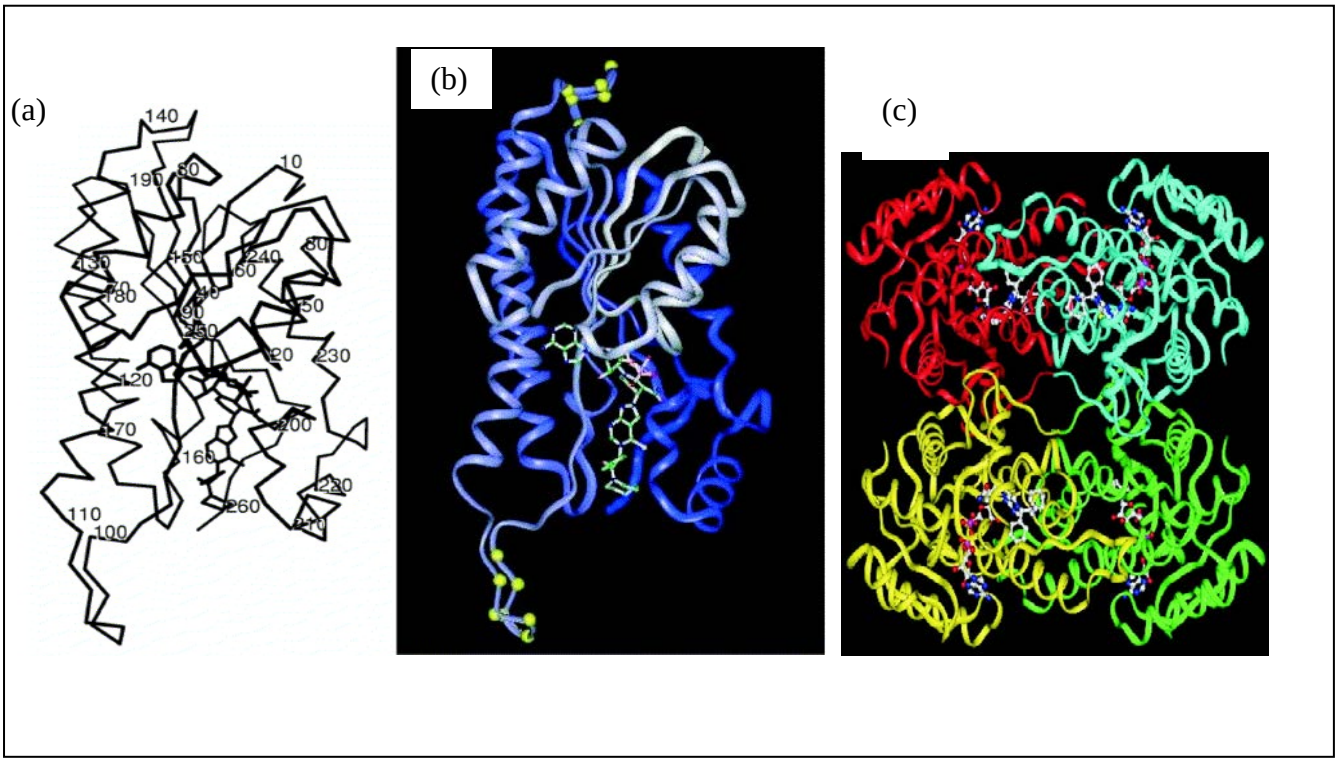
Las regiones más importantes de esta proteína se encuentran en las siguientes posiciones aminoacídicas:

- 1-34: señal de direccionamiento a la mitocondria (Shafqat et al., 2003). Aunque He et al., 2001 describieron que sólo la región aminoacídica 1-11 contienía dicha señal, igual que en la proteína homóloga de ratón (He et al., 2002). Dicha secuencia no se escinde al entrar en la mitocondria (He et al., 1998, 1999, 2001; Moeller and Adamski 2009).
- 12-37: dominio de unión al NAD^+ que contiene una región conservada rica en glicinas (p.Gly17, p.Gly21, p.Gly23), característica de la familia SDR (He et al., 1998), también presente en HSD10 de rata (Powell et al., 2000; He et al., 2001).
- p.Ser155, p.Tyr168 y p.Lys172, residuos conservados del centro activo (He et al., 1998; Kissinger et al., 2004), también presentes en la proteína homóloga de Drosophila (He et al., 1998), de rata y ratón (He et al., 2001; Powell et al., 2000; He et al., 2002). El centro activo tiene una gran flexibilidad pudiendo utilizar diversos sustratos: esteroides, ácidos grasos, ácidos biliares y xenobióticos (Opperman et al., 1999; He et al., 1998,1999, 2000, 2005(a); Shafquat et al., 2003; Yang et al., 2005 (b), 2007, 2011; Muirhead et al., 2010).
- 102-107 y 141-146: 2 regiones adicionales, no presentes en otros miembros de la familia SDR (Kissinguer et al., 2004), también presentes en HSD10 de rata (Powell et al., 2000). En 1997 Yan et al., demostraron que anticuerpos contra estas regiones inhibían la interacción de pβA con HSD10. Posteriormente, Lustbader et al., 2004 y Marques et al., 2006, describieron que en concreto la región 95-113 era muy importante para la unión de HSD10 con los residuos 13-22 del pβA.

- 204-219: región conservada movible característica de la familia SDR, son residuos adyacentes al centro activo que se encuentran desordenados en ausencia de sustrato (Powell et al., 2000).
- 256-261: residuos conservados implicados en la formación del tetrámero (Powell et al., 2000). Primero forma un homotetrámero y luego éste se une a otras 2 proteínas para formar la RNAasaP mitocondrial (Holzman et al., 2008).

HSD10 no posee ningún dominio que pueda ser glicosilado (Sambamurti et al., 1998)

Figura 1. Estructura de la proteína HSD10 humana.



(a) Monómero con las posiciones aminoacídicas. (b) Estructura tridimensional del monómero unido a cofactor, señalando con bolas verdes las 2 regiones específicas de HSD10 residuos 102-107 (parte superior) y residuos 141-146 (parte inferior). (c) Tetrámero unido al cofactor NAD^+ (*Kissinger et al., 2004*).

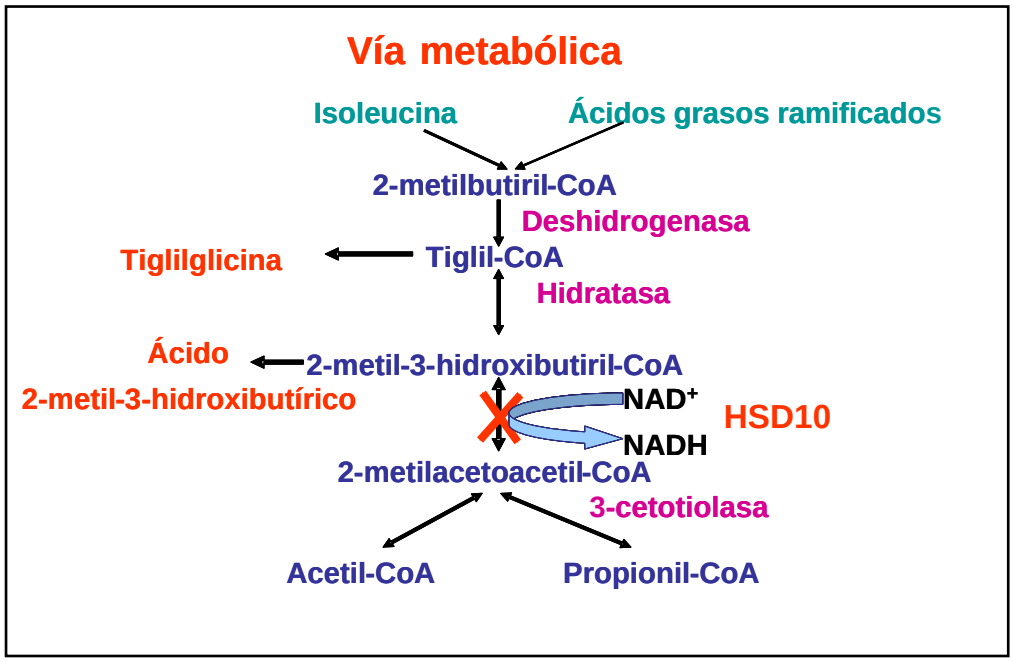
2.2.4. Deficiencia enzimática de HSD10

Según estudios previos realizados de todas las funciones que desempeña HSD10 la actividad 2-metil-3-hidroxiacil-CoA deshidrogenasa es la mayoritaria, ésta consiste en la siguiente reacción de oxidación reversible, la cual sólo se lleva a cabo con el isómero-L (He et al., 1999, 2000, 2005(b); Powell et al., 2000; Yang et al., 2009, 2011; Shafquat et al., 2003):

L-2-metil-3-hidroxiacil-CoA + NAD^+ = L-2-metil-3-oxoacil-CoA + NADH

Dicha actividad está implicada en el metabolismo de la isoleucina y de los ácidos grasos de cadena ramificada. En el año 2000 Zcshoscke et al., describieron el primer paciente con deficiencia de HSD10. Dicha deficiencia produce un acumulo de los sustratos: 2-metil-3-hidroxibutiril-CoA y tiglil-CoA en el metabolismo de la isoleucina. En consecuencia, se observa una elevada excreción de los correspondientes metabolitos (2M3HBA i TG) en orina (figura 2) (Zschocke et al., 2000; Ensenauer et al., 2002; Olpin et al., 2002; Poll The et al., 2004; Sass et al., 2004; Sutton et al., 2003; Rauschenberger et al., 2010; Seaver et al., 2011; Zschocke 2012, Fukao et al., 2014) y también puede observarse un aumento de 2-metil-3-hidroxi-butirilcarnitina (C5OH) y de tiglilcarnitina (C5:1) en plasma (Zschocke et al., 2000; Ensenauer et al., 2002; Sass et al., 2004; Poll The et al., 2004).

Figura 2. Vía metabólica de la isoleucina.



HSD10: 17β-hidroxiesteroide deshidrogenasa tipo 10.

Pero a parte de esta actividad enzimática, se han descrito otras propiedades cinéticas (He et al., 1998, 1999, 2000, 2005(a,b); Shafquat et al., 2003; Yang et al., 2005(a,b), 2009, 2011).

2.3. Gen *HSD17B10*

El gen que codifica para la proteína HSD10 se denomina *HSD17B10* (antiguamente, *HADH2*) (OMIM 300256), está localizado en el locus $X_p11.2$, abarca un total de 3,11 Kb, contiene 6 exones y el cDNA resultante de 786 pb codifica para los 261 aminoácidos (aa) que forman la proteína (He et al., 1998 y Ofman et al., 2003).

Se ha descrito que por "splicing" alternativo se obtienen 3 isoformas (Yang et al., 2007). La isoforma 1(ENST00000168216) contiene 261 aa y se ha localizado en pulmón (www.expasy.org). La isoforma 2 (ENST00000375304) no contiene los aa 190-198 de la isoforma 1, resultando una proteína de 252 aa. La isoforma 3 (ENST00000375298) se crea por la pérdida del exón 5, alterando la pauta de lectura, provocando un codón de parada prematuro y resultando en una proteína de 169 aa, de los cuales los 6 últimos difieren totalmente de las otras 2 isoformas. Las isoformas 2 y 3 se han localizado en cerebro y linfoblastos (Yang et al., 2007, www.expasy.org).

Se han encontrado 7 polimorfismos intrónicos, todos registrados en las bases de datos de SNPs (www.snpbrowser) y un polimorfismo en el promotor, rs12604014 (Yang et al., 2009).

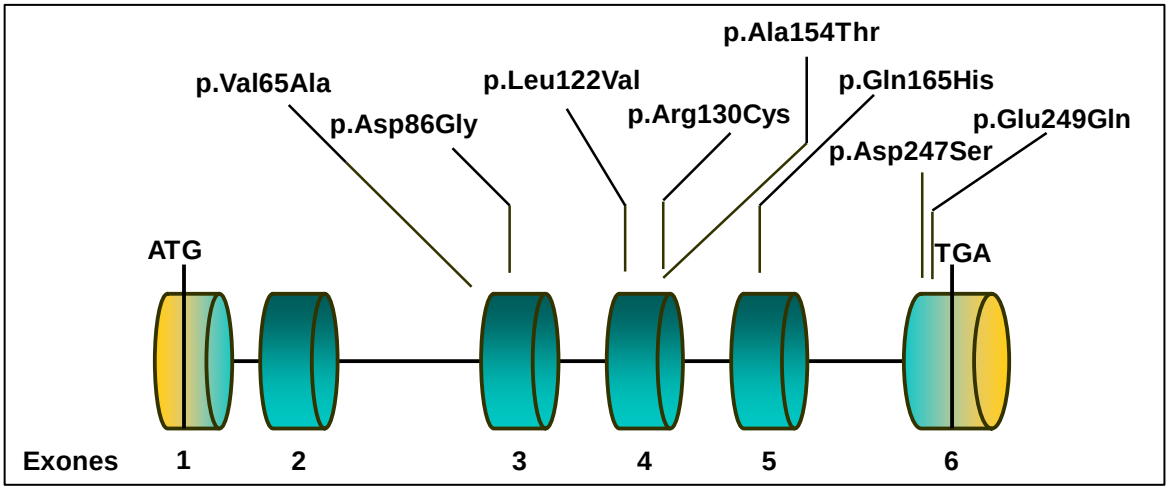
La deficiencia de HSD10 se hereda ligada al cromosoma X (Ofman et al., 2003) y se especula que de forma recesiva (Poll The et al., 2004). Sólo se han descrito 8 mutaciones de cambio de sentido (figura 3), la mutación c.388C>T (p.Arg130Cys) se ha encontrado en 7 de las 14 familias descritas. Mientras que el resto de las mutaciones c.194T>C (p.Val65Ala), c.257A>G (p.Asp86Gly), c.364C>G (p.Leu122Val), c. 460G<A (p.Ala154Thr), c.495A>C (p.Gln165His), c.740A>G (p.Asp247Ser) y c.776G>C (p.Glu249Gln) se han detectado en familias independientes (Ofman et al., 2003; Yang et al., 2009; Rauschenberger et al., 2010; Seaver et al., 2011; Fukao et al., 2014; Chatfield et al., 2015).

En 1999 Reyniers et al., describieron un nuevo síndrome neurológico ligado al cromosoma X, caracterizado por retraso mental moderado, coreoatetosis y trastorno del comportamiento. Recientemente, este síndrome se ha asociado a una mutación sinónima en *HSD17B10*: c.574C>A (p. Arg192Arg), que provoca la pérdida del exón 5 e introduce un codón de parada prematuro en el exón 6, produciendo un incremento de este tránscrito aberrante y una disminución del 60% del tránscrito correcto. Este síndrome se caracteriza por una clínica menos severa que la descrita en pacientes con deficiencia de HSD10, conservándose un 85% de la actividad enzimática (Lenski et al., 2007), mientras que en la deficiencia de HSD10 causada por mutaciones de cambio de sentido la actividad residual es inferior al 45% (Ofman et al., 2003).

Posteriormente Froyen et al., 2008, asociaron duplicaciones submicroscópicas de la región Xp11.22, que incluye el gen *HSD17B10*, con retraso mental en 6 familias, destacando que tanto la

disminución de HSD10 como su aumento puede interferir en el desarrollo cognitivo debido a un trastorno en el metabolismo de los neuroesteroides moduladores de los receptores de GABA.

Figura 3. Estructura del gen *HSD17B10*. Mutaciones descritas hasta el momento.



Es importante destacar que en 1998 Miller et al., describieron que el gen *HSD17B10* se escapaba de la inactivación del cromosoma X, que no fue confirmado posteriormente por Carrel et al., 2005 ya que sólo 1 de los 9 híbridos estudiados por estos autores se escapaba de la inactivación del cromosoma X.

El dominio N-terminal de HSD10, que incluye la región de unión al NAD^+, se encuentra codificado por los 3 primeros exones, mientras que el dominio C-terminal donde se une el sustrato, se encuentra codificado por los 3 últimos (He et al., 1998).

El estudio evolutivo de HSD10 indica que es un "ancient housekeeping" (Marques et al., 2006).

Por otro lado, se ha demostrado que en células de hepatocarcinoma, el factor C/EBPβ, implicado en procesos inflamatorios y de proliferación y diferenciación celular, se une al promotor de *HSD17B10* y regula su expresión (Rotinen et al., 2010).

2.3.1. Correlación genotipo-fenotipo

Sólo se han descrito 14 familias con esta deficiencia, lo cual hace difícil establecer una correlación genotipo- fenotipo, ya que sólo se conocen 8 mutaciones. Por un lado, parece ser que los pacientes con la mutación prevalente c.388C>T (p.Arg130Cys) presentan una actividad enzimática muy baja con una clínica muy severa (Zschocke et al., 2000; Ensenauer et al., 2002; Sass et al., 2004). La única hembra descrita también con esta mutación, muestra un fenotipo bioquímico y clínico más leve debido a la lionización del cromosoma X (Ensenauer et al., 2002). Se cree que dicha mutación (p.Arg130Cys) es la más prevalente porque la citosina en esa posición está metilada y la 5-

metilcitosina es propensa a pasar a timina por desaminación, lo que provoca que sea un "hotspot". Además el cambio de arginina por cisteína en la posición 130 de la proteína elimina puentes de hidrógeno y reduce la interacción "van der Waals" entre subunidades HSD10, lo que explicaría la baja actividad detectada en los pacientes con dicha mutación (Yang et al, 2013).

Por otro lado los pacientes con las mutaciones p.Leu122Val y p.Glu249Gln presentan una actividad residual mayor con una clínica más leve que la de los pacientes con la mutación prevalente (Poll The et al., 2004; Yang et al., 2009). Sin embargo, Rauschenberger et al., 2010 describen un paciente con la mutación p.Asp86Gly, con una clínica neurológica muy severa, pero con una actividad enzimática residual bastante elevada. Por el contrario, otro paciente con la mutación p.Gln165His, presenta un desarrollo cognitivo y motor normal con una actividad enzimática prácticamente indetectable, lo que parece indicar que la severidad clínica no está relacionada con la actividad enzimática residual.

2.4. Fisiopatología

A nivel metabólico, la excreción de los ácidos 2M3HBA y TG se encuentra elevada, aunque no se ha descrito que la acumulación de dichos metabolitos pueda ser tóxica (Poll The et al., 2004). Sin embargo, se ha demostrado que niveles altos de 2M3HBA inhiben el complejo IV de la cadena respiratoria mitocondrial (Rosa et al., 2005). Además, en la mayoría de los pacientes se ha observado un incremento de ácido láctico, tanto en plasma como en líquido cefalorraquídeo (Zschocke et al., 2000; Ensenauer et al., 2002; Olpin et al., 2002; Sass et al., 2004), habiéndose atribuido en algún caso a una deficiencia secundaria del complejo I o IV de la cadena respiratoria mitocondrial, debido a la alteración secundaria de la relación NAD^+/NADH (Ensenauer et al., 2002; Olpin et al., 2003).

Es importante destacar que en la deficiencia de 3-cetotiolasa (figura 2) se acumulan los mismos metabolitos en cantidades muy similares, en cambio los pacientes no presentan deterioro neurológico. Por tanto, parece que la causa de patología en la deficiencia de HSD10 reside a otro nivel (Poll The et al., 2004) y se deduce que estos compuestos no deben ser la causa de la neurodegeneración (Zschocke et al., 2000; Yang et al., 2009; Rauschenberger et al., 2010).

HSD10 parece tener un papel fisiológico importante, el gen *HSD17B10* está altamente conservado en animales (Marques et al., 2006) y parece indispensable para la vida, ya que el "Knock-out" (KO) de *Drosophila* tiene un trastorno del desarrollo mitocondrial letal (Torroja et al., 1998). Por otro lado, en 2010 Rauschenberger et al., demostraron mediante modelos de ratón KO y "Knock-down" de *Xenopus*, que HSD10 es esencial para la estructura e integridad funcional de la mitocondria y que esta integridad no estaría relacionada con la actividad enzimática en el

metabolismo de la isoleucina. Por otro lado, se observó que la pérdida completa de la proteína HSD10 en embriones de ratón era letal en estadios muy tempranos de la embriogénesis. Recientemente, se ha descrito que el fallo energético mitocondrial observado en pacientes con HSD10 era debido a un procesamiento erróneo del RNA mitocondrial (Chatfield et al, 2015; Vilardo and Rossmanith 2015).

Otros autores postulan que los efectos adversos en el desarrollo cognitivo probablemente sean debidos a un desequilibrio en el metabolismo de los neuroesteroides (Yang et al., 2009; Yang et al., 2014).

2.5. Tratamiento y pronóstico

El tratamiento consiste básicamente en una dieta hipoproteica o bien restringida en isoleucina. La evolución de los pacientes después del tratamiento ha sido variable, de 6 pacientes que fueron tratados, 3 se estabilizaron clínicamente y la excreción de metabolitos se normalizó (Zschocke et al., 2000; Ensenauer et al., 2002; Olpin et al., 2002), en uno de ellos sólo se normalizó la excreción de ácidos pero no se observaron cambios en su estado clínico (Ensenauer et al., 2002) y en otro no se evidenció ninguna mejoría (Sutton et al., 2004).

La esperanza de vida es muy variable, así como el grado de afectación neurológica. Se conoce el caso de un paciente que murió a los 3 años de vida (Zschocke et al., 2000), aunque en el otro extremo se ha descrito un paciente que sólo presenta un retraso intelectual a los 23 años de edad (Olpin et al., 2003).

En 2010 Rauschenberger et al., hipotetizan que el acumulo de metabolitos no son la causa los síntomas clínicos y que el tratamiento de estos pacientes debería dirigirse hacia la reducción del estrés oxidativo, el mantenimiento de la homeostasis mitocondrial y la administración de cofactores y vitaminas.

2.6. Pruebas diagnósticas

Ante una sospecha clínica de una deficiencia de HSD10 se procede al diagnóstico mediante el estudio de ácidos orgánicos en orina, de acilcarnitinas y aminoácidos en plasma (Zschocke et al., 2000) y posterior confirmación mediante la determinación de la actividad enzimática en fibroblastos cultivados y/o mediante el estudio mutacional del gen *HSD17B10* (Ofman et al., 2003). El perfil de ácidos orgánicos se caracteriza por un incremento de 2M3HBA y TG (Zschocke et al., 2000; Ensenauer et al., 2002; Olpin et al., 2002; Poll The et al., 2004; Sass et al., 2004; Sutton et al., 2003). Se ha de realizar un diagnóstico diferencial entre la deficiencia HSD10 y la deficiencia de 3-cetotoliasa, ya que presentan la misma alteración en el perfil de ácidos orgánicos, a excepción

del incremento de ácido 2-metil-acetoacético que sólo se observa en la deficiencia de 3-cetotiolasa, aunque no siempre está presente (Zschocke et al., 2000). En algunos casos el perfil de acilcarnitinas en plasma muestra un aumento de C5OH y C5:1 (Zschocke et al., 2000; Ensenauer et al., 2002; Sass et al., 2004; Poll The et al., 2004).

La actividad HSD10 se determina en fibroblastos cultivados (Zschoke et al., 2000; Ensenauer et al., 2002; Olpin et al., 2002; Ofman et al., 2003) y el análisis mutacional se realiza en DNA genómico o en cDNA (Ofman et al., 2003).

3.-IMPLICACION DE HSD10 EN ENFERMEDADES NEURODEGENERATIVAS

3.1. Implicación de HSD10 en la enfermedad de Alzheimer

Yan et al., 1997 describieron que HSD10 era una proteína de unión al pβA, que se encontraba sobre-expresada en neuronas afectadas por la enfermedad de Alzheimer, especialmente en zonas próximas a los depósitos de pβA. De acuerdo con las observaciones previas, se ha encontrado sobre-expresión de HSD10 en hipocampo de ratones transgénicos para la enfermedad de Alzheimer (He et al., 2002), en astrocitos de pacientes con enfermedad de Alzheimer esporádico y de pacientes con síndrome de Down y Alzheimer (He et al., 2005). Sin embargo, los estudios de Frakowiack et al., 2001 se contradicen con las anteriores observaciones, ya que en cerebro de pacientes con la enfermedad Alzheimer, HSD10 no colocalizaba con pβA y en las zonas donde había más pβA se observaba una menor expresión de HSD10. Wen et al., 2002 describieron que HSD10 se encontraba en abundancia en las placas β-amiloideas en cerebro de ratones transgénicos para la enfermedad de Alzheimer, pero no en cerebro humano con dicha enfermedad. Sin embargo, Hovorkova et al., 2008 observaron una lateralización de la expresión de HSD10 en hipocampo de pacientes con la enfermedad de Alzheimer y esquizofrenia, observando una mayor expresión de HSD10 en el lado izquierdo del cerebro. También se ha descrito que en líquido cefalorraquídeo la proteína HSD10 disminuye con la edad, en cambio en pacientes con AD esta proteína se encuentra elevada (He et al. (b), 2005; Kristofikova et al., 2015).

Diversos estudios demostraron que la región aminoacídica 12-24 del pβA inhibía la actividad 3-hidroxiacil-CoA deshidrogenasa de HSD10 y que dicha región era necesaria para la agregación de pβA 13-22. También se observó que la unión pβA-HSD10- se producía en una región distinta al centro catalítico de HSD10 (Yan et al., 1999; Opermann et al., 1999; Salim et al., 2000).

Estudios posteriores realizados por Lustbader et al., en el 2004 demostraron que la proteína HSD10 interactuaba dentro de la mitocondria con el pβA, y esta interacción provocaba un cambio en su conformación inhibiendo la unión a su cofactor, NAD^+, causando a su vez la pérdida de su función

catalítica, promoviendo la formación de especies reactivas de oxígeno (ROS) y en consecuencia, provocando una disfunción mitocondrial y apoptosis celular. La interacción de pβA-HSD10 se ha confirmado también en otros estudios (Yan et al., 2007). De acuerdo con las observaciones anteriores Yan et al., 2000 (b) demostraron que en células de neurblastoma y en COS con sobre-expresión de HSD10, en presencia de pβA producían radicales libres y aldehídos mientras que en ausencia de pβA, HSD10 ejercía un función citoprotectora, aumentando la supervivencia celular. Además, la inducción de estrés isquémico a los ratones que sobre-expresan HSD10 muestran una mejora neurológica respecto a los "wild-type" (Wt).

Murakami et al., 2009, describieron que HSD10 detoxificaba 4-HNE, aldehído que previamente ya se había encontrado incrementado en cerebros de pacientes con la enfermedad de Alzheimer (Sayre et al., 1997; Williams et al., 2006). Por otro lado, Tackuma et al., 2005 demostraron que los ratones doble transgénicos, con sobre-expresión de HSD10 y de la proteína precurora de pβA (AβPP): tg mut AβPP/HSD10, tenían un incremento del estrés celular y una disfunción mitocondrial. Estos hallazgos no se observaban en los ratones transgénicos que sólo sobre-expresaban HSD10 o en los que sólo sobre-expresaban AβPP.

En otros estudios relacionados con Alzheimer y HSD10 se observa que la actividad 17β-hidroxisteroide deshidrogenasa de HSD10 es la única descrita capaz de inactivar estrógenos en cerebro, los cuales son necesarios para la conexión sináptica, y su deficiencia probablemente provocaría defectos en aprendizaje y en la memoria (He et al., 2002). Por tanto, HSD10 podría modular el efecto neuroprotector de los estrógenos y podría estar relacionada con la neuroinflamación presente en la enfermedad de Alzheimer (Yan et al., 1999; Yang et al., 2005 (b)). Por otro lado, dado que HSD10 oxida alopregnanolona su sobre-expresión podría crear déficit de la misma y su efecto en la proliferación neuronal y en la resistencia a la neurodegeneración sería menor (He et al. (a), 2005).

Diversos autores (Milton et al., 2001; Lustbader et al., 2004; Xie et al., 2006; Yao et al., 2007; Ren et al., 2008; Yao et al., 2011; Lim et al., 2011) están llevando a cabo investigaciones que tienen como objetivo inhibir la interacción entre HSD10 y pβA, siendo HSD10 una diana terapéutica para la enfermedad de Alzheimer. Recientes estudios demuestran que inyectando a los ratones transgénicos para la enfermedad de Alzheimer una modificación del péptido inhibidor de la interacción pβA-HSD10 descrito por Lustbader et al., 2004 (Tat-mito-ABAD-DP) se produce una mejora en el aprendizaje y en la memoria de los animales transgénicos.

3.2. Implicación de HSD10 en la enfermedad de Parkinson

En cerebros post-mortem de enfermos de Parkinson y en cerebro modelo de ratón para esta enfermedad se observa una reducción en la expresión de HSD10. Si se inyecta la neurotoxina 1-metil-4-fenil-1,2,2,6-tetrahidropiridina (MPTP) en ratones transgénicos que sobre-expresan HSD10, se observa un aumento de la fosforilación oxidativa, de la producción de ATP y disminución de la apoptosis celular, en comparación con los ratones WT. Así, HSD10 protege contra la neurodegeneración en la enfermedad de Parkinson (Przedborski et al., 2004; Tieu et al., 2004).

OBJETIVOS

Dada la neurodegeneración de los pacientes con deficiencia de HSD10 y dada la implicación de esta proteína en la enfermedad de Alzheimer nos planteamos tres objetivos:

1. Estudio de pacientes con deficiencia de HSD10

1.1 -Desarrollar una metodología para la cuantificación de los metabolitos clave para el diagnóstico de esta enfermedad y búsqueda de nuevos biomarcadores.

1.2 -Puesta a punto de la determinación enzimática de HSD10 en fibroblastos cultivados y búsqueda de mutaciones en el gen *HSD17B10* para la confirmación diagnóstica.

2. HSD10: estudios de expresión

2.1 -Estudios de inactivación del cromosoma X para poder demostrar que el gen *HSD17B10* no escapa a la inactivación del cromosoma X.

2.2 -Análisis de expresión en pacientes con deficiencia de HSD10, con el fin de mejorar la comprensión fisiopatológica de esta enfermedad.

3. Implicación de HSD10 en la enfermedad de Alzheimer

3.1 -Determinar la actividad enzimática HSD10 y estudiar la expresión de dicha proteína en necropsias de cerebro con la enfermad de Alzheimer.

3.2 -Averiguar si la inhibición de la interacción pβA-HSD10, pudiera ser utilizada como diana terapéutica para la enfermedad de Alzheimer.

RESULTADOS

INFORME SOBRE LA CONTRIBUCIÓN DE LA DOCTORANDA A LAS PUBLICACIONES COMPRENDIDAS EN ESTA TESIS DOCTORAL

ARTICULO 1

Título: 2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency: an X-linked inborn error of isoleucine metabolism that may mimic a mitochondrial disease.

Autores: Perez-Cerda C, García-Villoria J, Ofman R, Sala PR, Merinero B, Ramos J, García-Silva MT, Beseler B, Dalmau J, Wanders RJ, Ugarte M, Ribes A.

Revista: Pediatric Research 2005; 58 (3): 488-491.

Factor de impacto (JCR Science Edition): 2,673 (1er cuartil)

Aportación de la doctoranda: La doctoranda participó en el diseño del estudio y realizó el estudio bioquímico y molecular de una de las dos familias que aquí se describen. Además elaboró el primer borrador y participó en la redacción final del artículo.

ARTICULO 2

Título: Study of patients and carriers with 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency: Difficulties in the diagnosis.

Autores: García-Villoria J, Navarro-Sastre A, Fons C, Pérez-Cerdá C, Baldellou A, Fuentes-Castelló MA, González I, Hernández-Gonzalez A, Fernández C, Campistol J, Delpiccolo C, Cortés N, Messeguer A, Briones P, Ribes A.

Revista: Clinical Biochemistry 2009; 42 (1-2): 27-33.

Factor de impacto (JCR Science Edition): 2,450 (1ercuartil)

Aportación de la doctoranda: La doctoranda participó en el diseño del estudio y realizó la totalidad del trabajo experimental presentado en este artículo. Además elaboró el primer borrador y participó en la redacción final del manuscrito.

ARTICULO 3

Título: Urinary tiglylcarnitine (C5:1) as biochemical marker in 17β-hydroxysteroid dehydrogenase 10 (HSD10) deficiency.

Autores: García-Villoria J and Ribes A.

Revista: Artículo en preparación.

Aportación de la doctoranda: La doctoranda ha participado en el diseño del estudio y ha realizado la totalidad del trabajo experimental presentado en este artículo. Además ha elaborado el primer borrador y está participando en la redacción final del manuscrito.

ARTICULO 4

Título: X-inactivation of *HSD17B10* revealed by cDNA analysis in two female patients with 17β-hydroxysteroid dehydrogenase 10 deficiency.

Autores: García-Villoria J, Gort L, Madrigal I, Fons C, Fernández C, Navarro-Sastre A, Milà M, Briones P, García-Cazorla A, Campistol J, Ribes A.

Revista: European Journal of Human Genetics 2010; 18(12):1353-1355.

Factor de impacto (JCR Science Edition): 4,319 (1^{er} cuartil)

Aportación de la doctoranda: La doctoranda participó en el diseño del estudio y realizó la totalidad del trabajo experimental presentado en este artículo, excepto los estudios de metilación en el locus del receptor de andrógenos. La doctoranda elaboró el primer borrador y participó en la redacción final del manuscrito.

ARTICULO 5

Título: Microarray expression analysis in patients with 17β-hydroxysteroid dehydrogenase 10 (HSD10) deficiency.

Autores: García-Villoria J, Blázquez E, Matalonga L, Gort L, Ribes A.

Revista: Artículo en preparación.

Aportación de la doctoranda: La doctoranda ha participado en el diseño del estudio y ha realizado la totalidad del trabajo experimental presentado en este artículo, a excepción de la clonación y expresión de las proteína recominante HSD10 con las diferentes mutaciones. Además ha elaborado el primer borrador y está participando en la redacción final del manuscrito.

ARTICULO 6

Título: 17β-hydroxysteroid dehydrogenase (HSD10) as a therapeutic target in Alzheimer disease**.**

Autores: García-Villoria J, Pascual R, Blázquez E, Matalonga L, Messeguer A, Delpiccolo C, Farrera-Sinfreu J, Fernandez-Carneado J, Ponsati B , Ferrer-Montiel A, Ribes A.

Revista: Journal of Alzheimers Disease (artículo enviado a la revista, pendiente de aceptación)

Factor de impacto (JCR Science Edition): 4,174 (2^{o} cuartil).

Aportación de la doctoranda: La doctoranda participó en el diseño del estudio y realizó la totalidad del trabajo experimental presentado en este artículo, a excepción de la clonación y expresión de la proteína recombinante de las isoformas 1 y 2 de HSD10 que se llevó a cabo en la Universidad Miguel Hernández de Elche. Además elaboró el primer borrador del artículo y participó en la redacción final del manuscrito.

ANEXO

De forma paralela al desarrollo de esta tesis doctoral, la doctoranda colaboró en nuevos estudios realizados en un paciente con deficiencia de HSD10, en el Hospital Sant Joan de Déu de Barcelona. Los resultados se recogieron en un artículo que se presenta en forma de anexo.

ARTICULO 7

Título: Undetectable levels of CSF amyloid-β peptide in a patient with 17β-hydroxysteroid dehydrogenase deficiency.

Autores: Ortez C, Villar C, Fons C, Duarte ST, Pérez A, García-Villoria J, Ribes A, Ormazábal A, Casado M, Campistol J, Vilaseca MA, García-Cazorla A.

Revista: Journal of Alzheimers Disease 2011; 27(2):253-7.

Factor de impacto (JCR Science Edition): 4,174 (2^{o} cuartil).

Aportación de la doctoranda: La doctoranda realizó el estudio bioquímico y genético del paciente descrito. Además participó en a redacción final del manuscrito.

Barcelona, 20 de Mayo 2016

Conformidad del director y tutor de la tesis:

Dra. Antonia Ribes Rubió Dr. Francesc Villarroya Gambau

PRESENTACIÓN DE LOS RESULTADOS

El trabajo desarrollado en esta tesis doctoral se centra en mejorar el diagnóstico de pacientes con deficiencia HSD10, entender la fisiopatología de la enfermedad y estudiar la implicación de la proteína HSD10 en la enfermedad de Alzheimer.

De acuerdo con los objetivos planteados, los resultados se han estructurado en 3 capítulos que incluyen los seis artículos de esta tesis

1. **ESTUDIO DE PACIENTES CON DEFICIENCIA DE HSD10:**
   Artículos 1, 2 y 3
2. **HSD0: ESTUDIOS DE EXPRESIÓN**
   Artículos 4 y 5
3. **IMPLICACIÓN DE HSD10 EN LA ENFERMEDAD DE ALZHEIMER**
   Artículo 6

1.- ESTUDIO DE PACIENTES CON DEFICIENCIA DE HSD10

ARTÍCULO 1

Título: 2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency: an X-linked inborn error of isoleucine metabolism that may mimic a mitochondrial disease.

Autores: Perez-Cerda C, García-Villoria J, Ofman R, Sala PR, Merinero B, Ramos J, García-Silva MT, Beseler B, Dalmau J, Wanders RJ, Ugarte M, Ribes A.

Revista: Pediatric Research 2005; 58 (3): 488-491.

PMID: 16148061

RESUMEN

Deficiencia de 2-metil-3-hidroxibutiril-CoA (MHBD): un error congénito del metabolismo de la isoleucina que puede mimetizar una enfermedad mitocondrial.

Describimos los tres primeros pacientes españoles con deficiencia de MHBD o HSD10, pertenecientes a dos familias. Dos de los pacientes eran varones y presentaban una acidosis láctica severa, lo que sugería una encefalopatía mitocondrial. El tercer paciente, hermana de uno de los varones, presentaba una menor severidad clínica. El perfil de ácidos orgánicos de todos los pacientes mostró un aumento de ácido 2-metil-3-hidroxibutírico (2M3HBA) y de tiglilglicina (TG), aunque el perfil de la hermana inicialmente sugería más bien una deficiencia de la cadena respiratoria mitocondrial.

Se cuantificaron los metabolitos característicos de esta entidad mediante cromatografía de gases-espectrometría de masas con monitorización de los iones específicos para cada compuesto En una familia detectamos una mutación de cambio de sentido, no descrita previamente: c.740A>G (p.Asp247Ser). En la otra familia se encontró una mutación de cambio de sentido: c. 388C>T (p.Arg130Cys), descrita previamente. Los dos pacientes varones fallecieron, uno de ellos a pesar del tratamiento dietético con restricción de isoleucina. En el caso de la hermana, la enfermedad permaneció estable tras un año de tratamiento. Actualmente la paciente tiene 14 años, presenta retraso mental leve no progresivo.

El hallazgo de distintos grados de severidad clínica en una familia demuestra que existe un patrón clásico de herencia ligada al cromosoma X. Los síntomas clínicos junto con la presencia de acidosis láctica encontrada en algunos pacientes mimetizan una enfermedad mitocondrial, por

lo que es necesario realizar el diagnóstico diferencial de esta entidad con las enfermedades mitocondriales. Remarcamos que el diagnóstico de las heterocigotas podría perderse, ya que tanto el fenotipo clínico como el bioquímico pueden ser muy leves. Por ello, sería necesario mejorar el diagnóstico de las portadoras con el fin de prevenir futuros nacimientos de varones gravemente afectos.

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2-Methyl-3-Hydroxybutyryl-CoA Dehydrogenase (MHBD) Deficiency: An X-linked Inborn Error of Isoleucine Metabolism that May Mimic a Mitochondrial Disease

JUDIT GARCÍA-VILLORIA, ROB OFMAN, PEDRO RUIZ SALA, BEGOÑA MERINERO, JULIO RAMOS, MARIA TERESA GARCÍA-SILVA, BEATRIZ BESELER, JAIME DALMAU, RONALD J.A. WANDERS, AND MAGDALENA UGARTE

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ABSTRACT

We describe three patients, from two Spanish families, with 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency, a recently described X-linked neurodegenerative inborn error of isoleucine metabolism. Two of them are males with severe lactic acidosis suggestive of a mitochondrial encephalopathy, and the third is a female who was less severely affected, suggesting skewed X-inactivation. Molecular studies revealed a new missense mutation, 740A→G, in one family and a previously described mutation, 388C→T, in the other, causing the amino acid substitutions N247S and R130C, respectively. Both male patients died, one of them despite treatment with an isoleucine-restricted diet, but the disease has remained stable in the female patient after 1 y of treatment. (***Pediatr Res* 58: 488–491, 2005**)

Abbreviations
MHBD, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase
2M3HBA, 2-methyl-3-hydroxybutyric acid
TG, tiglylglycine

MHBD (EC 1.1.1.178) deficiency is a recently described X-linked inborn error in the metabolism of isoleucine (MIM 300256). MHBD is a mitochondrial enzyme that catalyzes the conversion of 2-methyl-3-hydroxybutyryl-CoA to 2-methyl-acetoacetyl-CoA. This disorder is characterized by normal early development followed by progressive loss of mental and motor skills. To our knowledge, only seven patients (1–6) including one female patient (3) have been reported. As expected in an X-linked disease, males show lower enzyme activity and a more severe clinical course. However, a 23-y-old man with a milder phenotype has also been described (4).

In addition to its role in isoleucine metabolism, MHBD seems to play a role in the pathogenesis of Alzheimer's disease (7) and it seems that the interaction of this protein with amyloid-β peptide-binding protein may induce mitochondrial dysfunction (8). The gene encoding MHBD, is named HADH2, and has been mapped to chromosome Xp11.2. It spans about 3.11 Kb and consists of six exons. Only two different mutations have been reported to date (9).

An isoleucine-restricted diet has been administered to some patients (1,3,4,6), which seems to stabilize the clinical progression of the disease.

Here we report three MHBD deficient patients, which adds new clinical, biochemical, and molecular data to expand our knowledge of this severe disease.

PATIENTS AND METHODS

Patient 1. Patient 1 is a female, born to nonconsanguineous healthy parents. Family history revealed a brother (patient 2) who died during the neonatal

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period due to lactic acidosis. The patient presented with psychomotor delay from the first months of life, walked at around 22 mo and, at 2 y and 5 mo, her gait was ataxic. Later on, speech delay and neurosensorial deafness were evident. During stress, she showed episodes of myoclonus. Brain MRI, performed at 2 y and 5 mo of age, was normal.

Biochemical studies revealed slight hyperlactatemia: 1.7–3.0 mM (control range, 1–2 mM) and microcytic anemia. Amino acids, carnitine, glucose, ammonia, creatin phospho kinase (CPK), and other routine biochemical parameters were normal. Urinary organic acid profile showed an increase of 2M3HBA, 99 mmol/mol creatinine (control range, 5–12), and TG, 21 mmol/mol creatinine (controls, <5). Lactate, fumarate, and 3-methylglutaconate were also increased. This profile initially suggested a complex I deficiency (10). Measurement of the respiratory chain activities in a muscle biopsy were normal (Unpublished results, performed by Dr. Ruitenbeek, Clinical Genetics Center, Nijmegen). At 9 y of age, an oral isoleucine loading test was performed (1); plasma isoleucine rose to 980 μM within the first 2 h (values for two independent controls: 757, 812) and was still high (911 μM) after 4 h (values for two independent controls: 286, 375). Urinary excretion of 2M3HBA and TG rose to 1285 and 74 mmol/mol creatinine, respectively, 6 h after isoleucine load (values for two independent controls: 53, 33 and 24, 25 respectively) (Fig. 1). These results pointed to MHBD deficiency and a low-protein, isoleucine (50 mg/kg/day) restricted diet was prescribed. Under this treatment, the concentration of 2M3HBA decreased to 27 mmol/mol creatinine, and TG, lactate, fumarate, and 3-methylglutaconate normalized. Enzymatic activity in fibroblasts showed an intermediate value, 3.81 nmol/min/mg protein (controls, 7.27 ± 1.16), whereas acetoacetyl-CoA thiolase activity was normal. At present, the patient is 10 y old and moderate psychomotor retardation persists.

Patient 2. Patient 2 was a male and was the first-born brother of patient 1. From the first hours of life, he presented with cyanosis and hypotonia. On the second day of life, cardiomegaly, probably due to congenital cardiopathy, was evident. Biochemical analyses revealed hypoglycemia (2.2 mM), metabolic acidosis, hyperlactatemia (8 mM), and hyperlactaturia. Serum biotinidase, pyruvate carboxylase, and respiratory chain activities in cultured skin fibroblasts were normal. This patient died at 2 mo of age, presumably due to lactic acidosis.

After the diagnosis of his sister, MHBD activity was measured in fibroblasts, which revealed a marked deficiency (1.5 nmol/min/mg protein), whereas acetoacetyl-CoA thiolase activity was normal.

Patient 3. Patient 3 was a male, the first-born child of nonconsanguineous parents. The mother has borderline learning difficulties attributable to a head injury. He presented with dehydration, hypoglycemia, and hypotonia at 2 h of life. At 3 mo, horizontal nystagmus and absence of reaction to visual stimuli were evident. At 7 mo of age, he had marked generalized hypotonia, episodes of hyperextension of the upper limbs, and myoclonus, which were controlled with carbamazepine treatment. At this age, EEG revealed a slow background activity with paroxystic pattern. Magnetic resonance imaging (MRI), performed at 8 mo of age, showed a slight frontotemporal atrophy, and MR spectroscopy demonstrated a high concentration of lactate in basal ganglia, cortex, and white matter. These results suggested a mitochondrial encephalopathy. Amino acids in blood and urine were normal, but blood lactate was consistently high (7.6–11 mM). Urinary organic acid profile showed high excretion of 2M3HBA (89–226 mmol/mol creatinine) and of TG (145–440 mmol/mol creatinine). Normal plasma-free carnitine but high esterified carnitine to free carnitine ratio (0.54, controls < 0.26) was detected. Plasma acylcarnitine profile showed high C5:1 (1.37 μM, controls < 0.04) and C5-OH species (0.26 μM, controls < 0.11). MHBD activity in cultured fibroblasts was markedly deficient (0.8 nmol/min/mg protein), whereas acetoacetyl-CoA thiolase activity was normal.

The patient was treated with a vegetarian and isoleucine-restricted diet (45 mg/kg/ d), carnitine (50 mg/kg/d) and carbamazepine. Urinary organic acid excretion decreased to 19 and 10 mmol/mol creatinine of 2M3HBA and TG, respectively, and plasma acylcarnitine profile normalized, but clinical deterioration did not stop, showing optic atrophy, progressive cortical subcortical atrophy and regression in his developmental milestones. He died at 18 mo of age due to a bronchospasm during the course of a catarrh.

Biochemical studies. Organic acids were analyzed by gas chromatography-mass spectrometry as TMS derivates. Plasma acylcarnitines were isolated by strong cation-exchange solid phase extraction (11), evaporated to dryness, and esterified with 50 μL of butanolic HCl (3 N). The butylated dry residue was dissolved in 10 μL of matrix (10 mg/mL methanolic 2,5-dihydroxybenzoic acid) and analyzed in a Bruker Reflex III MALDI-TOF mass spectrometer. MHBD in fibroblasts was measured in the reverse direction, as previously described (9).

Molecular studies. Mutation analysis at the cDNA level in the patients was performed by nucleotide sequencing, as previously described (9). Molecular studies were also performed at the genomic level using restriction fragment length polymorphism and *Dde*I and BglII restriction enzymes to detect 740A→G and 388C→T mutations, respectively. Expression of the mutant cDNA was performed as described in Ofman *et al.* (9).

RESULTS AND DISCUSSION

MHBD deficiency is a recently described X-linked inborn error in the metabolism of isoleucine (1). The diagnosis is carried out through the urinary organic acid profile showing elevated excretion of 2M3HBA and TG, without concomitant increase of 2-methylacetoacetate, and is confirmed by measuring MHBD activity in cultured skin fibroblasts (1). So far, only six male patients and one female patient have been described (1–6); all are summarized in Table 1 and compared with the patients reported here.

Our patients 2 and 3 and patient 4, previously reported by Zschocke (1), presented symptoms in the first hours of life. All of them died at an early age, the death of patient 4 was reported by Poll-The *et al.* (2; personal communication from J. Zschocke). The first symptoms in the remaining patients presented over a range of 9 mo to 6 y of age, and all of them are alive with variable degrees of neurologic sequelae.

The most common clinical symptom was speech delay (Table 1); it was observed even in patients with a less severe form of the disease, such as patient 1. Visual and hearing alterations, hypotonia, and epilepsy are other common symptoms. Brain MRI revealed cerebral atrophy in five out of seven reported patients. Concerning our patients, the MRI was normal in patient 1 in accordance with a less severe form of the disease, whereas patient 3 showed frontotemporal atrophy.

Among the few described patients there is a clear phenotype difference between males and females. The natural history of the disorder in males follows a neurodegenerative course (Table 1), although onset of regression appears to be variable (4). In contrast, the two female patients described to date, patient 1 (this report) and patient 6 (3), do not show regression but do show mild to moderate developmental delay.

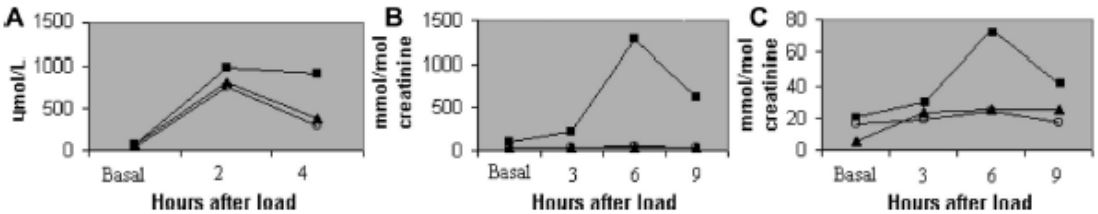

Figure 1. Plasma isoleucine (*A*) and urine organic acids, 2M3HBA (*B*) and TG (*C*) after an oral isoleucine load in patient 1 (*solid square*) and two independent controls (*open circle*, control 1; solid triangle, control 2).

Table 1. *Relevant clinical biochemical and molecular data in patients with MHBD deficiency described to date, in comparison with patients described here*

Patient no. (ref.)	Sex/age of onset	Clinical symptoms	Brain MRI	Present status	Blood or CSF lactate	MHBD activity (nmol/min/mg protein)	Mutation	Amino acid change
Patient 1 (this report)	F/3–4 mo	Psychomotor retardation, speech delay, hearing loss, hyperkinetic behavior	Normal	Psychomotor retardation (10 y)	Increased	3.8*	740A/G	N247S
Patient 2 (this report)	M/1 d	Metabolic acidosis, hypoglycemia, hypotonia, cyanosis, cardiomegalia	ND	Dead (2 mo)	Increased	1.5*	740A/G	N247S
Patient 3 (this report)	M/1 d	Dehydration, hypoglycemia, hypotonia, nystagmus, no reaction to visual stimuli, restlessness, epilepsy	Frontotemporal atrophy	Dead (18 mo)	Increased	0.8*	388C/T	R130C
Patient 4 (1)	M/2 d	Metabolic acidosis, hypoglycemia, hyperammonemia, speech delay, progressive loss of motor skills, epilepsy, hypotonia, near blindness	Frontotemporal atrophy	Dead (more than 2 y)	Increased	0.02†0.6*	388C/T	R130C
Patient 5 (2)	M/19 mo	Developmental delay, spastic diplegia, mild dysmorphic features, speech delay	Periventricular white matter abnormalities	NR	NR	0.7*	364C/G	L122V
Patient 6 (3)	F/15 mo	Psychomotor and speech delay	Mild frontoparietal cortical atrophy	Psychomotor retardation (7 y)	Normal	1.0*	388C/T	R130C
Patient 7 (3)	M/14 mo	Developmental regression, speech delay, dystonia, hypotonia, blindness, retinal degeneration and optic atrophy, hypertrophic myocardiopathy of the left ventricle	Severe cerebral atrophy and occipital infarctions	Severely retarded (7 y)	Increased	0.6*	388C/T	R130C
Patient 8 (4)	M/ 6y	Developmental regression, dysarthria, poor co-ordination, dystonia	Arnold-Chiari type I malformation (probably an incidental finding)	Moderately intellectually impaired (23 y)	Increased	0.89 *	NR	NR
Patient 9 (5)	M/9–10 mo	Loss of acquired motor skills, hypotonia, lack of head control, impaired hearing, convulsions, retinitis pigmentosa, nystagmus, microcephaly	Mild frontotemporal atrophy	Severely retarded (5 y)	Increased	0.0†1.8*	388C/T	R130C
Patient 10 (6)	M/21 mo	Mild hypotonia, developmental regression, seizures, cortical blindness, grade II/VI systolic murmur, nonintelligible words	Normal	Severe neurological impairment (8 y)	Normal	0.0*	NR	NR

ND, not done; NR; not reported.
* MHBD activity measured in fibroblasts in the reverse direction with 2-methyl-acetoacetyl-CoA as substrate (9), control values: 7.1 ± 0.8.
† MHBD activity measured in fibroblasts adding tigly-CoA as substrate (1), control values 1.48 ± 0.1.

Except for patient 2, from whom no urine sample was available, our patients presented with an increased excretion of 2M3HBA and TG. This excretion was less pronounced in patient 1, for that reason an oral isoleucine loading test was performed (Fig. 1), showing evidence of a block at the level of MHBD, which was confirmed by enzymatic and molecular studies. Enzymatic studies revealed that patient 1 presented the highest MHBD residual activity described to date, which correlates well with the mild clinical and biochemical phenotype (Table 1), but there are obvious differences in residual enzyme activity depending on the method (see patients 4 and 9 in Table 1), which need to be clarified in the future.

Molecular studies in family 1 revealed a new missense mutation in exon 6, 740A→G, which resulted in a replacement of asparagine at position 247 with serine, N247S. It was found in heterozygous form in patient 1 and in her asymptomatic mother, and in hemizygous fashion in her brother (patient 2). Expression studies of the mutant cDNA revealed absence of enzyme activity, confirming the pathogenic nature of the mutation (data not shown). Patient 3 presented a previously described mutation, 388C→T (9), which was also inherited from his mother, who has borderline learning difficulties. This mutation, the commonest among the described patients (Table 1), produced a reduced amount of protein (9).

Our results concerning treatment are somewhat puzzling. The isoleucine restriction did not show any benefit in patient 3 despite normalization of the organic acid profile, but as in other reported patients (1,3,4), the disease remained stable in patient 1 during 1 y of treatment. Therefore, unlike the treatment of other inborn errors of the same metabolic pathway, the use of an isoleucine-restricted diet would not be enough to treat this disease. Lactic acidosis, which is present in almost all patients, might be involved in the etiopathogenesis of the disease and it could be of value to attempt other therapies such us electron acceptors (vitamins) or cofactors (coenzyme Q10) to prevent lactate production (12). However, it remains to be seen whether treatment may improve outcome in presymptomatic patients, as this disease could probably be identified in the neonatal screening programmes by tandem mass spectrometry.

MHBD protein has been described as a multifunctional enzyme, and it has been hypothesized that, in addition to its function in the isoleucine metabolism, MHBD might play an important role in the pathogenesis of Alzheimer's disease (9). This protein appears to have an essential physiologic role in mitochondria, and mutational inactivation of the homologous gene in *Drosophila* resulted in a lethal phenotype (13). It has recently been demonstrated that human ABAD (also known as ERAB and MHBD) and amyloid-β peptide-binding protein directly interact in mitochondria in Alzheimer's disease, and that this interaction inhibits ABAD activity, thus promoting leakage of reactive oxygen species (ROS), mitochondrial dysfunction and cell death (8). The increased concentration of lactate, detected in almost all patients, could be in line with the latter observations or with the secondary complex I deficiency, as has been detected in some patients (3,4). It is noteworthy that, some years ago, a similar organic acid profile was proposed as a metabolic marker for primary complex I deficiency (10); therefore, the differential diagnosis should not only be made with acetoacetyl-CoA thiolase deficiency, but also with mitochondrial respiratory chain deficiencies.

In conclusion, the description of several degrees of clinical severity within a family supports the classical pattern of X-linked inheritance of this disease. Clinical symptoms as well as the impressive lactic acidosis found in some patients may mimic a mitochondrial disease. The slight clinical and biochemical phenotype in our patient 1 allow us to speculate that the diagnosis of some females might easily be missed. An accurate diagnosis of females is important to prevent the birth of affected offspring. Another consideration to take into account is the possibility of finding adults with neurodegenerative disease brought on by MHBD deficiency.

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ARTÍCULO 2

Título: Study of patients and carriers with 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency: Difficulties in the diagnosis.

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RESUMEN

Estudio de pacientes y portadoras con deficiencia de 2-metil-3-hidroxibutiril-CoA deshidrogenasa (MHBD): dificultades en el diagnóstico.

En este artículo se describen 7 pacientes pertenecientes a 6 familias españolas con deficiencia de HSD10. Analizamos el perfil de ácidos orgánicos en orina, determinamos la actividad de HSD10 en fibroblastos cultivados, realizamos estudios de expresión de la proteína mediante "western blot" y estudios genéticos de los 7 pacientes.

Todos los pacientes presentaron incremento de ácido 2-metil-3-hidroxibutírico (2M3HBA) y de tiglilglicina (TG) en orina, la única heterocigota estudiada mostraba una excreción más leve de los mismos, como a menudo sucede en las enfermedades ligadas al cromosoma X. Cabe destacar que en uno de los varones hemicigotos la excreción de ácidos fue prácticamente normal debido a que ya estaba tomando dieta hipoproteica en el momento del análisis. El estudio de este último caso nos puso en alerta sobre la posibilidad de que los pacientes con dieta baja o pobre en proteínas pudieran pasar desapercibidos al diagnóstico.

Para evidenciar mejor el defecto en las heterocigotas se realizó una sobrecarga de isoleucina con la posterior cuantificación de 2M3HBA y TG en orina. Este test falló en demostrar la condición de heterocigota de una de ellas. Sin embargo, si a este estudio añadíamos la cuantificación del ácido etilhidracrílico (EHA) el test mostraba la alteración en todas las heterocigotas.

A partir de estos resultados se sugiere la cuantificación de EHA, como una valiosa ayuda para el diagnóstico de heterocigotas. A diferencia de lo que había sido descrito por otros autores las portadoras o heterocigotas muestran la variabilidad bioquímica clásica de una enfermedad ligada al cromosoma X.

La confirmación diagnóstica de la deficiencia de HSD10 se puede llevar a cabo mediante la determinación enzimática. Sin embargo, hasta el momento ningún autor utilizaba el sustrato

directo para su valoración y los resultados diferían según el método utilizado (Tabla 1 del artículo 1 de esta tesis). Por este motivo, uno de nuestros objetivos fue la puesta a punto de la determinación de la actividad enzimática de HSD10 en fibroblastos cultivados con el sustrato directo: 2-metil-3-hidroxibutiril-CoA, con el fin de poder ofrecer un diagnóstico más exhaustivo. Este sustrato no es comercial, pero fue sintetizado por el grupo del Dr. Messeger del CSIC de Barcelona. Se obtuvieron los valores de referencia analizando 8 líneas de fibroblastos de individuos control y se realizaron estudios de linealidad y de precisión. Se valoró la actividad enzimática de 6 pacientes (5 varones y 1 hembra), también se valoró la actividad enzimática de la madre heterocigota de uno de los pacientes. Todos mostraron una clara deficiencia de HSD10, exceptuando la actividad de la madre portadora que fue normal, lo que se correlacionaba con una clínica muy leve.

Con el estudio molecular en las 6 familias se identificaron 4 mutaciones de cambio de sentido, dos de ellas no descritas previamente (p.Pro210Ser y p.Arg226Gln). Dicho estudio permitió detectar 7 portadoras más. Únicamente una de las mutaciones (p.Arg130Cys) provocaba una disminución en los niveles de HSD10.

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CLINICAL BIOCHEMISTRY

Study of patients and carriers with 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency: Difficulties in the diagnosis

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Abstract

Objectives: To search for biochemical and molecular markers for the diagnosis of patients and carriers with 2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency.

Design and methods: Organic acids in urine, MHBD activity in fibroblasts, immunoblotting and molecular studies were performed in seven patients. Seven carriers were also studied.

Results: Under low protein diet or poor feeding all the patients showed only a slightly altered organic acid profile. Measurement of 2-methyl-3-hydroxybutyric acid and tiglylglycine after an isoleucine loading test, failed to demonstrate the carrier status of one patient. However, measurement of 2-ethylhydracrylic acid (EHA) was positive in all the carriers tested. MHBD activity was clearly deficient in males and in one female patient. We identified four missense mutations, two of them were novel.

Conclusions: Quantification of EHA may be of help for the diagnosis of the heterozygous condition. The carrier females showed the classical biochemical variability of X-linked diseases due to random X-chromosome inactivation.

Keywords: MHBD deficiency; *HADH2*; ERAB; 17HSD10; ABAD

Introduction

2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) is a recently described mitochondrial enzyme involved in the degradation pathway of isoleucine and branched-chain fatty acids [1]. In addition, MHBD plays a role in the pathogenesis of Alzheimer's disease [2] and its interaction with amyloid-β peptide may induce mitochondrial dysfunction [3]. Moreover, MHBD seems to be involved in the metabolism of sex steroid

Abbreviations: MHBD, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase; 2M3HBA, 2-methyl-3-hydroxybutyric acid; TG, tiglylglycine; EHA, 2-ethylhydracrylic acid.

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hormones and neuroactive steroids [4], and in the detoxification of cytotoxic aldehydes [5].

Only 11 patients with MHBD deficiency have been described to date [1,6–12]. MHBD deficiency is an X-linked defect characterized by progressive neurodegeneration in males [1,6–12], and only two affected females have been described, both with psychomotor and speech delay [7,11]. Clinically, this defect differs from other organic acidurias of the same metabolic pathway, and only two patients with MHBD presenting with metabolic acidosis have been reported [1,11].

The diagnosis of this disease is carried out through the urinary organic acid profile showing elevated excretion of 2-methyl-3-hydroxybutyric acid (2M3HBA) and tiglylglycine (TG), and is confirmed by measuring MHBD activity in cultured skin fibroblasts or by mutational studies. It is remarkable that the diagnosis of some females could easily be missed due to their slightly altered organic acid profile. In addition, both the organic acid profile and the increase of plasma lactate found in some patients could mimic a mitochondrial disease [11]. The gene encoding for MHBD (*HADH2*) has been mapped to chromosome Xp11.2, spans about 3.11 Kb and consists of 6 exons. To date, three different missense mutations have been reported: p.R130C, p.L122V [13] and p.N247S [11]. In addition, one silent mutation (p.R192R) in *HADH2* has been associated to a new syndromic form of X-linked mental disease characterized by mild mental retardation, choreoathetosis, and abnormal behavior [14].

HADH2 has been reported as one of the few genes that escapes X-inactivation [15]. However, we found some discrepancies against such lack of inactivation during the study of the mothers and other female relatives of seven MHBD deficient patients.

Here we report the biochemical and molecular findings of six MHBD deficient families, which expand our knowledge of this severe disease.

Material and methods

Sample collection and patients

Urine or blood samples from seven patients with MHBD deficiency, from their respective mothers and from three maternal female relatives of two patients were collected. MHBD activity was measured in eight control cell lines and in fibroblasts of six index cases and in one of the carrier mothers.

An isoleucine loading test was performed to evidence the carrier status of the mother, the maternal aunt and the maternal grandmother of one of the patients according to the methodology of Zschocke et al. [1], and the same test was also performed in patient 1.2. We used as reference values those obtained from two females with no mutations in *HADH2*. All the samples were obtained according to the declaration of Helsinki.

The relevant clinical data of all the patients are summarized in Table 1; three of the patients have previously been reported [11,12].

Methods

Biochemical studies

Organic acids in urine were analyzed as TMS-derivates by gas chromatography–mass spectrometry as previously described [16].

Protein concentration was determined by the method of Lowry, using bovine serum albumin as standard.

The activity of MHBD in fibroblasts was done spectrophotometrically by measuring the absorbance at 340 nm during 15 min at 25 °C, as previously described [17], except that we used 2-methyl-3-hydroxybutyryl-CoA as substrate, which is not commercially available and was synthesized by one of us. Briefly, a suspension of 2-methyl-3-hydroxybutyrate potassium

Table 1
Relevant clinical data of patients with MHBD deficiency diagnosed by our group

Patients/(reference)	Sex/age of onset	Clinical symptoms	Brain MRI	Present status (age)	Family history
Patient 1.1 [11]	M/1 d	Metabolic acidosis, hypoglycemia, hypotonia, cyanosis and cardiomegalia	ND	Dead (2 m)	Brother of patient 1.2 Normal mother
Patient 1.2 [11]	F/3–4 m	Psychomotor retardation, speech delay, hearing loss, myoclonus and hyperkinetic behavior	Normal	Severe neurological impairment (13 y)	Sister of patient 1.1
Patient 2 [12]	M/10 m	Psychomotor and developmental delay, hypotonia, microcephaly, poor visual contact and multiorganic failure	Frontotemporal atrophy	Dead (17 m)	Mother with slight mental retardation
Patient 3 (this report)	M/3 m	Developmental regression, hypotonia, peculiar face, and macrocephaly	ND	Dead (4 m)	Mother and maternal grandmother with mild mental retardation
Patient 4 (this report)	M/6 m	Developmental regression, hypotonia, seizures, cortical blindness, myoclonus and microcephaly	ND	Severe neurological impairment (20 m)	Mother and maternal aunt had borderline learning difficulties
Patient 5 (this report)	M/1 d	Developmental regression, metabolic acidosis, hypoglycemia, hyperammoniemia, anemia, trombopenia, coagulopathy, hepatic dysfunction, myoclonus, seizures and hypertrophic miocardiopathy	Isquemic lesions in nucleus	Dead (7 m)	Normal mother
Patient 6 (this report)	M/14 m	Developmental regression, ataxia, myoclonus, nistagmus, optic atrophy and retinopathy	Normal	Severe neurological impairment (24 m)	Mother borderline with learning disabilities

ND= not done.

Table 2
Biochemical and molecular data of patients with MHBD deficiency

Patients	Blood lactate (mmol/L)	Urinary organic acids (mmol/mol creatinine)			MHBD activity (nmols/mgprot/min)	Mutation	Deduced effect on protein
		2M3HBA	TG	EHA			
Patient 1.1 (M)	8	ND	ND	ND	0.22	c.740A>G	p.N247S
Patient 1.2 (F)	3	99	21	↑	0.27	c.740A>G	p.N247S
Patient 2 (M)	7.6	49	154	↑	0.05	c.388C>T	p.R130C
Patient 3 (M)	8.3	262	59	50	ND	c.628C>T	p.P210S
Patient 4 (M)	↑	116	181	12	0.05	c.388C>T	p.R130C
Patient 5 (M)	19	323	168	↑	0.27	c.677G>A	p.Q226R
Patient 6 (M)	↑	54	30	18	0.5	c.628C>T	p.P210S
Mother Patient 6	ND	ND	ND	ND	1.4	c.628C>T	p.P210S
Control values	<2 (n=50)	<12 (n=50)	<5 (n=50)	<7 (n=50)	Mean=1.4 (SD=0.43; n=8)		

(F)=Female; (M)=male; N.D.=not done.
↑: increased as assessed by the organic acid profile, no quantitative values were available.

salt (15 mg, 96 mol, supplied by Prof. E. Brunet, Autonomous University of Madrid, Spain) in tetrahydrofuran (2 mL), was treated with ethyl chloroformate (55 µL, 63 mg, 576 µmol) under an inert atmosphere to form the corresponding anhydride. The mixture was stirred vigorously at room temperature for 1 h. The solvent and excess of reagent were evaporated and the dry residue was dissolved in 1 mL of *t*-butyl alcohol. CoA (SIGMA) was added to this solution as the sodium salt (50 mg, 65 µmol) dissolved in 1 mL of 0.4 M $NaHCO_3$, pH 8.3, and the mixture was allowed to react for 10 min at room

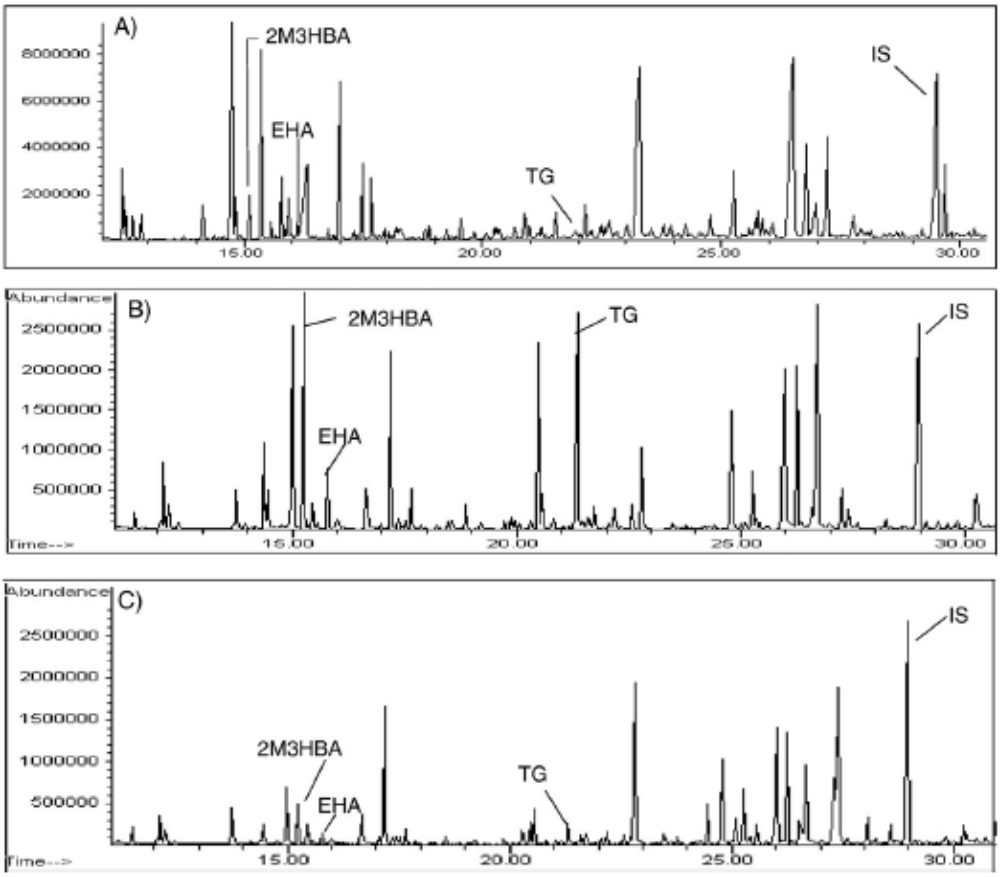

Fig. 1. Organic acids: A) profile of control urine, B) severe MHBD profile, and C) MHBD profile under low protein diet.

temperature. After acidification with 200 µl of 1 M HCl, solvents were removed by evaporation under vacuum and the new residue was dissolved in 0.5 mL of 25 mmol/L NH_4AcO containing 0.5% acetic acid, and purified by semipreparative HPLC (using a 150×10 mm, 5 µm, reverse-phase X-Terra C_{18} column, eluting with 25 mmol/L NH_4AcO-CH_3CN mixtures containing 0.1% acetic acid at 5 mL/min as mobile phase and monitoring at 260 nm), to give 31 mg of the expected product as a mixture of distereomers (61% yield). The purity of the product was estimated as >95% by HPLC. HRMS: Calcd. for $C_{26}H_{45}N_7O_{18}P_3S$ (M+H), 868.1755; found, 868.1744.

Molecular studies

Genomic DNA was extracted from whole blood or fibroblasts using standard protocols. The 6 exons and intron boundaries of *HADH2* gene were PCR-amplified using self-designed oligonucleotides and standard protocols. PCR products were directly sequenced. Primer sequences and PCR conditions are available upon request.

Gene nucleotide numbering was according to sequence RefSeq NM_004493 with +1 as A of the ATG start codon. The ATG codon represents +1 for the amino acid numbering according to MHBD protein sequence NP_004484.

Western-blotting analysis

For western-blotting, 100 µg of total protein from fibroblasts homogenates were loaded and analyzed by SDS-PAGE (12% polyacrylamide). We used anti-ERAB of rabbit (SIGMA) as primary antibody and anti-rabbit-peroxidase as secondary antibody.

Results and discussion

Metabolite studies

MHBD deficiency is a recently described X-linked inborn error in the metabolism of isoleucine, and only nine males [1,6–12], and two female patients [7,11], have been reported to date.

Patients with MHBD deficiency show a peculiar organic acid profile in urine, characterized by an increase of 2M3HBA and TG [1,6–12]. In addition, lactate has been reported to be increased in some patients [1,7–9,11,12], which has been attributed to a secondary deficiency of complex I or complex IV of the mitochondrial respiratory chain [7,8]. In fact, complex IV has been demonstrated to be inhibited by high amounts of 2M3HBA, which is the metabolite that accumulates in this

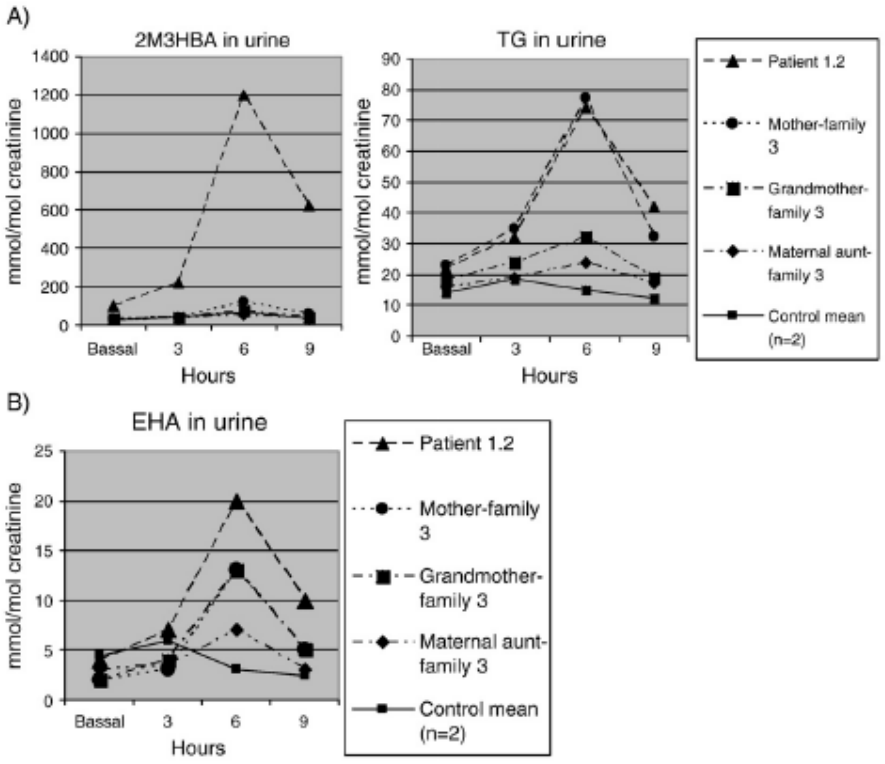

Fig. 2. Oral isoleucine loading test: A) quantification of 2M3HBA and TG, and B) quantification of EHA.

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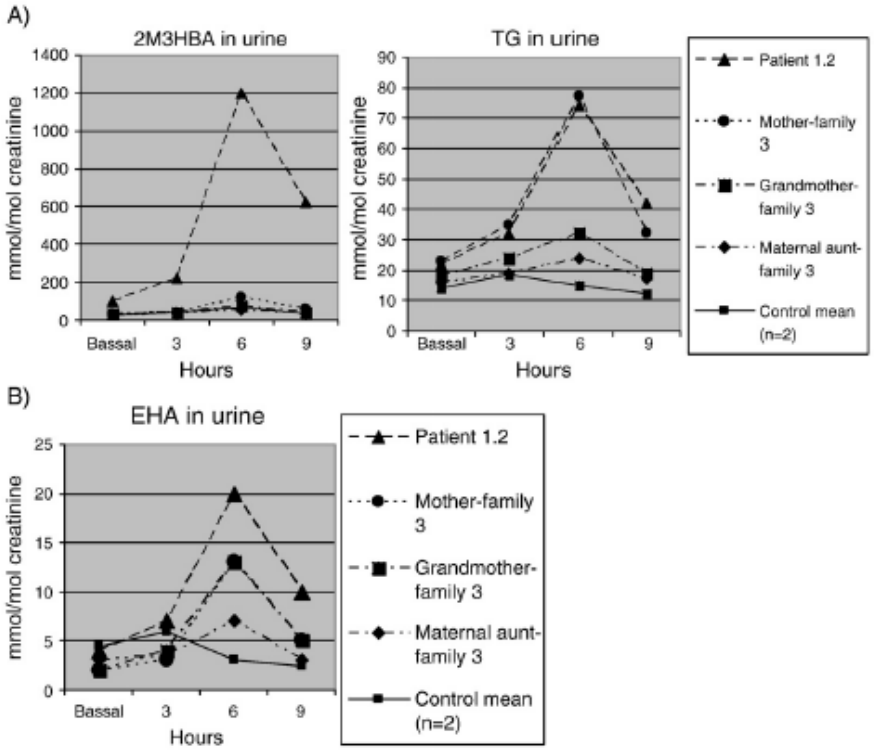

Fig. 2. Oral isoleucine loading test: A) quantification of 2M3HBA and TG, and B) quantification of EHA.

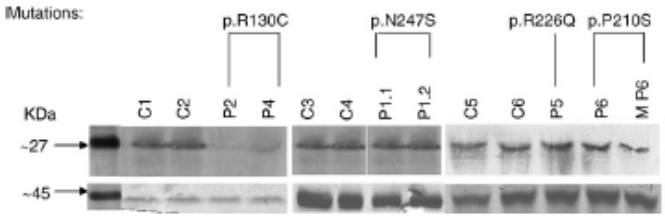

Fig. 4. Immunoblot analyses. Immunoblot analysis of MHBD protein (~27 kDa). Amounts of 100 μg protein of cultured skin fibroblasts from control subjects (C1, C2,C3,C4,C5,C6) and patients with MHBD deficiency (P1.1, P1.2, P2, P4, P5, P6) and mother of patient 6 (M P6) were loaded. PDH-E1α was analyzed as reference protein (~40 kDa).

Molecular and immunoblotting studies

Molecular studies allowed us to identify four missense mutations (Table 2), two of them (p.P210S and p.R226Q) were novel. It is noteworthy that two unrelated families presented mutation p.R130C, previously reported as the commonest mutation for this disease [13], (Fig. 3). All our patients, but patient five, inherited the corresponding mutation from their mothers (Fig. 3). The substitution p.R226Q of patient five was not found in genomic DNA of his mother and therefore, we assume that it was originated *de novo* as often happens in X-linked diseases.

To determine the effect of these mutations on the protein expression or stability, immunoblotting studies in cultured skin fibroblasts were performed. Results showed that the amount of protein was normal for all patients, except for those carrying the substitution p.R130C (Fig. 4). This change is located within a conserved domain and expression studies in *E. coli* showed that it leads to a fully inactive enzyme [13]. In fact our patients carrying the substitution p.R130C present severe MHBD deficiency, about 4% of controls (Table 2), and decreased amount of protein (Fig. 4), which is in agreement with the data previously reported [13]. In addition, all the reported male patients with this mutation [1,7,9,11,12], including ours, present a severe phenotype.

Expression results in *E. coli* for the substitution p.N247S, previously reported by our group, showed absence of enzyme activity [11]. Moreover, this substitution has also been predicted as an intolerant change, despite the two amino acids being uncharged polars (http://coot.embl.de/PolyPhen/). Expression studies of the two novel mutations p.R226Q and p.P210S have not been performed, but several lines of evidence suggest that these mutations may be pathogenic: 1) no other mutations were found in the patients, 2) the nucleotide changes were not present in more than 100 control chromosomes analyzed, and 3) both amino acid residues are highly conserved among different species and among other acyl-CoA dehydrogenases (http://coot.embl.de/PolyPhen/).

It is important to take into account that patients with mutations p.N247S, p.R226Q and p.P210S have a milder enzyme deficiency than patients with mutation p.R130C (Table 2). However, the mild biochemical phenotype of these patients does not prevent the development of a severe phenotype, as all the male patients died at an early age or were severely affected (Table 1). Ofman et al. [13] reported a patient with high residual MHBD activity and a mild mutation (p.L122V) and hypothesized that these data could account for his milder clinical presentation, but in our experience there is no clear genotype–phenotype correlation.

In addition, the novel silent mutation p.R192R has been associated with a new X-linked syndrome characterized by mild mental retardation, choreoathetosis, and abnormal behavior. Studies in two of the affected males revealed a reduction (60–70%) of the amount of MHBD protein and an almost normal activity (80%–90% of controls), probably due to a misregulated splicing process [14]. It is also possible that the clinical features are unrelated to the MHBD function [21], and other functions of the same protein have to be explored to understand the physiopathology of this disease.

As expected in an X-linked disease, males are more severely affected than females. The six male patients included in our study presented with symptoms before 10 months of age, all of them with neurodegeneration, while the female carriers and the female previously described [7] presented a variety of symptoms: from borderline learning difficulties to psychomotor and speech delay, which is in agreement with an X-linked inheritance with incomplete dominance. In addition, our molecular and enzymatic studies suggest that *HADH2* does not escape X-inactivation, contrary to what had previously been reported [15].

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Erratum

Erratum to "Study of patients and carriers with 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiency: Difficulties in the diagnosis" [Clin. Biochem. 42 (2009) 27–33]

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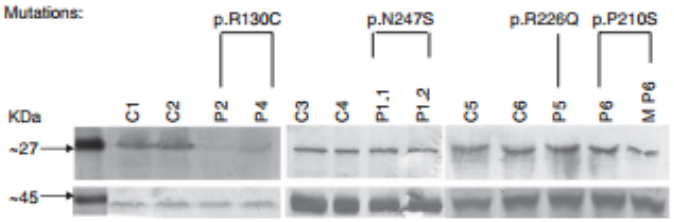
In Fig. 4, by error the Western blot of mutation p.N247S did not belong to the mentioned patients. A new Fig. 4 to reflect this is included.



The patients do not exhibit differences with respect to controls. Therefore, the text of the paper is not affected by this error. The authors wish to apologize to readers for this oversight.

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ARTÍCULO 3

Título: Urinary tiglylcarnitine (C5:1) as biochemical marker of 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (HSD10) deficiency.

Autores: García-Villoria J and Ribes A.

Revista: artículo en preparación.

RESUMEN

Nuevos marcadores bioquímicos en la deficiencia de 2-metil-butiril-CoA deshidrogenasa (HSD10).

Dado que el diagnóstico de pacientes con HSD10 puede ser difícil, en especial en las heterocigotas, decidimos realizar un estudio retrospectivo en muestras de pacientes ya diagnosticados, con el fin de buscar nuevos marcadores bioquímicos. Previamente se había descrito que los pacientes con deficiencia HSD10 presentaban un aumento de 2-metil-3-hidroxi-butirilcarnitina (C5-OH) y de tiglilcarnitina (C5:1) en plasma. Sin embargo, estas alteraciones no se observaron en ninguno de nuestros pacientes.

Decidimos valorar el perfil de acilcarnitinas en orina mediante espectrometría de masas en tándem en 4 pacientes, en muestras con dieta normal, con dieta restringida en isoleucina y tras una dieta rica en proteínas. Adicionalmente, analizamos las muestras de orina de 2 portadoras tras una sobrecarga de isoleucina.

Los 4 pacientes (3 varones y una heterocigota) mostraron un incremento significativo de C5:1, mientras que C5OH sólo se halló incrementada en uno de los pacientes. Los pacientes que presentaban una marcada excreción de ácido 2-metil-3-hidroxibutírico (2M3HBA) y de tiglilglicina (TG), también presentaban una mayor excreción de C5:1. Después de una dieta restringida en isoleucina, en 2 de los pacientes, el perfil de ácidos orgánicos prácticamente se normalizó y la C5:1 todavía se hallaba incrementada. En la muestra de un paciente tras un aporte proteico extra el incremento de C5:1 fue más evidente.

Estudiamos las muestras de orina de 2 portadoras tras una sobrecarga de isoleucina. En una de ellas la cuantificación de 2M3HBA y TG en orina no mostró el defecto, únicamente cuando se cuantificó el EHA se puso en evidencia la alteración (artículo2). En cambio, ambas mostraron un aumento de las acilcarnitinas C5:1 y C5-OH en todas las muestras.

Se propone la acilcarnitina C5:1 en orina como nuevo marcador bioquímico para esta enfermedad, que junto con el perfil de ácidos orgánicos en orina pueden contribuir a mejorar el diagnóstico de esta entidad.

Urinary tiglylcarnitine (C5:1) as biochemical marker of 17β-hydroxysteroid dehydrogenase type 10 (HSD10) deficiency

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Abstract

The diagnosis of HSD10 deficiency can be difficult. We conducted a retrospective study of samples' patients already diagnosed, in order to search for new biochemical markers. Had previously described that patients could present an increase of 2-methyl-3-hydroxy-butirilcarnitina (C5-OH) and tiglilcarnitina (C5:1) in plasma. However, these alterations were not observed in any of our patients.

We decided to evaluate the profile of urinary acylcarnitines by tandem mass spectrometry in 4 patients, in samples with normal diet, with isoleucine restricted diet and after a diet rich in protein. Additionally, we analyze urine samples of 2 carriers after overload isoleucine.

4 patients (3 men and one heterozygote) showed a significant increase of C5: 1, while C5OH was increased only in one patient. Patients that showed marked excretion of 2-methyl-3-hydroxybutyric acid (2M3HBA) and tiglilglicina (TG), also had higher excretion of C5:1. After a restricted isoleucine diet, in 2 patients, the organic acids profile was normalized and C5:1 was still increased. In the sample of a patient after an extra protein intake increased C5:1 was more evident.

The study of urine samples from 2 carriers after overload isoleucine, showed in one of them a normal excretion of 2M3HBA and TG, only when the EHA was quantified the defect was evident. Instead, both showed an increase of C5:1 and C5-OH in all studied samples.

C5:1 in urine is proposed as a new biochemical marker for this disease, which together with the profile of organic acids in urine, can contribute to improve the diagnosis of this entity.

Keywords: MHBD deficiency; HSD10; HADH2; ERAB; HSD17B10; ABAD

Introduction

2-Methyl-3-hydroxybutyryl-CoA dehydrogenase (HSD10) deficiency (MIM 300256) is an inborn error of isoleucine degradation pathway. In males this disorder is characterized by normal early development followed by progressive neurodegeneration [1-9]. However, as this defect is an X-linked disease [10], female patients present a variety of symptoms, from borderline learning difficulties to psychomotor and speech delay [2,7,9].

The diagnosis of this deficiency is carried out through the urinary organic acid profile showing elevated excretion of 2-methyl-3-hydroxybutyric acid (2M3HBA), ethylhydracrylic acid (EHA) and tiglylglycine (TG), and is confirmed by measuring HSD10 activity in fibroblasts or by molecular studies in *HSD17B10* gene [1-7,9]. In addition, an increase in plasma of 2-methyl-3-hydroxybutyrylcarnitine (C5-OH) and tiglylcarnitine (C5:1) have been detected in some patients [1,2,5,6].

It is noteworthy that the abnormalities in the organic acid profile of female patients and some male patients under low protein diet or poor feeding can be less pronounced. This fact makes difficult the diagnosis of this disorder [9]. To evidence the block in isoleucine pathway, an oral isoleucine loading test can be performed [1,2,6,9]. However, the test failed to demonstrate the carrier status in one female previously described by our group [9].

Only 15 families with HSD10 deficiency have been described to date. Therefore, it seems that this disease is less frequent than other X-linked defects or that new diagnosis might be missed if only testing for organic acids is done. For that reason, we performed a retrospective study in six patients previously described [9] to look for additional biochemical markers to improve the diagnosis. Results are reported here.

Materials and methods

Patients

We analyzed the urine acylcarnitine profile of four index patients (female 1, male 1, male 2 and male 3) with HSD10 deficiency and two female carriers (female 2 and female 3). These patients belong to four unrelated families, previously described by García-Villoria et al. 2009. Moreover, we studied a urine sample from female 1 and male 2 after an isoleucine restricted diet (post-treatment), and another one from male 3, after a high protein intake; 33 urine samples collected from healthy children were used as controls.

Methods

Biochemical studies

Urine acylcarnitine profiles were analyzed by positive electrospray ionization tandem mass spectrometry (ESI-MS/MS) in a Waters-Micromass model Quattro microTM API (Manchester, UK), as previously described [11], except that the internal standards used were: d_9-free carnitine, d_3-acetylcarnitine, d_3-propionylcarnitine, d_3-butyrylcarnitine, d_9-isovalerylcarnitine, d_6-glutarylcarnitine, d_3-hexanoylcarnitine, d_3-octanoylcarnitine, d_3-decanoylcarnitine, d_3-lauroylcarnitine, d_3-myristoylcarnitine, d_3-palmitoylcarnitine, d_3-octadecanoylcarnitine (Neobase Non-derivatized MSMS kit) that were purchase from PerkinElmer, Finland. The semi-quantification for each acylcarnitine species against the internal standards was achieved using Neolynx software (Waters) and then was expressed as mmol/mol of creatinine.

Reference ranges were established using 33 urine samples from healthy children.

Statistical methods

Statistical studies for urinary C5:1 and C5-OH acylcarnitines in controls and patients were performed using a non parametric test, U-Mann-Whitney, by SPSS® software (version 14.0 for Windows®).

Results and discussion

Patients with HSD10 deficiency show an organic acid profile in urine, characterized by an increase of 2M3HBA, EHA and TG. However, this peculiar profile can be only slightly altered in female as well as in male patients under low protein diet or poor feeding [9]. For that reason, we tried to find new and more robust biochemical markers for the diagnosis of HSD10 deficiency.

We retrospectively studied the acylcarnitine profile in urine from four patients previously described by our group [9]. Female 1 presented a slight increased 2M3HBA, TG and EHA in the organic acid profile [9], but urinary C5:1 was over control range (table 1). Male 1 and Male 2 presented the characteristic organic acid profile of HSD10, as well as a marked increased of C5:1, but an increase of C5-OH was only observed in Male 1 (table 1). On the other hand, Male 3 (under low protein diet) presented an almost normal qualitative organic acid profile [9], and a slight increase of urinary C5:1, while C-5OH was within the control range. However, the increase of these urinary acylcarnitines was more evident when they were analyzed in Male 3 after a high protein intake, (table 1). We had the opportunity to analyze Female 1 and Male 2 after an isoleucine restricted diet. Upon this treatment the organic acid profile and C5-OH normalized but the urinary C5:1 remained slightly high. U Mann-Whitney statistical test showed a p-value of 0.012 for C5:1, while it was of 0.14 for C5-OH. Therefore, significant differences between patients and controls for C5:1 were found, while differences were not significant for C5-OH.

We previously reported that the characteristic organic acid profile after an isoleucine load failed to identify one female carrier [9]. However, now we have analyzed the acylcarnitine profile in the same urine samples and found that both C5:1 and C5-OH were increased in all the samples and the highest amounts, were found 6 hours post-load (figure 1). Therefore, these acylcarnitines together with quantification of EHA [9] are useful for the diagnosis of HSD10 deficiency.

An increase of C5-OH and C5:1 in plasma have been detected in some patients [1,2,5,6]. We did not found any increase of these acylcarnitines in plasma of any patient. However, these two acylcarnitines were found to be increased in urine, particularly C5:1. As it has been reported in glutaric type I [11], it appears that the analysis of acylcarnitines in urine is a useful tool for the diagnosis.

To sum up, the retrospective study of urine samples from patients with HSD10 deficiency showed a significant increase of C5:1 in urine, even in samples after treatment with low protein diet and also can be of help for the diagnosis of the heterozygous condition. Therefore, we propose C5:1 as a new biochemical marker of HSD10 deficiency.

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Table 1. Quantitative results of urinary acylcarnitines compared with urinary organic acids.

	Urinary Organic acids (mmol/mol creatinine)			**Urinary acylcarnitines (mmol/mol creatinine)**	
Patients	**2M3HBA**	**TG**	**EHA**	**C5:1**	**C5:OH**
Female 1 pre-treatment	99	21	↑	1.97	1.07
Female 1 post-treatment	27	Indetectable	Normal	0.96	0.58
Male 1	262	59	↑	10.2	3.4
Male 2 pre-treatment	116	181	12	5.89	0.86
Male 2 post-treatment	42	49	1.8	0.52	0.19
Male 3	54	30	18	1.31	0.56
Male 3 after protein intake	48	33	17	1.97	1.07
Median controls **Control range**	<12 (n=50)	<5 (n=50)	<7 (n=50)	0.17 0.02 - 0.42 (n=33)	0.26 0.02 – 1.21 (n=33)

↑: Increased as assessed by the organic acid profile, no quantitative values were available

2M3HBA: 2-methyl-3-hydroxybutyric acid; C5:1: tiglylcarnitine; C5:OH: 2-methyl-3-hydroxybutyrilcarnitine; EHA: ethylhydracrilic acid; TG: tiglylglycine.

Figure 1. Quantification of tiglylcarnitine (C5:1) and 2-methyl-3-hydroxybutyrilcarnitine (C5-OH) in patients with HSD10 deficiency.

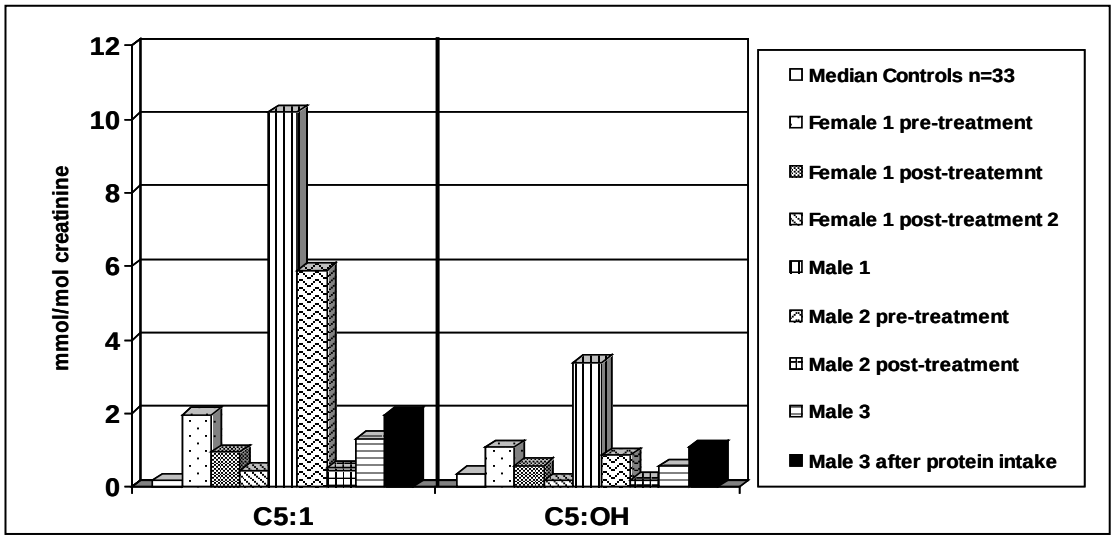
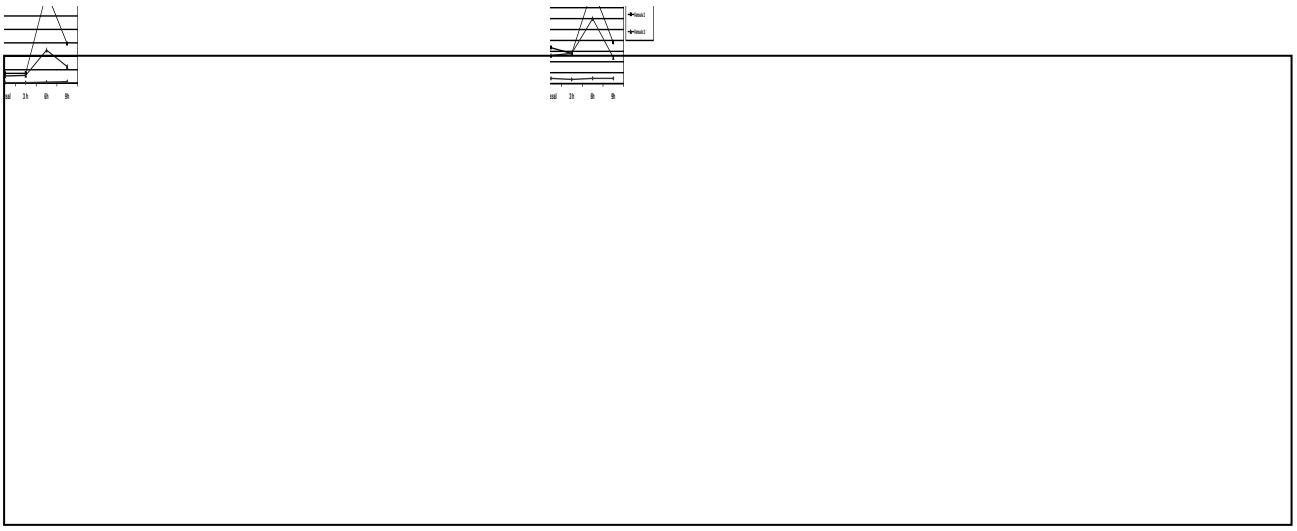


Figure 2. Quantification of tiglylcarnitine (C5:1) and 2-methyl-3-hydroxybutyrilcarnitine (C5-OH) after an isoleucine loading test.



2. HSD10: ESTUDIOS DE EXPRESION

ARTÍCULO 4

Título: X-inactivation of HSD17B10 revealed by cDNA analysis in two female patients with 17β-hydroxysteroid dehydrogenase 10 deficiency.

Autores: García-Villoria J, Gort L, Madrigal I, Fons C, Fernández C, Navarro-Sastre A, Milà M, Briones P, García-Cazorla A, Campistol J, Ribes A.

Revista: European Journal of Human Genetics 2010; 18(12):1353-1355.

PMID: 20664630

RESUMEN

Inactivación del cromosoma X en *HSD17B10* demostrado por análisis de cDNA en dos heterocigotas con deficiencia de 17β-hidroxiesteroide deshidrogenasa 10.

17β-hidroxiestroide deshidrogenasa 10 (HSD10) es un enzima mitocondrial implicado en la degradación de la isoleucina y de los ácidos grasos de cadena ramificada. El gen *HSD17B10* que codifica para HSD10, ha sido publicado como uno de los pocos genes que se escapan de la inactivación del cromosoma X. Estudios previos de nuestros pacientes no mostraban un patrón de herencia que pudiera responder a la afirmación previa. Por ello, obtuvimos cDNA a partir de fibroblastos cultivados de 8 líneas celulares control, 2 pacientes hemicigotos y 2 portadoras (una con fenotipo severo y otra con una leve afectación clínica), se secuenciaron y se cuantificaron mediante PCR a tiempo real (qRT-PCR).

La cuantificación relativa (RQ) del cDNA de *HSD17B10* de las líneas celulares control no difería entre varones y hembras controles, lo que indicaba que la dosis génica de mRNA de *HSD17B10* era la misma en ambos sexos. En fibroblastos de la portadora que presentaba un fenotipo severo, sólo se identificó el alelo mutado mediante secuenciación del cDNA, que posteriormente se confirmó por RQ del cDNA de *HSD17B10*. Estos resultados estaban en concordancia con una inactivación desfavorable del cromosoma X. Se descartó un síndrome de Turner. Los estudios de metilación en el locus del receptor de andrógenos en DNA genómico, mostraron un patrón de inactivación del cromosoma X preferencial.

Por otro lado, la portadora con una leve afectación clínica mostró la presencia de ambos alelos, mutado y wild-type, en la secuenciación del cDNA, que también fue confirmado por RQ del cDNA de *HSD17B10*. Los estudios de metilación no mostraron un patrón de inactivación del cromosoma X preferencial lo que indicaba una inactivación al azar del cromosoma X.

En conclusión, estos resultados sugieren que el gen HSD17B10 no se escapa de la inactivación del cromosoma X.

European Journal of Human Genetics (2010) 18, 1353–1355

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SHORT REPORT

X-inactivation of *HSD17B10* revealed by cDNA analysis in two female patients with 17β-hydroxysteroid dehydrogenase 10 deficiency

Judit García-Villoria[1,2], Laura Gort[1,2], Irene Madrigal[2,3], Carme Fons[2,4], Cristina Fernández[1,2], Aleix Navarro-Sastre[1,2], Montserrat Milà[2,3], Paz Briones[1,2,5], Angeles García-Cazorla[2,4], Jaume Campistol[2,4] and Antonia Ribes*[,1,2]

17β-Hydroxysteroid dehydrogenase 10 (HSD10) is a mitochondrial enzyme involved in the degradation pathway of isoleucine and branched-chain fatty acids. The gene encoding HSD10, *HSD17B10*, has been reported as one of the few genes that escapes X-inactivation. We previously studied two female patients with HSD10 deficiency, one of them was severely affected and the other presented a mild phenotype. To elucidate as to why these two carriers were so differently affected, cDNA analyses were performed. The *HSD17B10* cDNA of eight control cell lines, two hemizygous patients and two carriers was obtained from cultured fibroblasts, amplified by PCR and sequenced by standard methods. All *HSD17B10* cDNAs were quantified by real-time PCR. In the fibroblasts of the female patient who presented with the severe phenotype, only the mutant allele was identified in the cDNA sequence, which was further confirmed by relative quantification (RQ) of *HSD17B10* cDNA. This is in agreement with an unfavourable X-inactivation. The other female patient, with slight clinical affectation, showed the presence of both mutant and wild-type alleles in the cDNA sequence, which was confirmed by RQ of *HSD17B10* cDNA in fibroblasts. This is in line with normal X-inactivation and the expression of both alleles in different cells (functional mosaicism). RQ results of *HSD17B10* cDNA did not differ significantly between male and female controls, which indicate that the genetic doses of mRNA of *HSD17B10* was the same in both sexes. In conclusion, these results suggest that the *HSD17B10* gene does not escape X-inactivation as has been reported previously.
European Journal of Human Genetics (2010) **18,** 1353–1355; doi:10.1038/ejhg.2010.118; published online 28 July 2010

Keywords: HSD10 deficiency; HADH2; *HSD17B10*

INTRODUCTION

17β-Hydroxysteroid dehydrogenase 10 (HSD10) is a mitochondrial enzyme involved in the degradation pathway of isoleucine and branched-chain fatty acids.[1] This enzyme has also been found to be involved in the metabolism of sex steroid hormones, neuroactive steroids and in the detoxification of cytotoxic aldehydes.[2,3] HSD10 deficiency (OMIM 300256) is an X-linked defect caused by mutations in the *HSD17B10* gene. Clinically, the great majority of male patients show normal early development followed by progressive loss of mental and motor skills.[1,4–11] However, three patients were identified who presented symptoms in the first days of life.[1,11] It has recently been shown that symptoms of these patients are unrelated to accumulation of metabolites in the isoleucine pathway and that the neurological handicap can be associated with an imbalance in neurosteroid metabolism[12] or to defects in general mitochondrial function.[13] In addition, the splice variant c.574C>A of *HSD17B10* gene has been associated with a new syndromic form of X-linked mental retardation, choreoathetosis and abnormal behaviour.[14]

The *HSD17B10* gene has been mapped to chromosome Xp11.2[15] and has been reported as one of the few genes that escapes X-inactivation.[16] To date, 10 female patients with HSD10 deficiency have been described presenting a variety of symptoms, from borderline learning difficulties to psychomotor and speech delay.[5,9,11] We previously studied two of these female patients. One of them was heterozygous for the p.N247S mutation and was severely affected, whereas the other was heterozygous for the p.P210S mutation and presented a slight clinical affectation.[11] To elucidate as to why these two female patients were so differently affected, we performed *HSD17B10* cDNA quantitative analysis in both female patients and in control fibroblasts, the results of which are reported here.

MATERIALS AND METHODS

Material

Skin biopsies from patients of two unrelated Spanish families with HSD10 deficiency were obtained: family 1 consisting of a male patient (1IIM) and his carrier sister (1IIF), both with a severe phenotype (Figure 1a); family 2 consisting of a male patient (2IIM), with a severe phenotype, and his heterozygous mother (2IF), with a slight clinical affectation (Figure 1a). Both families have been described previously.[11] Eight cell lines (four males and four females) from our cell bank were used as controls.

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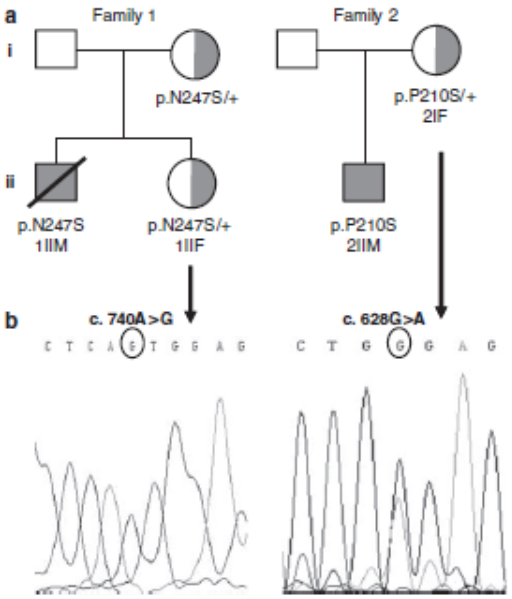

Figure 1 Pedigree (**a**) and cDNA sequence (**b**) of families 1 and 2.

All the samples were obtained according to the Declaration of Helsinki and informed consent was signed by all the patients or their parents.

Molecular studies

cDNAs were obtained from cultured fibroblasts, were amplified by PCR and sequenced using standard protocols and oligonucleotides designed in-house (sequences available upon request). All *HSD17B10* cDNAs were quantified by the StepOnePlus real-time PCR System using the Comparative Ct (ΔΔCt) method from StepOne software v2.0 (Applied Biosystems, Foster City, CA, USA). The Primer Express 3.0 software (Applied Biosystems) was used to design two sets of primers and probes to differentiate wild-type (Wt) and mutant (Mut) alleles corresponding to mutations p.N247S (c.740A>G) and p.P210S (c.628C>T) of the *HSD17B10* gene. We used two different endogenous controls: glyceraldehyde 3-phosphate dehydrogenase (PN4310884E) and cyclophilin A (PPIA, PN4310883E) (Applied Biosystems). As additional control, a mixed pool of four healthy male cDNAs was used in each analysis.

Gene nucleotide numbering was carried out according to sequence RefSeq NM_004493 with +1 as A of the ATG start codon. The ATG codon represents +1 for the amino-acid numbering according to the HSD10 protein sequence NP_004484.

X-inactivation studies

The androgen-receptor locus (*AR*) methylation assay was performed in genomic DNA of female carriers as described previously.[17] If the *AR* locus was uninformative, skewing was assessed at *FMR1* locus.[18] Briefly, genomic DNA (300 ng) was digested with 5 U of HpaII (both for the *AR* and *FMR1* assays) in a total volume of 20 μl. For each sample, an undigested control was prepared. We define the pattern of X-chromosome inactivation as skewed when the inactivation percentage was over 80%.

Statistical methods

Statistical studies for the analyses of relative quantification (RQ) in male and female controls were performed using the non-parametric two-related sample Wilcoxon's test, with the SPSS software (version 14.0 for Windows).

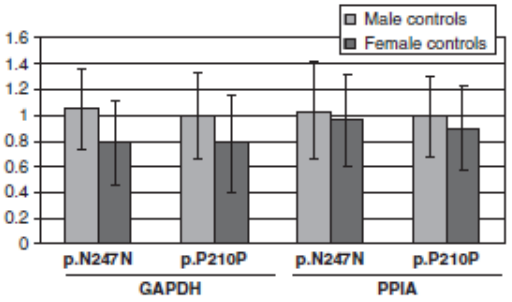

Figure 2 RQ of *HSD17B10* Wt probes named as p.N247N and p.P210P, with two distinct endogenous controls (glyceraldehyde 3-phosphate dehydrogenase and cyclophilin A) in four male and four female controls. The bars represent the mean of four controls and the error bars represent the mean ± SD.

RESULTS AND DISCUSSION

HSD17B10 has been reported as one of the few genes that escapes X-inactivation,[16] which predicts that female carriers would not be affected. However, 10 female carriers with HSD10 deficiency have been described so far, presenting different degrees of clinical affectation, which is in agreement with an X-linked inheritance with different degrees of X-inactivation.[11]

To elucidate whether *HSD17B10* cDNA doses differed between both sexes, we performed RQ of Wt *HSD17B10* cDNA alleles in four female and four male controls (Figure 2). Results of the Wilcoxon statistical test did not show any significant difference between the doses in both sexes, considering the two endogenous controls (*P*-value=0.07). Therefore, these results are in favour of an X-linked disease that does not escape X-inactivation.

We previously studied two unrelated female patients with different degrees of clinical affectation.[11] The female of family 1 (1IIF), like her brother (1IIM), presented a severe phenotype with psychomotor and speech delay, and a clear deficiency of HSD10 activity in fibroblasts.[11] When we sequenced her *HSD17B10* cDNA, it seemed that only the Mut allele was identified (Figure 1a). Results for the RQ of her *HSD17B10* cDNA (Figure 3a) showed that amplification levels of the Mut probe were much higher than those of the Wt probe and very similar to those of her brother (1IIM), independently of the endogenous control used (Figure 3a). To rule out a Turner syndrome, chromosome analysis was performed, which resulted in a normal karyotype (46,XX). Skewed X-inactivation was confirmed by methylation studies. Patient 1IIF was homozygous for *AR* locus, consequently this study was uninformative, but *FMR1* locus showed a skewed X-inactivation pattern (80/20). These results are in agreement with an unfavourable X-inactivation effect of *HSD17B10* gene in the analysed tissue. In addition, as the girl was severely affected, a similar unfavourable X-inactivation in other tissues could be expected.

The other female patient (2IF) showed a mild clinical affectation, with learning disabilities and HSD10 activity in fibroblasts within the control range.[11] *HSD17B10* cDNA sequencing showed the presence of both Mut and Wt alleles (Figure 1b). This observation was in agreement with the results of the RQ studies showing similar *HSD17B10* cDNA levels of both Wt and Mut probes, while we were only able to amplify the Mut probe in her severely affected son (2IIM) (Figure 3b). X-inactivation analysis showed a random X-inactivation pattern for *AR* locus in patient 2IF. These results suggest the presence

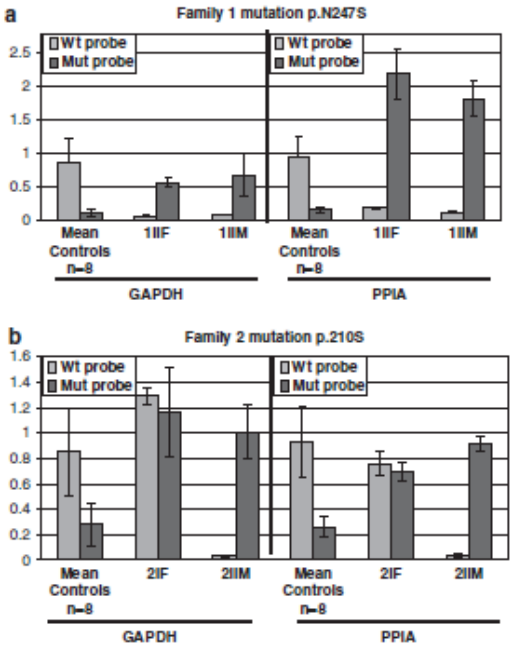

Figure 3 RQ of *HSD17B10* Wt and Mut probes with two distinct endogenous controls (glyceraldehyde 3-phosphate dehydrogenase and cyclophilin A) in patient and control cDNAs (**a**) family 1, (**b**) family 2. The bars of controls represent the mean of eight controls (four males+four females), performed in triplicate. The bars of patients represent the mean of triplicate measurements. The error bars represent the mean ± SD.

of both *HSD17B10* alleles in this female patient, which is in line with normal X-inactivation and the expression of both alleles in different cells (functional mosaicism). In addition, the normal enzymatic activity found in this female patient[11] might be due to lack of sensitivity of the enzymatic technique, or maybe there was enough dose of Wt *HSD17B10* mRNA to produce enough HSD10 protein to obtain normal activity.

However, we did observe that the amplification responses were different for each probe when they were corrected by the two different endogenous controls (Figure 3). This could be explained by the low specificity of the probes and by the variability of the endogenous controls. However, in spite of it, the interpretation of the results did not change.

To summarise, here we present the results of *HSD17B10* cDNA analysis in two female carriers compared with two male patients and controls. The hypothesis that *HSD17B10* is inactivated in one of the X-chromosome is supported by the results in controls, which showed that doses of *HSD17B10* cDNA were the same in both sexes (Figure 2). RQ cDNA results for one of the female patient (1IIF), together with the enzymatic studies and the severe clinical presentation, were in agreement with an unfavourable X-inactivation effect. In addition, RQ cDNA results for the other female patient (2IF) seem to reflect the presence of a mosaicism in the studied tissue, which could explain the normal enzymatic activity and her mild phenotype. However, we cannot exclude that differences in disease severity between both female carriers is at least partly due to differences in the effect of the mutations, as the male patient with the p.N247S mutation died at age 2 months, while the patient with p.P210S mutation is alive at 4 years of age.

In conclusion, our results suggest that *HSD17B10* gene does not escape X-inactivation as reported previously.[16] Heterozygous female patients showed the classical biochemical and clinical variability of X-linked diseases due to random X-chromosome inactivation and the severity of the phenotype will depend on the total dose of Mut mRNA in different tissues as well as on the severity of the mutation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ARTÍCULO 5

Título: Microarray RNA expression in patients with 17β-hydroxysteroid dehydrogenase 10 (HSD10) deficiency.

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Revista: Artículo en preparación.

RESUMEN

Estudios de expresión en pacientes con deficiencia de HSD10.

La neurodegeneración que presentan los pacientes con deficiencia de HSD10 no se observa en las otras alteraciones de la misma vía metabólica, así por ejemplo la deficiencia de 3-cetotiolasa, presenta una excreción de ácidos orgánicos muy similar a la de la deficiencia de HSD10, acidosis metabólica y vómitos, pero sin neurodegeneración. Por lo tanto, parece ser que la severidad neurológica en la deficiencia de HSD10 no sería una consecuencia de la alteración en el metabolismo de la isoleucina, si no de otras funciones que desempeña dicha proteína.

En este estudio hemos realizado la expresión en *E.Coli* de las mutaciones detectadas en nuestros pacientes: p.Arg130Cys, p.Asn247Ser, p.Pro210Ser y p.Arg226Gln. Todas las mutaciones presentaron una deficiencia de la actividad enzimática, confirmándose así la patogenicidad de las mismas. Destacamos que la mutación p.Pro210Ser presenta la actividad residual más elevada, tanto en *E. Coli* como en fibroblastos de pacientes con dicha mutación.

Con el fin de aportar nuevo conocimiento a los mecanismos de patogenicidad obtuvimos muestras de RNA a partir de fibroblastos de 5 hemicigotos y 2 heterocigotas con deficiencia de HSD10 y de 5 varones control y analizamos estas muestras mediante microarrays de expresión de 54.000 sondas. El análisis preliminar reveló que los pacientes con deficiencia de HSD10 presentaban un patrón de expresión diferente al de los controles. Seleccionamos los genes que tenían un log ratio >2 o <-2 comparando pacientes con controles y aplicamos el test estadístico U Mann Whitney. Obtuvimos un patrón de expresión significativamente diferente en 31 genes. Para confirmar estos resultados analizamos la expresión, mediante PCR cuantitativa, en un 22% de estos genes. En todos los casos se confirmaron las diferencias de expresión excepto para uno de los genes, *IL18R;* La diferencia de expresión encontrada en 14 genes podría estar relacionada con los síntomas neurológicos, entre los que destacamos: la sobre-expresión de *BCHE* y la infra-expresión de *MAOA y MAOB*, ya que el mismo cambio de expresión se ha observado en la enfermedad de Alzheimer y podrían estar implicados en la neurodegeneración que presentan ambas entidades. También se observaron diferencias en la expresión de 5 genes que podrían estar

relacionados con la ceguera que padecen algunos de estos pacientes, 5 genes que podrían explicar los síntomas cardíacos y de 3 genes implicados en el desarrollo esquelético.

Microarray expression analysis in patients with 17β-hydroxysteroid dehydrogenase 10 (HSD10) deficiency

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Abstract

The neurodegeneration that presents the HSD10 deficient patients is not observed in other disorders in the same metabolic pathway, even presenting a similar profile of organic acids. Therefore, it appears that the neurological severity in deficiency HSD10 is not due to an alteration in the metabolism of isoleucine, if not to other functions performed by this protein.

We first demonstrated the pathogenesis of the mutations studied here by expression studies in *E.Coli*.

To provide new knowledge about the pathogenicity of this disease, we performed microarray expression in RNA samples from cultured fibroblasts of 5 hemizygous and 2 heterozygous HSD10 patients and 5 control men. Preliminary analysis revealed that patients deficient HSD10 had a different expression pattern than controls. We selected genes that had a log ratio> 2 or <-2 comparing patients and controls. After applying a statistical test, we obtained a significantly different expression patterns in 31 genes. To confirm these results we analyzed the expression of 22% of these genes by quantitative PCR. In all cases except for one, the different expression was confirmed; The different expression observed in 14 genes could be associated with neurological symptoms, among these: the over-expression of BCHE, and the under-expressión of MAOA and MAOB are also related to Alzheimer's disease and might be involved in the neurodegeneration that present both entities. In addition, we found a different expression pattern in 5 genes that might be related to blindness, 6 genes that could explain the cardiac symptoms and 3 genes involved in skeletal development.

Introduction

17β-hydroxysteroid dehydrogenase 10 (HSD10) is a mitochondrial enzyme encoded by *HSD17B10* gene mapping in chromosome Xp11.2. This enzyme belongs to the short-chain dehydrogenase/reductase (SDR) family. HSD10 is a moonlighting protein and multiple functions have been described for it. The activity of this protein depends on NAD^+ or NADH as cofactors. HSD10 is involved in the degradation pathway of isoleucine and branched-chain fatty acids (Zscoke et al. 2000). HSD10 has an important role in sex steroid metabolism with the oxidation of 17β-estradiol, the generation of 5α-dihydroxydrotestosterone and with a non-classical pathway of androgen synthesis. HSD10 also plays a role in neuroactive steroid metabolism with the oxidation of allopregnanolone and allotetrahydrodeoxycorticosterone and seems to be involved in neurosteroidogenesis (Yang et al. 2005). HSD10 can detoxify 4-hydroxy-2-nonenal, a cytotoxic aldehyde derived from lipid peroxides (Murakami et al. 2009) and is also a cardiolipin phospholipase (Boynton and Shimkets 2016).

It has been described that HSD10 is important for maintaining mitochondrial integrity (Tieu et al. 2004, Yan et al. 2005 and Rauschenberger et al. 2010). Moreover, HSD10 is a protein of the postsynaptic density (Laumonnier et al. 2007) and and is a member of two important mitochondrial complex enzymes: RNaseP (Holzmann 2008) and tRNA methyltransferase (Vilardo 2012).

In addition, HSD10 play a role in pathogenesis of Alzheimer's disease (Yan 1997, Opperman 1999, Yan 1999, Yan 2000, Wen 2002, He 2002, Lustbader 2004, Takuma 2005, Yan 2005, Hovorkova 2008).

HSD10 deficiency has been described as X-linked inborn error of isoleucine metabolism (OMIM 300256) caused by mutations in *HSD17B10* gene. To date, 21 families with this disease have been described (Zschocke et al. 2000, Ensenauer et al. 2002, Olpin et al. 2002, Poll The et al. 2004, Sass et al. 2004, Sutton et al. 2003, García-Villoria et al. 2009, Rauschenberger et al. 2010, Seaver et al. 2011, Zschocke 2012, Fukao et al. 2014, Chatfield et al. 2015). This defect is characterized by progressive neurodegeneration in males and with a variety of symptoms, from borderline learning difficulties to psychomotor speech delay in females. In addition, one splice variant c.574C>A of *HSD17B10* gene has been associated with a new syndromic form of X-linked mental retardation, choreoathetosis and abnormal behaviour (Lenski et al. 2007).

This organic aciduria is characterized by the increase of 2-methyl-3-hydroxybutyryic acid and tiglylglycine in urine, the same metabolites that are accumulated in β-Ketotyolase deficiency, the next enzymatic step in isoleucine degradation pathway. However, the patients with β-ketotyolase deficiency do not present neurodegeneration. Therefore, the severe clinical presentation of HSD10 deficiency differs from the phenotype of the other diseases in the same metabolic pathway. For this reason, the neurologic symptoms of this disease can be caused by deficiencies of the other functions of HSD10 rather than by its function in the isoleucine metabolism (Yang et al. 2009, Rauschenberger et al. 2010, Yang et al. 2014, Chatfield et al, 2015, Vilardo and Rossmanith 2015).

Many studies are necessary to understand the physiopathology of HSD10 deficiency. For that reason, microarray expression analyses in patients with HSD10 deficiency compared with controls were performed. Results are reported here. To demonstrate the pathogenesis of the mutations studied here, expression studies in *E.Coli* were done.

Methods

Sample collection and patients

Skin biopsies from seven patients with HSD10 deficiency were obtained (5 hemizygous and 2 hetrozygous). Five fibroblasts cell lines from our cell bank were used as controls.
All samples were obtained according to the declaration of Hensinki and informed consent was signed by all the patients or their parents.

RNA extraction and cDNA synthesis
Total RNA was obtained of all patients and controls by Quiagen extraction RNA Kit. Then, it was purified using the RNeasy Minikit (Qiagen, Mississauga, ON).
Total cDNA synthesis was performed with standard ptotocols using oligo-DT primers.

Expression studies and cloning of the different mutations
cDNA of one control and four patients with the different mutations (p.Arg130Cys, p.Asn247Ser, p.Pro210Ser and p.Arg226Gln) were cloned using pET directional TOPO Expresion kit (Invitrogen Life Science Technologies).

Biochemical studies
The enzymatic activities were preformed as described in García-Villoria et al. 2009. Immunoblot analysis was performed as previously described by García-Villoria et al. 2009 but using another antibody: anti-17BHSD10 of rabbit (SIGMA) instead of anti-ERAB.

Microarray expression analysis
Microarray expression studies were performed to identify genes that were differentially expressed in patients with HSD10 deficiency versus control cell lines. Analysis was performed by using the GeneChip Human Genome U133 plus 2.0 (affymetrix, Santa Clara, CA), which included 54.500 probes. Sequence clusters were created from the UniGene database (Build 133, April 20, 2001 and build 159, January 25, 2003) and then refined by analysis and comparison with a number of other publicly available databases, including the Washington University EST trace repository and the NCBI human genome assembly.

Lowess normalization was performed in data, and gene expression values were transformed to log2 net fluorescence intensity ratios for analysis. To assess the functional composition of genes that presented a significant difference between patients and controls, we used Gene ontology (GO) (http://www.geneontology.org/), Kyoto Encyclopedia of Genes and Genomes (KEEGG) pathway, and Genecards data base (http://www.genecards.org/).

Quantitative real time PCR (qRT-PCR)

For validation by qRT-PCR, seven genes were selected based on their statistical and biological significance. The total RNA used was the same used for microarray analysis. All mRNA were quantified by StepOne Plus^{TM} real-time PCR System using the comparative Ct (ΔΔCt) method from Step One software v2.0 (applied Biosystems, Foster City, Ca, USA). Sets of primers and probe for the amplification of the seven mRNA were purchase from Aplied Biosystems as TaqMan® Gene expression Assay: Hs00248543_m1 for *SYNE2* gene; Hs00385388_m1 for *COL14A1* gene; Hs00175381_m1 for *IL18R1* gene; Hs01106243_m1 for *MAOB* gene; Hs00170815_m1 for *POSTN* gene; Hs00216195_m1 for *SLC6A15* gene and Hs00992319_m1 for *BCHE* gene. *GAPDH*, PN4310884E (Applied Biosystems) was used as endogenous control. As additional control, cDNA from a mixed pool of 4 healthy individuals was used in each analysis. Target mRNAs and endogenous control reaction were run in separate wells in triplicate.

Statistical studies

Statistical analysis for the analyses of differential expression in microarray and relative Quantification (RQ) by real time PCR between patients and controls were performed using a non parametric two independent sample test, U Manny Whitney, by SPSS® software v.16. Results were deemed significant when p-value< 0.05.

Results

Expression studies of the different mutations

All mutations presented a deficiency of the enzymatic activity, confirming the pathogenic nature of these changes, see table 1.

Microarray expression analysis

Preliminary analysis reveals that patients with HSD10 deficiency showed a different pattern of expression than controls, as it can be observed with Dendrogram for clustering experiments and centred correlation and average linkage. Stresses that hemizygous and heterozygous patients with p.Asn247Ser mutation, are closer to the subset of controls (figure 1). On the other hand, the image obtained by the GEPAS software also showed a different pattern of expression in the control group (classA) and the group of patients (classB), see figure 2.

We first selected the genes that had a log ratio > 2 or < -2 compared with controls. Results showed a different expression profile in 42 genes. We then applied a non parametric statistical test for independent samples (U Mann Whitney test), and the number of genes showing

significant differences was reduced to 31 (table 2). We then choose 22% of these genes to confirm the results by *qRT-PCR* (figure 3). Results obtained by microarray analysis were confirmed for all the genes tested, except for *IL18R1*.

Discussion

The clinical presentation of HSD10 deficiency differs from other organic acidurias of the same metabolic pathway (β-ketotyolase and 2-methylbutyril-CoA deficiencies). HSD10 is a "moonlighting" protein that besides its function in isoleucine catabolic pathway is involved in other metabolic pathways, such as sex steroid metabolism and neuroactive steroid metabolism and maintenance of the mitochondrial integrity (Tieu et al. 2004, Yan et al. 2005 and Rauschenberger et al. 2010). Some authors speculate that the homosteosis alteration of neurosteroids is the main cause of neurodegeneration (Yang et al. 2009, Yang et al. 2014).

Moreover, HSD10 is a protein of the postsynaptic density (PSD), which is essential for the induction of neuronal plasticity and cognitive processes in animals (Laumonnier et al. 2007). Recently, it has been demonstrated that the mitochondrial energy failure in patients with HSD10 deficiency is due to defective mtDNA transcript processing (Chatfield et al, 2015, Vilardo and Rossmanith 2015).

However, the physiopathology of the disease is still unknown. For that reason, we perform RNA microarray analysis to gain insight into the mechanisms involved in the severe neurological clinical affectation.

We have confirmed the pathogenic nature of the four mutations that we detected in the 7 patients with HSD10 deficiency studied in this report, showing all mutations a deficiency of HSD10 activity (table1). Noting that, the mutation p.Pro210Ser showed the highest residual activity, as observed in cultured fibroblasts from patients with the mutation (García-Villoria 2009). However expression studies with p.Arg130Cys mutation revealed a high activity compare with the studies in the patients' fibroblasts with this mutation. This could be explained because patients with this mutation have reduced protein levels, as observed in Ofman et al 2003 and 2009 J García-Villoria and in figure 1, while the enzymatic activity in the expression studies was analysed with more protein levels. All the results are according to the studies performed by Vilardo and Rossmanith in 2015. They observed a reduction of the dehydrogenase activity, as well as methyltransferase activity with these 4 mutations, showing a highest activity the p.Pro210Ser and p.Arg130Cys mutations. In addition, they showed that the formation of a tetramer of HSD10 with mutations p.Asn247Ser and p.Arg226Gln is severely impaired and disrupts the interaction with TRMT10C, the methyltransferase subunit of the mitochondrial RNase P complex. In the case of

p.Arg130Cys mutation the formation of the tetramer is correct but there is a significant decrease in the interaction between HSD10 and TRMT10C. The mutation p.Pro210Ser did not show an altered tetramerization or interaction with TRMT10C; however, as discussed, p.Pro210 is located at the surface of the protein and an alteration of the region might possibly hamper the scaffolding function of HSD10 without necessarily changing the affinity for TRMT10C. They propose that the structural and functional alterations of HSD10 impair mitochondrial RNA processing and modification, leading to the mitochondrial dysfunction observed in HSD10 patients.

The microarrays study revealed a different expression pattern between patients and controls. The patients with mutations p.Asn247Ser present a patter more similar to controls, however, both male patients previously described with this mutation (Chatfield et al. 2015 and Garcia-Villoria 2009: the same patient in this report) presented a severe clinical presentation with lactic acidosis and early death. Therefore, different mutations could cause different effects in fisiopathology and different interactions with other proteins, as it was described by Vilardo and Rossmanith in 2015.

After performing a statistical test, we selected 31 genes with a significative different expression levels between patients and controls (table 2). Then we validate the expression results in 22% of the genes by qRT-PCR. All of them present the same expression differences respect to controls, except for IL18R1 gene.

We found a different expression of some genes that could be involved in the fisiopathology of the HSD10 deficiency.

Some genes that were found over-expressed were involved in:

- Immune response: *TNFS4*, also involved in cell proliferation. The cytokine encoded by this gene belongs to the tumor necrosis factor (TNF) ligand family and mediate adhesion of activated T cells to vascular endothelial cells.

- Signal transduction: *OZD3, DIRAS2, TRIB2* y *IL18R.* Noting that, the last gene was not confirmed by qtPCR studies.

1) Some genes could be related with the neurological symptoms.

1.1- 6 genes that were found under-expressed in patients:

- *MAOA* and *MAOB,* monoamine oxidase A, an enzyme that degrades amine neurotransmitters, such as dopamine, norepinephrine, and serotonin. In addition, are found bound to the outer membrane of mitochondria in most cell types in the body. Monoamine oxidase B plays an important role in the metabolism of neuroactive and vasoactive amines in the central nervous sysytem and peripheral tissues. Genetic deficiencies of these genes cause neurochemical and clinical phenotypes, with mental retardation, behaviour

alteration, blindness and loss of audition. Also have been realtated with Parkinson and Alzheimer diseases and neurodegeneration.

- *SEMA5A* encodes membrane proteins containing a semaphorin domain and several thrombospondin type-1 repeats. Members of this family are involved in axonal guidance during neural development. This gene has been implicated as an autism susceptibility gene. Also has been related with Parkinson disease.
- *NFASC* encodes an L1 family immunoglobulin cell adhesion molecule with multiple IGcam and fibronectin domains. The protein is involved in neurite outgrowth, neurite fasciculation, and organization of the axon initial segment (AIS) and nodes of Ranvier, on axons during early development. It would be related with myelination and peripheral nervous system development.
- *GRIA3,* glutamate receptor. L-glutamate is an excitatory neurotransmitter induces a conformation change, leading to the opening of the cation channel. Defects in *GRIA3* are the cause of mental retardation X-linked type 94 (MRX94) [MIM: 300699] with macrocephaly, seizures, myoclonic jerks, autistic behavior, asthenic body habitus, distal muscle weakness and hyporeflexia.
- *S1PR3* regulates cell proliferation, apoptosis and motilityand retraction of neurites.

1.2- 8 genes that were found over-expressed in patients:

- *BCL2* encodes an integral outer mitochondrial membrane protein that blocks the apoptotic death, controlling the permeability of the mitochondrial membrane. But we also found increase of *HRK*, which actives the apoptosis by interaction with suppressor apoptosis proteins, like BCL2. It has been described that HSD10 is important for the integrity of mitochondria (Zschocke 2010), so the overexpression of these proteins could be a compensatory effect of mitochondrial dysfunction observed in patients with HSD10 deficiency.

- *MDK,* Midkine is a retinoic acid-responsive gene concerned with prenatal development and neurite growth and with cell-cell signal in neurogensis.
- *SYNE2* encodes a nuclear outer membrane protein that binds cytoplasmic F-actin. This binding aids in the maintenance of the structural integrity of the nucleus, neuron migration, regulation of transcription, DNA-dependent nuclear envelope organization, establishment or maintenance of cell polarity, signal transduction, cell-cell signalling, brain development, learning or memory, negative regulation of cell growth, estrogen

receptor signaling pathway, positive regulation of apoptosis, positive regulation of gene-specific transcription neuroprotection, positive regulation of survival gene product expression.

- *SOX11* encodes a member of the SOX (SRY-related HMG-box) family of transcription factors involved in the regulation of embryonic development and in the determination of the cell fate. The encoded protein may act as a transcriptional regulator after forming a protein complex with other proteins. The protein may function in the developing nervous system and play a role in tumorigenesis.
- *SLC6A15* has structural characteristics of an Na(+) and Cl(-)-dependent neurotransmitter transporter. Also it can transport branched chain aminocids.
- *BHE*, butyrylcholinesterase contributing to the inactivation of the neurotransmitter acetylcholine and has a negative regulation of synaptic transmission. His expression has also been increased in Alzheimer's disease and increase the risk of developing Type 2 Diabetes Mellitus in this disease (Rao et al., Medical Hypotheses (2007) 69, 1272-1276). In addition, the increased levels of this protein are significantly correlated with the development of neuritic plaques of beta-amyloid and neurofibrillary tangles in the cortical and neocortical region.
- *PTPRD*, the protein encoded by this gene is a member of the protein tyrosine phosphatase (PTP) family. PTPs are known to be signaling molecules that regulate a variety of cellular processes including cell growth, differentiation, mitotic cycle, and oncogenic transformation. Studies of the similar genes in chicken and fly suggest the role of this PTP is in promoting neurite growth, and regulating neurons axon guidance.
- *ODZ3* involved in neuronal development and connectivity of the nervous system, as well as axonal addressing.

2) Some genes might be related with the blindness observed in these patients.

2.1- 3 genes that we found under-expressed in patients:

- *FOXL2*, which encodes a transcription factor that regulates apoptosis and is involved in ovarian development, eye morphogenesis in embryo, development extraocular and skeletal muscle and cell differentiation. Mutations in this gene are associated with ovarian failure and ptosis (MIM 110100).
- *MAOA* and *MAOB*, under-expression of these genes can cause blindness.

2.2- 2 genes that we found over-expressed in patients:

- *NHSL1*, mutations in this gene, which was found over-expressed in patients, is partnering with Nance-Horan syndrome (MIM 302350), linked to the X chromosome and characterized by congenital cataracts with a vision probe, nystagmus, dental anomalies, dysmorphia and mental retardation in some cases.
- *ODZ3* gene mutations, mentioned above, associated with microphthalmia (MIM 615 145) and characterized by poor vision microcornea probably sets because gene is involved in the development of the visual pathway.

3) Genes that may be associated with cardiac symptoms.

3.1- 4 genes that we found over-expressed in patients:

- *IL18R1*, which has not been confirmed by the study by RT-PCR, is related to cardiovascular risk (Tiret et al, 2005 Circulation 112: 643-650).
- *TNFSF4*, susceptibility gene myocardial infarction (MIM 608446), in patients has been found over-expressed.
- *ETV1* is an androgen receptor-regulated gene, and it could be related with the binding of HSD10 with androgen receptor α. Also could be implicated for their role in angiogenesis.
- *NHSL1*, as mentioned above, has also been associated with endotheliitis with hypertension and vascular disease with immune response in vascular endothelial cells.

3.2- 1 gene that we found under-expressed in patients:

- *SIPR3* involved in signal transduction and in the regulation of angiogenesis and vascular endothelial cell function. The Knock-out pattern for this gene has severe vascular defects.

4) Genes involved in the development of the skeleton, found over-expressed in patients: *POSTN*, *COL14A1* and *NHSL1*.

5) Also ST3GAL6 was found over-expressed in patients, which codes for a protein which transfers sialic acid at the terminal position of the carbohydrate groups of glycoproteins and glycolipids.

In conclusion it is confirmed that genetic changes observed in our patients are pathogenic and cause a decrease of HSD10 activity. Furthermore, we have identified 31 genes that present a significativaly diference in pattern expression in HSD10 deficient patients compared to controls. Some of them could be associated with the symptoms of patients with this deficiency. In addition we observed that HSD10 patients present expression alterations in 3 genes related with Alzheimer disease, may be they are involved in the nerodegenration of both diseases. It would be necessary to performe proteomic studies to demonstrate that the differences in the expression correlate with a change in protein levels.

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Table 1. Results of enzymatic activity of different mutations expressed in COS cells.

Mutation	% activity respect to "wild-type"
p.Arg130Cys	26
p.Asn247Ser	10
p.Pro210Ser	77
p.Arg226Gln	0.76

Table 2. Genes with a significant different expression between patients and controls.

Number-Gene name **O: overexpressed** **U: underexpressed**	**GO Molecular Function Description/ GO Biological Process**	**p-value**	**Mutations**
1-BCL2 (O)	Protein binding, transcription factor binding, transcription activator activity, sequence-specific DNA binding, protein heterodimerization activity, BH3 domain binding/ G1/S transition of mitotic cell cycle protein, cell morphogenesis, oxygen and reactive oxygen species metabolism, cell differentiation, apoptosis, response to oxidative stress, cell proliferation, etc...	0.008	p.Arg130Cys p.Pro210Ser p.Arg226Gln
2-BHE (O)	Beta-amyloid binding cholinesterase activity, serine esterase activity, hydrolase activity, enzyme binding/ cocaine metabolism	0.008	p.Asn247Ser p.Pro210Ser p.Arg226Gln
3-ETVI (O)	Transcription activator activity sequence-specific DNA binding/regulation of transcription, DNA-dependent transcription from RNA polymerase II promoter axon guidance, muscle organ development, mechanosensory behavior positive regulation of transcription	0.008	p.Arg130Cys p.Pro210Ser p.Arg226Gln
4-IL18R1 (O)	Transmembrane receptor activity, interleukin-1 receptor activity/ immune response signal transduction	0.008	p.Arg130Cys p.Pro210Ser p.Arg226Gln
5-LOC283454 (O)	?	0.008	p.Arg130Cys p.Pro210Ser p.Arg226Gln p.Asn247Ser
6-NHSL1 (O)	?	0.008	p.Arg130Cys p.Pro210Ser p.Arg226Gln
7-ODZ3 (O)	Signal transduction	0.008	p.Arg130Cys p.Pro210Ser p.Arg226Gln p.Asn247Ser
8-S1PR3 (U)	Lysosphingolipid and lysophosphatidic acid receptor activity	0.008	p.Arg130Cys

	signal transducer activity, G-protein coupled receptor activity, lipid binding/ cytokine production, inflammatory response signal transduction G-protein coupled receptor protein signaling pathway, elevation of cytosolic calcium ion concentration positive regulation of cell proliferation, anatomical structure morphogenesis, regulation of interleukin-1 beta production		p.Pro210Ser p.Arg226Gln
9-ST3GAL6 (O)	Sialyltransferase activity/ amino sugar metabolic process, glycolipid metabolic process, protein lipoylation	0.008	p.Arg130Cys p.Pro210Ser p.Arg226Gln p.Asn247Ser
10-TNFSF4 (O)	Tumor necrosis factor, receptor binding/ immune response signal transduction, cell proliferation positive regulation of cell proliferation	0.008	p.Arg130Cys p.Pro210Ser p.Arg226Gln
11-DIRAS2 (O)	Nucleotide binding, GTP binding/ small GTPase mediated signal transduction	0.016	p.Arg130Cys p.Pro210Ser p.Asn247Ser
12-KIAA1211 (O)	?	0.016	p.Arg130Cys p.Pro210Ser p.Asn247Ser
13-MDK (O)	Cytokine activity, growth factor activity, heparin binding/ regulation of cell cycle signal transduction, cell-cell signal neurogenesis, cell proliferation response to wounding cell differentiation adrenal gland development	0.016	p.Arg130Cys p.Pro210Ser p.Arg226Gln p.Asn247Ser
14-POSTN (O)	Protein binding, heparin binding/ skeletal development, cell adhesion	0.016	p.Arg130Cys p.Pro210Ser p.Arg226Gln
15-SLC6A15 (O)	Neurotransmitter:sodium, symporter activity/ neurotransmitter transport	0.016	p.Arg130Cys p.Pro210Ser p.Arg226Gln p.Asn247Ser
16-COL14A1 (O)	Extracellular matrix structural constituent protein binding/ phosphate transport, cell adhesion collagen fibril organization	0.032	p.Arg130Cys p.Pro210Ser
17-HRK (O)	Protein binding/induction of apoptosis, negative regulation of survival gene product expression, positive regulation of neuron apoptosis, cellular response to potassium ion starvation	0.032	p.Arg130Cys p.Pro210Ser p.Asn247Ser
18-MAOA (U)	Amine oxidase activity, oxidoreductase activity/ electron transport catecholamine metabolism behaviour neurotransmitter catabolism	0.032	p.Arg130Cys p.Pro210Ser p.Arg226Gln
19-MAOB (U)	Amine oxidase activity, oxidoreductase activity/ electron transport	0.032	p.Arg130Cys p.Pro210Ser p.Arg226Gln p.Asn247Ser
20-PAPLN (O)	Metalloendopeptidase activity, serine-type endopeptidase inhibitor activity peptidase activity	0.032	p.Arg130Cys p.Pro210Ser

21-PTPRD (O)	Protein tyrosine phophatase activity, transmembrane receptor protein tyrosine phosphatise activity, hydrolase activity/ protein amino acid dephosphorylation, phosphate metabolism, transmembrane receptor protein tyrosine signalling pathway	0.032	p.Arg130Cys p.Arg226Gln p.Asn247Ser
22-RRAGD (O)	Nucleotide binding, protein binding, GTP binding protein, heterodimerization activity	0.032	p.Arg130Cys p.Pro210Ser p.Arg226Gln
23-SYNE2 (O)	DNA binding transcription factor activity, steroid hormone receptor activity, actin binding, receptor activity ligand-dependent nuclear receptor activity, steroid binding, protein binding, lipid binding, hormone binding/ behavioral fear response, neuron migration, regulation of transcription, DNA-dependent nuclear envelope organization, establishment or maintenance of cell polarity, signal transduction, cell-cell signaling brain development, learning or memory, fibroblast migration negative regulation of cell growth, estrogen receptor signaling pathway, response to testosterone stimulus, positive regulation of apoptosis, positive regulation of gene-specific transcription neuroprotection, positive regulation of survival gene product expression	0.032	p.Arg130Cys p.Pro210Ser p.Arg226Gln
24-FOXL2 (U)	ovarian follicle development, extraocular skeletal muscle development, DNA fragmentation involved in apoptosis transcription, regulation of transcription, DNA-dependent induction of apoptosis, negative regulation of transcription female somatic sex determination, cell differentiation, positive regulation of apoptosis, positive regulation of caspase activity positive regulation of transcription, DNA-dependent positive regulation of transcription from RNA polymerase II promoter, embryonic eye morphogenesis	0.038	p.Arg130Cys p.Pro210Ser p.Arg226Gln
25-GRIA 3 (U)	regulation of receptor recycling transport, ion transport glutamate signaling pathway, response to lithium ion	0.038	p.Arg130Cys p.Pro210Ser p.Arg226Gln
26-RGS2 (U)	cell cycle spermatogenesis, regulation of G-protein coupled receptor protein signaling pathway, negative regulation of signal transduction, brown fat cell differentiation	0.038	p.Arg130Cys p.Pro210Ser p.Asn247Ser
27-SEMA5A (U)	patterning of blood vessels, cell adhesion cell-cell signalling, multicellular organismal development, nervous system development, axon guidance, cell differentiation	0.038	p.Arg130Cys p.Pro210Ser p.Arg226Gln
28-NFASC (U)	cell adhesion, axon guidance, peripheral nervous system development, myelination, synapse organization	0.040	p.Arg130Cys p.Pro210Ser p.Arg226Gln p.Asn247Ser
29-GLIPR1 (U)	?	0.040	p.Arg130Cys p.Pro210Ser p.Arg226Gln

			p.Asn247Ser
30-SOX11 (O)	kidney development, transcription, multicellular organismal development, nervous system development, cell proliferation oligodendrocyte development, cell differentiation, positive regulation of transcription from RNA polymerase II promoter	0.045	p.Arg130Cys p.Pro210Ser p.Arg226Gln p.Asn247Ser
31-TRIB2 (O)	protein amino acid phosphorylation, negative regulation of protein kinase activity, positive regulation of proteasomal ubiquitin-dependent protein catabolic process, regulation of MAP kinase activity, negative regulation of interleukin-10 biosynthetic process, negative regulation of fat cell differentiation	0.045	p.Arg130Cys p.Pro210Ser p.Arg226Gln

The seven genes outlined in yellow have been tested by real-time PCR.

(O): over-expressed

(U): under-expressed

Figure 1. Dendrogram for clustering experiments, using centered correlation and average linkage.

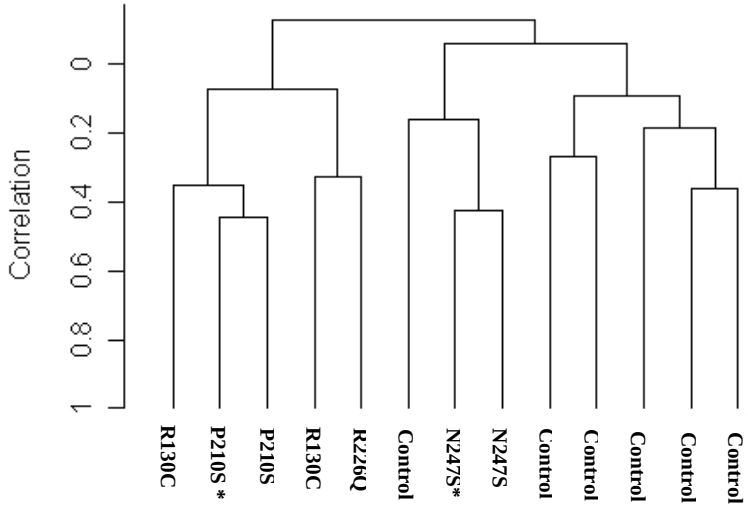


Figure 2. GEPAS image

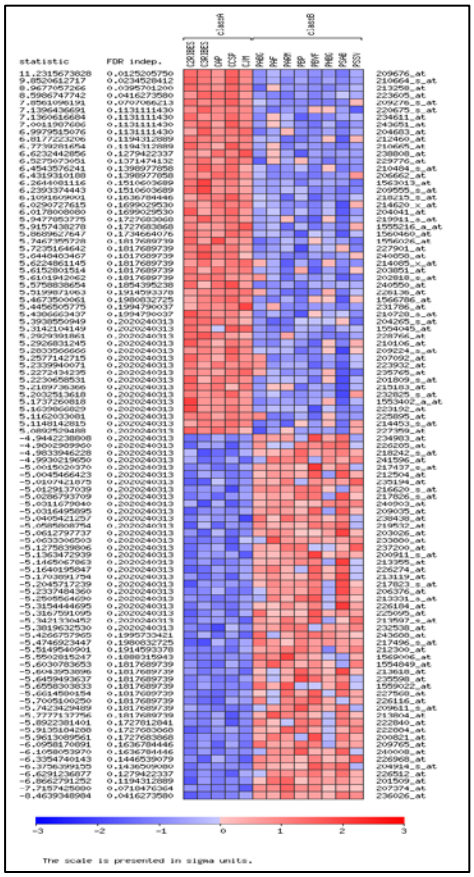
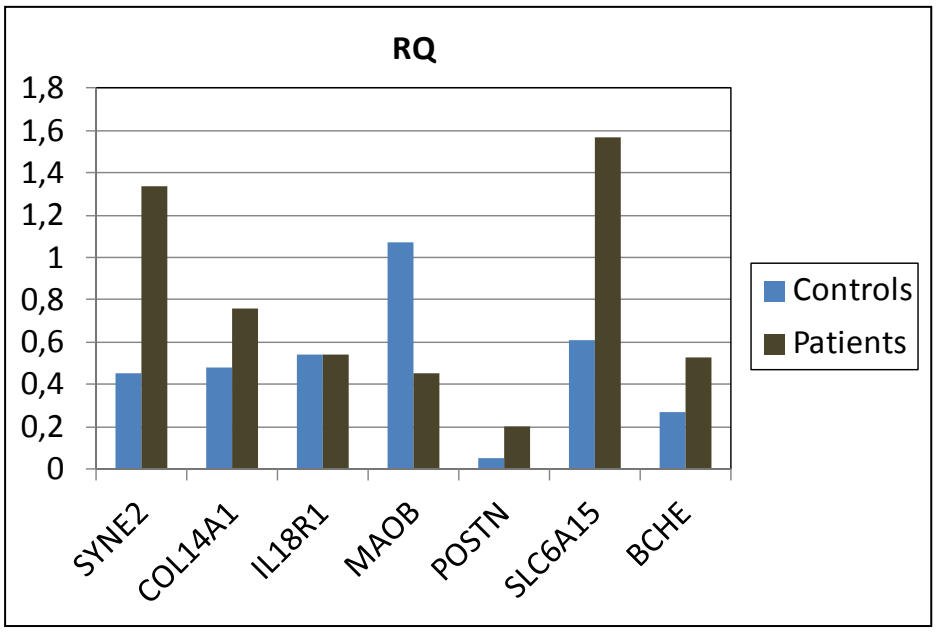


Figure 3. Mean of the RQ results of patients and controls of the 7 genes studied by Real time PCR.



3.- IMPLICACION DE HSD10 EN LA ENFERMEDAD DE ALZHEIMER

ARTÍCULO 6

Título: 17β-hydroxysteroid dehydrogenase (HSD10) as a therapeutic target in Alzheimer's disease.

Autores: García-Villoria J, Pascual R, Blázquez E, Matalonga L, Messeguer A, Delpiccolo C ML, Ferrer-Montiel A, Ribes A.

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RESUMEN

17β-hidroxiesteroide deshidrogenasa tipo 10 (HSD10) es una enzima mitocondrial multifuncional implicada en el metabolismo de la isoleucina, ácidos grasos de cadena ramificada, hormonas esteroideas sexuales, esteroides neuroactivos, y en la detoxificación de aldehídos citotóxicos. La deficiencia de HSD10 se caracteriza por neurodegeneración. HSD10 también se ha relacionado con la enfermedad de Alzheimer (EA), debido a su capacidad de interaccionar con el péptido beta-amiloide (pßA). Se sugiere que dicha interacción es una de las causas de la disfunción mitocondrial en la EA. Actualmente, algunos autores centran la búsqueda de tratamientos para la EA en la inhibición de la interacción de pßA-HSD10.

Nos propusimos explorar si la actividad HSD10 pudiera ser una diana terapéutica para la EA. Previamente realizamos estudios por "wester-blot" en homogenados de corteza cerebral humana procedente de pacientes con EA, pero no observamos sobre-expresión de HSD10, tal como se había descrito anteriormente. En cuanto a la actividad enzimática HSD10 encontramos disminución de la misma en homogenados de corteza parietal y occipital de pacientes con EA. Por otro lado, comprobamos que la incubación de homogenados de cerebro control con pßA provocaba una inhibición de la actividad HSD10, que se rescataba al incubar con elevadas concentraciones de NAD^+ (2- 8 mmol/L). Esta observación también fue corroborada en EA, es decir, al incubar con NAD^+ los homogenados de cerebro de pacientes con EA se recuperaba la actividad HSD10. Este hecho se podía explicar por una competición entre NAD^+ y pßA, ya que ambos interaccionan con HSD10 en la misma posición aminoacídica, por ello al incubar con elevadas concentraciones NAD^+ se inhibe la interacción pßA- HSD10. Por tanto, la medición de la actividad HSD10 podría reflejar un cambio en dicha interacción y podría ser utilizada como diana terapéutica para la EA. Con esta finalidad, obtuvimos la proteína recombinante HDSD10

isoforma 1, que es la que expresa mayoritariamente en cerebro humano, y medimos su actividad "in vitro" antes y después de la incubación con pßA, con y sin adición de NAD^+. Los resultados obtenidos fueron los mismos que al utilizar homogenados de cerebro humano. Por lo tanto, quedaba validado un sistema para cribar moléculas terapéuticas que pudieran inhibir la interacción pßA-HSD10 y restablecer la actividad HSD10. Se probaron 117 compuestos de una librería peptídica, Coenzima Q10 y otros antioxidantes, pero no se consiguió rescatar la actividad de HSD10. La actividad enzimática sólo se consiguió rescatar con NAD^+. Por lo que proponemos los precursores de NAD^+ como posible terapia coadyuvante para la EA. Estudios recientes apoyan nuestros resultados, ya que la administración de precursores de NAD^+ en ratones modelo para la EA, mejoran la disfunción mitocondrial así como la cognición.

Title: 17β-hydroxysteroid dehydrogenase (HSD10) as a therapeutic target for Alzheimer's disease

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Running title: HSD10 activity as therapeutic target in AD.

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Abstract

17β-hydroxysteroid dehydrogenase type 10 (HSD10) is a mitochondrial multifunctional enzyme and its genetic deficiency is characterized by neurodegeneration in males. HSD10 has also been associated to Alzheimer disease (AD), due to its ability to interact with the amyloid-β peptide (Aβ), suggesting that this interaction is one of the causes of Aβ toxicity and mitochondrial dysfunction in AD. Our aim was to analyze the HSD10 activity as a therapeutic target for AD. We found a decrease of HSD10 activity in AD brain's homogenates compared to controls that is not due to a lower expression of the enzyme. Similarly, the incubation of control brain's homogenates with Aβ inhibited HSD10 activity. This inhibition decreased by increasing NAD^+ concentration, in both AD brain's homogenates and control brain's homogenates exposed to Aβ, implying that HSD10 activity in the presence of Aβ reflects a change in the Aβ-HSD10 interaction. In vitro, using recombinant HSD10 of isoform 1, which is expressed in brain, we set a screening method to rescue HSD10 activity in the presence of Aβ. After testing more than 100 molecules, we found that only NAD^+ was able to rescue HSD10 activity from Aβ inhibition. Therefore, we hypothesize that NAD^+ or their precursors could be a plausible therapy to disrupt Aβ-HSD10 interaction and, consequently, a possible therapy for AD. In support of this tenet, it has recently been reported that treatment of AD mouse model with NAD^+ precursors improves mitochondrial function and cognition.

Keywords: Amyloid-β- peptide, HSD10 deficiency, Alzheimer's Disease, human brain samples, Aβ-HSD10 interaction, NAD^+ precursors.

Introduction

17β-hydroxysteroid dehydrogenase type 10 (HSD10) is a mitochondrial multifunctional enzyme involved in the metabolism of isoleucine and branched-chain fatty acids [1], sex steroid hormones and neuroactive steroids [2], and in the detoxification of cytotoxic aldehydes [3]. HSD10 has also cardiolipin phospholipase activity [4].

HSD10 is encoded by the *HSD17B10* gene that maps to chromosome Xp11.2 [5, 6]. Three isoforms are generated by alternative splicing: the isoform 1 of 261 aminoacids (aa)-ENST00000168216-, the isoform 2 of 252 aa-ENST00000375304- and the isoform 3 caused by exon 5 skipping- ENST00000375298-. Isoform 1 has been detected in lung, while isoform 2 and 3 have been detected in brain and lymphoblasts, being isoform 3 more unstable [7]. On the one hand, mutations in *HSD17B10* gene cause HSD10 deficiency (OMIM 300256), clinically characterized by progressive neurodegeneration in males [1, 8-14], while female carriers present a variety of symptoms: from borderline learning difficulties to psychomotor and speech delay [8, 13]. This variability is due to mosaicism of *HSD17B10* expression [15-17]. On the other hand, mutations in *HSD17B10* are associated to X-linked mental retardation, choreoathetosis and abnormal behaviour (MRXS10) [18], but clinical manifestations of MRXS10 are to some extent similar to the mildest case of HSD10 deficiency [9, 19]. Moreover, it has been described that *HSD17B10* gene is up-regulated in mental retardation [20].

In 1997, Yan et al. established a link between Alzheimer disease (AD) and HSD10 [21]. This protein has the ability to interact with the amyloid-β peptide (Aβ). *In vitro* studies revealed that the addition of Aβ inhibited HSD10 activity [22, 23]. Later on, it was demonstrated that the interaction occurred inside the mitochondria inducing a conformational change in the HSD10 structure which inhibits the binding of NAD^+ to the enzyme, causing generation of ROS and mitochondrial dysfunction [24, 25]. Several studies suggested that this interaction is one of the causes of Aβ toxicity and mitochondrial dysfunction in AD [21, 22, 24, 25-29]. Moreover, in one of our patients with a severe HSD10 deficiency, undetectable levels of Aβ in cerebrospinal fluid (CSF) have been found [30]. This result was in agreement with what is found in AD patients, that is, Aβ is very low in CSF [31, 32]. In addition, it has been reported that HSD10 is very important for mitochondrial integrity and cell survival [14] and is a member of two important mitochondrial complex enzymes: RNaseP [33] and tRNA methyltransferase [34]. Despite all this knowledge, the mechanisms by which neurodegeneration occurs in AD and the way in which Aβ might influence neurodegeneration are poorly understood. Several studies have been focused on the inhibition of Aβ-HSD10 interaction to find a treatment for this devastating disease [21, 24, 27, 29, 35-39].

Based on previous findings and on the development of an *in vitro* methodology for the measurement of HSD10 activity [13], we searched for compounds that rescue catalytic activity in AD brains as a therapeutic target for this neurodegenerative disease.

Materials and Methods

Samples

Occipital and parietal cortex of 25 autopsied AD human brains were classified by stages according to Braak&Braak, 11 samples at initial stages (I-II,III) and 14 samples at final stages (IV, V and VI); 8 age-matched controls were provided by the Neurologic Tissues Bank of Hospital Clinic, Barcelona (Spain). All the samples were taken according to declaration of Helsinki. The age of the individuals ranged from 37 to 87 years. Samples were collected between 2.5 and 24 hours post-mortem. After decapitation, mouse brain samples at 0, 12, 24, 48, 60, 85 and 110 hours post-mortem to study the stability of HSD10 and citrate synthase (CS) were obtained in accord with the guide of the Universidad Miguel Hernández, Elche (Spain).

Human and mouse brain samples were homogenated with 250 mmol/L sucrose, 2 mmol/L EDTA, 10 mmol/L Tris-HCl, and 50 U/mL heparin (SETH) and were centrifuged at 800g during 10 minutes. To prepare an enriched mitochondrial pellet the supernatant was again centrifuged at 10,000g during 10 minutes and was re-dissolved in SETH.

HSD10 activity in brain homogenates

The HSD10 activity was assayed using the synthetic and purified substrate 2-methy-3-hydroxybutyril-CoA (2M3HBCoA) as previously described [13] with some modifications, namely: the mitochondrial enrichment of the homogenates to abolish unspecific cytosolic NADH production, and the measurement of the activity in a spectrophotometer of 96-well plate (BGM-POLSTAR). Due to these modifications intra- and inter-assay precision as well as linearity studies were again measured with different concentrations of total protein, substrate and cofactor (NAD^+). Intra-assay variability was calculated by processing 6 independent samples of brain's homogenate within the same day. Inter-assay variability was calculated by processing 6 independent samples of brain's homogenate in 6 different days.

Western blot analyses

Western-blot analysis was performed as previously described [13], except that the primary antibody was rabbit anti-17βHSD10 (SIGMA) instead of rabbit anti-ERAB (SIGMA).

Amplification of cDNA encoding for human brain HSD10

Total RNA was obtained from a necropsy of a fresh human brain; cDNA of both isoform 1 and 2 of HSD10 were PCR amplified by standard protocols. A specific set of primers (available upon request) were used to be measured by RT-PCR.

HSD10 cloning and expression of recombinant protein.

The cDNA encoding the full sequence of the isoform 1 of HSD10 was cloned into the pET-22b (+) expression vector (Invitrogen) to express the protein with a C- terminal His-tag. The isoform 2 of HSD10 was obtained by inverse PCR from the HSD10/pET-22b (+) construction by removing the sequence ATCCGGGTGATGACCATTGCCCCAGGT. Both constructs were verified by DNA sequencing. His-tagged HSD10 isoforms 1 and 2 were produced in *Escherichia coli* strain BL21. Protein expression was induced with 1 mmol/L isopropylthiogalactoside when the culture reached an OD_{600} of 0.6−0.8 for 24 h at 37°C. Cells were harvested 4 h after induction and centrifuged at 6000 rpm in a Beckman Coulter J2-HS centrifuge for 15 min. Cell pellets were lysed by sonication in PBS, 20 mmol/L imidazole buffer 10 times in ice with bursts of 45 s at maximal power (model 102-C, Branson), interleaved with 15 s in ice. The homogenate was centrifuged at 15000 rpm for 50 min in a Beckman Coulter J2-HS centrifuge. His-tagged recombinant proteins were purified from cell lysate supernatants by affinity chromatography. Supernatants were incubated with a Ni-NTA Agarose matrix (Qiagen) during 4 h at 4ºC with constant shaking. After washing, pure recombinant HSD10 protein was eluted with 150 mmol/L imidazole, 10% glycerol and PBS. Immediately after purification, the protein was subjected to a buffer change by dialysis using dialysis tubes (MWCO 12kDa, Sigma-Aldrich) in PBS with 10% glycerol to prevent precipitation. The protein purity was higher than 95%, as judged from SDS−PAGE gels. Protein concentration was determined after dialysis by the BCA method (Thermo Fisher Scientific) and aliquots were stored at -20ºC.

Study of the functionality of both recombinant isoforms

We analysed HSD10 activity of isoform 1 and isoform 2 and calculated the Km using three different substrates: 2M3HBCoA (synthesized by the group of Angel Messeguer, IQAC-CSIC, Barcelona), 17β-estradiol (SIGMA) and allopregnanolone (SIGMA) at different substrate concentrations: 100, 200, 400, 800, 1000 and 1200 µmol/L.

Inhibition of HSD10 activity by addition of Aβ

We analysed HSD10 activity in control brain's homogenates with the addition of different concentration of Aβ 1-40: $Aβ_{40}$ (BACHEM) (0.1, 0.2, 0.5, 1, 2, 5, 10 and 50 µmol/L) without previous incubation and pre-incubating at different times (0.5, 1, 3, 24 hours) at 25ºC. Linearity studies of the enzymatic activity of the recombinant protein at several protein concentrations (0.01, 0.002, 0.005, 0.01, 0.02, 0.03, 0.05, 0.1 mg/mL) were performed. Inhibition of HSD10 activity was studied at different $Aβ_{40}$ concentrations (0, 6, 12, 24, 50, 100, and 150 µmol/L). We also assayed HSD10 activity at different NAD^+ concentrations (2, 4, 8 and 16 mmol/L) in both, brain's homogenates and HSD10 recombinant protein (isoform 1).

Statistical methods

Statistical studies were performed using a non-parametric two independent sample test (U Mann Whitney), by SPSS® software v.16. Results were deemed significant when p-value was <0.05.

Results

Stability of HSD10 activity in post-mortem brain homogenates

The optimal experimental conditions to monitor HSD10 enzymatic activity within a linear range were: 0.3 mg/mL of total protein, 2 mmol/L of substrate and 2 mmol/L of NAD^+. The variation coefficients (CV) of intra- and inter-assays were 7% and 10%, respectively.

Next we performed stability studies, of both HSD10 and CS activities, in mice brain homogenates at different post-mortem hours (Fig. 1A and Fig 1B). CS was used as a control of the mitochondrial mass. As depicted in the Figure 1, HSD10 activity remained stable at least until 48 hours post-mortem, while the activity of CS was stable for longer time. In support of this result, statistical analysis using the non-parametric U Mann-Witney test did not show any significant difference in enzymatic activity at the post-mortem times tested.

HSD10 and CS activities in human AD brains

We had the opportunity to study HSD10 activity in occipital and parietal cortex of 25 AD brain samples. We did not observe any difference in the enzyme activity in these brain areas (data not shown). HSD10 activity was significantly lower in all AD stages, being more evident at advanced AD stages, as compared with controls. P-values for the HSD10 activity between AD and control brain homogenates, obtained with the non-parametric U Mann-Witney test, were 0.021 and 0.001 for initial and advanced stages, respectively (Figure 2A). CS activity did not differ significantly at initial stages, but it was lower at advanced stages (p-value was 0.07) (figure 2B).

HSD10 inhibition of control brain homogenates by the addition of Aβ.

When brain homogenates from controls were pre-incubated with 50 µmol/L $A\beta_{40}$ for 30 min at 25ºC, a 40% inhibition of HSD10 enzymatic activity was achieved. Degradation of the protein by incubation was ruled out because we did not observe significant differences when HSD10 was measured before or after pre-incubation with $A\beta_{40}$ vehicle (Figure 3).

NAD^+ rescues HSD10 activity inhibited by $A\beta_{40}$

We next evaluated the effect of NAD^+ on the HSD10 activity in control brain homogenates and upon exposure to $A\beta_{40}$, as well as on AD brain (Stage VI) homogenates. We investigated the effect of increasing the NAD^+ concentration (Figure 4). As seen, NAD^+, between 1 to 4 mmol/L, significantly increased HSD10 activity in all homogenates. Notably, in

AD homogenates the NAD^+ effect was not saturated and linearly increased between 4 to 16 mmol/L. At 1 mmol/L of NAD^+, HSD10 activity in AD homogenates was 0.5 nmol/mg prot x min (16% of that in control brain homogenates), while at 16 mmol/L NAD^+ the activity reached 1.5 nmol/mg prot x min, which represents a 36% of that present in control brain homogenates under the same conditions and 50% of control brain homogenates in the absence of NAD^+. Therefore, these findings indicate that NAD^+ was able to rescue HSD10 activity in AD brain homogenates, implying that NAD^+ may directly or indirectly disrupt the Aβ-HSD10 interaction leading to reactivation of HSD10 activity in AD brains. The higher concentration of NAD^+ needed by AD homogenates suggest a high Aβ content in these brains.

It should be noted that the lower HSD10 activity in AD brain homogenates is not due to a lower expression of the enzyme as we did not find differences in enzyme protein levels in the parietal and occipital cortex of AD brains compared to controls (Figure 5).

Functionality of HSD10 isoforms 1 and 2

HSD10 has two predominant isoforms (1 of 261 amino acids and 2 of 252 amino acids). Thus, we wanted to check if both enzyme isoforms were expressed in brain homogenates. For this task, we collected the mRNA from control brain homogenates and, using conventional PCR, we obtained cDNA that was sequenced. This strategy only rendered transcripts of isoform 1. Because isoform 2 was previously detected in brain homogenates [7], we questioned that our approach may have not the sufficient sensitivity to detect isoform 2 that have low expression. Thus, we next used RT-qPCR and were able to amplify both isoforms although isoform 1 was much more abundant than isoform 2. Both isoforms were cloned for enzymatic characterization.

The activity of the two isoforms with three different substrates was analyzed and the corresponding Km was obtained (Table 1). Results showed that isoform 2 was not functional when using 2M3HBCoA as a substrate, but it was functionally active when using 17β-estradiol and allopregnanolone, although with lower affinity than isoform 1, which was functional with the three substrates tested (Table 1).

Setting up a method to screen for possible therapeutic molecules for AD using the recombinant isoform 1 of HSD10 *protein.*

Having cloned the HSD10 enzyme from human brain, we reasoned that we could produce the recombinant protein to set a screening method suitable for searching disrupters of the Aβ-HSD10 complex, thus rescuing the enzymatic activity. We next produced and purified recombinant HSD10 isoform 1. This enzyme was active and sensitive to Aβ inhibition in a dose-dependent manner (Figure 6). The optimal linearity, when using 2M2HBCoA as substrate, was obtained at 0.02 mg/mL of protein. The best inhibition condition (80%) was obtained with 50

μmol/L $A\beta_{40}$ at a pre-incubation temperature of 4ºC for 30 minutes (figure 6). Under these conditions the Z-factor was 0.82, which indicates that this assay is appropriate for screening compounds.

To validate the method for compound screening and identification of potential therapeutic molecules, we first investigated if NAD^+ was able to rescue the activity of HSD10 inhibited by $A\beta_{40}$. As illustrated in Figure 7, NAD^+ was able to stimulate the enzymatic HSD10 activity of the recombinant protein in the presence $A\beta_{40}$ (Figure 7). Therefore, the designed screening method is able to detect promotors of HSD10 function.

Screening of a small peptide library (117 compounds, some of them synthesized by BCN PEPTIDES and Diverdrugs), along with coenzyme Q10 and other antioxidants did not result in restoration of HSD10 activity. Nonetheless, the screening method proves to be potent, sensitive and efficacious. It is anticipated that the screening of large chemical libraries will render the identification of active molecules.

Discussion

The intensive research directed towards the relationship between the moonlighting mitochondrial enzyme, HSD10, and AD pathogenesis are based on: i) the overexpression of HSD10 observed in human and murine AD brains [21, 26, 40-43]; and, ii) the finding that Aβ binds to HSD10 in mitochondria inhibiting its enzymatic activity, which promotes the generation of ROS, mitochondrial dysfunction, and causes neurotoxicity in AD brains [21-25]. Therefore, these findings indicate that HSD10 is centrally involved in Aβ-induced cell stress. In support of this tenet, transgenic mice overexpressing Aβ protein precursor (AβPP) and HSD10 in neurons show an increased generation of ROS, reduced production of ATP and reduced glucose usage. In addition, they also exhibit exaggerated spatial learning/memory deficit and neuropathological changes [25]. Therefore, it appears that disruption of Aβ-HSD10 complex may be an important therapeutic approach for AD [29, 44].

Contrary to other authors [21, 42], we did not observe an over-expression of HSD10 protein in AD brains. However, it would be in line with other studies showing that HSD10 is not over-expressed in brains of patients with AD [41]. Probably, all depends on the brain area studied, since in hippocampus of AD patients there is a lateralization of expression HSD10, noting increased expression of HSD10 on the left side of the brain [43].

A salient contribution of this report is that we investigated the activity of HSD10 in post-mortem human brain homogenates from AD patients and the consequences of exposing control brain homogenates to Aβ. We found that the enzymatic activity of HSD10 was notably reduced in human AD brain homogenates, from the initial stages of the disease, in line with these

observations, studies in transgenic mAβPP mice show that Aβ is first detectable inside the mitochondria before any significant extracellular deposition of Aβ occurs, and it seems that the mitochondrial dysfunction occurs at early stages of AD [45]. In addition, it has been described that the increased oxidative damage is an early event [46]. CS activity, used as a control of the mitochondrial mass, was only significantly low at the final stages. A decrease of CS has been also found in primary cortical neurons exposed to Aβ [47, 48]. Furthermore, the 40% decrease HSD10 activity observed in control homogenates exposed to the $A\beta_{40,}$ supports the hypothesis that the cause of HSD10 activity inhibition is an excessive Aβ content [22, 23, 29, 49].

On it has been reported that the interaction of HSD10 with Aβ inside the mitochondria inhibits the *in vitro* HSD10 activity by inducing a conformational change in HSD10 structure, which in turn inhibits the binding of its cofactor NAD^+ [24]. Thus, we reasoned that an increment of NAD^+ may disrupt the Aβ-HSD10 complex. Noteworthy, we found that HSD10 activity in the presence of Aβ could be significantly rescued by NAD^+. We observed an increase of HSD10 activity upon increasing the NAD^+ concentration in control and AD brain homogenates, as well as in control homogenates exposed to the Aβ. Interestingly, at high NAD^+ concentrations the increase of HSD10 activity in brain homogenates of AD continues to progress, while that of control brains remains unchanged. These results indicate that more NAD^+ is needed to stimulate the HSD10 activity in AD brains, probably due to the high Aβ content in these brains that inactivate the enzyme. Similar *in vitro* results were reported by nuclear magnetic resonance, where an increase of NAD^+ concentration inhibited the Aβ-HSD10 interaction [27]. Taken together, these findings suggest that disruption of Aβ-HSD10 interaction and rescue of HSD10 activity could be a therapeutic strategy for AD.

Based on the previous results, we decided to establish an in vitro therapeutic model to test compounds able to disrupt the Aβ-HSD10 interaction and rescue the enzymatic activity. First of all, we cloned and characterized the two most important isoforms of HSD10, namely isoform 1 of 261 amino acids, and isoform 2 of 252 amino acids. We found both isoforms in human brain, although isoform 1 was the most abundant, supporting the results of He XY et al. 1998 [5]. Recombinantly produced isoform 1 was active with the three substrates tested, i.e.: 2M3HBCoA, 17β-estradiol and allopregnanolone, showing the highest affinity for 2M3HBCoA. In marked contrast, isoform 2 was not functionally active when using 2M3HBCoA as substrate, and it showed significantly lower activity than isoform 1 with the other two substrates. Furthermore, we found that addition of Aβ to the recombinant isoform 1 inhibited 80% its enzymatic activity. Aβ-induced HSD10 activity of isoform 1 was rescued by NAD^+ incubation. Therefore, isoform 1 was

selected for setting an in vitro assay suitable for screening compound libraries using 2M3HBCoA as a substrate.

We tested the designed in vitro screening assay to test a set of 117 peptidic compounds, coenzyme Q10 and other antioxidants, but none of them were able to rescue HSD10 activity.

Previous studies support NAD^+ precursors as a new therapeutic opportunity for aging and its associated disorders, particularly neurodegenerative diseases [50]. NAD^+ plays a role in all cells as a substrate for signal transduction and as a cofactor in metabolic redox reactions. Nicotinic acid (NA) or nicotinamide (NAM), both present in the diet as vitamin B3, are the primary source of NAD^+. Other NAD^+ precursors such as nicotinamide riboside (NR) and nicotinamide mononucleotide (NMN) have been described to increase NAD^+ levels in various cell types and tissues of mice resulting in SIRT1 activation [51, 52]. Recently, it has been reported that the NMN–treated AD transgenic mice showed a decrease of AβPP levels, an increase of SIRT1, and an improvement of the mitochondrial morphology [52]. In addition, it has been demonstrated NMN is able to cross the blood-brain barrier and to be converted into NAD^+, thus increasing cellular NAD^+ levels in the brain [53]. Interestingly, in a cell culture model of Parkinson's disease, NMN intervention reduced apoptosis and restored intracellular levels of NAD^+ [54]. All these evidences, along with our findings indicate that NAD^+ or its precursors could be a possible therapy for AD that merits in vivo exploration.

HSD10 is a very important protein for mitochondrial integrity and cell survival [14] and is a member of two enzymatic complexes: mitochondrial RNaseP [33] and mitochondrial tRNA methyltransferase [34]. These authors reported that only the presence of HSD10, and not its enzymatic function, is the relevant fact. Moreover, they demonstrated that the inhibition of RNaseP and tRNA methyltransferase activity by Aβ is not mediated by Aβ-HSD10 interaction [49]. On the contrary, other studies supported that inhibition of HSD10 activity can disturb its cytoprotective role on neurons in response to cell stress [40, 55], or inhibit the detoxification of aldehydes [3], or interfere the effect of estrogens in neuroinflammation [2, 22, 42]. Such inhibition may also decrease the metabolism of peroxidized cardiolipin, which trigger cell apoptosis [4]. Moreover, patients with HSD10 deficiency present neurodegeneration independently if the protein is present or not [13], which strengthens the importance of HSD10 activity in neurodegeneration. In fact, a patient with HSD10 deficiency and severe neurodegeneration showed normal protein levels in fibroblasts (patient 6 in [14]) and absence of Aβ protein expression in CSF [30]. Similarly, Aβ protein is low in CSF of AD, and previous studies suggest that Aβ and AβPP may play important roles in the development, such as promoting synapse elimination [56, 57]. Probably the apparent capacity of HSD10 to bind and

trap Aβ protein could be explained by similar mechanisms both in HSD10 deficient patients and in patients with AD. It might be that the trapping of HSD10 by Aβ might also disrupt non-enzymatic functions of HSD10 that are required for mitochondrial integrity, as well as alter the binding of HSD10 to other proteins, as ciclophyline D, which would cause an increase in mitochondrial permeability and apoptosis [26], or to the α estrogen receptor [58] or to the postsynaptic density complex (PSD) involved in the maintenance of synaptic function [59]. In summary, we found that HSD10 activity is low in AD brain homogenates and that its activity can be rescued by NAD^+. We also observed that HSD10 activity decreases with the progression of the disease. Therefore, HSD10 activity should be taken into account as one of the targets to find a treatment for the mitochondrial dysfunction of AD.

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	Substrates		
	2-methyl-3-hydroxybutyril-CoA	17β-estradiol	Allopregnanolone
Isoform 1 (Km)	160	275	265
Isoform 2 (Km)	Non functional	490	300

Table 1. Km values of isoform 1 and 2 of 17β-hydroxysteroid dehydrogenase type 10 (HSD10) with different substrates. Values are the mean of duplicates.

Figure legends

Figure 1. Stability of 17β-hydroxysteroid dehydrogenase type 10 (HSD10) (A) and citrate synthase (CS) (B) activities in post-mortem mouse brains. Values are the mean of 3 independent samples. The error bars represent the standard deviation.

Figure 2. 17β-hydroxysteroid dehydrogenase type 10 (HSD10) (A) and citrate synthase (CS) (B) activities in parietal and occipital cortex of human brain of initial and final AD stages. Values are the mean of samples from different patients. The error bars represent the standard deviation. n= number analyzed of each group. *: Mann Whitney U test, p-value: 0.01-0.05; **: Mann Whitney U test p-value: <0.01.

Figure 3. Inhibition of 17β-hydroxysteroid dehydrogenase type 10 (HSD10) activity by the addition of Aβ to control brain homogenates. Values are the mean of duplicates.

Figure 4. Comparative studies of 17β-hydroxysteroid dehydrogenase type 10 (HSD10) activity in control brain homogenate ( ), the same homogenate with 50 µM Aβ incubation ( ) and AD brain homogenate at stage VI ( ) incubated with different NAD^+ concentrations. Values are the mean of duplicates.

Figure 5. Western-blot studies of 17β-hydroxysteroid dehydrogenase type 10 (HSD10) in parietal (A) and occipital cortex (B) of human AD brains of different stages.

Figure 6. Inhibition of the recombinant 17β-hydroxysteroid dehydrogenase type 10 (HSD10) activity with different concentrations of Aβ. Values are the mean of duplicates.

Figure 7. Recombinant 17β-hydroxysteroid dehydrogenase type 10 (HSD10) protein, measurement of the enzymatic activity with and without 50 µM Aβ incubation and, rescue of the activity by the addition of NAD^+. Values are the mean of duplicates.

Figure 1.

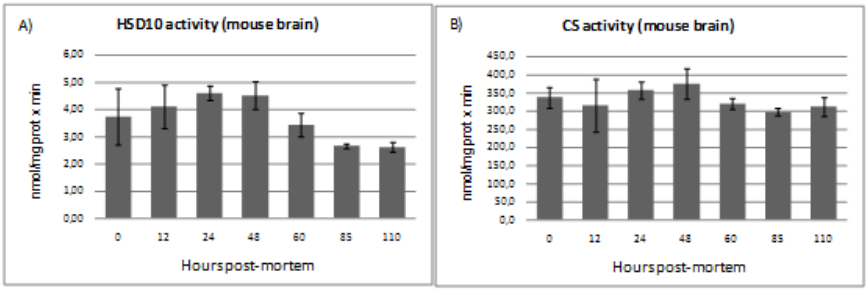


Figure 2.

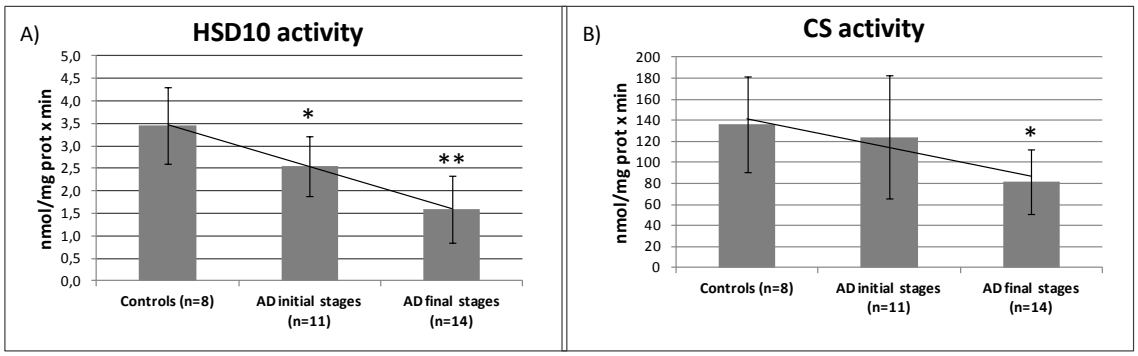


Figure 3.

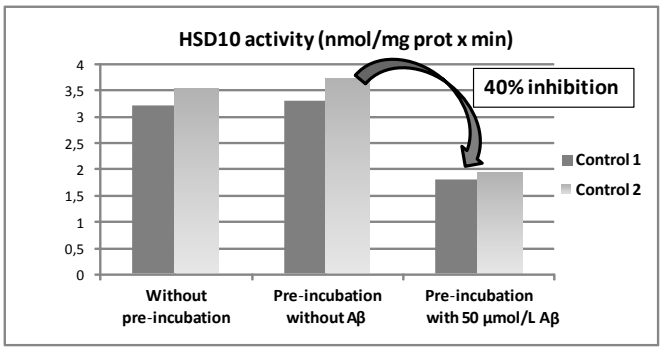


Figure 4.

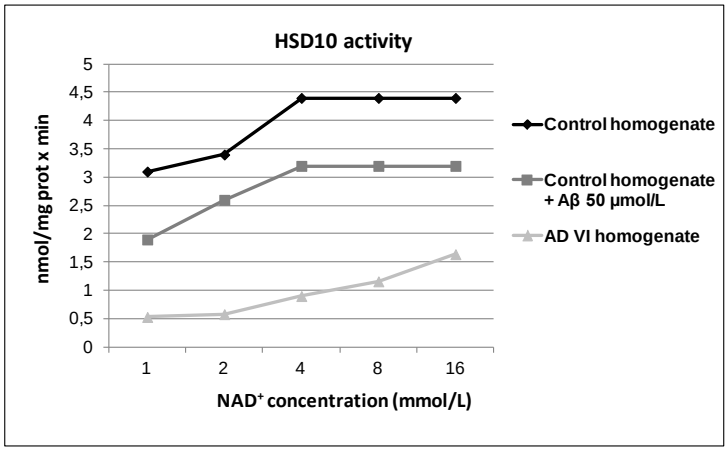


Figure 5.

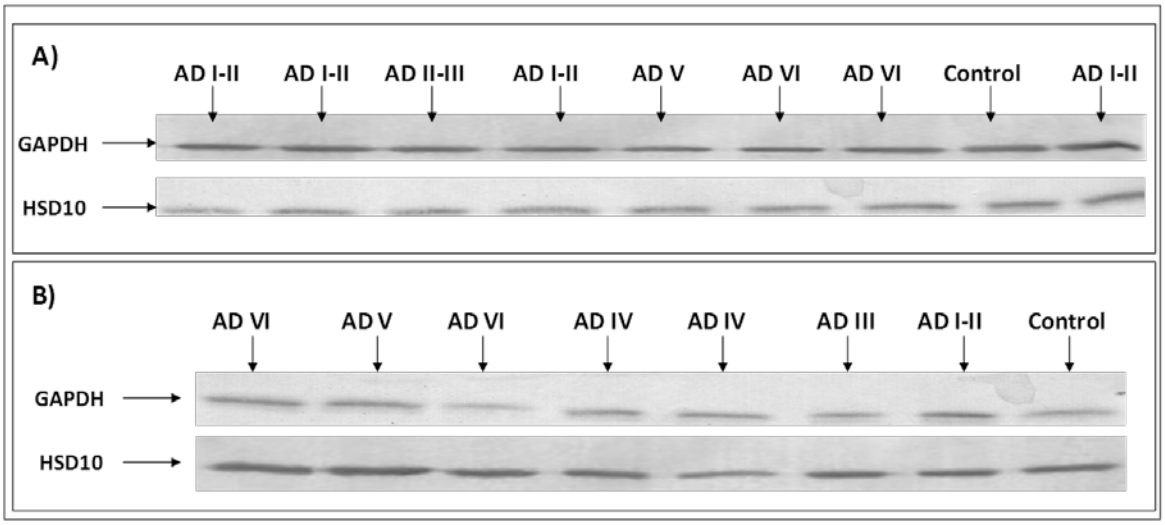


Figure 6.

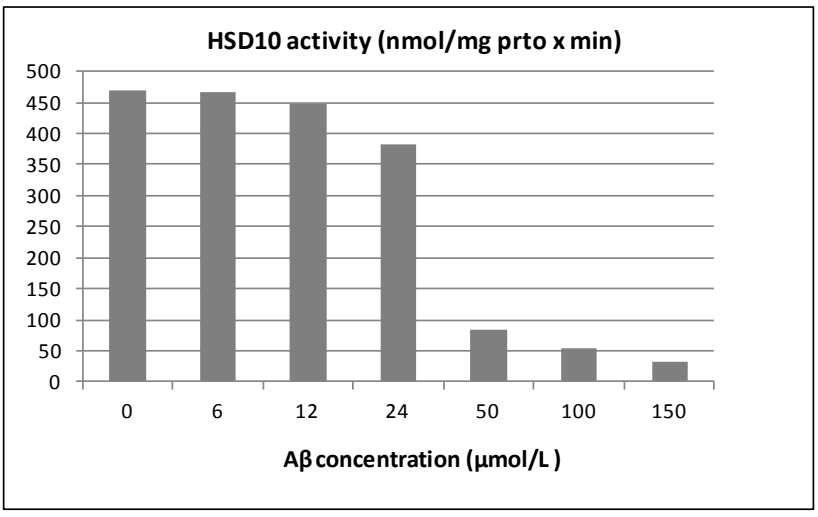
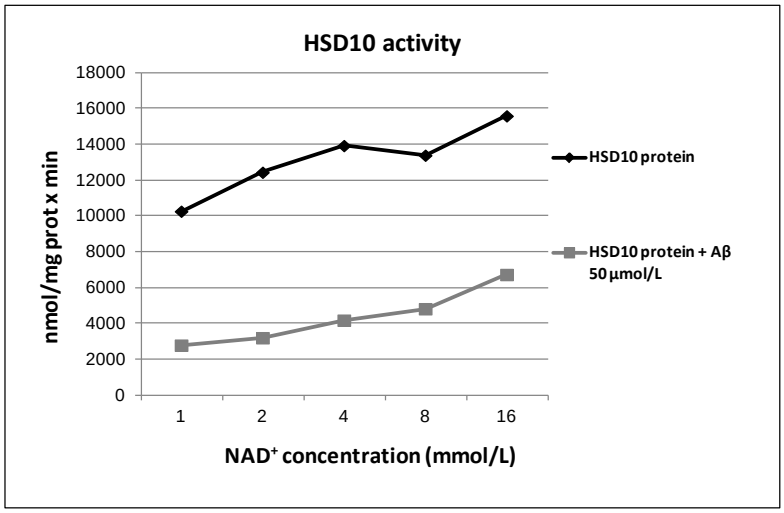


Figure 7.


HSD10 activity
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
nmol/mg prot x min
1
2
4
8
16
NAD+ concentration (mmol/L)
HSD10 protein
HSD10 protein + Aβ 50 µmol/L

RESUMEN
Y DISCUSIÓN GENERAL

1. ESTUDIO DE PACIENTES CON DEFICIENCIA DE HSD10

La deficiencia de HSD10 es una EMH que se enmarca en el metabolismo de la isoleucina y de los ácidos grasos de cadena ramificada (OMIM 300438). Esta enfermedad fue descrita por primera vez en el año 2000 por Zschocke et al. Clínicamente se caracteriza por neurodegeneración progresiva, que en general se inicia entre los 6-18 meses de vida. Su diagnóstico se lleva a cabo mediante el estudio de ácidos orgánicos en orina, mostrando una elevada excreción de ácido 2-metil-3-hidroxibutírico (2M3HBA) y tiglilglicina (TG). Posteriormente se confirma mediante estudio de la actividad enzimática HSD10 o mediante análisis molecular del gen *HSD17B10*. En la literatura sólo se han descrito 14 familias (15 hemicigotos y 6 heterocigotas) (Zschocke et al., 2000; Ensenauer et al., 2002; Olpin et al., 2002; Poll The et al., 2004; Sass et al., 2004; Sutton et al., 2003; Rauschenberger et al., 2010; Seaver et al., 2011; Zschocke, 2012; Fukao et al., 2014; Chatfield et al., 2015). En el transcurso de este estudio hemos realizado el diagnóstico de 6 familias españolas con dicha deficiencia. Para ello, tuvimos que desarrollar un método cuantitativo de los metabolitos clave en orina mediante cromatografía de gases-espectrometría de masas y un método para análisis enzimático de HSD10 en fibroblastos cultivados mediante espectrofotometría.

1.1.- Análisis de metabolitos

Entre los pacientes estudiados sólo 6 (5 varones y una mujer) mostraron un incremento significativo de 2M3HBA y TG y fue en estos 6 pacientes, en los que se confirmó la enfermedad mediante la determinación enzimática de HSD10 y el estudio molecular del gen *HSD17B10 (*tabla 1). Por tanto, la cuantificación de estos metabolitos nos permitió realizar una buena aproximación diagnóstica. La primera familia se describe en el **artículo 1** y el resto de familias en el **artículo 2**.

Cabe destacar que la dieta que siguen los pacientes puede dificultar el diagnóstico. Uno de los pacientes presentaba sólo un ligero incremento de estos metabolitos (paciente 6, tabla 1). Posteriormente, nos comunicaron que dicho paciente recibía una dieta hipo-proteica, pero al analizar una muestra tras un aporte proteico elevado se evidenció el defecto. Así, proponemos que antes de descartar una deficiencia de HSD10 es importante asegurar que el paciente siga una dieta normal y ante una sospecha clínica fundada es recomendable proseguir el estudio con la determinación de la actividad enzimática o con el estudio mutacional (**artículo 2**).

Tabla 1. Resultados bioquímicos y genéticos de los pacientes con deficiencia de HSD10.

Pacientes	Lactato en sangre (mmol/L)	Ácidos orgánicos (mmol/mol creatinina)			Actividad HSD10 (nmols/mg prot/min)	Genotipo	Efecto en la proteína
		2M3HBA	TG	EHA			
Paciente 1.1 (M)	8	NR	NR	NR	0.22	c.[740A>G]	p.Asn247Ser
Paciente 1.2 (F)	3	99	21	↑	0.27	c.[740A>G] + [=]	p.[Asn247Ser] +[=]
Paciente 2 (M)	7.6	49	154	↑	0.05	c.[388C>T]	p.Arg130Cys
Paciente 3 (M)	8.3	262	59	50	NR	c.[628C>T]	p.Pro210Ser
Paciente 4 (M)	↑	116	181	12	0.05	c.[388C>T]	p.Arg130Cys
Paciente 5 (M)	19	323	168	↑	0.27	c.[677G>A]	p.Arg226Gln
Paciente 6 (M)	↑	54	30	18	0.5	c.[628C>T]	p.Pro210Ser
Madre Paciente 6	NR	NR	NR	NR	1.4	c.[628C>T] + [=]	p.[Pro210Ser] + [=]
Valores de referencia	<2 (n=50)	<12 (n=50)	< 5 (n=50)	<7 (n=50)	Media = 1.4 (DS= 0.43; n=8)		

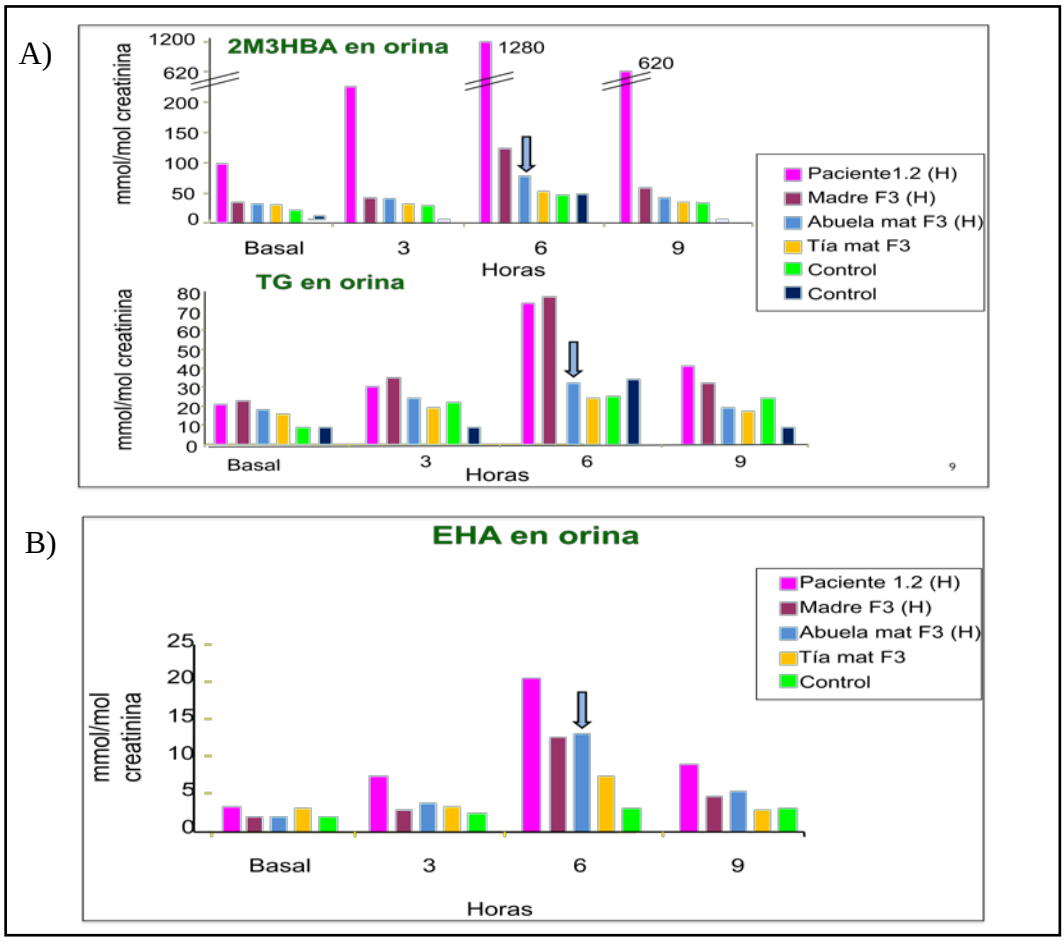
2M3HBA: ácido 2-metil-3-hidroxibutírico; EHA: ácido etilhidracrílico; F: sexo femenino; M: sexo masculino; NR: No realizado; SD: desviación estándar; TG: tiglilglicina; ↑: elevación comprobada solo cualitativamente.

Dado que la herencia de la enfermedad está ligada al cromosoma X, podría ocurrir que ante una lionización favorable, las portadoras presentaran una excreción más leve, de los metabolitos clave, que los varones hemicigotos. Este fue el caso de la paciente 1.2 (tabla 1). Por ello y por la experiencia anterior (Zschocke et al., 2000; Ensenauer et al., 2002), se decidió realizar un test de sobrecarga de isoleucina en 3 heterocigotas, pero este test fue negativo en una de las heterocigotas de la familia 3 (figura 4A).

Por otro lado, se había descrito que el ácido etilhidracrílico (EHA) se encontraba aumentado en esta enfermedad, ya que es un intermediario de la oxidación de isoleucina (Mamer et al., 1976). Al analizar retrospectivamente los resultados, observamos que tras la sobrecarga se evidenciaba

un incremento de dicho ácido en todas las heterocigotas (figura 4B), así como también en los casos índice previamente diagnosticados (tabla 1). Por tanto, la valoración del EHA junto con 2M3HBA y TG, puede ser de ayuda para el diagnóstico de portadoras. Un hecho similar sucede en otras enfermedades con herencia ligada al cromosoma X. Así en la deficiencia de ornitina transcarboamilasa, el test de sobrecarga de Allopurinol no siempre es positivo en las heterocigotas, pero la valoración del ácido orótico además de la oritidina aporta a la técnica una mayor sensibilidad (Grünewald et al., 2004).

Figura 4. Resultados de la valoración de ácidos orgánicos tras una sobrecarga de isoleucina.
A) Ácido 2-metil-3-hidroxibutírico (2M3HBA) y tiglilglicina (TG).
B) Ácido etilhidracrílico (EHA).



F: familia; H: Heterocigota; mat: materna.

Dada la necesidad de encontrar nuevos biomarcadores para esta entidad, decidimos estudiar el perfil de acilcarnitinas en 7 muestras de orina de nuestros pacientes. Se había descrito que los pacientes con deficiencia HSD10 podían presentar un aumento de 2-metil-3-hidroxi-

butirilcarnitina (C5-OH) y de tiglilcarnitina (C5:1) en plasma (Zschocke et al., 2000; Ensenauer et al., 2002; Sass et al., 2004; Poll The et al., 2004). Sin embargo, estas alteraciones plasmáticas no se observaron en ninguno de nuestros pacientes. Dada nuestra experiencia previa en la aciduria glutárica tipo I y dados los resultados publicados por Tortorelli et al., 2005 sobre esta entidad, decidimos valorar el perfil de acilcarnitinas en orina mediante espectrometría de masas en tándem (HPLC-MS/MS). Se analizaron las muestras de orina de 4 pacientes con dieta estándar, 2 pacientes con dieta restringida en isoleucina y 1 paciente tras una dieta rica en proteínas (tabla 2). Los 4 pacientes (3 varones y una mujer) mostraron un incremento significativo de la acilcarnitina C5:1. Los pacientes que presentaban una marcada excreción de 2M3HBA y TG, también presentaban una mayor excreción de C5:1. Después de una dieta restringida en isoleucina la acilcarnitina C5:1 permanece aumentada (tabla 2). Cabe señalar que en la muestra del paciente 6 con dieta hiperproteica el incremento de C5:1 fue mucho más evidente (tabla 2).

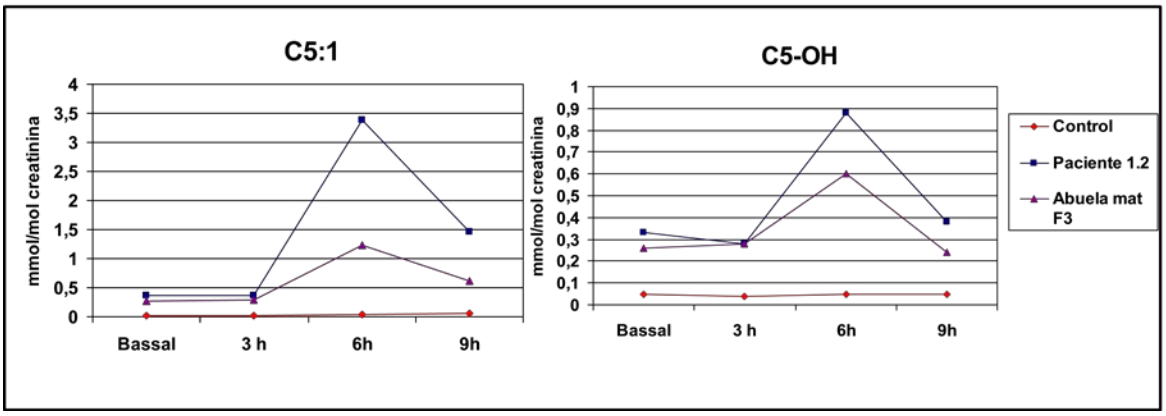
Tabla 2. Resultados del análisis de acilcarnitinas en orina comparado con los resultados de ácidos orgánicos.

	Ácidos orgánicos en orina (mmol/mol creatinina)			**Acilcarnitinas en orina (mmol/mol creatinina)**	
	2M3HBA	**TG**	**EHA**	**C5:1**	**C5:OH**
Pacientes pre-tratamiento					
Paciente 1.2 (F)	99	21	Aumentado	2.05	1.05
Paciente 3 (M)	262	59	Aumentado	10.2	3.4
Paciente 4 (M)	116	181	12	5.89	0.86
Paciente 6 (M)	54	30	18	1.31	0.56
Pacientes post-tratamiento					
Paciente 1.2 (F) (Restricción isoleucina)	27	Indetectable	Normal	0.96	0.58
Paciente 4 (M) (Restricción isoleucina)	42	49	1.8	0.52	0.19
Paciente 6 (M) (Aporte proteico)	48	33	17	1.97	1.07
Media de los controles Valores de referencia	<12 (n=50)	<5 (n=50)	<7 (n=50)	0.17 0.02 - 0.42 (n=33)	0.26 0.02 – 1.21 (n=33)

2M3HBA: ácido 2-metil-3-hidroxibutírico; C5:1: tiglilcarnitina; C5:OH: 2-metil-3-hidroxibutirilcarnitina; EHA: ácido etilhidracrílico; F: sexo femenino; M: sexo masculino; TG: tiglilglicina.

El análisis de acilcarnitinas tras sobrecarga de isoleucina en las muestras de orina de las portadoras (Figura 5) también fue positivo incluso en una de ellas en que el test fue negativo con la cuantificación de ácido orgánicos.

Figura 5. Valoración de tiglilcarnitina (C5:1) y 2-metil-3-hidroxibutirilcarnitina (C5OH) en orina tras una sobrecarga de isoleucina.



Mat:materna; F: familia.

Así, la acilcarnitina C5:1 en orina, se ha encontrado significativamente elevada en todas las condiciones estudiadas, por ello proponemos su valoración como nuevo marcador bioquímico para esta enfermedad, que junto con el perfil de ácidos orgánicos en orina pueden contribuir a mejorar el diagnóstico de esta entidad (**artículo 3**).

Por otro lado, la presentación clínica y bioquímica de los pacientes con deficiencia de HSD10 puede mimetizar una enfermedad mitocondrial. Así pues, la heterocigota que diagnosticamos en el presente trabajo (paciente 1.2 del **artículo 1)**, presentó un perfil de ácidos orgánicos que inicialmente sugirió una deficiencia del complejo I de la cadena respiratoria mitocondrial, ya que en esta entidad se ha descrito un perfil de ácidos orgánicos similar (Bennett et al., 1993), pero el estudio de las actividades de la cadena respiratoria en músculo fue normal. A posteriori, se orientó el diagnóstico hacia una deficiencia de HSD10 y se realizó un test de sobrecarga de isoleucina con lo que se evidenció el defecto, que fue confirmado mediante estudio enzimático y molecular (tabla 1). La historia familiar de esta paciente (hermano fallecido a los dos meses de vida por una acidosis láctica severa) dio pié a pensar que su hermano debía tener el mismo diagnóstico. Esta sospecha se confirmó por el estudio enzimático y molecular en la necropsia (paciente 1.1, tabla1). Este y otros estudios (Ensenauer et al., 2002; Olpin et al., 2002, Rosa et al, 2005), indican que la deficiencia de HSD10 podría mimetizar una enfermedad mitocondrial, por

lo que es necesario incluir dicha deficiencia en el diagnóstico diferencial de los trastornos de la cadena respiratoria mitocondrial (**artículo 1**).

El tratamiento para esta entidad consiste básicamente en una dieta hipoproteica o bien restringida en isoleucina. La evolución de los pacientes después de dicho tratamiento no ha sido favorable (Zschocke et al., 2000; Ensenauer et al., 2002; Olpin et al., 2002; Sutton et al., 2004). Es muy posible que la acidosis láctica que presentan estos pacientes pudiera estar involucrada en la etiopatogenia de esta enfermedad y quizás el tratamiento con aceptores de electrones, podría ser beneficioso (**artículo 1**). El grupo del Dr.Zscocke (Rauschenberger et al., 2010) apoya nuestra sugerencia de tratar a los pacientes con cofactores y vitaminas, especialmente en periodos de fiebre e infecciones, para mantener la homeostasis mitocondrial.

1.2.-Actividad HSD10 en fibroblastos y búsqueda de mutaciones en el gen *HSD17B10*

La confirmación diagnóstica de la deficiencia de HSD10 se puede llevar a cabo mediante la determinación enzimática. Se han descrito dos métodos, dependiendo de si se utiliza como sustrato tiglil-CoA (Zschocke et al., 2000) o acetoacetil-CoA (Ofman et al., 2003), que son los dos únicos sustratos comerciales. Uno de nuestros objetivos consistió en poner a punto de la determinación de la actividad enzimática de HSD10 en fibroblastos cultivados con el sustrato directo: 2-metil-3-hidroxibutiril-CoA (2M3HBCoA), con el fin de poder ofrecer un diagnóstico más preciso y realizar otros estudios como veremos más adelante. Este sustrato lo sintetizó el grupo del Dr. Messeger del CSIC de Barcelona.

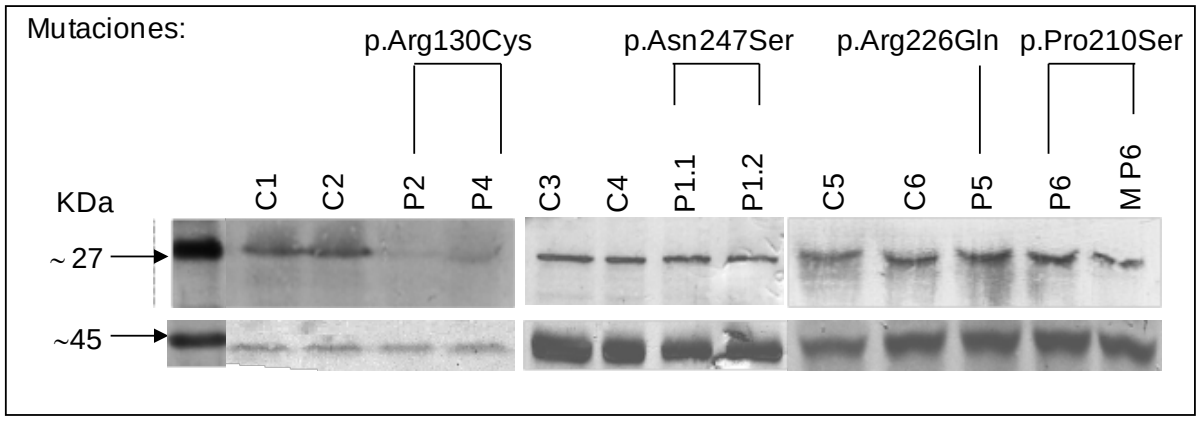
La metodología para la valoración enzimática fue fundamentalmente la descrita por Wanders et al., 2001 aunque utilizando 2M3HBCoA como sustrato lo que supuso varias modificaciones. La reacción enzimática fue lineal en un rango de 0,3-0,5 mg/mL y la precisión intra-día e inter-día fue 7% y 10% respectivamente. Se valoró la actividad enzimática en fibroblastos cultivados de 6 pacientes (5 varones y 1 mujer) y de una madre heterocigota (tabla 1). Todos los pacientes mostraron una clara deficiencia de HSD10 (<35 % de actividad residual), exceptuando la madre heterocigota que presentó una actividad normal.

Se realizó el estudio molecular en los 6 pacientes (5 varones y una mujer) y detectamos 4 mutaciones (tabla 1), tres de ellas nuevas (p.Pro210Ser, p.Ans247Ser y p.Arg226Gln) así como otra mutación descrita previamente: p.Arg130Cys. Todos los pacientes excepto uno, heredaron la mutación de sus madres. En los estudios familiares se detectaron otras 7 portadoras, las cuales presentaban un retraso mental leve o dificultades en el aprendizaje (**artículo 2**).

Se ha descrito que la mutación p.Arg130Cys es la más prevalente en esta enfermedad. Este hecho probablemente es debido a que la citosina en esa posición se encuentra metilada dando

lugar a 5-metilcitosina, y ésta tiene tendencia a convertirse en timina por desaminación, lo que provoca que sea un "hotspot". Además el cambio de arginina por cisteína en la posición 130 de la proteína elimina puentes de hidrógeno y reduce la interacción de "van der Waals" entre subunidades HSD10, lo que explicaría la baja actividad detectada en los pacientes con dicha mutación (Yang et al., 2013) y lo que provocaría una inestabilidad de la proteína, siendo esta mutación la única que prococa una reducción de los niveles de HSD10 (figura 6). De la misma forma Ofman et al., 2003, observaron también disminución de proteína en otros pacientes con la misma mutación y en los estudios de expresión.

Figura 6. "Western blot2 en fibroblastos cultivados de pacientes con deficiencia de HSD10.



C:control; P:paciente; MP: madre de paciente.

Los pacientes con la mutación p.Arg130Cys presentan una actividad enzimática baja con una clínica muy severa (Zschocke et al., 2000; Ensenauer et al., 2002; Ofman et al., 2003; Sass et al., 2004), mientras que los pacientes con las mutaciones p.Leu122Val y p.Glu249Gln muestran una actividad residual más elevada y una clínica más leve (Ofman et al., 2003; Poll The et al., 2004; Yang et al., 2009). Sin embargo, en nuestros pacientes no se ha podido establecer una clara correlación fenotipo-genotipo (**artículo 2**). Otros autores postulan que la severidad clínica no estaría relacionada con la actividad enzimática residual (Rauschenberger et al., 2010).

Una de las mutaciones nuevas hallada en una de nuestras familias: p.Asn247Ser, ha sido descrita en otro paciente (Chatfield et la., 2015).

Todos los paciente incluidos en este estudio presentaron síntomas antes de los 10 meses de vida, todos ellos con neurodegeneración progresiva, mientras que las 8 heterocigotas presentaban una variedad de síntomas: desde leves dificultades en el lenguaje hasta un severo retraso psicomotor, lo que estaría de acuerdo con una herencia ligada al cromosoma X con dominancia incompleta y no con una herencia recesiva como se había especulado anteriormente (Poll The et al., 2004).

Los estudios de estos pacientes aportan la descripción del 37% de las familias publicadas hasta el momento y amplia el conocimiento sobre la variabilidad fenotípica y bioquímica que pueden presentar las heterocigotas.

1.3 Estudios de expresión en *E.Coli*

Para demostrar la patogenicidad de las mutaciones detectadas en nuestros pacientes realizamos estudios de expresión en *E.Coli* mediante mutagénesis dirigida, todas las mutaciones presentaron una deficiencia en la actividad enzimática (tabla 3). La mutación p.Pro210Ser presentó la actividad residual más elevada, tal como se observaba en fibroblastos de los pacientes con dicha mutación (tabla 1). Sorprendentemente, los estudios de expresión de la mutación p.Arg130Cys revelaron una mayor actividad residual (tabla 3), en comparación con la severa deficiencia observada en fibroblastos de los pacientes con dicha mutación (tabla 1). Estos resultados en línea con los publicados previamente (Rauschenberger et al., 2010; Vilardo and Rossmanith 2015).

Tabla 3. Actividad enzimática de la proteínas HSD10 recombinantes con las diferentes mutaciones.

Mutación	% actividad enzimática respecto a la proteína WT
p.Arg130Cys	26
p.Asn247Ser	10
p.Pro210Ser	77
p.Arg226Gln	0.76

WT: wild-type

No se realizaron los estudios previstos para el rescate de actividad de los mutantes detectados ya que los estudios paralelos de Vilardo et al., 2012 no ofrecían ninguna duda acerca de la patogenicidad de las mismas. Identificaron HSD10 como un componente del complejo mitocondrial tRNA methyltransferasa junto con la proteína tRNA methyltransferase 10C (TRMT10C) y en 2015 Vilardo and Rossmanith realizaron estudios de expresión en *E. Coli* de los mismos cambios encontradas en nuestros pacientes. Estos autores observaron una reducción tanto de la actividad deshidrogenasa como de la actividad metiltransferasa en todos los cambios estudiados. Además, demostraron una disminución de la formación del tetrámero y una disrupción de la unión de HSD10 con TRMT10C en las mutaciones p.Asn247Ser y p.Arg226Gln En la mutación p.Arg130Cys la formación del tetrámero era correcta, pero la interacción

HSD10-TRMT10C estaba disminuida. La mutación p.Pro210Ser no mostró alteración de la tetramerización, ni de la interacción con TRMT10C. Sin embargo, la prolina en la posición 210 de HSD10 se encuentra en la superficie de la proteína y una alteración de esta región, podría obstaculizar el plegamiento correcto de dicha proteína sin cambiar necesariamente la afinidad por TRMT10C.

Estos autores concluyen que, una alteración estructural o funcional de HSD10 provocaría un mal procesamiento o modificación del RNA mitocondrial, lo que a su vez daría lugar a la disfunción mitocondrial y podría explicar las alteraciones mitocondriales observadas en estos pacientes (Vilardo and Rossmanith 2015).

2. HSD10: ESTUDIOS DE EXPRESIÓN.

2.1- Estudios de inactivación del cromosoma X.

En 1998 Miller et al., describieron que el gen *HSD17B10* se escapaba de la inactivación del cromosoma X; por lo tanto, las portadoras no deberían presentar síntomas. Sin embargo, las 6 portadoras publicadas previamente (Ensenauer et al., 2002; Rauschenberger et al., 2010; Zschoke, 2012), así como las 8 descritas en este trabajo presentan diferentes grados de afectación clínica: desde asintomáticas hasta dificultades en el aprendizaje, en el lenguaje y retraso psicomotor. Esta diferencia en la afectación clínica estaría de acuerdo con una herencia ligada al cromosoma X con diferentes grados de inactivación del cromosoma X.

En los inicios de este trabajo estudiamos dos portadoras, una de ellas, heterocigota para la mutación c.740A>G (p.Asn247Ser), con una afectación clínica severa y una actividad enzimática deficiente, mientras que otra, heterocigota para la mutación c.628C>T (p.Pro210Ser), no tenía apenas afectación clínica y la actividad enzimática se hallaba dentro de los valores control (tabla 1). Para explicar esta diferencia realizamos estudios a nivel de cDNA a partir de fibroblastos cultivados de las dos heterocigotas mencionadas y los dos hemicigotos correspondientes a ambas familias y se compararon con 4 mujeres y 4 varones control**.** El estudio de la dosis génica del cDNA de *HSD17B10* entre los controles de ambos sexos (tabla 4), no mostró ninguna diferencia, ya que los valores de cuantificación relativa (RQ) fueron muy similares con las sondas Wt (tabla 4). Estos resultados estarían a favor de que *HSD17B10* no se escapa de la inactivación del cromosoma X (**artículo 4**).

Tabla 4. Cuantificación de cDNAs de *HSD17B10* por cuantificación relativa (RQ) mediante PCR a tiempo real, utilizando 2 sondas, una con el nucleótido wild-type (Wt) y otra con el nucleótido mutado (Mut) para las mutaciones p.Asn247Ser y p.Pro210Ser. Se utilizaron 2 controles endógenos: gliceraldehido 3-fosfato deshidrogenasa (GAPDH) y ciclofinila A (PPIA).

RQ Mutación p.Asn247Ser	GAPDH		PPIA	
	Wt c.740A	Mut c.740G	Wt c.740A	Mut c.740G
Paciente 1.2 (F)	0,06	0,57	0,18	2,20
Paciente 1.1 (M)	0,07	0,68	0,12	1,83
Hombres control n=4 Media (rango)	1,05 (0,73 -1,36)	0,12 (0,03 -0,19)	0,99 (0,70 -1,57)	0,17 (0,12 -0,21)
Mujeres control N=4 Media (rango)	0,7 (0,45 -1,13)	0,10 (0,05 -0,16)	0,88 (0,64 -1,37)	0,15 (0,12 -0,21)

RQ Mutación p.Pro210Ser	GAPDH		PPIA	
	Wt c.628C	Mut c.628T	Wt c.628C	Mut c.628T
Madre paciente 6 (F)	1,29	1,16	0,76	0,69
Paciente 6 (M)	0,03	1,01	0,03	0,91
Hombres control n=4 Media (rango)	0,99 (0,61 -1,29)	0,31 (0,09 -0,52)	1,01 (0,77 - 1,43)	0,27 (0,17 -0,38)
Mujeres control n=4 Media (rango)	0,7 (0,46 -1,2)	0,25 (0,12-0,47)	0,83 (0,62 -1,26)	0,25 (0,2 -0,36)

F: sexo femenino; M: sexo masculino.

Se secuenció por "Sanger" el cDNA *HSD17B10* de la paciente 1.2 con la mutación p.Asn247Ser y sólo se detectó el alelo mutado (Mut). Estos resultados se confirmaron mediante RQ, y se vio que los niveles del alelo Mut eran mucho más elevados que los del alelo Wt, con un patrón de expresión similar al de su hermano hemicigoto, (paciente 1.1, tabla 4). Se descartó un síndrome de Turner y se realizaron estudios de metilación en el *locus* del receptor de andrógenos en DNA genómico. Los resultados mostraron un patrón de inactivación del cromosoma X preferencial

(Dra. Milá. Hospital Clínic). Estos resultados apoyaban una inactivación del cromosoma X desfavorable en el tejido analizado y probablemente esto mismo podría esperarse en otros tejidos, lo que explicaría la severidad clínica de esta heterocigota.

Por el contrario, la secuenciación "Sanger" del cDNA de *HSD17B10* de una heterocigota con la mutación p.Pro210Ser, mostró la presencia de ambos alelos Wt y Mut. Estos resultados se confirmaron por RQ, mostrando unos niveles similares de ambos alelos (tabla 4). Los estudios de metilación en el *locus* del receptor de andrógenos, mostraron un patrón de inactivación del cromosoma X al azar, indicando la presencia de mosaicismo en las células del tejido analizado, lo que explicaría la leve sintomatología clínica de esta portadora. Por otro lado, especulamos que la actividad enzimática normal de esta heterocigota, podría ser debida a que los niveles del alelo Wt puedieran ser suficientes para producir la cantidad de proteína necesaria.

Queremos señalar que las respuestas de la amplificación por RT-PCR fueron diferentes según el control endógeno utilizado, este hecho se podría explicar por la baja especificidad de las sondas y por la variabilidad de dichos controles. Sin embargo, la interpretación de los resultados fue la misma, independientemente del control utilizado.

No se puede excluir que la diferencia clínica entre ambas portadoras pudiera deberse al diferente efecto de las mutaciones, ya que el paciente hemicigoto para la mutación p.Asn247Ser falleció a los 2 meses de vida y los estudios de expresión de esta mutación demuestran una disminución de la formación del tetrámero de HSD10 y una disrupción de la unión de HSD10 con TRMT10C, provocando una deficiencia tanto de la actividad deshidrogenasa de HSD10, como de la actividad metiltransferasa (Vilardo and Rossmanith 2015). En cambio, el paciente hemicigoto para la mutación p.Pro210Ser, falleció a los 4 años de edad y los estudios de expresión revelaron que no había una alteración en la tetramerización o en la interacción con TRMT10C, aunque dicha mutación podría alterar el plegamiento correcto de HSD10 (Vilardo and Rossmanith 2015).

En conclusión, estos resultados sugieren que el gen *HSD17B10* no se escapa de la inactivación del cromosoma X como se había publicado anteriormente (Miller et al., 1998). Los resultados obtenidos estarían en la misma línea que los obtenidos por Carrel et al., 2005. Estos autores observaron que sólo en 1 de 9 híbridos el gen *HSD17B10* se escapaba de la inactivación del cromosoma X. Las heterocigotas estudiadas presentan la variabilidad clínica y bioquímica clásica de una enfermedad ligada al cromosoma X y la severidad clínica dependerá de la dosis total del alelo mutado en los diferentes tejidos.

2.2- Análisis de la expresión génica en pacientes con deficiencia de HSD10, mediante el estudio de microarrays de expresión.

La presentación clínica de los pacientes con deficiencia de HSD10 es muy diferente de las otras acidurias orgánicas de la misma vía metabólica, tal como la deficiencia de 3-cetotiolasa, en la que también se observa un aumento de los mismos metabolitos que en la deficiencia de HSD10, sin embargo estos pacientes no presentan deterioro neurológico. En cuanto a la patogénesis de la enfermedad, se ha demostrado que niveles elevados de 2M3HBA, metabolito que se acumula en la deficiencia de HSD10, inhibe el complejo IV de la cadena respiratoria mitocondrial (Rosa RB 2005). Sin embargo, este metabolito también se acumula en la deficiencia de 3-cetotiolasa, enfermedad en la que los pacientes no presentan afectación neurológica. Por tanto, parece que la causa de patología en la deficiencia de HSD10 reside a otro nivel (Poll The et al., 2004) y se deduce que estos compuestos no deben ser la causa de la neurodegeneración (Zschocke et al., 2000; Yang et al., 2009; Rauschenberger et al., 2010).

HSD10 parece tener un papel fisiológico importante: el gen *HSD17B10* está altamente conservado en animales (Marques et al., 2006) y parece indispensable para la vida, ya que el "Knock-out" (KO) de *Drosophila* es letal (Torroja et al., 1998). Por un lado, en 2010 Rauschenberger et al., demostraron, mediante modelos de ratón KO y "Knock-down" de *Xenopus*, que HSD10 era esencial para la estructura e integridad funcional de la mitocondria, y que esta integridad no estaría relacionada con la actividad enzimática en el metabolismo de la isoleucina. Además, se observó que la pérdida completa de la proteína HSD10 en embriones de ratón era letal en estadios muy tempranos de la embriogénesis. También se ha descrito que esta proteína es un componente de dos complejos enzimáticos mitocondriales: RNasaP (Holzmann et al., 2008) y tRNA metiltransferasa (Vilardo et al., 2012), esenciales para la traducción de proteínas, por lo que una disfunción a este nivel explicaría la neurodegeneración de estos pacientes (Vilardo and Rossmanith 2015). Por otro lado, se ha descrito que HSD10 es una proteína del complejo de densidad postsináptica (PSD), esencial para la inducción de la plasticidad neuronal y para el proceso cognitivo en animales (Laumonnier et al., 2007). Otros autores postulan que la fisiopatología de esta enfermedad podría estar relacionada con el papel de HSD10 en la oxidación de los neuroesteroides, función esencial para el funcionamiento de las neuronas gabaérgicas (He et al., 2005 (a, b), Yang et al., 2009; Yang et al., 2011).

Para obtener una idea del mecanismo de patogenicidad, decidimos realizar estudios de microarrays de expresión. Se obtuvo el RNA de 5 pacientes hemicigotos y 2 heterocigotas con deficiencia de HSD10 y 5 líneas celulares control, que se analizaron en la unidad de expresión

del Hospital Clínic de Barcelona utilizando el "GeneChip Human Genome U133 plus 2.0" (Affymetrix, Santa Clara, CA) con 54.000 sondas. Los resultados revelaron que los pacientes con deficiencia de HSD10 presentaban un patrón de expresión diferente al de los controles. Se seleccionaron los genes que mostraban un log ratio >2 o <2 respecto a los controles. Se observó una diferencia en la expresión de 42 genes. Tras aplicar un test estadístico no paramétrico, se obtuvo una diferencia de expresión significativa en 31 de estos genes.

Destacamos algunas de las diferencias de expresión entre pacientes con deficiencia de HSD10 y controles.

Genes que podrían tener relación con los síntomas clínicos y la neurodegeneración

-Sobre-expresión de:

BCL2, que codifica para una proteína de membrana mitocondrial externa supresora de apoptosis. Al mismo tiempo se ha encontrado sobre-expresión de *HRK*, que codifica para una proteína que activa la apoptosis al unirse con otras proteínas supresoras de la apoptosis, como BCL2. Este puede ser un mecanismo de compensación de la disfunción mitocondrial observada en los pacientes con deficiencia de HSD10.

MDK, gen implicado en el desarrollo y crecimiento de la neurita e importante para la transducción de señal entre células. .

SYNE2, la proteína para la que codifica está implicada en la migración de neuronas, desarrollo cerebral, aprendizaje, memoria y en la regulación de la expresión de genes relacionados con supervivencia celular.

SOX11, implicada en la regulación del desarrollo embrionario del sistema nervioso.

SLC6A15, codifica para un transportador de neurotransmisores y de aminoácidos ramificados Na(+) y Cl(-)-dependiente, especialmente de isoleucina, leucina y valina.

PTPRD **y** ***ODZ3*** implicados en el desarrollo neuronal y en el direccionamiento axonal. Ésta sobre-expresión podría ser debida a una respuesta compensatoria de la neurodegeneración.

BCHE, butirilcolinesterasa que contribuye a la inactivación del neurotransmisor acetilcolina con regulación negativa de la transmisión sináptica. Cabe destacar que su expresión también se ha visto aumentada en la enfermedad de Alzheimer (EA), los niveles incrementados de esta proteína se correlacionan con el desarrollo de las placas neuríticas de péptido β-amiloide (pβA) y con los ovillos neurofibrilares.

- **Infra-expresión** de genes implicados en la neurogénesis, en el direccionamiento axonal o en la sinapsis: ***NFAS, SIPR3, SEMA5A*** (gen también relacionado con Parkinson) y ***GRIA3*** *(*que codifica para una proteína cuya deficiencia causa retraso mental). ***MAOA*** y ***MAOB***, la

deficiencia de las proteínas para las que codificas se asocia con retraso mental, neurodegeneración y también con las enfermedades de Parkinson y Alzheimer.

Genes que podrían estar relacionados con ceguera:

-**Sobre-expresión**: de ***NHSL1*** y de ***ODZ3***, mutaciones en estos genes se asocian a defectos visuales. La sobre-expresión podría ser un mecanismo compensatorio.

-**Infra-expresión**: de ***FOXL2***, que codifica para un factor de transcripción que regula la apoptosis y está implicado en la morfogénesis ocular y en el desarrollo extraocular. De ***MAOA*** y ***MAOB***, una infra-expresión de estos genes puede provocar ceguera.

Genes que podrían estar relacionados con los síntomas cardíacos:

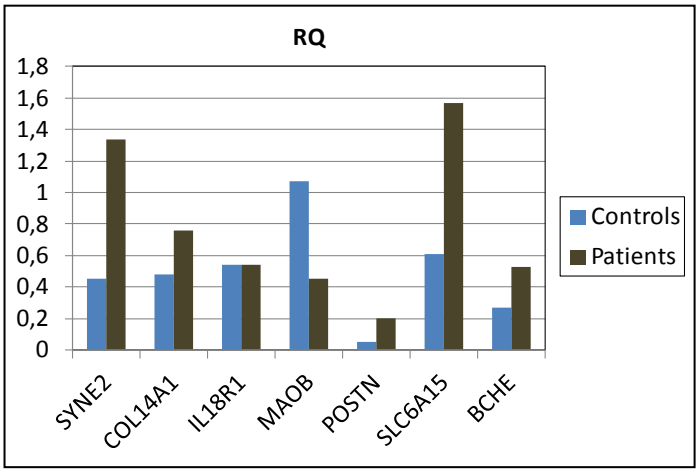
-**Sobre-expresión**: de ***TNFSF4***, gen de susceptibilidad al infarto de miocardio. ***NHSL1***, que se ha asociado con hipertensión y enfermedad vascular, con respuesta inmune en las células endoteliales vasculares. De ***ETV1***, la proteína para la que codifica podría estar implicada en la angiogénesis.

-**Infra-expresión**: de ***SIPR3*** implicado en la transducción de señal y en la regulación de la angiogénesis y de la función celular del endotelio vascular, el modelo KO para este gen presenta defectos vasculares graves.

Entre todos los genes anteriores los que podrían estar relacionados con la implicación de HSD10 en la EA sería la infra-expresión de ***MAOA* y *MAOB*** y la sobre-expresión de ***BCHE***; dichos genes podrían estar implicados en la neurodegeneración que presentan los pacientes con deficiencia de HSD10.

Posteriormente, para validar los resultados de expresión, se seleccionaron 7 genes (*SYNE2, COL14A1, IL18R1, MAOB, POSTN, SLC6A15 y BCHE)* y se realizaron estudios de expresión mediante qRT-PCR por RQ. Se confirmaron todas las diferencias de expresión excepto para el gen *IL18R* (figura 7) **(artículo 5)**.

Figura 7. Media de la cuantificación relativa (RQ) de la expresión de los 7 genes estudiados por real time PCR.



En un futuro se deberían realizar estudios de proteómica para confirmar si la variación en la expresión de estos genes, también causa una variación en la cantidad de proteína para las cuales codifican. Por otro lado, los estudios de proteómica podrían ayudar a explicar cambios en la interacción con otras proteínas dependiendo de la mutación.

3. IMPLICACIÓN DE HSD10 EN LA ENFERMEDAD DE ALZHEIMER

3.1- Determinación de la actividad enzimática HSD10 y estudios de expresión en necropsias de cerebro de enfermos de Alzheimer

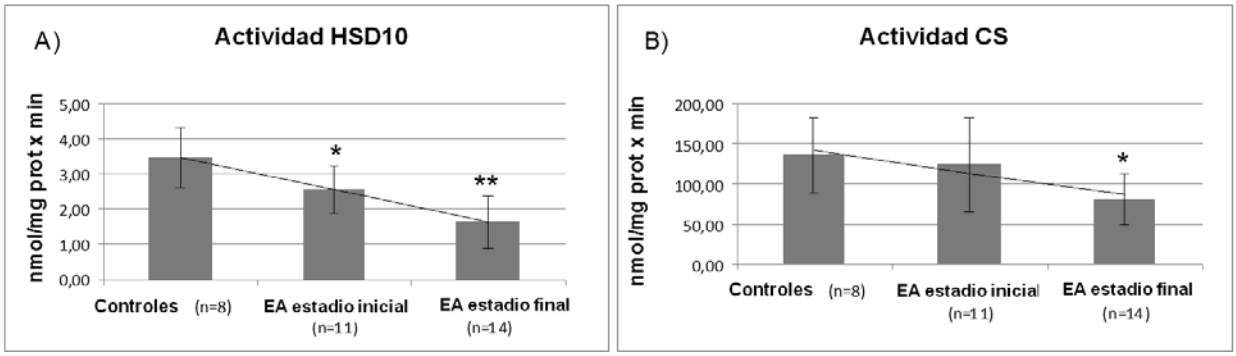
La investigación que se lleva a cabo actualmente sobre la relación entre el enzima multifuncional mitocondrial, HSD10, y la patogénesis de la EA se basa en: i) la sobre-expresión de HSD10 observada en cerebros de pacientes con EA y en modelos de ratón para dicha enfermedad (Yan et al., 1997; He et al., 2002, 2005; Yan et al., 2000; Wen et al., 2002; Yan et al., 2005; Hovorková et al., 2008); y, ii) el hallazgo de que el pβA se une a HSD10 en la mitocondria causando la inhibición de su actividad catalítica, promoviendo la generación de especies reactivas de oxígeno (ROS), disfunción mitocondrial, neurotoxicidad y apoptosis celular (Yan et al., 1997; Yan et al., 1999; Opperman et al., 1999; Lustbader et al., 2004; Takuma et al., 2005).

Se obtuvieron 25 muestras de autopsias de cerebro de pacientes con EA (corteza parietal y occipital), 11 de las muestras en el estadio inicial de la enfermedad (I- III) y 14 de ellas en estadio final (IV-VI) y 8 muestras de individuos control, del banco de tejidos neurológicos de

nuestro hospital. No pudimos disponer de muestras de hipocampo, zona del cerebro más afectada en la EA, sin embargo, estudiamos la corteza parietal y occipital dado que se había demostrado la presencia de pβA y de HSD10 en estas áreas del cerebro (Yan et al., 1997). Se puso a punto la determinación enzimática de HSD10 en homogenados de cerebro utilizando fundamentalmente la misma metodología que habíamos utilizado anteriormente para los fibroblastos, aunque con algunas modificaciones: el enriquecimiento en mitocondrias para suprimir la producción de NADH citosólico inespecífica y la medición de la actividad en un espectrofotómetro de placas de 96 pocillos. Las mejores condiciones se obtuvieron con 0,3mg/mL de proteína total, 2mmol/L de sustrato purificado y 2mmol/L de NAD^+. El coeficiente de variación intra e inter-día fue 7% y 10%, respectivamente.

El estudio de actividad HSD10 en corteza occipital y parietal no mostró diferencias significativas por lo que decidimos unir los resultados de ambas áreas. Un resultado importante de este trabajo fué el hallazgo de una disminución notable de la actividad HSD10 en homogenados de cerebro de pacientes con EA desde las etapas iniciales (figura 8A). Esta observación estaría en línea con los resultados obtenidos por otros autores que describen la afectación mitocondrial como un evento temprano en la EA. Así, estudios en ratones transgénicos que sobre-expresan la proteína precursora de pβA (AβPP) muestran que el pβA se detecta primero dentro de la mitocondria antes de que tenga lugar cualquier depósito extracelular y que la disfunción mitocondrial se produce en las primeras etapas de la EA (Caspersen et al., 2005). Por otro lado, se ha descrito que el daño oxidativo también ocurre al inicio de la enfermedad (Nunomura et al., 2001). Por el contrario, la actividad citrato sintasa (CS), que se utiliza como un control de la masa mitocondrial, se halló significativamente disminuida sólo en las etapas finales de la EA (figura 8B). Una disminución de CS también se ha observado en neuronas corticales primarias expuestas a pβA (Kim et al., 2002; Casley et al., 2002).

Figura 8. Actividad enzimática de A) HSD10 y de B) citrato sintasa (CS) en necropsias de cerebro de individuos control y de pacientes con enfermedad de Alzheimer (EA).



Dado que las muestras de cerebro humano se obtuvieron entre las 2,5 h y las 24 h post-mortem, decidimos realizar estudios de estabilidad de ambas proteínas en cerebro de ratón. Los resultados mostraron que ambas proteínas eran estables al menos hasta las 48 horas post-mortem (**artículo 6**), por lo que se podía descartar que la disminución de actividad no se debía a un problema de inestabilidad de las proteínas.

Cabe señalar que la menor actividad de HSD10 en homogenados de cerebro de EA no se debe a una menor expresión de la enzima, ya que no se encontraron diferencias en los niveles de proteína con respecto a los controles (**artículo 6**). Estos resultados no estarían de acuerdo con la sobre-expresión de HSD10 observada tanto en cerebro de pacientes con EA, como en cerebros modelo de ratón para dicha enfermedad (Yan et al., 1997; He et al., 2002; He et al., 2005 (b)). Sin embargo, sí estarían en línea con otros estudios que demuestran que HSD10 no está sobre-expresada en cerebro de pacientes con EA (Wen et al., 2002). Probablemente, todo ello dependerá de la zona del cerebro estudiado, así en hipocampo de pacientes con la EA existe una lateralización de la expresión de HSD10, observando una mayor expresión de HSD10 en el lado izquierdo del cerebro (Hovorkova et al., 2008).

3.2.-Estudio de la inhibición de HSD10 por el pβA

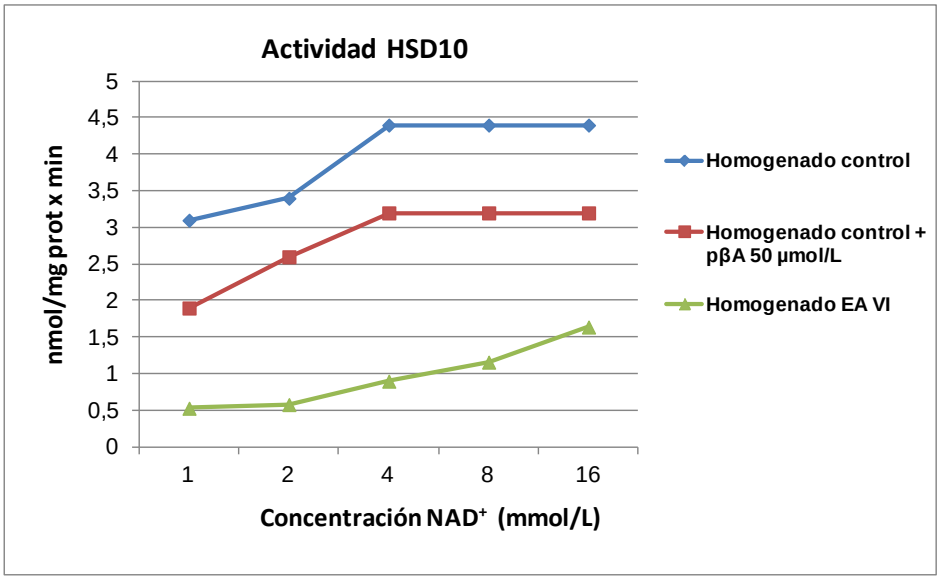
Diversos estudios demostraron que la región aminoácidica 12-24 del pβA inhibía la actividad catalítica de HSD10 (Yan et al., 1999; Opperman et al., 1999; Salim et al., 2000; Lustbader et al., 2004; Takuma et al., 2005).

En nuestros estudios encontramos una disminución del 40% de la actividad HSD10 en homogenados de cerebro control expuestos a pβA (**artículo 6**), apoyando la hipótesis de que la causa de la inhibición de dicha actividad se debe a un exceso de pβA (Opperman et al., 1999; Yan et al., 1999; Lim et al., 2011; Vilardo et al., 2013). Cabe destacar que para conseguir una inhibición de HSD10 es necesario una concentración de pβA a nivel µmol/L, mientras que la constante de disociación para la unión pβA a HSD10 es 60 nmol/L. Otros estudios demuestran que sólo la forma oligomérica de pβA puede unirse a HSD10 (Yan et al., 2007; Vilardo and Rossmanith 2013).

Se ha descrito que HSD10 interacciona con el pβA dentro de la mitocondria causando la inhibición de la actividad a través de la inducción de un cambio conformacional en su estructura, que a su vez inhibe la unión de su cofactor NAD^+ (Yan et al., 1999; Opperman et al., 1999; Salim et al., 2000; Lustbader et al., 2004; Takuma et al., 2005; Yan et al., 2007). Así, presumiblemente un incremento de NAD^+ puede perturbar el complejo pβA-HSD10. De hecho,

nosotros hemos observado un aumento de la actividad HSD10 al aumentar la concentración de NAD^+ en homogenados de cerebro (figura 9). En particular, en homogenados de cerebro con EA se observa un incremento lineal al aumentar la concentración de NAD^+ hasta 16 mmol/L, mientras que en cerebro control la actividad se satura con 4 mmol/L de NAD^+. Por lo tanto, hemos demostrado que NAD^+ era capaz de rescatar la actividad HSD10 en homogenado de cerebro, lo que implica que NAD^+ puede alterar directa o indirectamente la interacción pβA-HSD10. Estos resultados están de acuerdo con los estudios realizados por resonancia magnética nuclear en los que se demuestra que un incremento de la concentración de NAD^+ inhibe la interacción pβA-HSD10 (Yan et al., 2007).

Figura 9. Rescate de la actividad HSD10 por NAD^+ tras la inhibición por el péptido beta-amiloide (Aβ) en homogenados de cerebro control y de un paciente con enfermedad de Alzheimer (AD) en estadio VI.

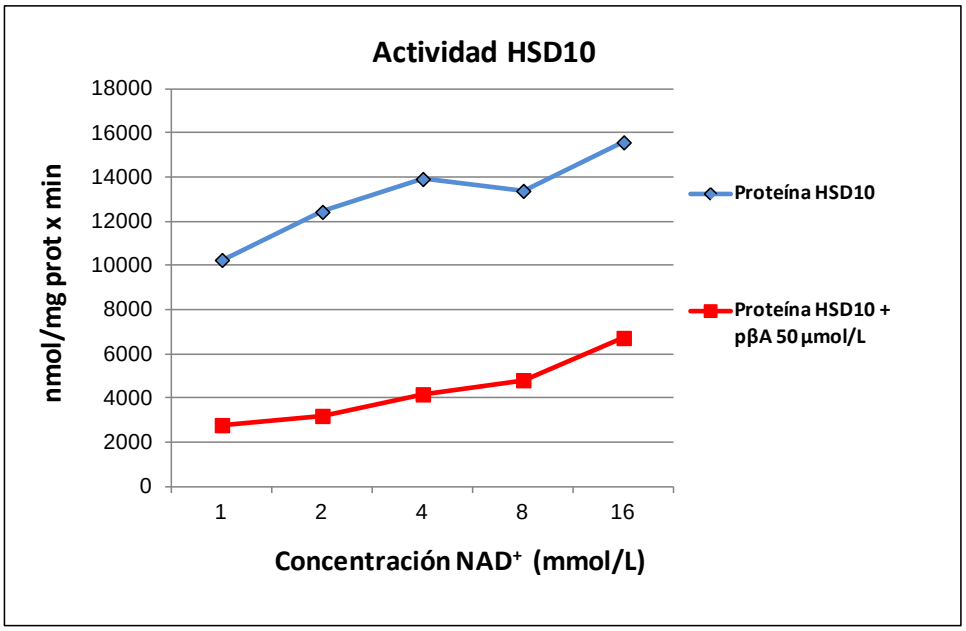


.3.- Inhibición de la interacción pβA-HSD10, como diana terapéutica para la enfermedad de Alzheimer.

Parece ser que la proteína HSD10 está implicada en la inducción de estrés celular por el pβA. En línea con esta hipótesis, las neuronas de ratón doble transgénico que sobre-expresa tanto la proteína precursora del pβA (AβPP) como HSD10 se observa un incremento de radicales libres, una reducción de la formación de ATP y de la utilización de glucosa. También se observa un mayor déficit de memoria y más dificultades en el aprendizaje, en comparación con el ratón transgénico que sólo sobre-expresa la proteína AβPP o sólo sobre-expresa la proteína HSD10

(Yan et al., 2000; Takuma et al., 2005). Por lo tanto, parece que la interrupción de complejo pβa-HSD10 podría ser una diana terapéutica importante para EA (Chen et al., 2006; Lim et al., 2011). En base a los resultados anteriores, decidimos establecer un modelo terapéutico "in vitro" para probar compuestos capaces de interrumpir la interacción pβA-HSD10 y rescatar a la actividad enzimática. En primer lugar, clonamos y caracterizamos las dos isoformas más importantes de HSD10, es decir, la isoforma 1 de 261 aminoácidos, y la isoforma 2 de 252 aminoácidos. Encontramos ambas isoformas en cerebro humano, aunque la isoforma 1 fue la más abundante (**artículo 6**), apoyando los resultados de He et al., 1998. La isoforma 1 de HSD10 fue activa con los tres sustratos probados: 2M3HBCoA, 17β -estradiol y alopregnanolona, mostrando mayor afinidad por 2M3HBCoA. Contrariamente, la isoforma 2 no era funcionalmente activa cuando se utilizaba 2M3HBCoA como sustrato, y mostró una actividad significativamente más baja que la isoforma 1 con los otros dos sustratos (**artículo 6**). Además, la incubación de la isoforma 1 con pβA 50 µmol/L inhibía un 80% la actividad HSD10. Dicha actividad podía ser rescatada con NAD^+ (figura 10). En estas condiciones de inhibición el valor de Z fue de 0,82, lo que indicaba que nuestro sistema tenía una buena sensibilidad (**artículo 6**). Por tanto, se seleccionó la isoforma 1 para establecer un ensayo "in vitro" adecuado para el cribado de librerías de compuestos, utilizando 2M3HBCoA como sustrato.

Figura 10. Rescate de la actividad HSD10 tras la inhibición con péptido beta-amiloide (pAβ).



Con dicho ensayo probamos un conjunto de 117 compuestos peptídicos, coenzima Q10 y otros antioxidantes, pero ninguno de ellos fue capaz de rescatar la actividad HSD10.

Se han descrito muchos inhibidores de la interacción pβA-HSD10: un péptido antisentido que contenía la región aminoacídica 99-108 de HSD10, este péptido inhibía la interacción pβA-HSD10 en neuronas humanas, reduía la muerte celular e inhibía la agregación de pβA (Opperman et al., 1999; Milton et al., 2001). Un péptido comprendiendo la región 92-120 de la proteína HSD10 (ABAD-DP), que en cultivo de neuronas parecía inhibir la interacción pβA-HSD10 ya que se observaba una disminución del estrés oxidativo (Lustbader et al., 2004). La inyección de una modificción de éste último péptido: Tat-mito-ABAD-DP a ratones modelo para la EA, producía una mejora en el aprendizaje y en la memoria de estos animales (Yan et al., 2007, Yao et al., 2007; Ren et al., 2008; Yao et al., 2011). También se han descrito otros inhibidores de esta interacción: anticuerpos contra las regions específicas de HSD10 (100-116 and 133-147) (Yan et al., 1997), AG18051 que se une a la cavidad el sitio activo de HSD10 y reacciona con el NAD^+ para formar una aducto covalente (Lim et al, 2011; Kissinger et al., 2004) y pequeñas moléculas (Xie et al., 2006; Valasani et al., 2013).

Dado que NAD^+ es capaz de interrumpir la interacción pβA-HSD10 y rescatar la actividad de HSD10, creemos que dicho compuesto o sus precursores podrían ser una posible terapia coadyuvante para la EA.

Estudios posteriores apoyan los precursores de NAD^+ como nueva oportunidad terapéutica para el tratamiento del envejecimiento y sus trastornos asociados, en particular para las enfermedades neurodegenerativas (Verdin, 2015). NAD^+ desempeña un papel importante en todas las células como sustrato para la transducción de señales y como cofactor en las reacciones redox del metabolismo. El ácido nicotínico (NA) o nicotinamida (NAM), ambos presentes en la dieta como vitamina B3, son la fuente principal de NAD^+. Se han descrito otros precursores de NAD^+ como ribósido nicotinamida (NR) y mononucleótido de nicotinamida (NMN), que aumentan los niveles de NAD^+ en varios tipos de tejidos y de células de modelo de ratón para la EA, provocando la activación de SIRT1 (Mouchiroud et al., 2013; Long et al., 2015). SIRT1 es una sirtuina nuclear, que desacetila histonas, regula la biogénesis de la mitocondria y la respuesta celular ante una estrés, mejorando la homeostasis mitocondrial (Mouchiroud et al., 2013). Recientemente, se ha reportado que los ratones transgénicos para la EA tratados con NMN muestran una disminución de los niveles de AβPP, un aumento de SIRT1, y una mejora de la morfología mitocondrial (Long et al., 2015). Además, se ha demostrado que NMN es capaz de atravesar la barrera hematoencefálica para ser convertido en NAD^+, aumentando así los niveles de NAD^+ celular en cerebro (Spector and Johanson, 2007). Curiosamente, en un modelo celular

de la enfermedad de Parkinson, la adición de NMN provoca una reducción de la apoptosis y restaura los niveles intracelulares de NAD^+ (Lu L et al., 2014). Todas estas evidencias, junto con nuestros resultados indican que el NAD^+ o sus precursores podría ser una posible terapia para la EA, que creemos merece ser explorar "in vivo".

HSD10 es una proteína muy importante para la integridad de la mitocondria y la supervivencia celular (Rauschenberger et al., 2010) y es un miembro de dos complejos enzimáticos: RNasaP mitocondrial (Holzmann et al., 2008) y metiltransferasa tRNA mitocondrial (Vilardo et al., 2012). Estos autores describieron que sólo la presencia de HSD10, y no su función enzimática, es importante para estos dos complejos. Por otra parte, demostraron que la inhibición de la actividad RNasaP y tRNA metiltransferasa por pβA no está mediada por la interacción de pβA-HSD10 (Vilardo and Rossmanith, 2013). Por el contrario, otros estudios apoyan que la inhibición de la actividad HSD10 puede perturbar su función citoprotectora en neuronas en respuesta al estrés celular (Yan et al., 2000; Tieu et al., 2004), o inhibir la detoxificación de aldehídos, en especial de 4-HNE, aldehído que se encuentra incrementado en pacientes con EA (Murakami et al., 2009), o interferir en el efecto de los estrógenos en la neuroinflamación (Yan 1999, 2002, Yang et al., 2005 (a)), o inhibir la oxidación de neuroesteroides, esenciales para la función de las neuronas gabaérgicas (Yang et al., 2009, 2011). Tal inhibición puede también disminuir el metabolismo de la cardiolipina peroxidada, lo que desencadenaría la apoptosis celular (Boynton and Shimkets 2015).

Además, los pacientes con deficiencia de HSD10 presentan neurodegeneración independientemente si la proteína está presente o no (figura 6), lo que refuerza la importancia de la actividad enzimática de HSD10 en la neurodegeneración. De hecho, un paciente con deficiencia HSD10 con neurodegeneración severa mostró niveles normales de esta proteína en fibroblastos y ausencia de pβA en líquido cefalorraquídeo (Ortez C et al., 2011- **artículo 7 - anexo-**). Del mismo modo, los niveles de pβA están disminuidos en el líquido cefalorraquídeo de paceintes con EA, y estudios previos sugieren que pβA y AβPP pueden desempeñar un papel importante en el desarrollo y en la eliminación de la sinapsis (Zetterberg et al., 2010; Priller et al., 2006). Probablemente, la capacidad de pβA para atrapar HSD10 podría explicarse por mecanismos similares tanto, en pacientes con deficiencia de HSD10, como en pacientes con EA. Podría ser que la captura de HSD10 por pβA pudiera interrumpir las funciones no enzimáticas de HSD10 que son necesarias para la integridad mitocondrial, así como alterar la unión de HSD10 a otras proteínas, como a la ciclofilina D, lo que causaría un aumento de la permeabilidad mitocondrial y apoptosis (Yan et al., 2005; Muirhead et al., 2010) y alterar la función de HSD10 en el poro de permeabilidad mitocondrial (MPT) (Tieu et al., 2004; Rauschenberger et al., 2010).

Además esta interacción podría alterar la unión al receptor α de estrógenos (Jazbutyle et al., 2009) o al complejo de densidad postsináptica (PSD) que participa en el mantenimiento de la función sináptica (Laumonnier et al., 2007).

En resumen, se encontró que la actividad HSD10 se hallaba disminuida en los homogenados de cerebro de pacientes con la EA y que su actividad podía ser rescatada por la adición de NAD^+. También se observó que la actividad HSD10 disminuye con la progresión de la enfermedad. Por lo tanto, la actividad HSD10 debe ser considerada como uno de los objetivos para encontrar un tratamiento que pueda mejorar la disfunción mitocondrial en la EA.

CONCLUSIONES

1. El desarrollo de la metodología anlítica para el estudio de metabolitos clave, enzimático y molecular, ha permitido el diagnóstico de 6 hemicigotos y 8 heterocigotas, que representa el 37% de los pacientes con deficiencia de HSD10 descritos hasta el momento.
2. La valoración cuantitativa del ácido etilhidracrílico en orina, junto con la valoración de los ácido orgánicos clave para esta entidad, ayuda al diagnóstico de las portadoras.
3. Se propone la cuantificación de la tilglilcarnitina como nuevo biomarcador para la deficiencia de HSD10, con el fin de obtener una mayor sensibilidad diagnóstica.
4. Con el estudio molecular se identificaron 3 mutaciones de cambio de sentido nuevas y otra ya diagnosticada previamente. Todas ellas se heredaron, excepto una. La patogenicidad de dichas mutaciones ha sido demostrada.
5. Se ha demostrado que el gen *17βHSD10* no se escapa de la inactivación del cromosoma X.
6. Los estudios de microarrays de expresión mostraron diferencias significativas respecto a los controles en el patrón de expresión en 31 genes, algunos de los cuales podrían explicar la sintomatología clínica de los pacientes.
7. Se demuestra que la isoforma 1 de 261 aminoácidos es la que se expresa mayoritariamente en cerebro y es funcional con los 3 sustratos estudiados.
8. Se ha detectado una deficiencia de HSD10 desde los estadios iniciales de la EA en cerebro de pacientes, lo que podría ser una de las causas de disfunción mitocondrial en la EA.
9. El péptido β-amiloide (pβA) inhibe la actividad de HSD10 tanto en homogenado de cerebro control, como en el modelo “in vitro” con la proteína recombinante de la isoforma 1.
10. Se ha obtenido un sistema “in vitro” para el estudio de competición entre el pβA y otros compuestos, siendo ésta una posible diana terapéutica para la EA.
11. La inhibición de la actividad catlítica de HSD10 por el pβA puede ser rescatada por la adición de NAD+. Creemos que los precursores de NAD+ podrían ser considerados como posible terapia coadyuvante en la EA.
12. Hemos demostrado que el estudio de la proteína HSD10, cuya deficiencia causa una enfermedad minoritaria, puede aportar nuevas herramientas para el estudio de enfermedades más comunes como la enfermedad de Alzheimer.

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ANEXO

ARTÍCULO 7

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Short Communication

Undetectable Levels of CSF Amyloid-β Peptide in a Patient with 17β-Hydroxysteroid Dehydrogenase Deficiency

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Abstract. 17β-hydroxysteroid dehydrogenase 10 (HSD10) deficiency is a rare X-linked inborn error of isoleucine catabolism. Although this protein has been genetically implicated in Alzheimer's disease pathogenesis, studies of amyloid-β peptide (Aβ) in patients with HSD10 deficiency have not been previously reported. We found, in a severely affected child with HSD10 deficiency, undetectable levels of Aβ in the cerebrospinal fluid, together with low expression of brain-derived neurotrophic factor, α-synuclein, and serotonin metabolites. Confirmation of these findings in other patients would help elucidating mechanisms of synaptic dysfunction in this disease, and highlight the role of Aβ in both early and late periods of life.

Keywords: Amyloid-β peptide, cerebrospinal fluid, childhood, HSD10 deficiency, inborn errors of metabolism, neurotransmitters, synaptic proteins

INTRODUCTION

17β-hydroxysteroid dehydrogenase (HSD10) is a multifunctional mitochondrial enzyme with complex roles [1, 2]. HSD10 deficiency is an X-linked disease (MIM300256) caused by mutations in *HSD17B10* gene [3]. This disease is characterized by a clinical picture very different from other organic acidurias: patients do not develop metabolic crises, but they follow a neurodegenerative course associated with mitochondrial dysfunction [4], progressive loss of skills, epilepsy, optic atrophy, retinopathy, deafness, and movement disorders [2].

HSD10 is a protein implicated in the pathogenesis of Alzheimer's disease (AD) [5, 6]. Although HSD10 has an affinity for amyloid-β peptide (Aβ), no studies

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regarding *in vivo* quantification of Aβ have been reported in patients with HSD10 deficiency. Moreover there are no studies concerning normal cerebrospinal fluid (CSF) Aβ values in the pediatric age. We aimed to analyze the expression of Aβ in the CSF of an affected child, compared to a control pediatric population. In order to gather more information about synaptic mechanisms in this disease, neurotransmitters, brain-derived neurotrophic factor (BDNF; associated with dendritic growth, and serotoninergic transmission) and α-synuclein (AS; associated with neurotransmitter release, dopaminergic modulation and neurodegeneration) were also included in our study.

PATIENT AND METHODS

Clinical report

The patient is the first child of healthy non-consanguineous parents. Pregnancy, peripartum, and first year of life were uneventful (normal psychomotor development: he could walk with support and was able to say some words). At 13 months, and within the context of fever and diarrhea, he developed psychomotor regression (inability to sit unsupported, loss of normal use of hands), and disclosed abnormal fast erratic ocular movements, non-epileptic myoclonus, and irritability. Plasma ammonia and amino acids were normal but lactate concentration was up to 3 mmol/l (NV: 0.66–1.88 mM/l). Brain MRI was normal. Because of the initial symptoms, opsoclonus-myoclonus syndrome was suspected and dexamethasone (0.8 mg/kg/d IV for 6 days) was started. Although the patient showed slight improvement, ocular fundus revealed optic atrophy and a determination of urine organic acids revealed elevation of 3-hydroxy-2-methylbutyryic (48 mmol/mol creatinine; CV 5–12) and tiglylglycine (44 mmol/mol creatinine; CV <5). HSD10 activity in fibroblast was 0.5 nmols/mg protx min (C.V. 1.4 ± SD 0.43) and a new missense mutation was detected in *HSD17B10* gen (c.628 C > T; p.P210 S) [7]. The mother was confirmed to be a carrier. Despite low isoleucine diet, the global outcome was very poor. At 3 years the child was unable to walk due to severe motor dyspraxia and choreoathetosis. Abnormal ocular movements and severe cognitive delay were present. At 5 years of age, the patient died of pneumonia leading to sepsis, multi-organ failure, and mitochondrial dysfunction (hyperlactacidemia, abundant Kreb's cycle metabolites in urine). Autopsy was not authorized.

CSF samples

CSF samples from our patient and controls were obtained by lumbar puncture as previously described [8]. First ten drops were used for routine cytochemical/microbiological studies and the rest immediately stored at −80°C until further analysis. The study of controls was performed in 30 subjects (age range: 21 days–5 years; average: 0.9 years, 16 boys, 14 girls) whose CSF samples were submitted under suspicion of central nervous system infection. Exclusion criteria were diagnosis of viral or bacterial meningitis, a chronic neurological condition, hyperproteinorrachia, and hematic or xanthochromic CSF.

Biogenic amine metabolites (3-orthomethyldopa, 3-methoxy-4-hydroxyphenylglycol, HVA, 5hydroxytryptophan, and 5-HIAA) and pterins (neopterin and biopterin) were analyzed by HPLC with electrochemical and fluorescence detection. Results were compared with our reference values [8].

Western blot analysis was performed for each protein (BDNF, Aβ, AS). Twenty μL of CSF was loaded on to the gel and proteins were separated on 10% sodium dodecyl sulphate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Amersham™ Hybond™–ECL; GE Healthcare). Membranes were incubated in TBST buffer (0.02 M Tris-base, pH 7.6, 0.8% NaCl, 0.1% Tween 20) supplemented with 5% dried skimmed milk for 60 min to block non-specific binding. Anti-BDNF extracellular loop (1 : 500; Santa Cruz Biotechnology ®), anti-Aβ (1 : 500; Santa Cruz Biotechnology ®) and Anti AS (1 : 500; Santa Cruz Biotechnology®) antibodies were added, and the preparations were incubated at 4°C overnight. The membranes were washed three times with TBST buffer and then incubated with appropriate anti-rabbit (1 : 3,000; Promega®) or anti-mouse (1 : 5,000; Promega®) IgG secondary antibodies at room temperature for 1 h. The blots were then washed six times with TBST and prepared with ECL (Pierce® ECL Western Blotting Substract; Thermo Scientific) for developing. Relative levels of each protein were quantified by measuring optical densities (OD) of the corresponding bands with Quantity One® V 4.3.1 software.

CSF total protein concentration was measured by standard automated procedures in an Architect ci8200 analyzer (Abbott, USA).

Samples were obtained in accordance with the Helsinki Declaration of 1964, as revised in 2000. The ethical committee of the Hospital Sant Joan de Déu approved the study. Statistical analysis (linear regres-

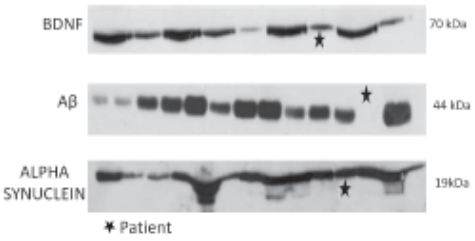

Fig. 1. Western blots of proteins in the patient and in controls.

sion; significance level: $p < 0.05$) was performed with the SPSS 19.0 program.

RESULTS

In our patient, CSF 5-hydroxyindolacetic acid concentration was low (133 nmol/L; C.V. 170–490) whereas dopamine metabolites were within normal limits (HVA: 360 nmol/L; C.V.: 344–906). BDNF, Aβ, and AS were detected in the CSF of the patient and controls, at the expected molecular weight (Fig. 1). CSF total protein concentration values in patients and controls were within normal limits according to different age ranges [9]. Linear regression showed no correlation between age and any of the studied proteins (Aβ: p:0,1; BDNF: p:0,2; AS: p:0,1). The expression of BDNF and AS in the CSF of our patient showed low values with respect to the control group, particularly when compared with age-matched controls (Fig. 2.). The expression of Aβ was undetectable in the patient whereas it was clearly present in all subjects of the control group. Given its negativity, it was repeated twice in two CSF samples (at 3 and 4.5 years), disclosing the same result.

DISCUSSION

HSD10 deficiency is a neurodegenerative disease with a complex pathophysiology. Furthermore, HSD10 is a protein that may mediate the neurodegeneration of AD through its apparent capacity to bind Aβ (1). Due to this affinity, we hypothesized that CSF Aβ concentration in patients with HSD10 deficiency could be abnormal and contribute to the neurobiology of this disorder. The most striking feature of this study is the lack of expression of Aβ in this patient in several samples obtained at different ages.

HSD10 catalyzes the conversion of 2-methyl-3-hydroxybutyryl-CoA to 2-methylacetoacetyl-CoA in the isoleucine degradation pathway, and is active against a broad range of substrates in diverse pathways such as steroid metabolism, $GABA_A$ receptors, and the oxidation of other substrates (hydroxyacyl-CoAs, 2-methyl-3-hydroxyacyl-coas) [1, 2]. Consequences of HSD17B10 gene mutations seem to be unrelated to accumulation of toxic metabolites in the isoleucine pathway but rather are caused by a non-enzymatic effect triggering mitochondrial disintegration and apoptosis [4].

Several studies indicate that mitochondria plays an important role in the development of AD, and this effect could be caused by a direct interaction of Aβ with MHBD (2-methyl-3-hydroxybutyryl-CoA dehydrogenase) [5–7]. HSD10 has a unique loop D (residues 95–114) that binds Aβ [1]. In AD brain tissue, Aβ accumulates in mitochondria. Conversely its concentration is low in CSF. HSD17B10 gene mutation in our patient could have modified this binding and promote post-synaptic Aβ trapping, thereby explaining the apparent absence of Aβ in the CSF.

Little is known about the neurobiology of Aβ in childhood. Recent studies suggest that Aβ and its precursor protein (AβPP) may play important roles in development such as promoting synapse elimination [10, 11], pruning neurites [12], and restricting mature forms of LTP in glutamatergic synapses [13]. In the rodent hippocampus, Aβ has a maximum expression during the period of most intense synaptogenesis and synaptic elimination [14]. Therefore, persistently low synaptic levels of Aβ in our patient could have impaired synaptic balance and glutamatergic transmission. Furthermore, increased post-synaptic Aβ levels could have disrupted synaptic plasticity and promoted apoptosis [15–18].

BDNF is associated with dendritic growth, synaptic transmission [19], and the development of the GABAergic and monoaminergic system [20]. In our patient, BDNF low expression argues in favor of low dendritic density and support reduced CSF concentration of 5-HIAA, as this neurotrophin regulates serotonin system development. Concerning AS, this is a presynaptic protein that binds to the SNARE complex [21] and is involved in regulation of vesicle pools. In fact, its deletion causes a reduction in the reserve pool size [22], leading to impaired long-term potentiation and synaptic plasticity. Synaptic accumulation of AS has been related to dopaminergic loss [23]. In our patient AS showed very low expression which perhaps might explain why CSF dopaminergic metabolites were not reduced as expected at high AS synaptic levels.

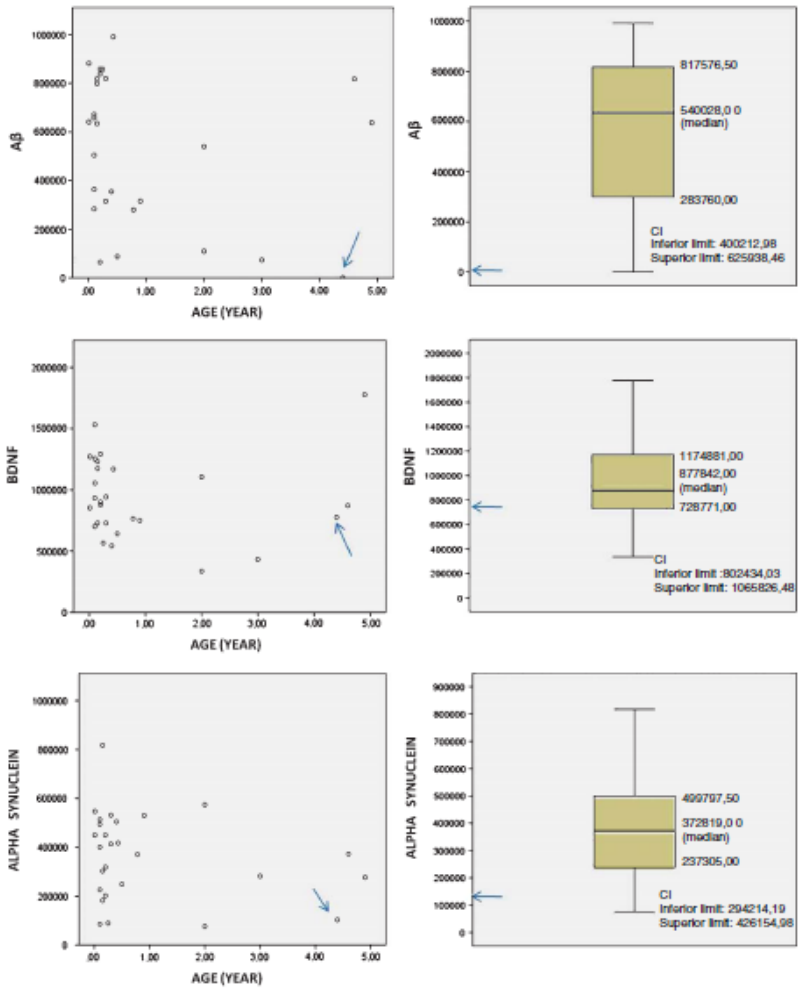

Fig. 2. Distribution of Aβ, BDNF and AS values in the patient (arrows) and in controls. Units on the left are optical densities. CI: confidence interval.

In summary, we report a patient with HSD10 deficiency, undetectable CSF Aβ expression, and low BDNF and AS levels, which probably disrupted critical developmental functions, contributing to impaired synaptic plasticity, low serotoninergic transmission and apparently preserved dopaminergic function.

Confirmation of these findings in other patients would help elucidating mechanisms of synaptic dysfunction in this disease, and highlight the need for

further studies to better understanding of the role of Aβ in both early and late periods of life.

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